Characterizing the Development of Secondary Hyperparathyroidism and Vitamin D Resistance in Experimental Chronic Kidney Disease

by

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Abstract

The parathyroid gland (PTG) responds to alterations in calcium and phosphate *via* secretion of parathyroid hormone (PTH) that acts on kidneys and bone to regulate mineral homeostasis. Hallmarks of chronic kidney disease (CKD) include increased retention of phosphate (PO_4) and decreased renal production of 1,25-dihydroxyvitaminD_3 (calcitriol) from its precursor, 25-hydroxyvitaminD_3 (calcifediol). Hyperphosphatemia and vitamin D deficiency in CKD stimulate excessive production and secretion of PTH, generating maladaptive consequences to the structure and function of bone and blood vessels, pathologies which are associated with increase mortality in the CKD population.

Calcitriol is a mainstay therapy for treatment of secondary hyperparathyroidism (SHPT) that develops in CKD. A major issue with therapy includes the development of calcitriol resistance and increased vascular calcification. Modifications to the PTG that hinder its sensitivity to calcium (Ca) and PO_4 are hypothesized to be involved in the development of calcitriol resistance, renal osteodystrophy and vascular calcification in SHPT which is associated with increased risk of bone fracture and cardiovascular burden. The research contained herein, involving *in vivo* animal studies as well as *in vitro* isolated PTG studies, sought to characterize the development of calcitriol resistance and SHPT in CKD, particularly with regard to changes in response mediated by excess PO_4.

The major findings indicate that high dietary PO_4 attenuates calcitriol mediated suppression of PTH to acute and chronic treatment of calcitriol. Calcitriol treatment (CTx) in both CKD and non-CKD animals showed reduced gland responsiveness and persistent lowering of basal PTH secretion *in vitro*.

Overall the findings emphasize the clinical significance of PO_4 restriction for enhancing the effectiveness of strategies which target PTH lowering and suggest caution in the use of calcitriol

for the treatment of SHPT. A common approach for management of calcitriol resistance is to increase doses of calcitriol during therapy. This should be avoided as prolonged treatment appears to be able to persistently impact PTG response to alterations in Ca and PO_4 both in non-CKD and experimental CKD animals resulting in impaired mineral homeostasis.

Co-Authorship

The research presented in this thesis has been performed and written by Lok Hang Lee (LHL) with the following co-authorships and technical assistance (see below). Dr. Michael A. Adams and Dr. Rachel M. Holden were supervisors of LHL and assisted with the conception and design of the research, performing statistical analysis, and developing the discussion of all experiments.

Chapter 2: Co-authored by Mandy E. Turner, Dr. Cynthia M. Pruss, Kimberly Laverty, Emilie C. Ward, Dr. P. Martin Petkovich, Dr. Rachel M. Holden, and Dr. Michael A. Adams.

LHL, MET, and KL dosed and sampled animals, and performed health checks, MET and KL conducted animal sacrifices, LHL, ECW and CMP conducted biochemical measurements. LHL, MET, RMH, MPP and MAA designed the study, analyzed the results and prepared the manuscript.

Chapter 3: Co-authored by Mandy E. Turner, Austin P. Lansing, Kimberly Laverty, Emilie C. Ward, Dr. Cynthia M. Pruss, Dr. P. Martin Petkovich, Dr. Rachel M. Holden, and Dr. Michael A. Adams

LHL, MET, CMP, RMH, MMP and MAA designed the study.

MET and KL were in charge of surgical interventions. MET, KL, LHL and AL dosed and sampled animals and performed health checks. MET and KL conducted animal sacrifices and retrieval of parathyroid glands for culture. LHL conducted the parathyroid gland culture preparation and protocol. LHL, MET, and ECW conducted biochemical measurements. LHL, MET, RH, MPP, and MAA designed the study, analyzed the results and prepared the manuscript.

Chapter 4: Co-authored by Mandy E. Turner, Sarah M. Taylor, Dr. P. Martin Petkovich, Dr. Rachel M. Holden, and Dr. Michael A. Adams.

SMT dosed and performed health checks, MET and KL conducted animal sacrifices and retrieval of parathyroid glands for culture. LHL conducted the parathyroid gland culture preparation and

protocol. LHL, SMT, MET, and ECW conducted biochemical measurements. LHL, SMT, MET, RH, MPP, and MAA designed the study, analyzed the results and prepared the manuscript.

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Ca	calcium
CaSR	calcium sensing receptor
Calcifediol	25-dihydroxyvitamin D_3
Calcitriol	1,25-dihydroxyvitamin D_3
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease mineral bone disorder
CTx	calcitriol treatment
CT-Ab	calcitriol-abrupt
CT-Grad	calcitriol graduated
CVD	cardiovascular disease
CYP27B1	cytochrome P450 27B1 1.14.15.18 (1-alpha hydroxylase)
CYP24A1	cytochrome P450 24A1 1.14.15.16 (24 hydroxylase)
DMEM-F12	Dulbecco's modified Eagle's medium/Nutrient Mixture F-12
eGFR	estimated glomerular filtration rate
ESRD	end stage renal disorder
FGF-23	fibroblast growth factor 23
FGFR1	fibroblast growth factor receptor 1
HA	hydroxyapatite
HPD	high phosphate diet or high dietary phosphate
KDIGO	Kidney Disease Improving Global Outcomes
LPD	low phosphate diet
OT	off-treatment
PIM	parathyroid hormone inhibitory condition/media
PRD	phosphate restrictive diet
PSTM	parathyroid hormone stimulatory condition/media
PTG	parathyroid gland
PTH	parathyroid hormone
PTHR1	parathyroid hormone receptor 1
Runx2	runt related transcription factor 2
SHPT	secondary hyperparathyroidism
PO_4	phosphate

NaPi-IIa/c	sodium-phosphate cotransporters 2a and 2c
TRPV 5	transient receptor potential vanilloid 5 cation channel
TRPV6	transient receptor potential vanilloid 6 cation channel
UnTx CKD	untreated CKD
VC	vascular calcification
VDR	vitamin-D receptor
VDRA	vitamin-D receptor agonist
VSMC	vascular smooth muscle cells

Chapter 1

General Introduction

1.1 Chronic Kidney Disease

Chronic kidney disease (CKD) is a sustained and progressive decline in kidney function that affects 11-13% of the global population[1]. CKD is a consequence of pathogenic processes that occur with conditions such as obesity, diabetes, hypertension, glomerulonephritis, and urologic diseases[2]. CKD can be divided into 5 stages that are classified by a decline in estimated glomerular filtration rate (eGFR) (Stage 1 for eGFR less than 90ml/min/1.73m^2 to Stage 5 for eGFR less than 15ml/min/1.73m^2) and an increase in urinary albumin to creatinine ratio[2]. Reduced renal function consequently results in decline in the regulation of blood pressure, excretion of waste, and regulation of mineral homeostasis. Due to the complexity of the disease and precipitating factors, CKD patients face increased mortality risk with increased disease severity and progression, particularly with respect to cardiovascular disease (CVD)[3].

1.2 CKD and Cardiovascular Disease

Reduced eGFR and albuminuria are both major independent predictors of cardiovascular morbidity and mortality[4-6]. Patients suffering from CKD are 60% more likely to develop CVD compared to healthy individuals. In fact, CKD patients are more likely to die from CVD than from progression to dialysis-requiring end stage renal disease (ESRD)[6-7]. The Framingham risk score (a value which predicts an individual's risk of developing CVD) incorporates a number of risk factors (e.g. gender, age, total cholesterol level, blood pressure and smoking history)[8] but only partially explains the prevalence of CVD in CKD patients.[9] Non-traditional factors such as disorders of mineral metabolism and secondary hyperparathyroidism (SHPT) have determined to be more

common contributors to the CVD burden in patients of CKD[10,11]. Although the contributions of other risk factors are still being evaluated, CKD-induced development of left ventricular hypertrophy and ectopic calcification, most notably vascular calcification (VC), are clearly important players[12].

1.3 Vascular Calcification

Development of VC results in a circulation with decreased compliance, increased pulse wave velocity, and increased afterload to the heart, all of which promote increased risk of stroke and adverse cardiac events.[12] In CKD, the main type of VC that occurs is termed Monckeberg's sclerosis and involves the deposition of calcium-phosphate (Ca-PO_4) crystals in the form of hydroxyapatite (HA) within the medial layer of vessels, causing vascular damage.[13] The presence and severity of arterial calcification within the population of ESRD patients is emerging as an important predicative risk factor for CVD and mortality[13,14]. Monckeberg's sclerosis develops in early stages of CKD and is defined by mineral depots which form circumferentially within the elastic lamellae of the medial vascular layer.[15,16] Uremia-related VC can contain various forms of Ca and PO_4 containing crystals including hydroxyapatite (HA) (Ca and PO_4 in a 10:6 ratio), whitlockite (Ca, magnesium and PO_4 crystal in a 9:1:6 ratio) as well as others.[17] Pathogenesis of this type of VC in CKD develops when there is a reduction in inhibitors of calcification (e.g magnesium, fetuin-A, and matrix-gla (γ-carboxyglutamate) protein), a dysregulation of Ca and PO_4 homeostasis, or both.[18,19, 20]

Dysregulation of PO_4 homeostasis in particular has been shown to play a fundamental role in the onset and progression of calcification within the circulation[21-23]. Both Ca and PO_4 are obtained through the diet with the mechanisms of regulation described below. Once in the circulation, both minerals localize to bone which acts as a short-term depot. Past studies have shown that high circulating PO_4 levels are a key driver for the development of VC in CKD.[21- 24] PO_4 acts

as both a key signaling molecule and an essential component for the progression of VC pathogenesis, although the exact mechanisms of initiation and progression are still under speculation[23,24]. VC had been previously hypothesized to develop through passive deposition of HA but has now been recognized to be an active process.[16,26-29] It is currently believed that trans-differentiation of vascular smooth muscle cells (VSMC) to an osteogenic phenotype is an important event in the pathogenesis of VC.[25] For example, the initiation of osteoblast differentiation has been marked by expression of key transcription factor runt related transcription factor 2 (Runx2). As such, cells expressing RUNX2 are found almost exclusively in bone tissue.[30] Many studies have found that cells expressing both RUNX2 and VSMC specific markers (e.g. smooth muscle actin) are located at the site of calcification in the vasculature suggesting that trans-differentiation is occurring at these sites. [26-29]

1.4 Mineral Homeostatic Mechanisms

Calcium and PO_4 are involved in a myriad of bodily functions including maintenance of skeletal structure, extra and intra-cellular signaling and muscle contraction. Homeostasis of both Ca and PO_4 is dependent on their storage in bone and levels in circulation which are regulated by dietary intake, intestinal absorption, renal excretion, and bone remodeling. Regulation of circulating Ca and PO_4 is highly dependent on 1,25-dihydroxyvitamin D_3 (calcitriol) and on the hormones parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23).[31]

Handling of Ca and PO_4 begins in the small intestines where both minerals are absorbed by active transport and passive diffusion.[31,32] When luminal concentrations are high, Ca is transported from the lumen into intestinal cells *via* the transient receptor potential vanilloid 6 cation channel (TRPV6),[33,34] while PO_4 is transported by the sodium- PO_4 co-transporter type 2b (NaPi-IIb) and the sodium dependent PO_4 transporters (Pit 1 and 2).[35,36] Calcitriol increases absorption of Ca

through induction of both TRPV5 and 6, and upregulation of Ca binding protein calbidin-D_{9k}[37], as well as increasing PO_4 absorption through upregulation of NaPi-IIb.[38,39] PTH also increases intestinal absorption of both Ca and PO_4 but does so indirectly by increasing the formation of calcitriol by renal 1α-hydroxylase (CYP27B1).[40] Conversely, FGF-23 functions to downregulate luminal NaPi-IIb and renal CYP27B1.[41] In contrast to Ca regulation, circulating PO_4 is not as tightly controlled, and can rise with high dietary PO_4 (HPD).

In the kidney, reabsorption of Ca and PO_4 are substantially regulated by PTH. PTH signaling *via* the PTH receptor 1 (PTHR1) upregulates the expression of Ca transporting proteins including TRPV5 and calbindin[42] and can directly mitigate cytosolic Ca concentrations through activation of adenylyl cyclase resulting in increased Ca reabsorption.[43] In an acute setting, PO_4 reabsorption is significantly regulated by PTH.[44] When PTH binds to PTHR1 the result is downregulation of sodium- PO_4 cotransporters 2a and 2c (NaPi-IIa/c) within the proximal tubule.[45] This effect would reduce PO_4 reabsorption thereby increasing its excretion. FGF-23 maintains long-term control of PO_4 reabsorption within the kidney that occurs similarly *via* downregulation of NaPi-IIa/c.[46] FGF-23 has also been determined to independently increase Ca reabsorption in the kidney possibly through modulation of kidney responsiveness to PTH.[47]

The skeleton represents a major source as well as a depot for the total body flux of both Ca and PO_4.[48] Bone remodeling is a crucial lifelong process for maintaining skeletal integrity that requires balance between bone resorption and bone formation. In the bone, PTH can result in one of two outcomes. When PTH elevation is sustained over a longer period, osteoclasts are stimulated to release PO_4 and Ca into the circulation.[49] However, when PTH is elevated acutely, signaling directly increases osteoblast differentiation and survival resulting in increased bone mineralization.[50] The actions of calcitriol on bone are similar to PTH such that chronic high levels

of vitamin-D can cause increased bone resorption whereas short-term elevation can increase bone formation.[51] The effects of FGF-23 on bone are currently not well known, however it has been determined that FGF-23 is secreted by osteocytes in response to high circulating levels of PO_4 and vitamin-D.[41]

As indicated above, the parathyroid gland (PTG) plays an important role in mineral homeostasis as it coordinates functions in the kidney, intestine, and bone to modulate changes in the levels of Ca and PO_4. PTH secretion is stimulated when circulating levels of Ca are low as well as when circulating PO_4 is elevated.[52] Changes in Ca are sensed *via* binding of Ca directly to the calcium sensing receptor (CaSR) to block PTH release[53,54]. This sensing mechanism of Ca by the PTG follows a set-point behavior, such that changes in the regulation of Ca by PTH effectively provides a negative feedback loop for the control of further PTH secretion.[55,56] PO_4 has also been determined to be a non-competitive antagonist of the CaSR that regulates the active-inactive conformation of the receptor.[57] When circulating PO_4 is high, the CaSR is displaced from its active form, allowing secretion of PTH.[57] It has also been observed to increase the stability of PTH mRNA.[58,59] Finally, production and secretion of PTH is downregulated when calcitriol binds to and signals *via* the PTG vitamin-D receptor (VDR)[60-62] or when FGF-23 binds to and signals *via* fibroblast growth factor receptor 1 (FGFR1)'s on PTG cells.[63]

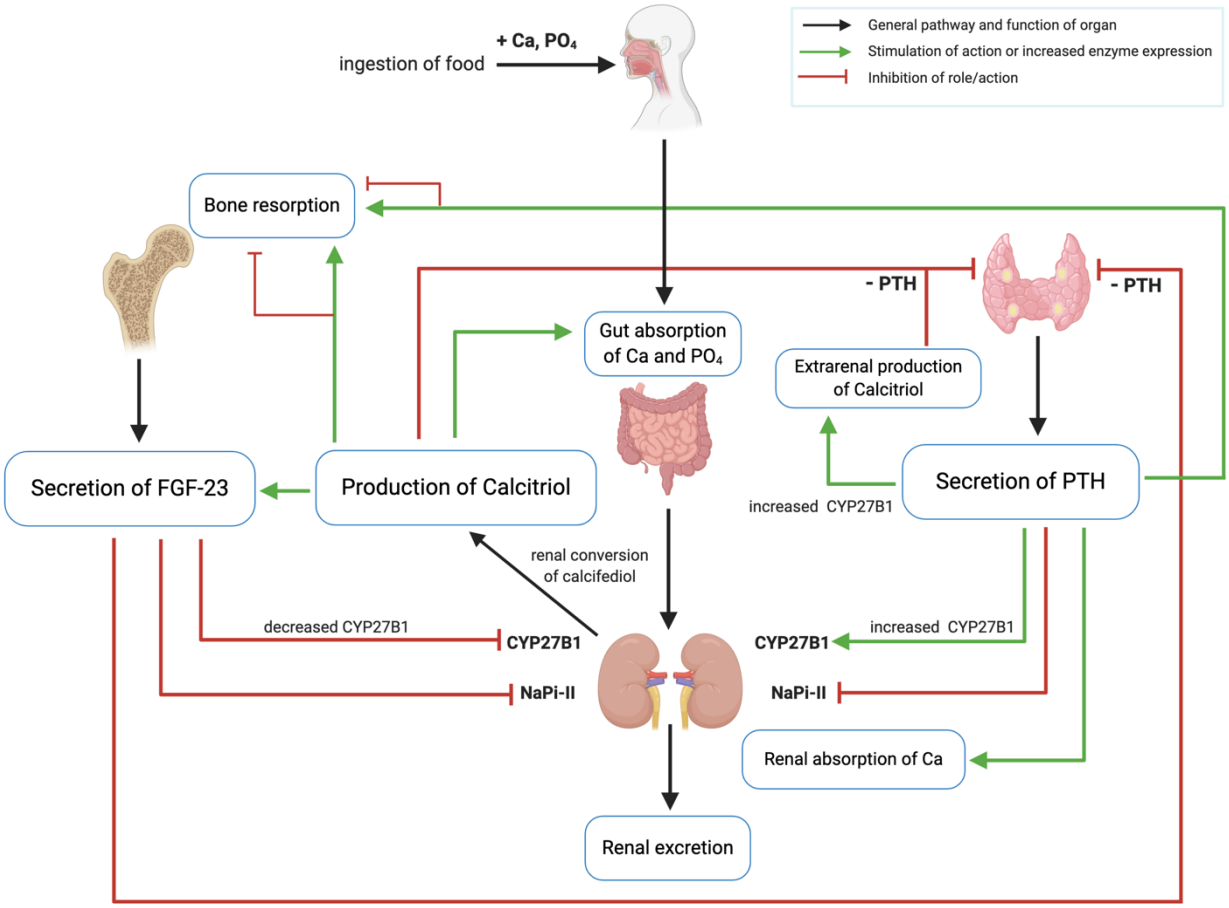

Figure 1-1 Mineral homeostasis in normal physiology

The black arrows represent the general pathway and function of the organ. Green arrows show stimulation of an action and red arrows show inhibition of an action. Parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23) and calcitriol are all important hormones involved in the coordination of gut absorption, bone remodeling, and renal excretion of these minerals as well as the regulation of each other by stimulating or inhibiting hormone production or secretion. The main role of PTH is to increase circulating calcium (Ca) and decrease circulating phosphate (PO_4). PTH increases serum Ca by increased gut absorption, bone resorption and increased production of calcitriol through increased expression of 1-α hydroxylase (CYP27B1). At low doses PTH increases bone remodeling while high doses promote bone resorption. PTH decreases circulating PO_4 *via* inhibition of the NaPi-II, resulting in increased renal excretion of PO_4. Increased production of calcitriol results in increased circulating Ca and PO_4 by increased gut absorption and *via* bone resorption at high doses. Calcitriol stimulates the secretion of FGF-23 and inhibits PTH secretion and production. Finally, FGF-23 is mainly responsible for promoting the excretion of PO_4 through inhibition of NaPi-II. FGF-23 can also indirectly decrease circulating Ca levels *via* inhibiting the production of calcitriol and PTH secretion. Created at Biorender.com

1.5 Vitamin-D Metabolism

Vitamin D is a multifunctional signaling molecule that regulates the body's levels of Ca, PO_4, and magnesium, among other functions. The biosynthesis and breakdown of vitamin D is largely dependent on multiple cytochrome P450 enzymes (CYP).[64] The first step in the biosynthetic pathway occurs in the epidermal layer of the skin where UVB radiation catalyzes the reaction of 7-dehydrocholesterol to cholecalciferol.[65] Cholecalciferol is then carried in circulation by the vitamin D binding protein (DBP) to the liver where vitamin D 25-hydroxylase and, to a lesser extent, sterol 27-hydroxylase, carry out hydroxylation reactions at position 25, resulting in the formation of 25-hydroxyvitamin D_3 (calcifediol).[66] Circulating levels of 25 hydroxyvitamin-D (calcifediol), a weak vitamin D-receptor (VDR) agonist (VDRA), are currently used to assess an individual's prevailing vitamin D status.[67] Hydroxylation of calcifediol is then carried out by 1-α-hydroxylase (CYP27B1) at position 1, resulting in the formation of calcitriol.[68] This primarily occurs in the kidney, but also occurs in other non-renal tissue to a lesser extent.[69] Once synthesized, calcitriol travels through the circulation to its effector organs, including the intestines, kidney, bone, parathyroid glands, and other less significant sites of action. Due to the highly potent nature of calcitriol, the synthesis and breakdown of this molecule are tightly regulated. Circulating levels of calcitriol are 1/1000th of the circulating levels of its precursor calcifediol.[70] Breakdown of calcitriol occurs through further hydroxylation at the 24 position *via* 24-hydroxylase (CYP24A1) to form 1,24,25- hydroxyvitamin D3.[71] CYP24A1 is also capable of catalyzing the 24-hydroxylation of calcifediol, forming 24,25-hydroxyvitamin D3 which is then excreted.[71] By undergoing 24-hydroxylation, excess calcifediol may be removed from the available pool capable of acting as a substrate for more production of calcitriol.[64,71] Production of calcitriol is impacted by the actions of PTH and FGF-23.[71] PTH increases the production of calcitriol through upregulation of CYP27B1 and downregulation of CYP24A1 mRNA[72] while FGF-23 performs the opposite effects on the same enzymes to decrease production of calcitriol.[41]

The interactions between PTH, calcitriol and FGF-23 form a complex multi-tissue feedback system that facilitates PO_4 and Ca regulation. Therefore, when hormonal control of PTH, vitamin-D, and FGF-23 are impaired, such as in CKD, the result can be the development of a mineral disorder and its associated consequences.

1.6 Mineral Dysregulation in CKD

Within the CKD population, a subset of patients develop chronic kidney disease mineral bone disorder (CKD-MBD), which is characterized as the systemic disorder of mineral and bone metabolism concomitantly with uremia.[4] The degree of Ca and PO_4 dysregulation worsens with CKD progression.[5] In CKD-MBD, CKD patients lose the capacity to excrete excess PO_4 with the decline in functional renal mass. As levels of circulating PO_4 begin to rise, both PTH and FGF-23 rise concomitantly in an attempt to regain PO_4 homeostasis, eventually the increased levels of both hormones become pathological and yet inadequate for control of mineral balance.[73] The levels of calcitriol decline as a result of the reduced functional kidney mass and because elevated FGF-23 decreases the expression of renal CYP27B1.[74] As indicated above, calcitriol is responsible for Ca absorption in the gut,[34] such that decreased calcitriol levels can cause hypocalcemia, which further stimulates the secretion of PTH.[52] Collectively, increased circulating PO_4, and decreased circulating Ca and vitamin D contribute to the development of secondary hyperparathyroidism (SHPT), characterized as persistent elevations in PTH. In the initial stage of CKD, the PTG cells have been found to proliferate in response to these stimuli, leading to diffuse parathyroid hyperplasia.[75] If the PTG continues to be stimulated, cell division becomes uncontrolled resulting in the formation of nodules containing more tightly packed cells.[76,77] The development of this nodular PH is associated with downregulation of VDR, CaSR, and FGFR1 which further aggravates the impaired control of SHPT.[77-79]

PO_4 can also contribute indirectly to the development of VC via impacting levels of PTH and FGF-23. Both of these hormones become elevated in the presence of high circulating PO_4 in an attempt to regain normophosphatemia.[73] When the effect is prolonged, increased levels of PTH can result in increased bone resorption, an action that further contributes to the increased levels of circulating Ca and PO_4.[80] Elevated FGF-23, is emerging as a possible predictor of aortic, coronary, and peripheral vascular calcification severity in dialysis patients and may be released by the vasculature early in CKD.[81] Elevations in both phosphaturic hormones therefore puts patients with CKD-MBD at a higher risk for developing VC.[13] Taken together, understanding the mechanisms of mineral homeostasis is crucial for determining how VC is developed and attenuate the cardiovascular complications associated with CKD.

Moreover, dysregulation of these key signaling molecules has an important impact on bone health. As discussed above, PTH, vitamin-D and FGF-23 are essential for regulating bone formation and turnover. Thus, when circulating levels are either too high or too low, metabolic processes in bone become disrupted. For example, persistent elevations in PTH can cause pathological dissolution of bone termed renal osteodystrophy, a process that promotes increased risk of skeletal fractures and disability in CKD patients.[80] The reclassification of renal osteodystrophy into CKD-MBD highlights the impact of abnormal mineral metabolism and signaling in contributing to the development of bone abnormalities, extra-skeletal calcification and increasing morbidity and mortality within CKD patients.[4,13]

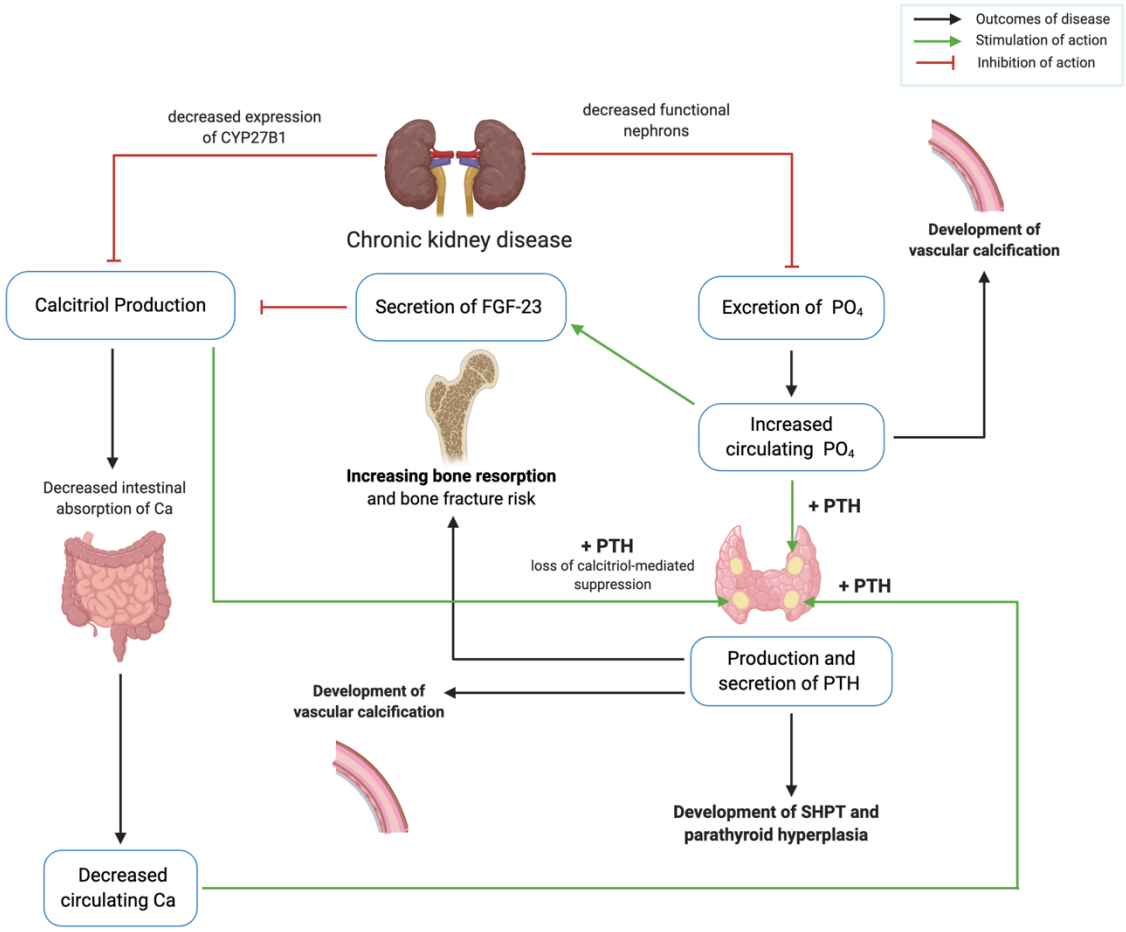

Figure 1-2 Dysregulation of mineral homeostasis in CKD

The black arrows show the general movement of the minerals once it is ingested. Green arrows represent stimulation of an action and red lines represent inhibition. Dysregulation of mineral homeostasis in chronic kidney disease (CKD) results in phosphate (PO_4) retention, low levels of calcitriol, and hypocalcaemia that are all major stimulants for the development of secondary hyperparathyroidism (SHPT). Two major hallmarks of CKD include increased PO_4 retention and decreased production of calcitriol *via* renal 1-α hydroxylase. Increased PO_4 retention results in increased circulating PO_4 that stimulates the secretion of PTH and FGF-23, hormones which mitigate PO_4 excretion. Decreased production both directly and indirectly increases PTH secretion. Reduced levels of calcitriol result in decreased VDR mediated suppression of PTH and decreased intestinal absorption of Ca, resulting in decreased circulating Ca. Stimulation of PTH secretion and production causes the gland to proliferate in response to the sensed stimuli. The consequence is the development of SHPT and parathyroid hyperplasia which results in reduced gland responsiveness, renal osteodystrophy, and increased vascular calcification. Created at BioRender.com.

1.7 Vitamin-D and CKD

CKD has been shown to reduce levels of calcitriol in the circulation, as activation of its precursor, calcifediol, occurs primarily within the proximal tubule of the kidney, an organ whose overall function is progressively declining in CKD.[68,74] Reduced activity within the vitamin-D metabolome removes a major suppressor of both PTH production and secretion which often results in the development of SHPT.[76] As discussed above, SHPT as a result of dysregulated mineral metabolism, accelerates the development of vascular calcification and bone abnormalities.[82] As such, many patients with CKD are given vitamin-D analogs as a mainstay therapy for suppressing the PTG and mitigating the consequences of SHPT.[83]

Challenges faced during vitamin-D treatment include the accelerated development of VC and calcitriol resistance.[84-86,] Calcitriol and its analogs are thought to contribute to the development of VC because of both target effects on the vasculature and on mineral homeostasis.[84-85] Specifically, previous studies have been demonstrated that uremic animals given calcitriol are more prone to calcification,[79] and healthy animals given high doses of calcitriol (0.5ug/kg) subcutaneously also develop substantial calcification (M. Turner, personal communication). The other challenge with treatment, calcitriol resistance, occurs when there is a lack of PTH suppression despite continuous increases in VDRA dosage, a condition occurring in 20-30% of CKD patients receiving therapy.[86] A major mechanism which is hypothesized to cause resistance is the downregulation of functional receptors in the PTG in CKD,[77] with multiple studies demonstrating pan receptor downregulation with parathyroid hyperplasia.[77-79] Characterizing calcitriol resistance early, will allow for the generation of more nuanced dosing strategies of vitamin D receptor agonists in this population, preventing unnecessary SHPT and parathyroidectomies. Therefore, it is important to study the PTG thoroughly as a central mediator of mineral homeostasis and possible player in the pathological alterations of bone and vasculature function and structure observed in CKD-MBD.

1.8 Parathyroid Gland Cell Types

The human parathyroid gland is made up of two cell types, chief cells and oxyphil cells. Chief cells are responsible for the regulation of PTH production and secretion and play a major role in mineral homeostasis by responding to low circulating Ca and high circulating PO_4 acting on CaSR, as well as low circulating calcitriol acting on the VDR, respectively.[87] Although the potential functions of oxyphil cells are still being elucidated, it has been determined that oxyphil cells express PTH related peptide and PTH mRNA, and also express VDR and CaSR.[88,,89]. These phenotypes suggests oxyphil cells have a possible role in PTH secretion. In addition, oxyphil cells have been hypothesized to develop as a result of transition from chief cells. This transition concept is based, at least in part, on the observation of cell clusters within the parathyroid gland, which contain both chief and oxyphil cell characteristics.[90,91] The proportion of oxyphil cells in the PTG increases progressively with age in the CKD population in association with increased PTG mass.[89] In comparison to chief cells, oxyphil cells have been determined to have greater expression of CYP27B1, and CaSR which may be related to increased production of calcitriol for greater control of PTH secretion.[88-,91] Oxyphil cells have been determined to be prominent with the development of secondary hyperparathyroidism in CKD. In fact, the severity of SHPT is associated with increased number of oxyphil cells and is hypothesized to be involved in the development of calcitriol resistance.

1.9 Assessment of PTG responsiveness

In vitro models of PTGs using rat, bovine, or human tissue have revealed functional, metabolic, and genomic information regarding the PTG. Through these experimental models, physiological responses have been characterized including: the Ca setpoint (the level of Ca required to suppress PTH secretion by 50%), PTG responsiveness to alterations in calcitriol or PO_4, and the

inhibitory response to calcitriol in healthy PTGs[93-95]. However, few experiments have determined the effects on the PTG in response to manipulation of the Ca and PO_4 ratio in culture media.

Thus, a focus of the research in this thesis is to examine the impact of HPD, which is becoming increasingly prevalent in the North American diet in part because of increased food preservatives and overall food consumption[96], and calcitriol treatment (CTx), a mainstay therapy in CKD, in the alteration to PTG function. Characterizing alterations in the PTG will allow us to better understand the development of calcitriol resistance in the CKD population and potentially help to generate novel dosing strategies in which there is an enhanced safety profile of vitamin D agonists for treatment of SHPT.

1.10 Statement and Hypotheses

The overall working hypotheses are as follows:

1. CKD duration and HPD are both factors which can decrease the ability of the PTG to respond to vitamin D therapy.
2. Both experimental CKD and high dose CTx will induce the PTG to be less responsive to Ca and PO_4.

The studies in which these hypotheses were tested include:

Chapter 2:

The specific objective was:

1. To determine the effects of CKD duration and HPD on acute suppression of PTH by calcitriol.

Chapter 3:

The specific objectives in an experimental CKD model were:

1. To generate a viable model for assessing changes in PTH release in response to changes in Ca and PO_4 concentrations *in vitro.*

2. To determine the effects of vitamin D treatment on PTG responsiveness to changes in Ca and PO_4 concentrations *in vitro.*

Chapter 4:

The specific objectives were:

1. To determine whether prolonged treatment with high doses of calcitriol in non-CKD animals impacts the PTG and its ability to respond to alterations in Ca and PO_4 *in vitro*.

2. To determine whether there are any differences in the responsiveness of PTG to mineral concentrations between experimental CKD and the calcitriol-induced vascular calcification model.

Chapter 2

The effect of CKD duration and dietary phosphate on calcitriol efficacy in an experimental rat model

2.1 Abstract

SHPT is a prevalent disorder of CKD that develops as a result of impaired PO_4 excretion and calcitriol deficiency with disease progression. SHPT leads to increased bone resorption and is a risk factor for VC and cardiovascular disease in the CKD population. Calcitriol is commonly prescribed to overcome this deficiency and reduce SHPT, however resistance to suppression often occurs. The purpose of this study was to use an experimental model of CKD to characterize calcitriol resistance in the CKD population. The ability of single doses of calcitriol to acutely suppress PTH will be assessed as kidney function declines and dietary PO_4 is increased.

Experimental CKD was induced using a 0.25% adenine diet. An oral dose of calcitriol (160ng/kg) was given at baseline, one, three, and five weeks as CKD progressed on low (0.5%) dietary PO_4 (LPD), and at seven and eight weeks following a high (1.0%) dietary PO_4 intervention. At each dose, Ca, PO_4, PTH, and FGF-23 were assessed over a 24-hour period prior to and after CTx.

Creatinine was elevated by one week of dietary adenine ($p<0.01$) and stabilized at ~300uM. Despite providing a LPD, statistically significant increases in circulating PO_4 were detected. Throughout CKD generation (baseline to five weeks), the single dose of calcitriol consistently induced a PTH reduction by 24 hours. The responsiveness was maintained despite elevations in PTH from 79.6±25.8 pg/mL to 221.4±66.7 pg/mL by week 5. Following the increase to 1% dietary PO_4, PTH increased to 1207±176 pg/mL and 1342 ± 310 pg/mL, at 7- and 14-days, respectively. In this stage, PTH was no longer reduced at 24 hours following the dose of calcitriol. The response of PTH and FGF-23 was independent of changes in serum PO_4 and Ca.

Suppression of PTH by an acute dose of calcitriol was significantly attenuated by dietary PO_4 in the setting of stable experimental CKD. These findings suggest that limiting dietary PO_4 in the CKD population is critical to limiting mineral bone disease and facilitating treatment effectiveness in this population.

2.2 Introduction

PO_4 dysregulation is a major characteristic of CKD with disease progression.[3] High circulating PO_4 has been determined to correlate with increased cardiovascular risk in both healthy individuals and patients with CKD.[97-99] PO_4 dysregulation in CKD is managed clinically *via* a three-pronged approach including a PO_4 restrictive diet (PRD), providing oral PO_4 binder regimens, and more regular dialysis treatment.[100] Compliance to PRDs has become more difficult with increased prevalence of bio-accessible, inorganic PO_4 containing food additives that are not always indicated on food labels, especially for processed foods.[93] In contrast to consumption of PO_4 from natural sources, PO_4 obtained from food additives lowers the ratio of Ca and PO_4 from a 1:1 to less than 0.6:1.[101] A lower ratio can then stimulate release of PTH.[52] Circulating PO_4 does not typically elevate abnormally until stage 4 of CKD (an eGFR below 20mL/min/1.73m^2), while levels of counterregulatory hormones responsible for PO_4 homeostasis (i.e. ↑PTH, ↑FGF23 and ↓calcitriol) are altered in earlier stages of renal disease.[4-6, 73] Therefore, normal levels of PO_4 do not preclude substantial underlying PO_4 dysregulation or off target effects related to high levels of PTH or FGF-23.[73] It is important not to dismiss excess dietary PO_4 and its associated health risks as potential contributors to disease burden.

SHPT is a common complication of CKD that affects the majority of patients with stage 3 or 4 CKD.[82,83] SHPT is characterized by persistent elevations in PTH and is hypothesized to be mediated by PTG cellular hypertrophy and hyperplasia.[102] SHPT is associated with impaired mineral homeostasis and alterations in calcitriol metabolism, which develop early in the course of CKD and become more pronounced with disease progression.[103] Levels of PTH are stimulated initially in response to increased circulating levels of PO_4 as a result of declining renal function. PO_4 has been determined to directly stimulate release of PTH and increase proliferation of the PTG *in vitro*.[24,44,52,57,94] PO_4 may also cause levels of PTH to rise indirectly *via* increased FGF-23, resulting in upregulated breakdown of calcitriol.[104] In excess, PTH can promote pathological

dissolution of bone, which causes disproportionate risk for bone fractures and increased development of extra-osseous soft-tissue calcification.[80] In fact, high PTH (>600pg/mL) has been linked to increased cardiovascular morbidities and mortality in dialysis patients.[105]

The current Kidney Disease Improving Global Outcomes (KDIGO) recommendations for correcting abnormalities in the vitamin-D metabolome and mineral-bone axis focus on the use of calcitriol and active VDRA to target SHPT in patients with stage 3 to 5 CKD.[83] Observational studies suggest that use of VDRAs is associated with reduced cardiovascular events, left ventricular hypertrophy, and increased survival in ESRD patients with SHPT.[106, 107] However, multiple clinical and animal studies have also suggested that CTx can also promote cardiovascular events *via* its effects on mineral homeostasis.[106-109, 111] For example, calcitriol has been determined to contribute to the development of vascular calcification in part *via* increased gut absorption of Ca and PO_4, creating a pro-mineralizing environment, and upregulation of several pro-calcification genes in the vasculature.[108, 109] Furthermore, calcitriol promotes secretion of FGF-23, a phosphaturic hormone which has been determined to stimulate left ventricular growth *via* FGFR on cardiomyocytes.[110] The discordant effects associated with VDRAs seem to be linked to dose, where low doses[106, 107] of VDRAs are protective while high doses increase cardiovascular burden.[108-109, 111]

Calcitriol resistance is an ongoing problem associated with VDRAs and has been hypothesized to be largely affected by high levels of circulating PO_4 and the development of parathyroid hyperplasia with disease.[86,112] Persistence of calcitriol resistance results in uncontrolled SHPT which necessitates prescription of higher doses of calcitriol, which in turn results in more adverse effects such as increased VC severity and necessitates parathyroidectomies.

The goal of this study is twofold; 1. to determine the onset of calcitriol resistance and how drug efficacy may be impacted with progression of CKD and SHPT and 2. to determine if HPD can impact calcitriol responsiveness in an experimental animal model. Characterization and earlier

detection of calcitriol resistance will allow for the generation of more nuanced dosing strategies for VDRAs in this population and increase their safety profile.

2.3 Methods

Experimental CKD Model:

All animal procedures were performed in accordance to the guidelines set by the Canadian Council on Animal Care and approved by the Queen's Animal Care Committee. Male Sprague-Dawley rats (n=7; 14 weeks old; 400-450g) (Charles River Laboratories Inc., Sherbrooke, Quebec) were acclimated for one week prior to the start of the experiment. Each animal was individually housed and maintained on a 12 hour-light-dark cycle throughout the duration of the study. In the first week of experimentation and during acclimatization, animals were given a control diet containing 0.5% PO_4, 1% Ca, 0.05% magnesium, 0.2 mg/kg vitamin K, 1 IU/g cholecalciferol, and 6% protein (Harlan Teklad Corp., WI, USA: TD150555) with 10g of food provided twice daily in the morning at 8:30am and afternoon at 4:30pm, and water *ad libitum*. The Harland Teklad diet is designed as a standard rodent chow with a fixed formulation of minerals and proteins essential for normal growth, maintenance, and reproduction. Progressive renal failure was induced using dietary adenine models as previously described in Shoberi et. al (2013).[113] In short, the adenine diet which is identical to the control diet with the exception of an added 0.25% adenine (Harlan Teklad Corp., WI, USA: TD150558) was given to the animals at 18 weeks of age with the same daily scheduled feeds of 20g/day as the control diet and water *ad libitum*. CKD progression and severity were monitored through weekly measurements of serum creatinine (QuantiChrom Corp., BioAssay Systems, Hayward, CA, USA). As creatinine is a waste product normally excreted by healthy kidneys, rises in serum levels serve as an indicator for decline in renal function. Animals were removed from adenine after 4.5 to 5.5 weeks based on the level of serum creatinine, greater than 300μM, which mimics creatinine levels of individuals with moderate CKD severity. From this point

onward, all animals were maintained on the control diet (Harlan Teklad Corp., WI, USA: TD150555) until 7 weeks of experimentation when all the animals are subsequently switched onto a HPD (1% PO_4) until their sacrifice date (week 8). The 1% PO_4 diet was a combination of the animal control diet described above and a 1mL solution of sucralose PO_4 gel containing the equivalent of 0.5% PO_4. This protocol was done in this manner to ensure that changes in mineral homeostasis were due to disease progression and not adenine.

Animal body weight, food and water intake was monitored daily throughout the experiment by weighing remaining food and water bottles. If a body weight loss of 15% or greater was reached, subcutaneous fluids (saline) and nutritional supplementation (DietGel Boost, Clear H_2O Inc., PA, USA) was provided.

Calcitriol Dosages and Blood Sampling Protocol

A relatively high clinical dose of calcitriol (160ng/kg) was provided orally to CKD induced rats (N=7) at week 0 prior to the induction of CKD and at weeks 1, 3, 5, 7, and 8 of CKD. Biweekly measurements were performed according to the Canadian Council on Animal Care Sampling Standard of Operations guideline which dictates sampling blood volume limits. Sampling from the saphenous vein was done with a 22G needle and microcapillary blood collection tubes (3 whole blood, 5 heparinized plasma). Whole blood was left at room temperature for at least 45 minutes and heparinized whole blood was collected on ice prior to centrifugation to generate serum and plasma, respectively. Samples were centrifuged at 9,000 RPM for 20 minutes at 4°C. Ca, PO_4, PTH, and FGF-23 was measured from each blood sample collection timepoint. Ca, PO_4, PTH, and FGF-23 response to each calcitriol dose was assessed over a time course 24-hour period prior to and for 24 hours after dosing at week 0 (prior to the induction of CKD), and at weeks one, three, five ,7, and 8 of CKD. Blood samples prior to baseline (24hr) allowed us to account for daily fluctuations of serum mineral and plasma hormone levels in drug-response investigations (Fig.2-1). The blood

sampling timepoints (Fig 2-1.) were chosen based on calcitriol's non-genomic (operating through phosphoinositides, Ca^{2+}, cyclic guanosine monophosphate, and MAP kinase)[37,39,115] and genomic (regulation of transcription)[60] mechanisms of action[114] which act acutely and chronically and evidence of PTH suppression at all selected time points from previous studies within the Adams/Holden Lab.

Blood and Tissue Biochemistry

Circulating creatinine (QuantiChrom Corp., BioAssay Systems, Hayward, CA, USA), Ca, and PO_4 (Heresztyn et. al) were evaluated spectrophotometrically (SynergyHT Microplate Reader, BioTek Instruments Inc., Winooski, VT), with previously described modifications by McCabe et. al (2013).[113] Ca and PO_4 were measured using the O-cresolphthalein and malachite green methods respectively. Complexes are formed between O-cresolphthalein and Ca to form a dark pink purple colour that is detected at 540nm for both standards and blood samples. The malachite green method relies on the ability of alkaline and protein phosphatases to use phosvitin (phosphorylated protein) as a substrate. The product of this reaction is detected colorimetrically by measuring the green complex formed between PO_4 and the malachite green and ammonium molybdate reagent. The absorbance of this complex was measured for both standards and blood samples at 650nm. Plasma concentrations of intact PTH and intact C-term FGF-23 will be measured using ELISAs (Immutopics Inc., San Clemente, CA, USA) as per the manufacturer's protocol.

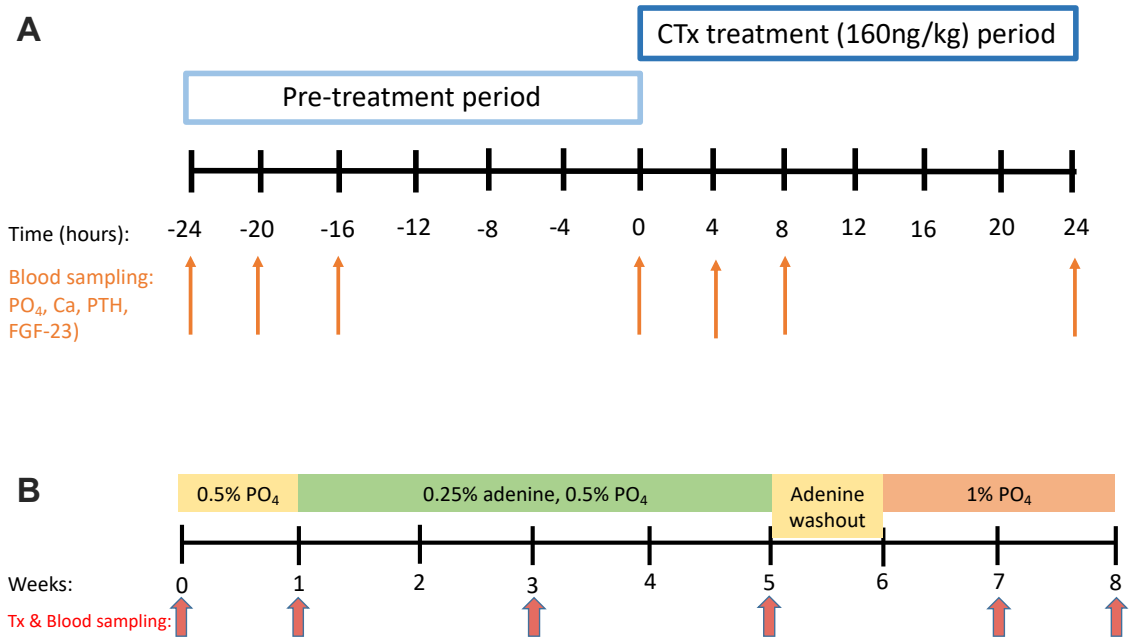

Figure 2-1: Experiment schematic

A) Dosing and sampling protocol. **B)** Induction of chronic kidney disease (CKD) and secondary hyperparathyroidism (SHPT). The orange arrows in Fig 2-1A each timeline represents a blood sample taken for measurements of calcium (Ca), phosphate (PO_4), parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23) at each time point. Measurements were done twice 1. 24 hours prior to dosing and 2. 24 hours after a 160ng/kg dose of calcitriol was given. This protocol was carried out at week 0 prior to the induction of CKD and at weeks 1, 3, and 5 of CKD on low dietary phosphate, 7, and 8 of CKD on high dietary PO_4 indicated by the red arrows in Fig 2-1B.

Animal Euthanasia Protocol:

Animals were anesthetized with inhaled isoflurane (5% isoflurane, 2% O_2) and sacrificed *via* cardiac puncture and exsanguination. Various tissues were collected (34) including: arteries, veins, kidney, liver, pancreas, lung. Tissues were patted dry, weighed, and placed in 1N HCl (50uL/mg tissue) for one week at room temperature to facilitate demineralization and solubilization into solution. The residual undissolved tissues, devoid of minerals, were removed from the acid to generate an acid homogenate for analysis of tissue mineral concentrations (data not shown).

Statistical Analysis:

Calcium, phosphate, PTH, and FGF-23 data will be presented as mean (standard deviation) and relative % change from the average of the untreated time course (24 hours prior to receiving 160ng/kg of calcitriol). Relative % changes were calculated to assure that calcitriol mediated effects on the circulating biomarkers are not a result of circadian rhythms. Mixed effects analysis and *post-hoc* Dunnet's multiple comparisons test were used to evaluate changes in time measurements for both raw data and relative % change. Comparisons were made from baseline prior to receiving CTx throughout the study at each week of the experiment in order to assess the effects of calcitriol on Ca, PO_4, PTH, and FGF-23 with CKD duration and high dietary PO_4. Comparisons of longitudinal measurements in creatinine, Ca, PO_4, PTH, and FGF-23 between experimental weeks (0, 1, 3, 5, 7, and 8 weeks) will each be assessed separately using a two-way ANOVA or mixed effects model with Geisser-Greenhouse corrections and *post-hoc* Dunnett's multiple comparisons test to determine changes in circulating biomarkers with increasing CKD severity.

2.4 Results

CKD Progression

Experimental CKD was induced in Sprague Dawley rats (n=7) using dietary adenine to cause progressive nephron crystallization and damage. Figure 2-2 depicts the elevations in serum creatinine over the course of the experiment. Serum creatinine was elevated after only one week of dietary adenine (103.45 ± 16.65μM, $p<0.01$) compared to baseline (47.37 ± 6.19 μM). Animals were removed from adenine and switched back onto a LPD by week 4.5 when all of the animals achieved creatinine levels of 250-400 μM (Mean of 329.15 ± 82.01 μM,), indicative of moderate to severe CKD. Creatinine levels remained somewhat stable after one week of HPD (week 7: 265.99 ± 85.69 μM) but were then significantly elevated at week 8 (369.34±84.57 μM, $p<0.05$) compared to the previous week (Fig. 2-2), potentially in association with some weight loss with disease progression.

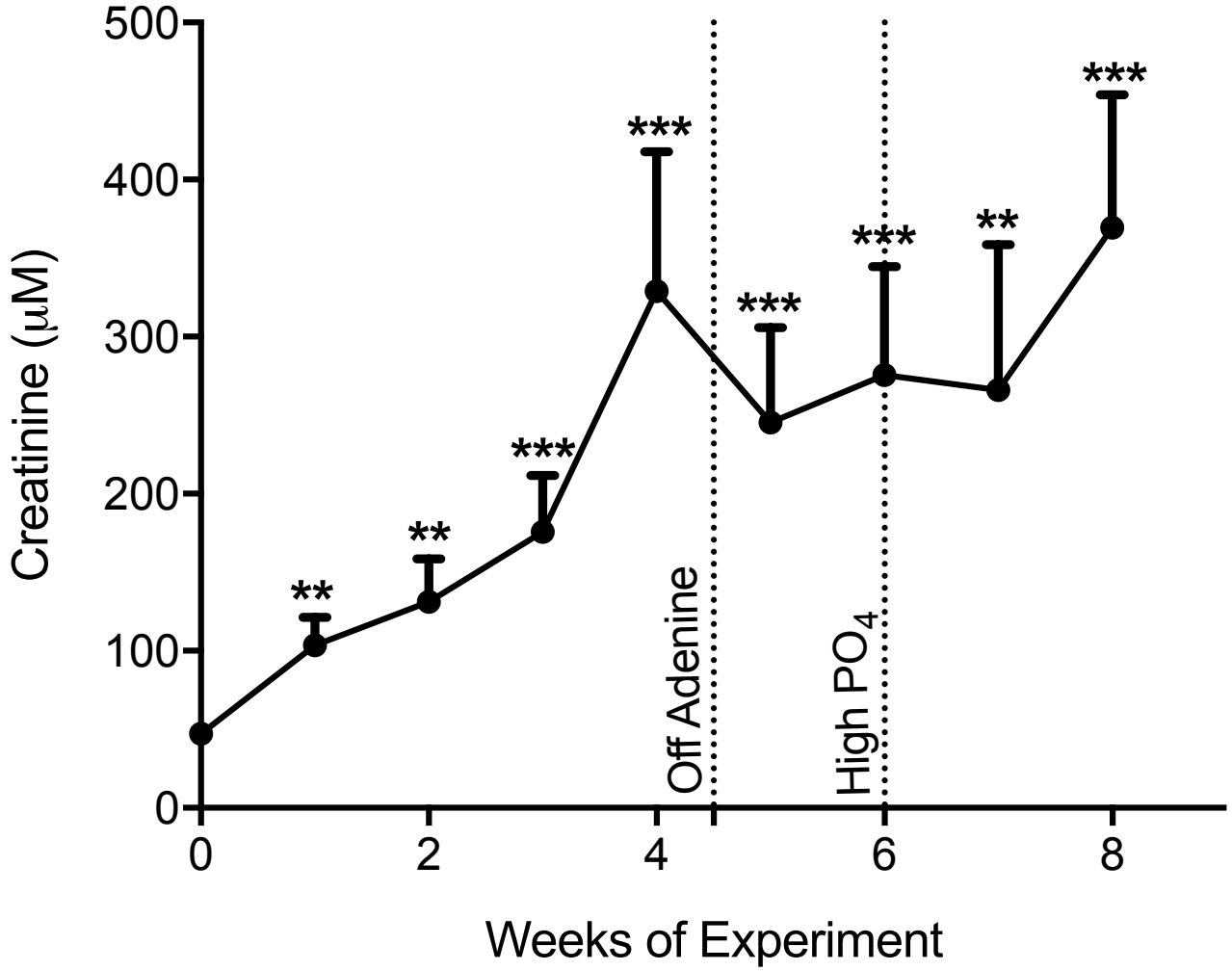

Figure 2-2 Longitudinal assessment of chronic kidney disease severity *via* serum creatinine

Raw data was measured from serum weekly for assessment of CKD severity (N=7). Data represented as mean +/- SD. Values at each week of the graph were taken from measurements at the untreated time course (-24hr) of the experiment. Difference between weeks of experiment are compared using a two-way ANOVA and *post-hoc* Dunnet's multiple comparisons test. Stars denote differences between each measured timepoint compared to baseline (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Longitudinal Assessments of Mineral and PTH Responses

Despite some trends during the HPD, the analysis of variance did not indicate significant increases overall in circulating levels of Ca and PO_4 (Fig. 2-3A, Fig. 2-3B) over the 8-week time course. In contrast, PTH levels elevated with progression of CKD and with HPD intervention. PTH increased ~three-fold from week 0 (99.92 ± 51.65 pg/mL) to week 5 (322.32 ± 170.25pg/mL, $p<0.0001$) and ~ten-fold from week 0 to week 7 (1385.4 ± 1147 pg/mL), one week after HPD (Figure 2-3C). PTH levels at week 7 (1385.4 ± 1147 pg/mL) were significantly elevated compared to circulating PTH at weeks 1 (137.94 ± 54.29 pg/mL, $p<0.0001$) , 3 (173.12 ± 59.76 pg/mL, $p<0.0001$) , and 5 (322.32 ± 170.25pg/mL, $p<0.0001$). FGF-23 also increased with CKD progression with a 17-fold increase in plasma FGF-23 from week 0 (381.7 ± 32.15 pg/mL) to week 5 (662.38 ± 340.78 pg/mL, $p<0.0001$) and a ~ forty-fold increase from baseline to week 7 (14,486 ± 7695.9 pg/mL, $p<0.0001$) (Fig. 2-3D). FGF-23 levels at week 7 (14,486 ± 7695.9 pg/mL) were significantly elevated compared to circulating FGF-23 at weeks 1 (), 3 (), and 5 (662.38 ± 340.78 pg/mL, $p<0.0001$).

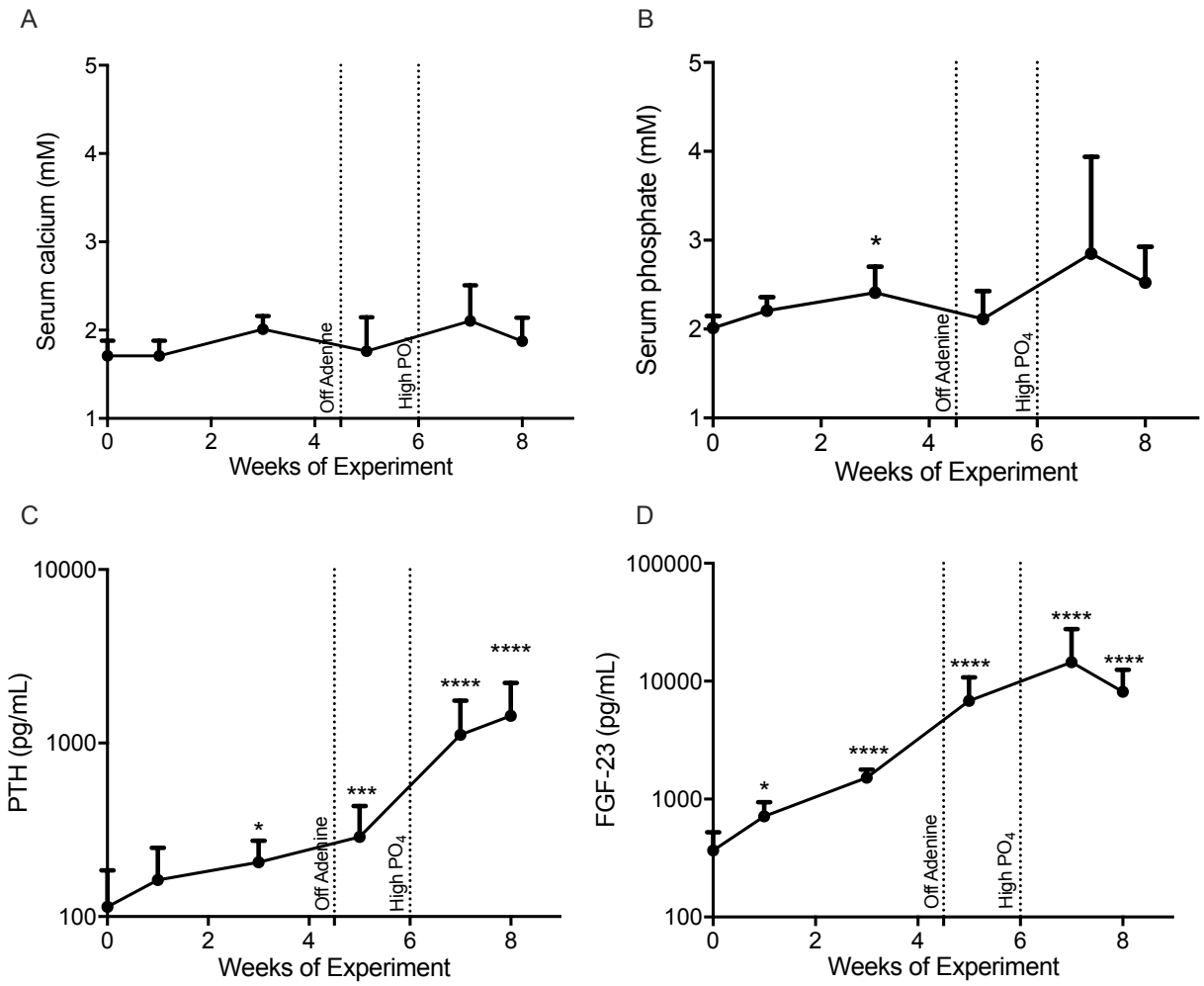

Figure 2-3 Longitudinal measurements of circulating mineral and hormone levels in experimental CKD

A) calcium, **B)** phosphate, **C)** parathyroid hormone (PTH), and **D)** fibroblast growth factor 23 (FGF-23) (N=7). Raw data plotted from baseline (prior to dosing) measures of calcium, phosphate, PTH and FGF-23 at week 0 (prior to induction of CKD), and weeks 1, 3, 5, 7, and 8 of CKD (N=7). Data represented as mean +/- SD. Difference between weeks of experiment are compared using two-way ANOVA with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. These comparisons were done on the logged values of PTH and FGF-23. Stars denote differences between timepoints compared to week 0 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

The levels of Ca during the first 24 hours prior to CTx were not markedly altered either throughout the progression of CKD or during the HPD phase. Administration of calcitriol had a minimal effect on circulating Ca before the induction of CKD (Baseline) and at weeks 1, 3, and 7 of CKD. However, administration of calcitriol did induce significant elevations in circulating Ca at weeks 5 and 8. At week 5 calcium levels rose from baseline (0hr, 1.69 ± 0.41 mM) to 4 hours (2.11 ± 0.44mM, $p<0.05$, (Fig. 2-4) and at week 8 serum calcium rose significantly from baseline (0hr, 1.87 ± 0.27mM) to a similar level at 4 and 8 hours (2.31 ± 0.18mM, $p<0.01$ and 2.32 ± 0.22mM, $p<0.05$), respectively .

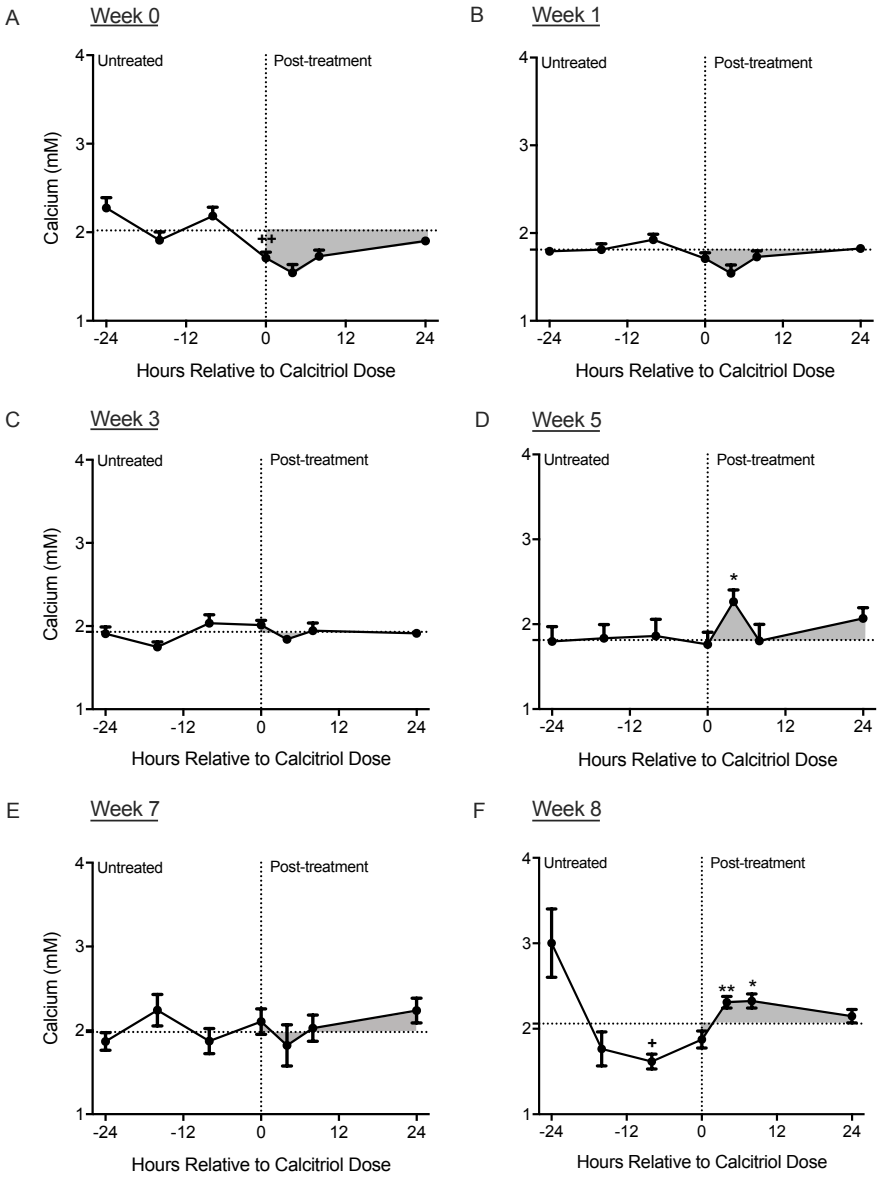

Figure 2-4 Circulating calcium in CKD animals from 24 hours prior to 24 hours after calcitriol treatment

A) Week 0**, B)** Week 1, **C)** Week 3, **D)** Week 5, **E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7). The horizontal line represents the average value of the untreated time course from -24hr to 0hr. The vertical line represents the baseline prior to receiving the 160ng/kg dose of calcitriol. Data represented as mean +/- SD. Difference between timepoints 24 hours prior to and 24 hours after calcitriol treatment were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Crosses denote differences between time points within the untreated time course and -24hr (+ $p<0.05$). Stars denote differences between timepoints within the post-treatment time course compared and time 0hr (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Administration of calcitriol did not produce consistent effects on circulating PO_4 throughout the experiment when compared to baseline values prior to CTx. However, there was a significant rise on week 5 at 24 hours (2.94 ± 0.55 mM, $p<0.05$) compared to baseline (2.11 ± 0.31 mM), an effect which was not a common trend in other data (Fig. 2-5). In addition, there was a significant decline in PO_4 at 4 to 8 hours in the untreated time course which did not occur by week 8, which was two weeks of high dietary phosphate.

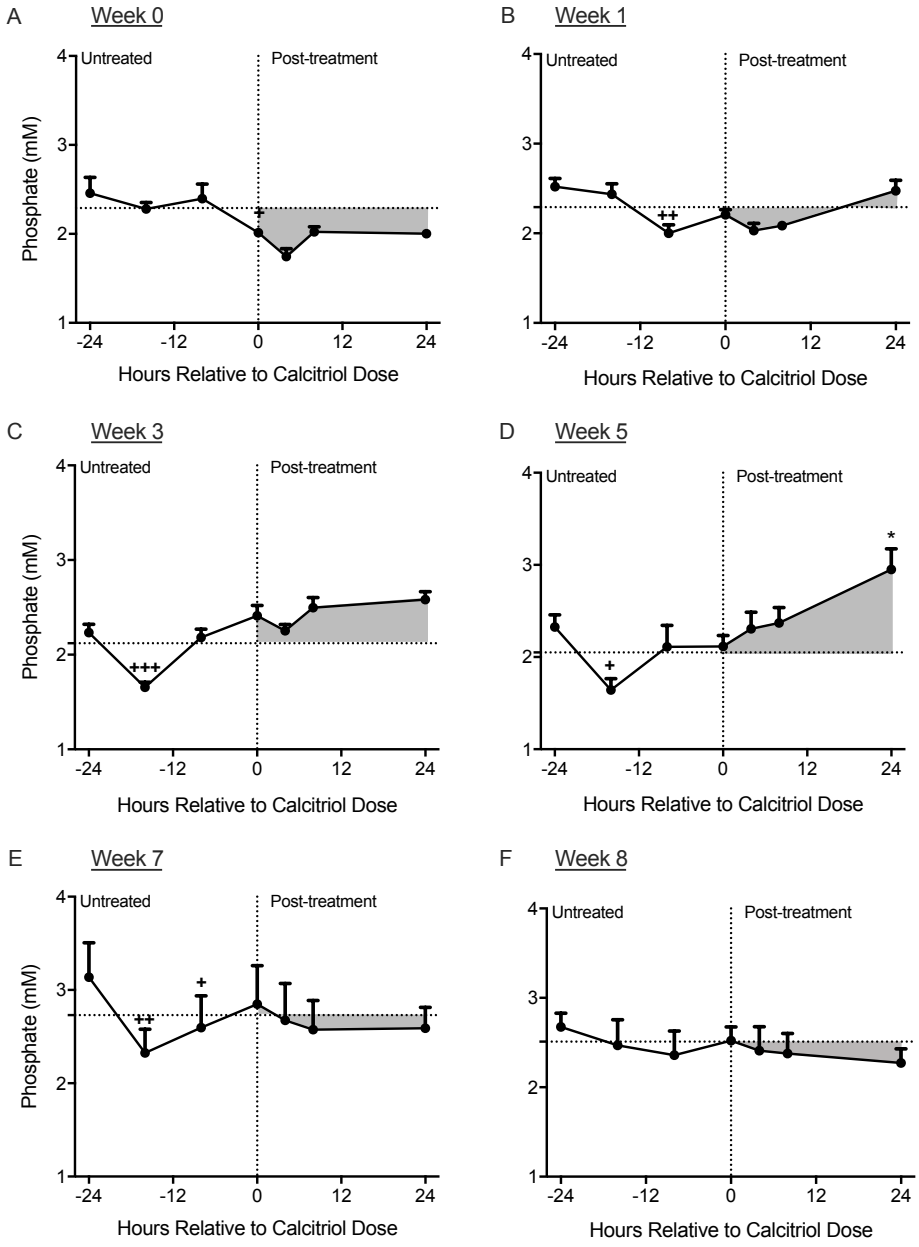

Figure 2-5 Circulating phosphate in CKD animals from 24 hours prior to 24 hours after calcitriol treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7). The horizontal line represents the average value of the untreated time course from -24hr to 0hr. The vertical line represents the baseline prior to receiving the 160ng/kg dose of calcitriol. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Crosses denote differences between time points within the untreated time course and -24hr (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$). Stars denote differences between timepoints within the post-treatment time course compared and time 0hr (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Basal PTH (99.9 ± 51.65 pg/mL) rose significantly at week 1 (137.9 pg/mL ± 54.29pg/mL) and continued to rise at significantly at week 5 (322.3± 170.25, $p<0.05$) even while animals were consuming a LPD. The level of PTH at week 0 was then elevated more than tenfold post HPD in weeks 7 (1385.4 ± 1147.0, $p<0.0001$) and 8 (1671.7 ± 703.9, $p<0.0001$) (Fig. 2-3C). A single dose of calcitriol in healthy rats (week 0) was capable of significantly suppressing PTH at 24 hours at baseline as well as in weeks 1 and 3 of CKD (49%, 29% and 33%) whereas with increased CKD severity plus high dietary phosphate (week 7 and 8) there was no significant calcitriol-induced suppression of PTH. (Fig. 2-6)

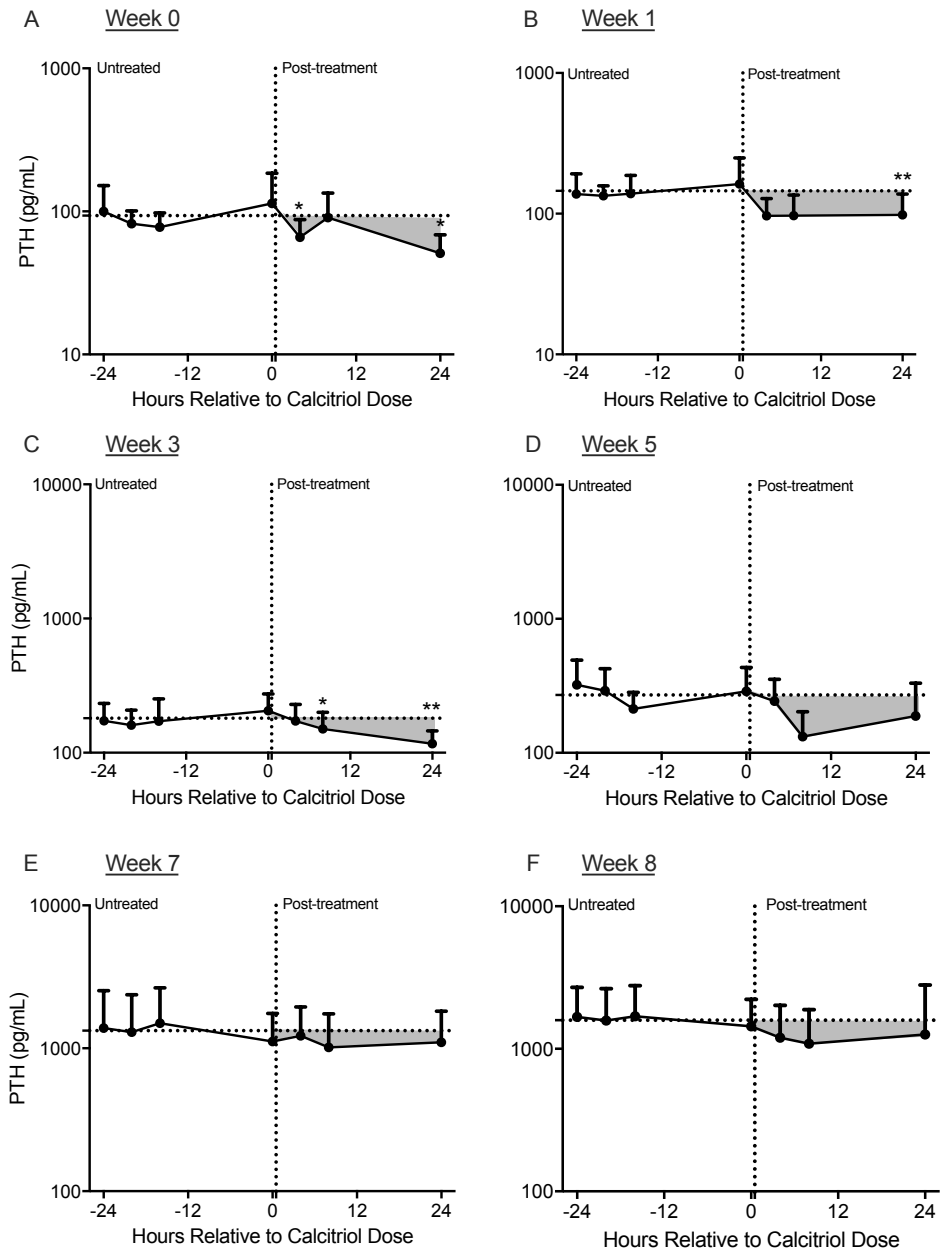

Figure 2-6 Circulating PTH in CKD animals from 24 hours prior to 24 hours after calcitriol treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7)**.** The horizontal line represents the average value of the untreated time course from -24hr to 0hr. The vertical line represents the baseline prior to receiving the 160ng/kg dose of calcitriol. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model on log transformed data with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Stars denote differences between timepoints within the post-treatment time course compared and 0hr (* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Basal FGF-23 (381.7 ± 32.15 pg/mL) rose at week 1 (662.4 pg/mL ± 340.78 pg/mL, $p<0.05$) and continued to increase at week 3 (1417.2 ± 497.92 pg/mL, $p<0.05$) and week 5 (6460.6± 3404.89 pg/mL, $p<0.001$) (Fig. 2-3D). The level of FGF-23 at week 0 prior to the induction of CKD, was elevated by 38-fold in week 7 (14486.2 ± 7695.9, $p<0.0001$) and 30-fold in week 8 (11326.8 ± 5792.11, $p<0.0001$) with increased dietary PO_4 (Fig. 2-7). At week 0, FGF-23 levels declined significantly by approximately 25% between -24 and -20 hours of the untreated time course, an effect which was lost after the CTx. This transient decline in FGF-23 levels during the pre-treatment day was lost with the onset of CKD. Overall, administration of a single dose of calcitriol had minimal effects on FGF-23. (Fig 2-7)

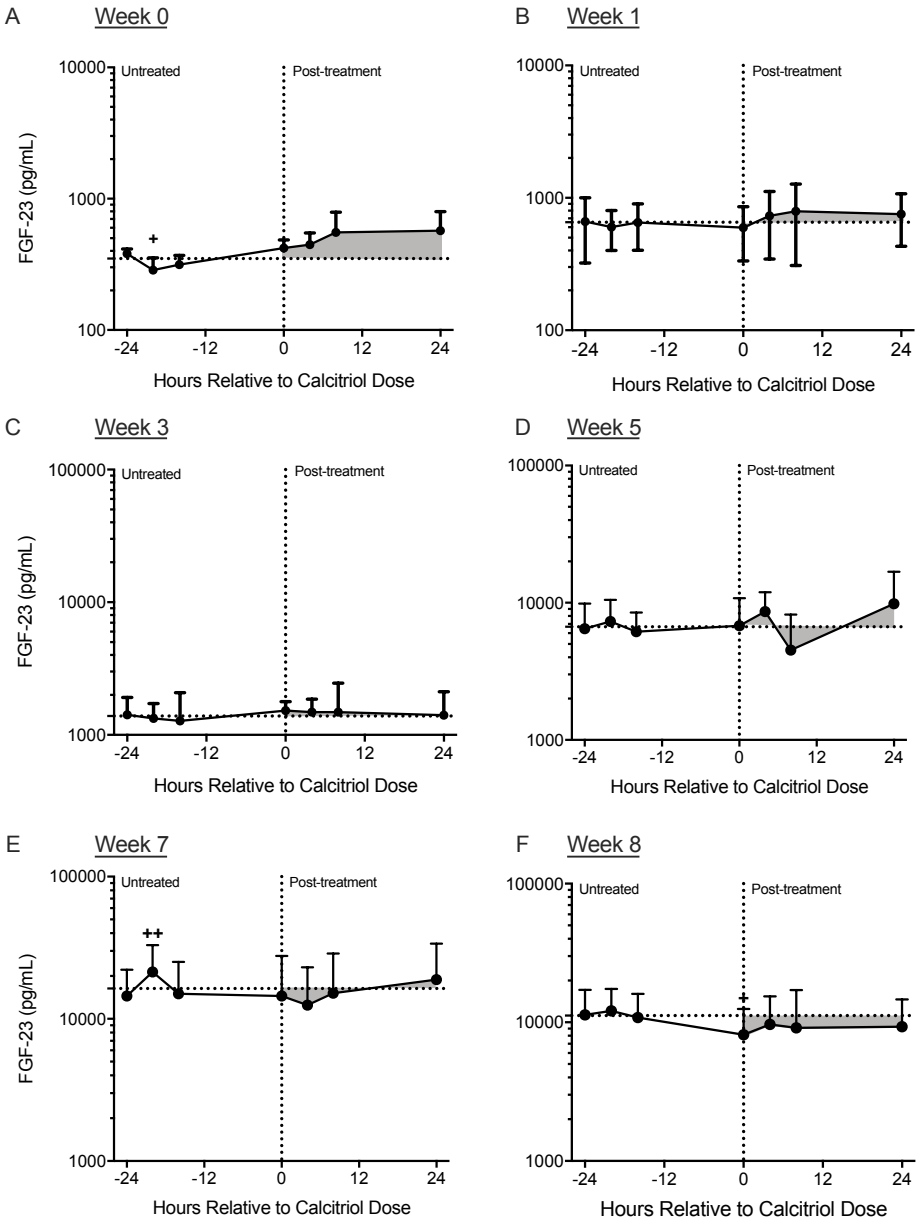

Figure 2-7 Circulating FGF-23 in CKD animals from 24 hours prior to 24 hours after calcitriol treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7). The horizontal line represents the average value of the untreated time course from -24hr to 0hr. The vertical line represents the baseline prior to receiving the 160ng/kg dose of calcitriol. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model on log transformed data with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Crosses denote differences between timepoints within the untreated time course compared to -24hr (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$). Stars denote differences between timepoints within the post-treatment time course compared and time 0hr (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Response to therapy is independent of mineral and hormone circadian rhythm

Relative changes between the area under the curve of the untreated time course (-24hr, -20hr, -16hr, and 0hr) and each time point in the post-treatment time course (4hr, 8hr, and 24hr) were assessed in order to account for time based fluctuations in Ca, PO_4, PTH, and FGF-23. A 0% change indicates a lack of difference between the level of the mineral (Ca, PO_4) or hormone (PTH, FGF-23) achieved post calcitriol (160ng/kg) compared to the untreated time course. Calcitriol had minimal effects on Ca despite normalizing the animals to their own untreated time course prior to the induction of CKD and at weeks 1, 3, and 7 of CKD. Similar to analysis of the raw data, calcitriol administration showed elevated Ca levels at weeks 5 and 8. At week 5 circulating Ca was elevated at 4 hours ($p<0.05$) and at week 8, Ca was elevated at each time point (4hr, 8hr and 24hr, $p<0.05$) measured post-treatment compared to baseline (Fig. 2-8).

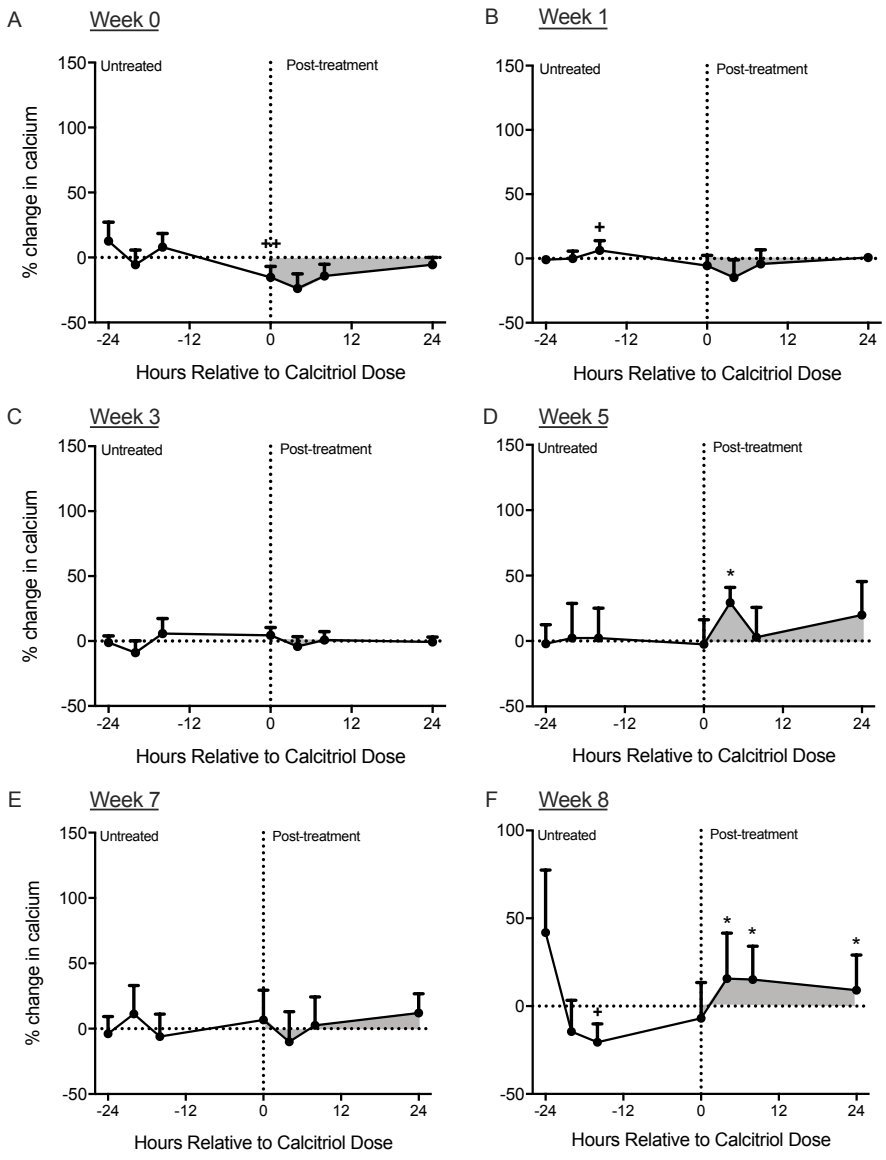

Figure 2-8 Relative change in serum calcium in CKD animals from 24 hours prior to 24 hours after treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7)**.** Percent change was calculated in CKD animals between the average of the untreated time course to each timepoint 24 hours prior to dosing and 24 hours after dosing. The horizontal line represents a 0% change between the average values of the untreated time course and value at each time point. The vertical line represents the baseline of the post-treatment time course. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Crosses denote differences between measured time points within the untreated time course and -24hr (+ $p<0.05$). Stars denote differences between measured timepoints of the post-treatment time course compared and time 0hr (* $p<0.05$).

Calcitriol had minimal effects on PO_4 despite normalizing the animals to their own untreated time course with the exception of week 5 where phosphate levels rose by 55.3 ± 38.1%, $p<0.0001$, at 24 hours (Fig. 2-9). Prior to CTx, PO_4 levels declined significantly by 4 hours (week 3, $p<0.0001$; week 5, $p<0.05$; and week 7, $p<0.01$) or 8 hours (week 1, $p<0.05$; and week 7, $p<0.05$) during weeks 1-7. This decline in PO_4 was not seen at week 8 (Fig. 2-9).

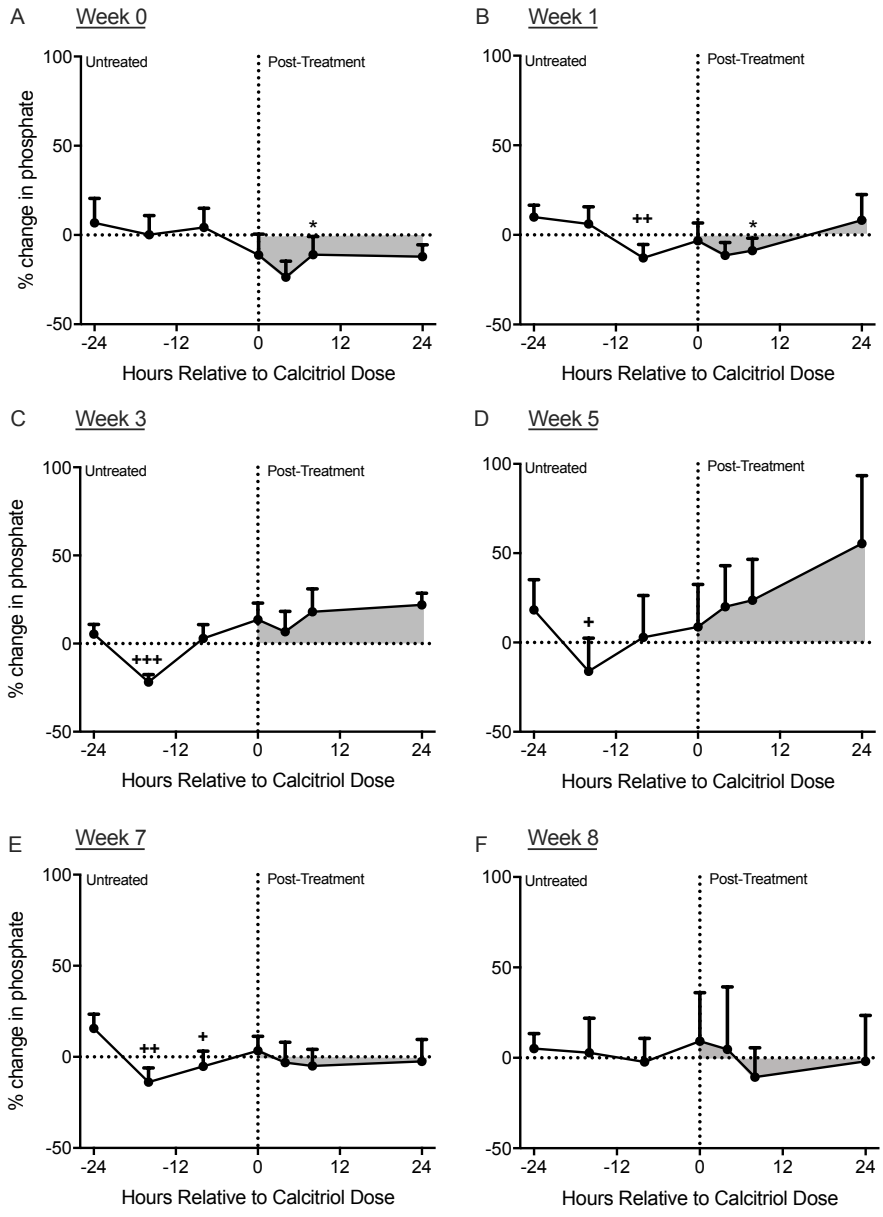

Figure 2-9 Relative change in serum phosphate in CKD animals from 24 hours prior to 24 hours after treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7)**.** Percent change was calculated in CKD animals between the average of the untreated time course to each timepoint 24 hours prior to dosing and 24 hours after dosing. The horizontal line represents a 0% change between the average values of the untreated time course and value at each time point. The vertical line represents the baseline of the post-treatment time course. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Crosses denote differences between measured time points within the untreated time course and -24hr (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$). Stars denote differences between measured timepoints of the post-treatment time course compared and time 0hr (* $p<0.05$).

Significant reductions in PTH following single dose calcitriol were achieved prior to the induction of CKD (baseline) and at weeks 1, 3, and 5 of CKD compared to baseline at 8 hours (week 3= -14.5 ± 23.4%, $p<0.05$; week 5=-54.6 ± 25.8%, $p<0.01$) and at 24 hours (baseline= -41.9 ± 19.8%, $p<0.05$; week 1= -34.4 ± 14.7%, $p<0.05$; week 3=-33.1 ± 16.0%, $p<0.01$) from baseline levels. Significant calcitriol mediated suppression of PTH was attenuated with increased dietary PO_4 at week 7 and 8 (Fig. 2-10).

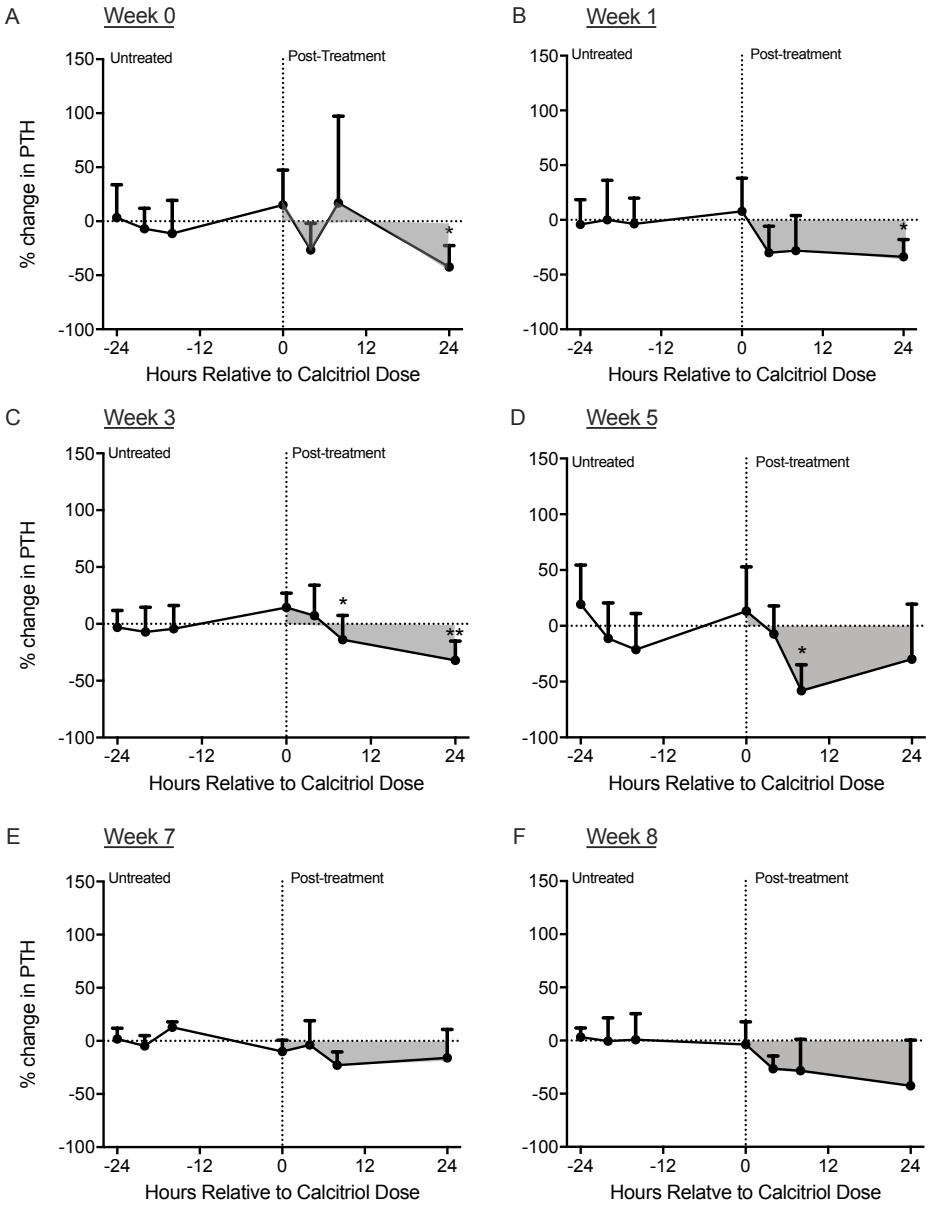

Figure 2-10 Relative change in plasma PTH in CKD animals from 24 hours prior to 24 hours after treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7). Percent change was calculated in CKD animals between the average of the untreated time course to each timepoint 24 hours prior to dosing and 24 hours after dosing. The horizontal line represents a 0% change between the average of the untreated time course and value at each time-point. The vertical line represents the baseline of the post-treatment time course. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Stars denote differences between timepoints within the post-treatment time course compared and time 0hr (* $p<0.05$, ** $p<0.01$).

Overall, calcitriol had minimal effects on FGF-23 throughout the experiment (Fig. 2-11). A small but significant decline in FGF-23 levels occurred at week 0 but only prior to CTx. This decline in FGF-23 on the pre-treatment day was lost with progression of CKD.

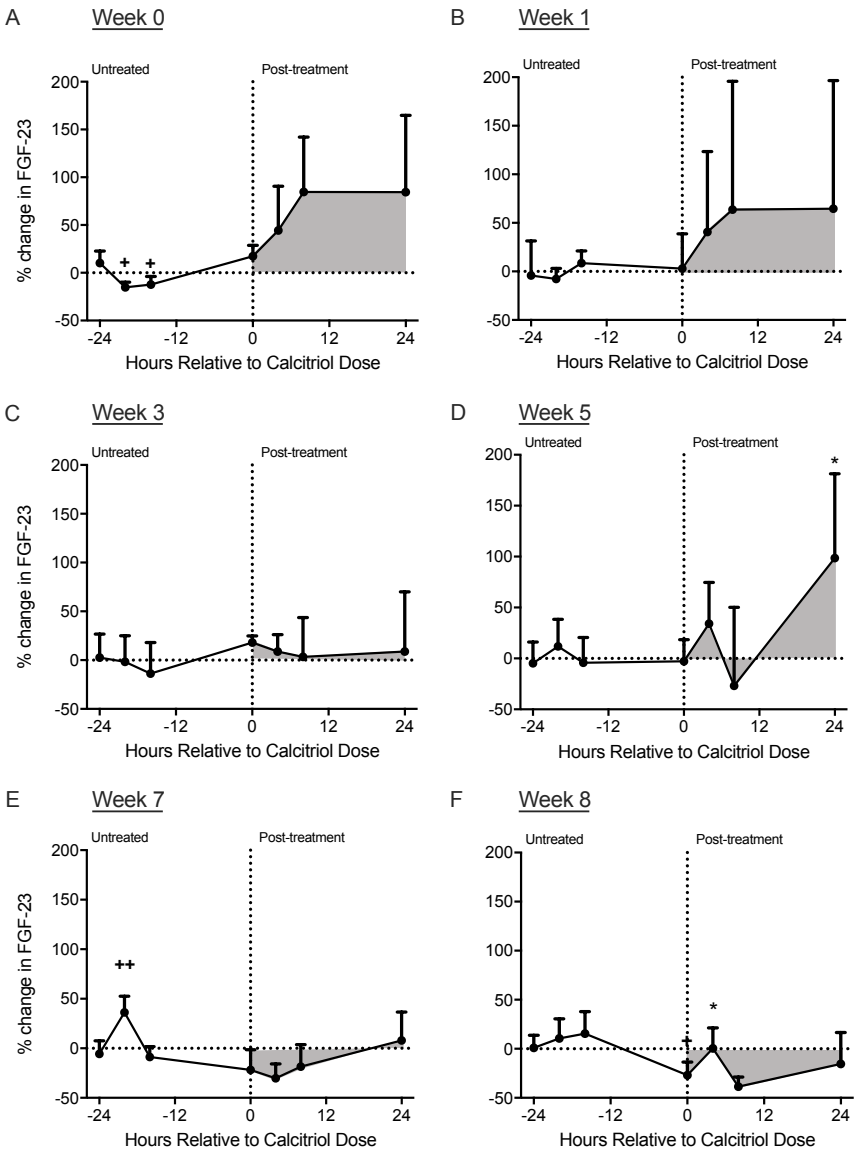

Figure 2-11 Relative change in plasma FGF-23 in CKD animals from 24 hours prior to 24 hours after treatment

A) Week 0**, B)** Week 1**, C)** Week 3**, D)** Week 5**, E)** Week 7 and **F)** Week 8 of chronic kidney disease (N=7). Percent change was calculated in CKD animals between the average of the untreated time course to each timepoint 24 hours prior to dosing and 24 hours after dosing. The horizontal line represents a 0% change between the average of the untreated time course and value at each time-point. The vertical line represents the baseline of the post-treatment time course. Data represented as mean +/- SD. Difference between timepoints were compared using a mixed effects model with Geisser-Greenhouse correction and *post-hoc* Dunnet's multiple comparisons test. Stars denote differences between 0hr and all timepoints (4hr, 8hr, and 24hr) within post -treatment time course (* $p<0.05$). Crosses denote differences between -24hr and all timepoints within untreated time course (+ $p<0.05$, ++ $p<0.01$).

2.5 Discussion

The effects of CKD severity and HPD on acute calcitriol mediated suppression of PTH were assessed using an experimental model of CKD. The major finding was that acute calcitriol-induced suppression of PTH was significantly attenuated with progressing CKD, and even more so with concomitant HPD, despite circulating levels of Ca, PO_4, and FGF-23 that were not markedly altered by the single dose of calcitriol.

Although calcitriol is often used, but loses effectiveness, in the management of SHPT in CKD patients the mechanisms mediating alterations in intestinal absorption of Ca, homeostatic regulation of circulating Ca and bone resorption are not fully elucidated[39]. Intestinal absorption of Ca is proposed to occur predominantly *via* calcitriol enhanced transcription of Ca binding protein, calbindin D_{9k} and intestinal plasma membrane Ca pumps.[37,115,116] The actions of calcitriol have also been determined to involve non-genomic pathways *via* activation of signaling molecules such as phospholipase A1 and kinases, MAP and protein kinase C which allow for more rapid and tight regulation of bodily processes including regulation of circulating Ca.[114] In the present study, it is possible that there were only minimal alterations in circulating Ca because of the actions of other control mechanisms that were not altered by the disease state or the treatment.

Administration of calcitriol did not result in significant elevations of circulating PO_4. Levels of circulating PO_4 were not markedly elevated throughout the experiment despite the increasing CKD severity and the HPD. The lack of significant hyperphosphatemia in this study was not anticipated as increased circulating PO_4 levels is normally a hallmark of CKD in this model[2.4,97]. One explanation could be that the severity of the disease was not sufficient to overwhelm the compensatory mechanisms and thereby maintain circulating PO_4 homeostasis. That is, PTH and FGF-23 levels did rise markedly as would be expected, as part of the adaptation response to declining renal function.[73] These hormones are widely acknowledged to promote renal excretion of PO_4 by inhibiting the activity and/or expression of renal NaPi-IIa and Na-PiIIc in the proximal

tubules which act to reabsorb PO_4 in the nephrons.[45,46] The finding that there was no significant further progression of PTH and FGF-23 between weeks 7 and 8 when animals were receiving HPD suggests the additional elevation was again sufficient to compensate for the increased supply of PO_4. However, the loss of the transient decline in circulating PO_4, that occurred at 4-8 hours during the pre-treatment period, suggests that the duration of the HPD intervention could be an important factor in the effect. Other studies have shown a blunted decline in circulating PO_4 in CKD patients after only 5 days of HPD.[117,118] The loss of this transient change in circulating PO_4 in experimental CKD combined with HPD could also be the result of changes in the non-renal clearance of PO_4, although the mechanisms involved remain to be resolved[119].

Alterations in hormone levels with calcitriol were only observed with PTH. FGF-23 levels were not statistically different between the calcitriol pre-treatment and treatment day while PTH was suppressed starting at 4 hours. Throughout the CKD induction period (weeks 1, 3, and 5) and at week 0 (healthy), PTH levels began to fall at 4 hours and was significantly reduced by 24 hours after dosing. This response was lost starting at week 7, the beginning of HPD. Loss of response with HPD suggests that changes in dietary PO_4 is a major player for the attenuation of calcitriol responsiveness and promotion of resistance to calcitriol therapy.

During the initial stages of CKD, the PTGs are well-known to secrete increased levels of PTH in response to the kidneys declining capacity to excrete PO_4[94, 120-122]. Previous CKD studies have shown that the increased PTH secretion is associated with increased cell proliferation, leading to the development of diffuse parathyroid hyperplasia.[75,76,] In some studies, it has been proposed that the burden of hypocalcemia, vitamin-D deficiency and greater PO_4 retention are all involved in stimulating the development of nodular parathyroid hyperplasia.[75,123] This type of glandular hyperplasia is associated with an overall reduction in glandular receptors, in particular, expression of VDR and CaSR which modulate calcitriol responsiveness. [77,78] HPD has previously been shown in experimental CKD models to increase PTG secretory function and volume[57,58,94, 120,122]. In a

study done by Hayakawa *et. al* (1999)[112] it was determined that hyperphosphatemia accelerated PTG proliferation and PTH secretion in SHPT hyperplasia independent of hypocalcaemia. PO_4 was also determined to directly increase expression of growth factors such as TGF-alpha that are elevated in hyperplastic glands.[120] Conversely, early restriction of dietary PO_4 was determined to be capable of preventing both PTH over secretion and parathyroid hyperplasia[121, 124]. The present experimental findings combined with previous evidence emphasize the importance of PO_4 management in the CKD population for preventing the development of SHPT and maintaining responsiveness to calcitriol therapy. [120,125]

A significant strength of the present study is the longitudinal evaluation of CKD with respect to calcitriol-induced effects at different stages of CKD severity and dietary PO_4. In addition, by having a pre-treatment period before an acute treatment enabled normalization of Ca, PO_4, FGF-23, and PTH levels to their normal rhythms when assessing calcitriol effectiveness. In contrast, a limitation of the current study was the relatively small sample size which prevented appropriate characterization of whether animals were responders and non-responders to calcitriol therapy during each week of the experiment. The use of adenine to generate this CKD model is complicated, at least in part, by the off-target effects of adenine on bone turnover. Adenosine triphosphate and other cyclic nucleotides derived from adenine have been determined to play a role in modulating bone cell function with increased stimulation of osteoclast activity and inhibition of osteoblast mediated bone mineralization.[126] The effects of adenine on bone have to be considered as they may impact Ca and PO_4 metabolism and its respective biomarkers independent of the severity of renal failure.[127] A part of this confounding influence is mitigated, in part, by stopping adenine treatment once CKD is established, a protocol which provides an adenine-free period in the study[113]. Few studies have used this approach to mitigate the adenine-induced complications[113,119,127,128]

In conclusion, this study determined that a relatively short-term exposure to HPD played a significant role in attenuating the capacity for calcitriol-induced suppression of PTH in an experimental model of CKD. This effect was produced in a manner which appeared to be independent of changes in circulating Ca and PO_4 and FGF-23. Parathyroid hyperplasia is speculated to contribute to the calcitriol resistance that developed after 1 week of HPD and will be the subject of future studies. The consequences of calcitriol resistance include the requirement to increase the dose used of VDRA, and progressive mineral bone disorder that is associated with reduced quality of life.[5-7] Characterizing the mechanisms which underlie the onset and development of calcitriol resistance will potentially help to improve the use of vitamin-D analogues in the clinical setting and thereby prevent increased progression of secondary hyperparathyroidism and the necessity for parathyroidectomies in the CKD population.

Chapter 3

The effect of different calcitriol dosing strategies on parathyroid gland responsiveness

3.1 Abstract

MBD and VC are substantial contributors to increased cardiovascular disease burden in CKD patients. Vitamin-D receptor agonists (VDRAs), such as calcitriol are widely used for the management of SHPT associated with MBD. However, certain patients can develop resistance to vitamin-D therapies, and compensatory increased dosing may accelerate the development of VC. This study will determine whether specific calcitriol dosing strategies alter *ex vivo* PTG responsiveness to simulated hyperphosphatemia and hypercalcemia in adenine-induced experimental CKD. CKD animals (Sprague Dawley rats (n=27)), were either untreated (n=10) or treated with calcitriol at a maximal dosage of 20ng/kg/day from 4 to 9 weeks (n=17). Two dosing strategies for each therapy were employed: a graduated rise starting from week 1 (2 to 20ng/kg/day) (n=8), or an abrupt rise to the maximal dose at 4 weeks after CKD had been established (n=9). At sacrifice, PTGs were excised and placed in PTH-inhibitory media (PIM; DMEM with 2.4 mM Ca/1 mM, PO_4) for 2 hours followed by PTH-stimulating media (PSTM; DMEM with 0.6 mM Ca, 4 mM PO_4) to assess parathyroid hormone (PTH) responsiveness to inhibition with exposure to a hypercalcemic environment and stimulation with exposure to a simulated hyperphosphatemic environment. Treatment with calcitriol, given either abruptly or gradually, resulted in significantly suppressed PTH, elevated FGF-23 and increased degree of vascular calcification at sacrifice compared to untreated CKD. Maximum % PTH suppression and maximum % PTH stimulation calculated as the maximum response either inhibition or stimulation achieved with incubation in the PIM and PSTM respectively was not detected between groups. However, treatment with calcitriol either using the abrupt or gradual dosing strategy showed responder and non-responder phenotypes. This result suggests that gland responsiveness may only be impacted in the presence of calcitriol *in vivo*. It is possible that changes in glandular responsiveness *in vitro* is dependent on alterations in PTG receptors and not by changes in biomarkers of mineral homeostasis as a result of CTx.

3.2 Introduction

CKD-MBD is a classification of CKD associated with dysregulated mineral homeostasis, renal osteodystrophy, and extra skeletal calcification that results in increased bone fracture risk and cardiovascular mortality.[4] Vitamin-D insufficiency commonly measured as a level of calcifediol below 30ng/mL,[129] is a hallmark of CKD resulting from reduced conversion of calcifediol into calcitriol, the active form of vitamin-D due to reduced expression and/or activity of renal CYP27B1.[68] This vitamin-D deficiency typically results in hypocalcaemia, in part, because of the impact on intestinal absorption of Ca.[37,39] Decreased vitamin-D together with hypocalcemia stimulates PTH release and/or production, an effect which will work to restore Ca levels by stimulating bone resorption to release stores of both Ca and PO_4.[49,50,82] CKD is also associated with increased PO_4 retention as a result of kidney function decline. PTH acutely regulates circulating PO_4 by decreasing the expression and/or function of Na-Pi IIa/c in the kidney, preventing renal absorption of PO_4. [45] Vitamin-D deficiency, hypocalcemia, and loss of PO_4 excretion together stimulate the development of SHPT and parathyroid hyperplasia in CKD.[102,103] Dysregulation in mineral homeostasis worsens with progression of CKD leading to a continuous cycle of increasing SHPT and parathyroid hyperplasia.

The current Kidney Disease Improving Global Outcomes (KDIGO) recommendations for rectifying abnormalities in the vitamin D metabolome and mineral-bone axis focus on the use of calcitriol and other active vitamin D analogs for targeting severe and progressive hyperparathyroidism in patients without dialysis in stage 3-5 CKD.[83] Several observational studies and experimental models have associated the use of calcitriol with fewer and less severe cardiovascular events and left ventricular hypertrophy.[130-132] It has been suggested that activation of VDR protects cardiovascular health through modulation of the renin-angiotensin system and cardiac contractile function *via* Ca.[131,132,] Patients treated with calcitriol showed reduced mortality

compared to patients who were untreated.[83,106,] In fact, studies have also observed a slower progression of CKD with CTx.[19,106, 111,132, 133]

Although a number of clinical studies link CTx to survival, a number of clinical and animal studies have also linked CTx with increased development of vascular calcification (VC) *via* its impact on mineral homeostasis.[108,109] Some adverse effects associated with calcitriol treatment include hypercalcemia, hyperphosphatemia,[108,109] and over-suppression of PTH[109,134], which exacerbates the development of VC as well as generating resistance to calcitriol therapy.[86] Calcitriol resistance is clinically defined as failure of vitamin D treatment to suppress PTH levels to below 600pg/mL.[83] About 20-30% of patients with SHPT develop calcitriol resistance whereby vitamin D treatment becomes less effective at inhibiting PTG proliferation and PTH secretion and/or production.[86]

The PTG is the main target of calcitriol therapy and is also a major player in the development of calcitriol resistance.[86,106,128] Under healthy physiologic conditions, the rate of parathyroid cell proliferation is equal to the cells rate of apoptosis. However, in CKD, high PO_4, low Ca and low calcitriol results in PTG over-secretion which promotes cell-proliferation and the development of first diffuse and then nodular parathyroid hyperplasia with increasing CKD severity.[124] The nodular form of parathyroid hyperplasia is highly associated with the loss of VDR and CaSR in the PTG, which impairs response to circulating calcitriol, Ca, and PO_4.[57,77,79,] CTx has been determined to be able to regress the development of parathyroid hyperplasia, however the evidence for regression is limited. [135, 136] Calcitriol treatment early in CKD has been commonly avoided due to the risk of hypercalcemia, hyperphosphatemia and over-suppression of PTH.[108, 109, 134] A previous study comparing different dosing and frequency of calcitriol administration determined that all calcitriol treatment strategies regardless of the changes in treatment profile, lost the ability to maintain suppression of PTH long term. However, these measurements were done for *in vivo* biomarkers of mineral homeostasis only and did not look at the PTG's function

independently.[128] Assessment of the PTG *ex vivo* will enable the determination of whether calcitriol treatment impacts the PTG's ability to respond to circulating Ca and PO_4 and can correct for impaired Ca and PO_4 sensitivity that develops in CKD with SHPT.[57, 112,137]

To test whether differences in calcitriol dosing strategies have an effect on PTG responsiveness, this study examined the response of PTGs *ex vivo* from animals treated with calcitriol abruptly (20ng/kg/day) or gradually (2 to 20ng/kg/day). PTG responsiveness to inhibition in a simulated hypercalcemic environment and stimulation within a simulated hyperphosphatemic environment will be assessed by measuring differences in the suppression or stimulation of PTH between treatment groups. The hypothesis was that calcitriol mediated suppression *in vivo* would persist in the *in vitro* environment and that PTGs from animals treated with calcitriol abruptly would have impaired responsiveness to stimulation by high media PO_4 concentrations. This is because animals treated abruptly, will not receive calcitriol early in CKD progression to supplement calcitriol deficiency.

3.3 Methods

Surgical Model

All animal procedures were performed in accordance to the Canadian Council on Animal Care and approved by the Queen's Animal Care Committee. Male Sprague-Dawley rats (n=27; 14 weeks old; 400-450g) (Hilltop Lab Animals Inc. PA, USA) were acclimated for one week before the start of the experiment and were individually housed and maintained on a 12 hour-light-dark cycle throughout the duration of the study. Animals in this study were obtained from a different source than the previous chapter (Charles River Inc.) because the protocol in chapter 2 was new therefore we wanted to save on the experiment's price. Animals obtained from Hilltop Lab Animals are more expensive but show less variability between animals and are consistent with previous studies done in the lab. In the first week of experimentation and during acclimatization, animals

were given a control low-phosphate diet containing 0.5% PO_4, 1% Ca, 0.05% magnesium, 0.2 mg/kg vitamin K, 1 IU/g cholecalciferol, and 6% protein (Harlan Teklad Corp., WI, USA: TD150555) with food and water provided *ad libitum*. During this time a programmable infusion mini pump filled (SMP-200, iPRECIO Corp., Tokyo, Japan) with saline was implanted dorsolaterally in the subcutaneous space as per manufacturing instructions and monitored for 10 days prior to experimental intervention. Subcutaneous meloxicam was provided pre- and post-operatively for 3 days (2mg/kg loading, 1mg/kg maintenance) for analgesia. Topical flamazine (1%) was applied as necessary to skin lesions.

Experimental CKD Model

Progressive renal failure was induced after surgical recovery using dietary adenine as previously described in Shoberi et. al (2013) and in the previous chapter of this thesis. In short, a LPD (0.5%) supplemented with 0.25% adenine (Harlan Teklad Corp., WI, USA: TD150558) was provided at 20g/day and water *ad libtitum*. CKD progression and severity were monitored through weekly measurements of serum creatinine (QuantiChrom Corp, BioAssay Systems, Hayward, CA, USA). As creatinine is a chemical waste product normally excreted by healthy kidneys, rises in serum levels serve as an indicator for decline in renal function. Animals were removed from adenine after 4.5 to 5.5 weeks based on the level of serum creatinine, greater than 300μM, which mimics creatinine levels of individuals with moderate CKD severity.[113] All animals were maintained on a low PO_4 (0.5%) non-adenine diet (Harlan Teklad, WI, USA: TD150555) until 7 weeks of experimentation when they were switched onto HPD (1% PO_4) (Harlan Teklad Corp., WI, USA: TD150555) until their sacrifice date (week 9) (Fig. 3-1). This protocol was done in this manner to ensure that changes in mineral homeostasis were due to disease progression and not adenine.

Animal body weight, food intake was monitored daily throughout the experiment by weighing remaining food. If a body weight loss of 15% or greater was reached, subcutaneous fluids (saline) and nutritional supplementation (Boost, Clear H_2O Inc, PA, USA) was provided.

Experiment Groups and Dosing Protocol

Animals were stratified into three groups after surgical recovery based on body weight, Ca, PO_4, and PTH (Table 3-1). The group designations were as follows: into five groups; 1. untreated CKD (UnTx CKD), 2. calcitriol graduated (CT-Grad), 3. And calcitriol abrupt (CT-Ab). These following groups in order to determine whether 1. supplementation of calcitriol early in CKD could reduce mortalities associated with CKD progression and 2. replenish calcitriol that declines with disease. Animals in CT-Grad, started treatment at week 1. Pumps were programmed to infuse a proportional rise in calcitriol (CT) (2 to 20ng/kg/day) over 4 weeks. At week 4, animals in CT-Grad continued to receive the highest dose of CT, 20ng/kg/day until sacrifice (week 9). Rats in CT-Ab started treatment at week 4 of the experiment and received 20ng/kg/day of CT (Fig.3-1). The UnTx group maintained their diet throughout the experiment and received no additional treatments

Table 3-1 Experimental Group Stratification

	Group 1 (N=10)	Group 2 (N=8)	Group 3 (N=9)
	Untreated CKD	Calcitriol Graduated	Calcitriol Abrupt
Ca (mM)	2.43±0.19	2.54±0.16	2.40±0.18
PO_4 (mM)	2.10±0.15	2.16±0.27	2.08±0.16
PTH (pg/mL)	144.5±42.6	167.7±111.6	170.3±61.0
Body weight (kg)	0.434±0.014	0.454±0.017	0.453±0.013

Values presented as Mean±SD. Animals were stratified based on circulating calcium (Ca), phosphate (PO_4), parathyroid hormone (PTH), and fibroblast growth factor (FGF-23) into three treatment groups, untreated chronic kidney disease time control, calcitriol-graduated, and calcitriol abrupt. No significant differences were found between treatment groups in any stratification variable as assessed by one-way ANOVA with *post hoc* Tukey's multiple comparisons test between groups.

Blood Sampling and Sample Preparation

Blood was sampled every week for 9 weeks to assess alterations in circulating Ca, PO_4, PTH, and FGF-23. Blood samples were left at room temperature for at least 45 minutes to form serum and heparinized whole blood was also collected on ice prior to centrifugation to generate plasma, respectively. Samples were then centrifuged at 9,000 RPM for 20 minutes at 4°C and stored in a -80°C for assessment of the biochemical measurements mentioned above.

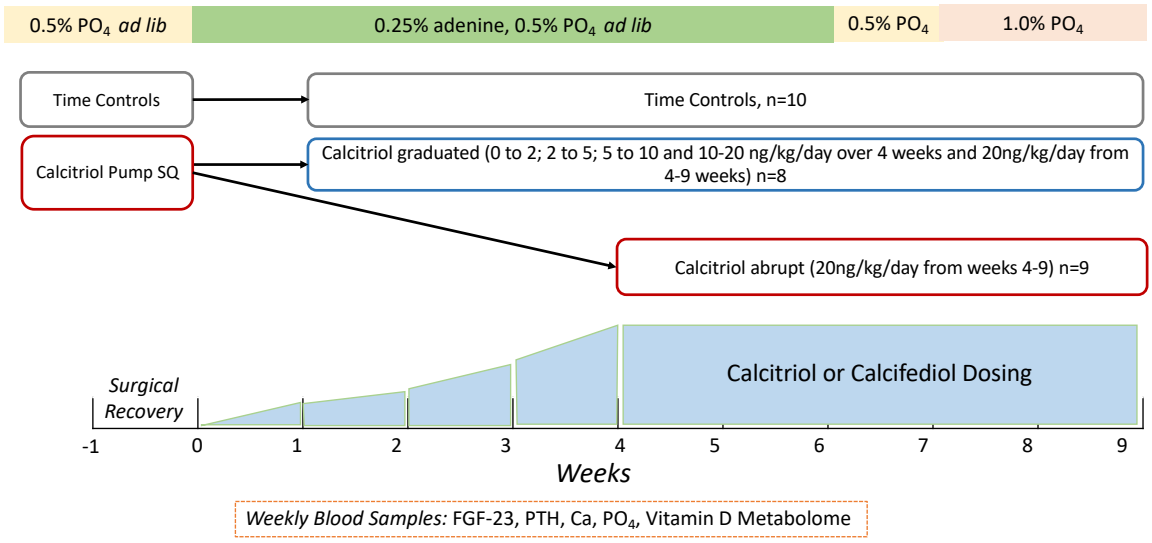

Figure 3-1 Study design and schematic

Animals were divided into three groups untreated chronic kidney disease (UnTx CKD) (N=10), calcitriol-graduated (CT-Grad) (N=8), and calcitriol-abrupt (CT-Ab) (N=9). Animals within the CT-Grad and CT-Ab groups were left to recover after iPRECIO pumps were inserted. UnTx CKD was left to recover after SHAM operations. Animals in the CT-Grad treatment group received 2ng/kg/day which progressively increased to 20ng/kg/day from weeks 1-4 and then 20ng/kg/day from weeks 4 to 9. Animals in the CT-Ab group were treated with 20ng/kg/day from week 4 to week 9. Progressive renal failure was induced with dietary adenine and then placed on an adenine wash out period. Animals were subsequently placed on a high phosphate diet to induce secondary hyperparathyroidism. Figure created by Mandy Turner 2019.

Incubation Protocol

PTGs from the experimental animals were carefully excised immediately after animals were sacrificed cardiac puncture and exsanguination. The glands were incubated in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM F-12) (ThermoFisher Co., ON, CAN) and a DMEM low Ca media at pH 7.7 in a 37°C, 5% CO_2 cell incubator for a total of 5 hours. Glands were placed in two conditions of media; 1. a PTH inhibitory condition (2.4mM Ca and 1mM PO_4) in DMEM F-12 for 2 hours (PIM) and 2. a PTH stimulating condition (0.6mM Ca, 4mM PO_4) in DMEM low calcium media for 2 hours (PSTM). The media was altered as required by the addition of calcium chloride and a 1:2 ratio of monobasic sodium phosphate and dibasic sodium phosphate. These values were assessed prior to the start of the experiment using spectrophotometric assays for Ca and PO_4. The cell plate was plated with 200uL of media per well. Media was collected every 30 minutes and placed into fresh media while tissues were removed at the end of the 4 hours period. These samples were stored at -80°C for PTH measurements (Fig 3-2.).

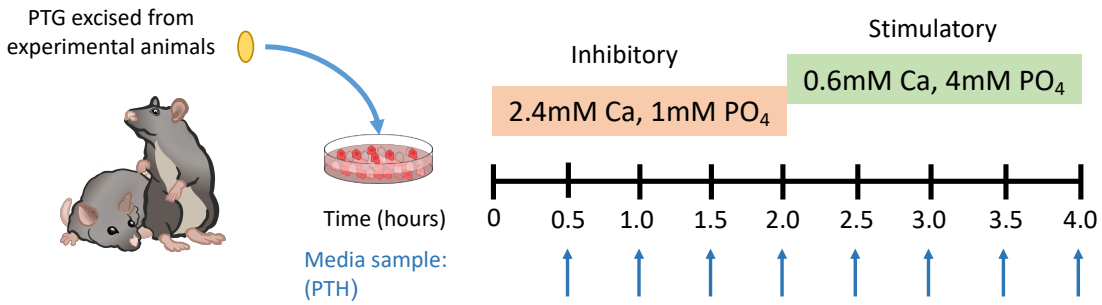

Figure 3-2 Parathyroid gland culture protocol

Glands were excised from untreated chronic kidney disease (CKD), and experimental CKD animals treated within the calcitriol graduated and abrupt groups. The orange section (2.4mM Ca, 1mM PO_4) represents the PTH inhibitory phase (PIM) and the green section (0.6mM Ca, 4mM PO_4) represents the PTH stimulation phase (PSTM). The blue arrows represent 30-minute 200µL media sample retrieval taken for assessment of PTG responsiveness. Glands were moved into fresh media PIM or PSTM every 30 minutes.

Biochemical, tissue and media measurements

Serum Ca and PO_4 were measured spectrophotometrically (SynergyHT Microplate Reader; BioTek Instruments Inc., Winooski, VT) from serum samples using the o-cresolphthalein complexone method, and the malachite green (Sigma-Aldrich Co., ON, CAN) method respectively[21]. Tissue Ca and PO_4 were measured per mg wet weight tissue from acid using the same methods with pH-matched standards. Creatinine was evaluated using the Jaffe method (QuantiChrom™ Corp., Creatinine Assay Kit, Bioassay Systems). Rat intact PTH, intact FGF-23, and secretions of PTH from PTG cultures were measured from plasma using ELISAs (Immunotopics Inc., CA, USA) following the method provided by the manufacturer. Calcitriol levels were similarly quantified using LC-MS/MS as previously established by Kaufmann *et. al* 2011.[138]

Statistical Analysis

Creatinine, Ca, PO_4, PTH, FGF-23 and calcitriol data will be presented as mean (standard deviation). PTH and FGF-23 will be presented in their logged form. Creatinine, Ca, PO_4, PTH, FGF-23 and calcitriol will as be assessed at each time point (week) of the experiment between treatment groups will be assessed using a one-way ANOVA with Tukey's *post hoc* corrections test due to variations in dietary PO_4, and dose of calcitriol at each week. Mixed effects analysis and *post-hoc* Dunnet's multiple comparisons test will be used to evaluate differences in creatinine, Ca, PO_4, PTH, FGF-23, and calcitriol within groups and between groups. The analyses compare week 0 to weeks 1-4 to assess the effects of calcitriol on the graduated treatment group (Pre-abrupt treatment), week 4 to week 7 to assess the effects of CTx with LPD and week 7 to 8 and 9 to assess the effects of calcitriol treatment with high dietary PO_4 HPD.

To assess vascular calcification across the vascular tree, tissue Ca (Figure 3-9A) and PO_4 (Figure 3-9B) content were measured in central (thoracic and abdominal aortae) and peripheral

(carotid, iliac, renal, superior mesenteric, celiac, and pudendal arteries) blood vessels. Tissue Ca and PO_4 between groups will be presented as individual data points and organized by tissue type. Differences in tissue minerals will be assessed using ordinary one-way ANOVA and *post-hoc* Tukey's multiple comparisons test.

Data regarding PTH secretion *in vitro* will be presented as mean (± standard deviation). Comparisons of PTH secretion at each timepoint in the PTG culture protocol between treatment groups will be made using a two-way repeated measures ANOVA with *post-hoc* Dunnett's multiple comparisons test to compare 0.5hr to 1hr and 2hr to assess the effects of inhibitory Ca and 2hr to 3hr and 4hr to assess the effects of stimulatory PO_4 conditions on PTG responsiveness.

Comparisons of maximum and minimum secretion, maximum % stimulation, and minimum % suppression between treatment groups will be made using ordinary one-way ANOVA with *post-hoc* Tukey's multiple comparisons test. The maximum secretion and minimum secretion of PTH was used to assess PTG responsiveness due to their frequency in pharmacological studies to determine the effects of a drug after its administration. The maximum and minimum values are the most relevant ones to use in this time course due to the limitations of PTG gland viability in the study which may impact PTH secretion over time. Maximum % suppression and maximum % stimulation was calculated as a % change between the maximum PTH achieved in the PSTM and the minimum PTH secreted in PIM or vice versa respectively.

Correlations between maximum and minimum PTH secretion, maximum % stimulation, and minimum % suppression were made between *in vivo* Ca, PO_4, PTH, and FGF-23 at week 9 to see if biomarkers of mineral homeostasis *in vivo* correlated with changes in responsiveness to Ca and PO_4 *ex vivo*. Correlations were done with Spearman's rho.

3.4 Results

Assessment of Progression of CKD

Mean serum creatinine (Fig 3-3) rose progressively in each group (untreated CKD (UnTx CKD), calcitriol graduated (CT-Grad), and calcitriol abrupt (CT-Ab)) from baseline that was less than 50 μM in each group until it stabilized at approximately week 5, when all the animals were off adenine, and group mean values were at or slightly above 300 μM. Creatinine levels between treatment groups were not significantly different from each other throughout the LPD period. Serum creatinine became further elevated between weeks 7 to weeks 8 and 9 on HPD with calcitriol treatment (Fig 3-4). Animals treated with calcitriol gradually (327.30 ± 102.91 μM, $p<0.01$) and abruptly (356.27 ± 41.22 μM, $p<0.01$) had significantly different creatinine compared to UnTx CKD at week 8 (252.98 ± 62.06 μM), likely resulting from increased calcitriol-induced calcification of the kidney (data not shown).

Assessing Vitamin-D Treatment

Measurements of CT were made in order to ensure that the animals received the appropriate treatment regimen throughout the duration of the experiment. Calcitriol levels in UnTx CKD rats progressively declined from baseline (86.8 ± 38.05 pg/mL) to levels below the detection limit of the assay (<5pg/mL) at week 5 ($p<0.001$), an effect that was sustained until the end of the experiment. Levels of calcitriol in CT-Grad declined at week 2 (20.8 ± 7.04 pg/mL) pre-treatment compared to baseline (115.6 ± 37.74 pg/mL, $p<0.01$) but rose back to baseline levels once CT was infused at week 4 (100.7 ± 11.79 pg/mL). Calcitriol in CT-Ab declined progressively until week 4 (5.2 ± 0.61 pg/mL, $p<0.0001$) and rose to a level slightly higher than baseline (102.9 ± 27.11 pg/mL) at week 5 (129.8 ± 41.66, $p<0.05$) after receiving treatment. Serum calcitriol in both the graduated ($p<0.001$) and abrupt treatment groups ($p<0.001$) declined significantly from week 7 to

week 9. This reason for the decrease is under investigation by ongoing studies to characterize changes in the vitamin-D metabolome (Fig 3-4).

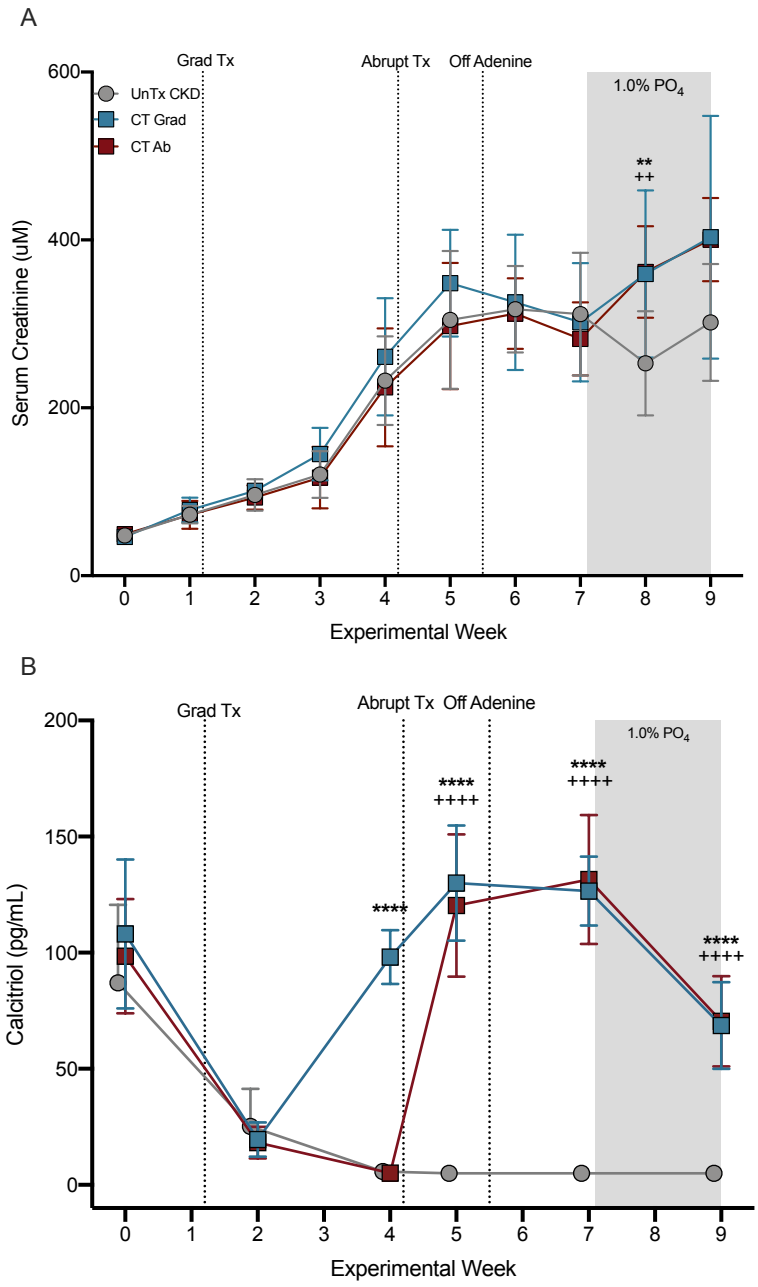

Figure 3-3 Longitudinal assessment of CKD severity and iPRECIO pump function

A) serum creatinine and **B)** calcitriol in chronic kidney disease (CKD) animals (N=27). The vertical dotted lines represent when the graduated group started receiving treatment of 2ng/kg/day, the abrupt group started receiving their treatment of 20ng/kg/day, and the animals went off adenine. The dark shaded region represents when the animals were switched onto HPD (1%). Data represented as mean +/- SD. Differences between untreated CKD, and calcitriol abrupt and graduated groups at each time point were compared using an ordinary one-way ANOVA with *post-hoc* Tukey's test for multiple comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Effects of Calcitriol Treatment In vivo

Circulating Ca levels (Fig 3-4) remained consistent throughout the experiment in UnTx CKD. Administration of calcitriol either gradually or abruptly caused a progressive increase in circulating Ca with a maximum elevation of ~1.3 x and 1.2x respectively achieved at week 6 compared to baseline in both treatment groups. This elevation was sustained at week 7 for both calcitriol-treated groups. This calcitriol-dependent increase in serum Ca was reversed toward untreated levels in both treatment groups once the animals were put on HPD.

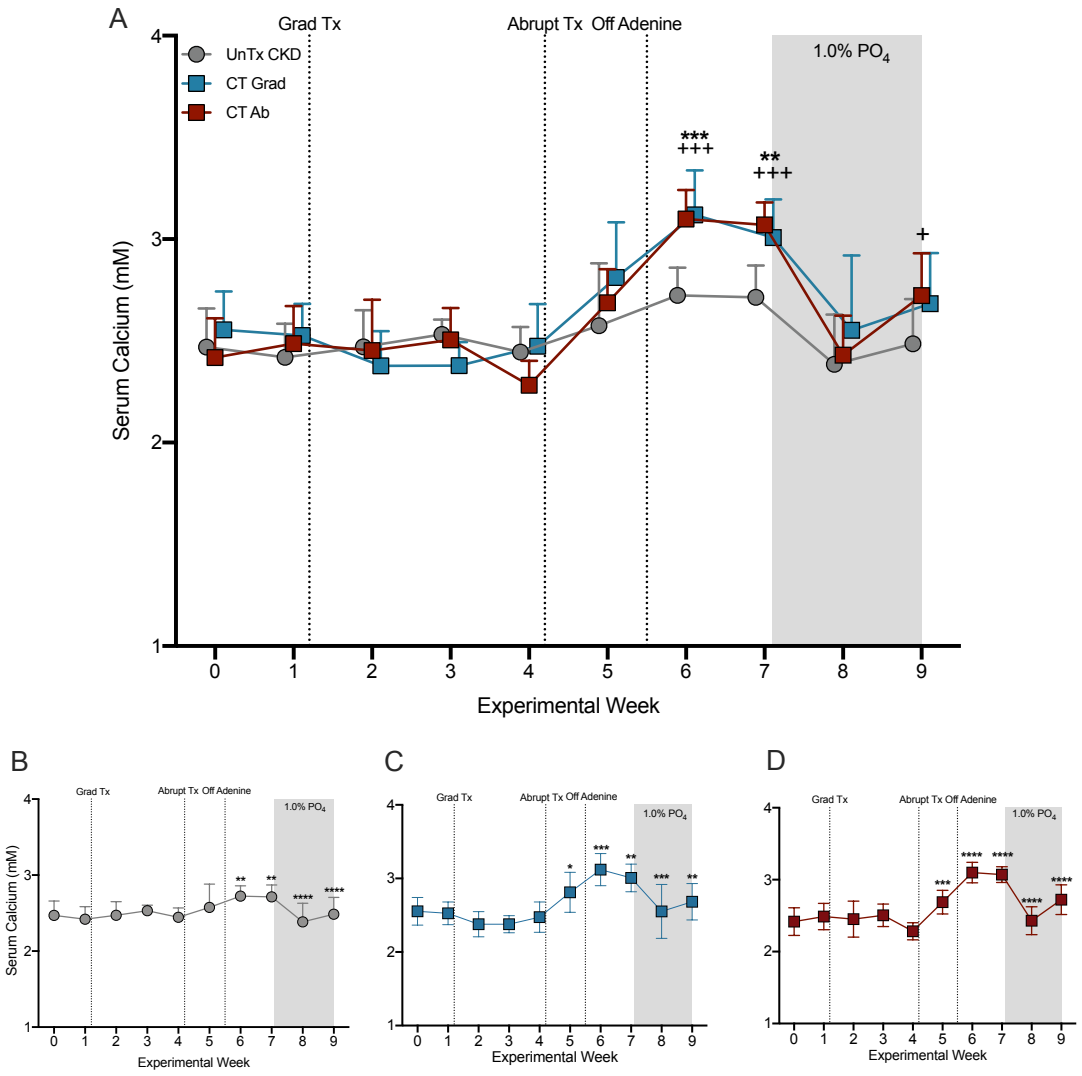

Figure 3-4 Longitudinal measurements of circulating calcium in CKD animals:

A) all treatment groups (N=27), **B)** untreated chronic kidney disease (UnTx CKD) (N=10), **C)** calcitriol-graduated (CT-Grad) (N=8) and **D)** calcitriol-abrupt (CT-Ab) (N=9). The vertical dotted lines represent when the graduated group started receiving treatment of 2ng/kg/day, the abrupt group started receiving their treatment of 20ng/kg/day, and the animals went off adenine. The dark shaded region represents when the animals were switched onto HPD. Data represented as mean +/- SD. A) Differences between UnTx CKD, and CT-Grad and Ab groups at each time point were compared using ordinary one-way ANOVA and *post-hoc* Tukey's test for multiple comparisons. B-D) Values at each time point were analyzed in three sections: 1. Pre-abrupt treatment (weeks 0-4), 2. LPD (weeks 4-7) and 3.HPD (weeks 7-9 using mixed effects model with Geisser-Greenhouse corrections and *post-hoc* Dunnet's multiple comparisons test to compare timepoints in section 1 (Pre-Ab) to week 0, section 2 (LPD) to week 4, and section 3 (HPD) to week 7. Stars denote comparisons made between UnTx CKD and CT-Ab and differences between timepoints within groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Crosses denote comparisons made between untreated CKD and CT-Grad (+ $p<0.05$, ++$p<0.01$, +++$p<0.001$, ++++$p<0.0001$).

Circulating PO_4 levels (Fig. 3-5) remained consistent between groups throughout the LPD period. Administration of gradual or abrupt CTx did not alter circulating PO_4 for the first 5 weeks. At week 6 and 7 CT-Grad had increased levels compared to the UnTx group. Circulating PO_4 was further elevated by nearly two-fold with HPD in all groups compared levels found on the LPD.

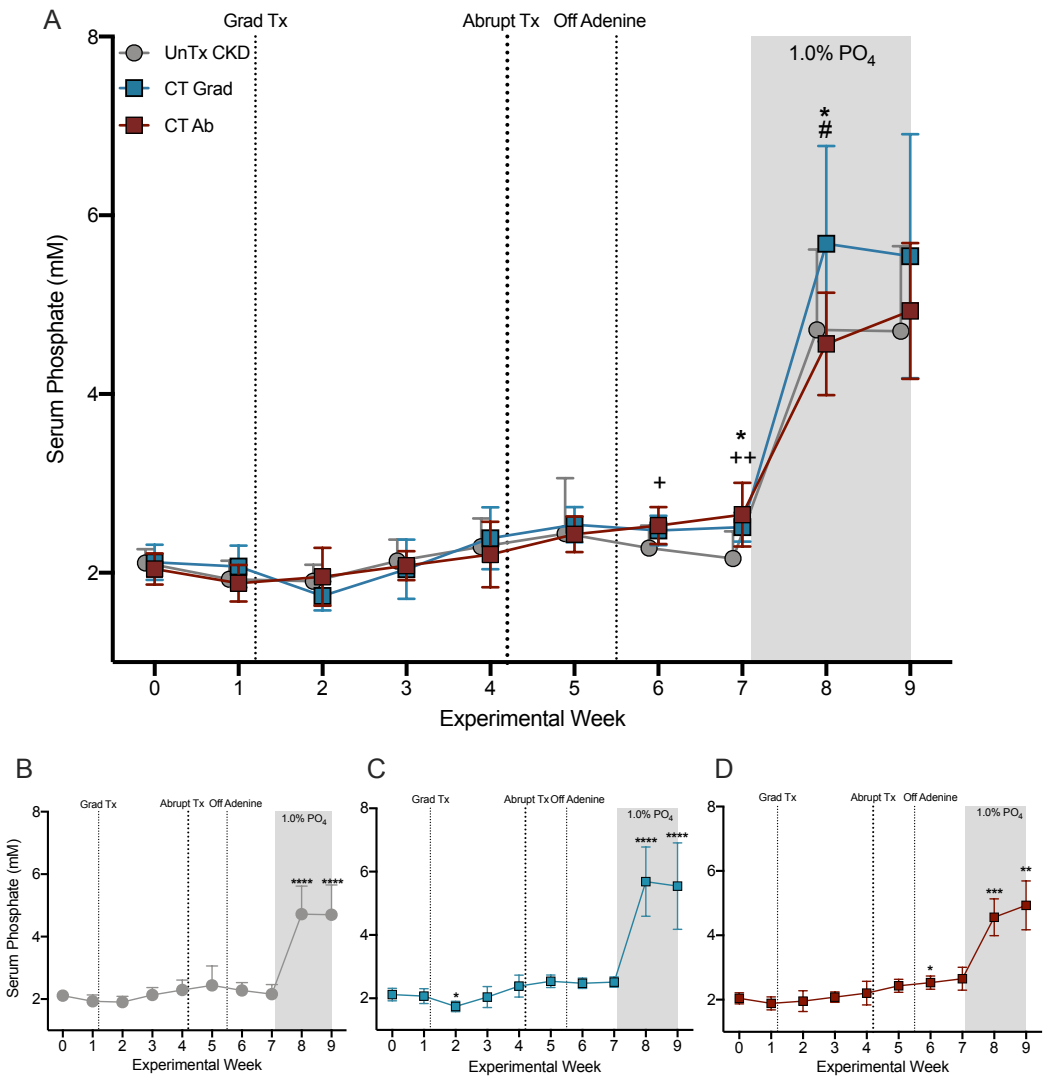

Figure 3-5 Longitudinal measurements of circulating phosphate in CKD animals:

A) all treatment groups (N=27), **B)** untreated chronic kidney disease (UnTx CKD) (N=10), **C)** calcitriol-graduated (CT-Grad) (N=8) and **D)** calcitriol-abrupt (CT-Ab) (N=9). The vertical dotted lines represent when the graduated group started receiving treatment of 2ng/kg/day, the abrupt group started receiving their treatment of 20ng/kg/day, and the animals went off adenine. The dark shaded region represents when the animals were switched onto HPD. Data represented as mean +/- SD. A) Differences between UnTx CKD, and CT-Grad and Ab groups at each time point were compared using ordinary one-way ANOVA and *post-hoc* Tukey's test for multiple comparisons. B-D) Values at each time point were analyzed in three sections: 1. Pre-abrupt treatment (weeks 0-4), 2. LPD (weeks 5-7) and 3. HPD (weeks 7-9 using mixed effects model with Geisser-Greenhouse corrections and *post-hoc* Dunnet's multiple comparisons test. Stars denote comparisons made between untreated CKD and CT-Ab and differences between timepoints within groups (* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$). Crosses denote comparisons made between UnTx CKD and CT-Grad (+ $p<0.05$, ++$p<0.01$). #s denote comparisons between CT-Grad and CT-Ab (# $p<0.05$)

Plasma PTH levels (Fig. 3-6) rose progressively and significantly throughout the experiment in the UnTx group on LPD and became markedly elevated by ~19x when put on HPD (2750.1 ± 1269.19 pg/mL, $p<0.0001$), compared to baseline (147.57 ± 43.65 pg/mL). Calcitriol mediated suppression of PTH in CT-Grad was evident in the progressive decline in PTH levels with increased calcitriol dosing. Calcitriol-induced PTH suppression was significant starting at week 4, achieved a maximum reduction compared to baseline at week 7 (42.42 ± 11.25 pg/mL, $p<0.01$). Administration of calcitriol abruptly at week 4 also showed a progressive lowering of PTH with a significant decline in week 7 (38.87 ± 25.72 pg/mL, $p<0.001$) compared to baseline (170.30 ± 61.02 pg/mL). Calcitriol mediated suppression of PTH was no longer present when the animals were on HPD starting at week 7. (Fig. 3-6)

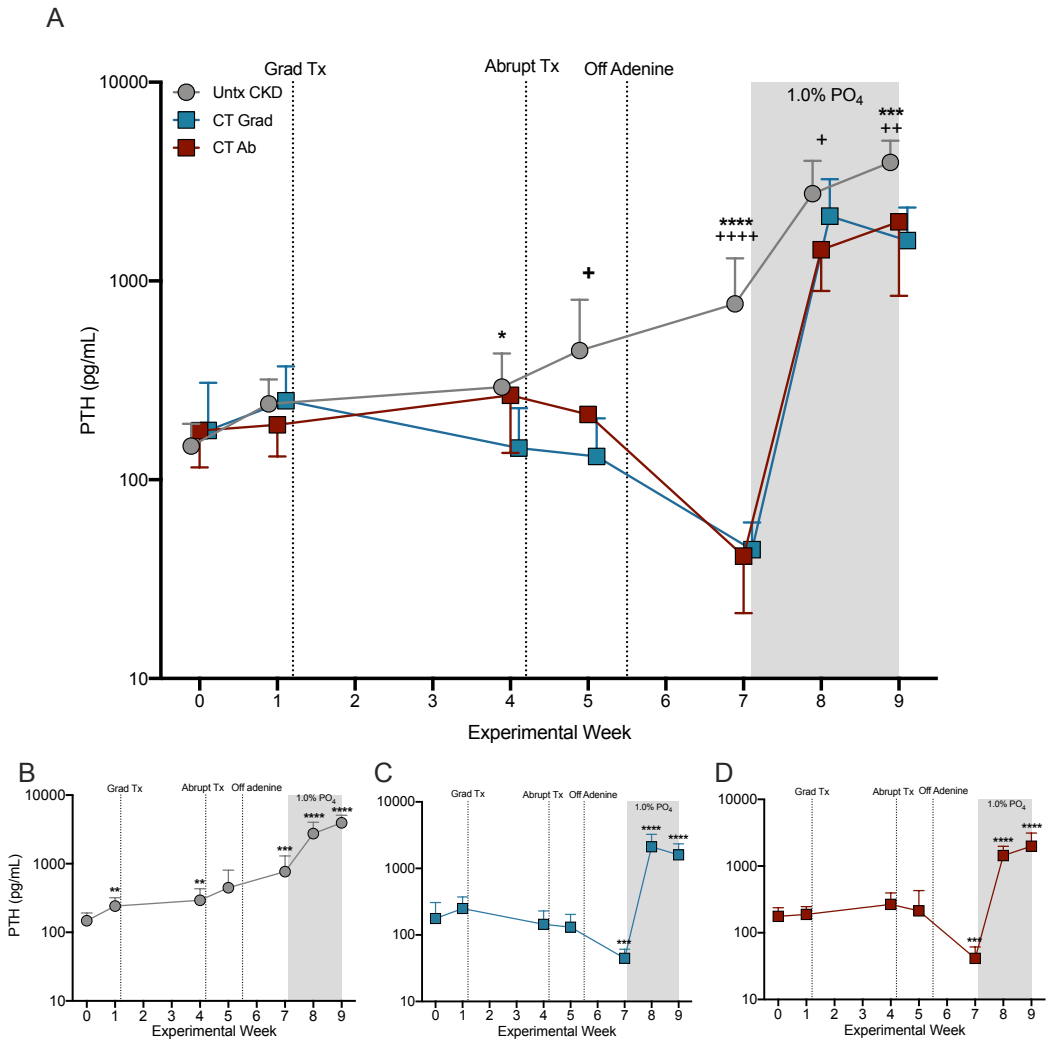

Figure 3-6 Longitudinal assessment of circulating PTH in CKD animals:

A) all treatment groups (N=27), **B)** untreated chronic kidney disease (UnTx CKD) (N=10), **C)** calcitriol-graduated (CT-Grad) (N=8) and **D)** calcitriol-abrupt (CT-Ab) (N=9). The vertical dotted lines represent when the graduated group started receiving treatment of 2ng/kg/day, the abrupt group started receiving their treatment of 20ng/kg/day, and the animals went off adenine. The dark shaded region represents when the animals were switched onto HPD. Data represented as logged mean +/- SD. A) Differences between UnTx CKD, and CT-Grad and Ab groups at each time point were compared using ordinary one-way ANOVA and *post-hoc* Tukey's test for multiple comparisons. B-D) Values at each time point were analyzed in three sections using logged values: 1. Pre-abrupt treatment (weeks 0-4), 2. LPD (weeks 5-7) and 3. HPD (weeks 7-9 using mixed effects model with Geisser-Greenhouse corrections and *post-hoc* Dunnet's multiple comparisons test to compare timepoints in section 1 (Pre-Ab) to week 0, section 2 (LPD) to week 4, and section 3 (HPD) to week 7. Stars denote comparisons made between UnTx CKD and CT-Ab and differences between timepoints within groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Crosses denote comparisons made between untreated CKD and CT-Grad (+ $p<0.05$, ++$p<0.01$, +++$p<0.001$, ++++$p<0.0001$).

Plasma FGF-23 (Fig 3-7) increased progressively with the increasing severity of CKD in the UnTx group such that by week 7 the levels were more than 14-fold above baseline levels. Placing the UnTx group on the HPD further increased FGF-23 levels 10-fold such that by 9 weeks they were more than 140 times baseline levels. In the CT-Grad and Ab treatment groups the FGF-23 levels were increased by 474 and 339 times at 7 weeks and then by 517 and 658 times at 9 weeks, respectively, when the two CT groups were on the HPD for two weeks. The levels of FGF-23 were significantly elevated in both CT groups from 7 weeks onward although the difference was less pronounced when all groups were on the HPD.

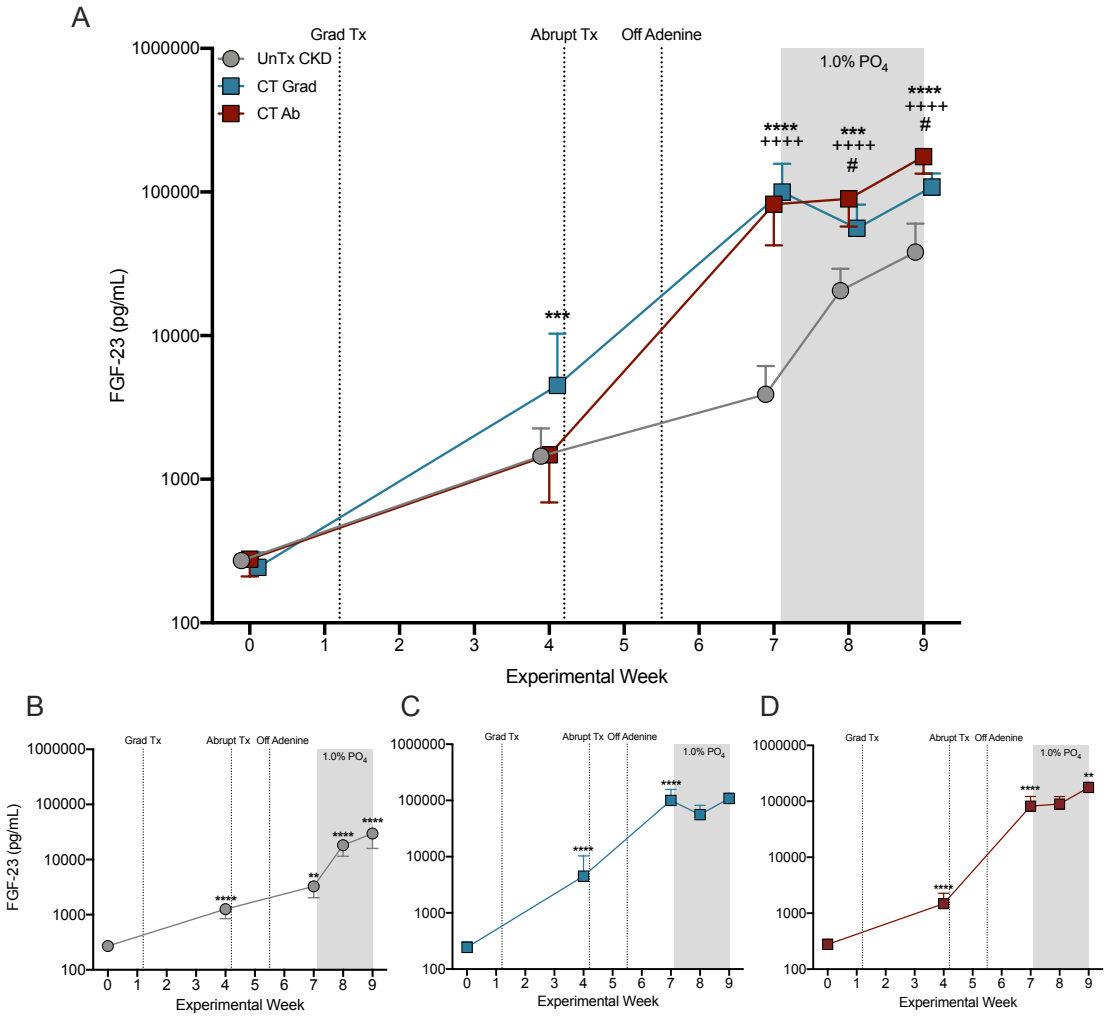

Figure 3-7 Longitudinal measurements of circulating FGF-23 in CKD animals:

A) all treatment groups (N=27), **B)** untreated chronic kidney disease (UnTx CKD) (N=10), **C)** calcitriol-graduated (CT-Grad) (N=8) and **D)** calcitriol-abrupt (CT-Ab) (N=9). The vertical dotted lines represent when the graduated group started receiving treatment of 2ng/kg/day, the abrupt group started receiving their treatment of 20ng/kg/day, and the animals went off adenine. The dark shaded region represents when the animals were switched onto HPD. Data represented as logged mean +/- SD. A) Differences between UnTx CKD, and CT-Grad and Ab groups at each time point were compared using ordinary one-way ANOVA and *post-hoc* Tukey's test for multiple comparisons. B-D) Values at each time point were analyzed in three sections using logged values: 1. Pre-abrupt treatment (weeks 0-4), 2. LPD (weeks 5-7) and HPD (weeks 7-9 using mixed effects model with Geisser-Greenhouse corrections and *post-hoc* Dunnet's multiple comparisons test to compare timepoints in section 1 (Pre-Ab) to week 0, section 2 (LPD) to week 4, and section 3 (HPD) to week 7. Stars denote comparisons made between UnTx CKD and CT-Ab and differences between timepoints within groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Crosses denote comparisons made between untreated CKD and CT-Grad (+ $p<0.05$, ++$p<0.01$, +++$p<0.001$, ++++$p<0.0001$). #s denote comparisons between CT-Grad and CT-Ab (# $p<0.05$)

Persistent calcitriol-induced vascular calcification

A similar degree of calcification was achieved between CT-Grad and CT-Ab groups with significantly elevated average tissue Ca (CT-Grad= 660.21 ± 340.69 nmol/mg tissue, CT-Ab= 883.78± 233.65 nmol/mg tissue) and tissue PO_4 (CT-Grad=493.43 ± 240.75 nmol/mg tissue, CT-Ab= 633.10± 148.77 nmol/mg tissue) compared to UnTx CKD (Ca= 483.61± 497.20 nmol/mg tissue, PO_4= 375.57 ± 353.59 nmol/mg tissue).

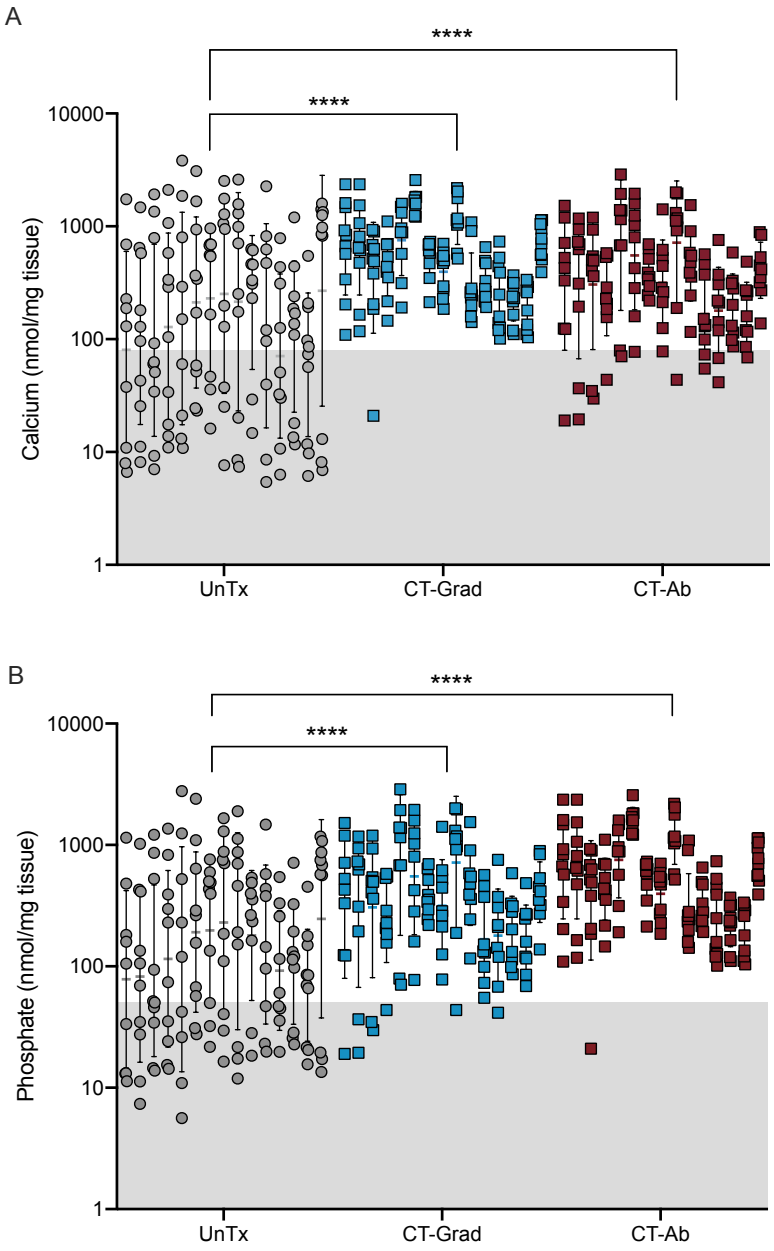

Figure 3-8 Vascular calcification status of untreated and calcitriol treated CKD

A) Tissue calcium (Ca) and **B)** phosphate (PO_4) levels in central and peripheral blood vessels of untreated chronic kidney disease (CKD) (N=10) and calcitriol graduated (N=8) and abrupt (N=9) CKD animals. Each column represents a blood vessel, left to right: thoracic aorta, abdominal aorta, carotid artery, iliac artery, aortic arch, superior mesenteric artery, pudental artery, renal artery, and celiac artery. The grey box indicates vessels which do not have histologically visible vascular calcification *via* von Kossa staining (80 nmol/mg Ca & 50 nmol/mg PO_4). One-way ANOVA on log-transformed data with post hoc Tukey's multiple comparisons test between experimental groups and untreated CKD (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

In vitro PTH secretion profile

All parathyroid glands (PTGs) from untreated CKD rats and animals in the CT-Grad and CT-Ab group were able to respond to inhibitory Ca and stimulatory PO_4. Glands surgically excised from UnTx CKD presented with significantly suppressed PTH compared the baseline PTH (0.5 hr) in PIM throughout the PIM time course ($p<0.001$). Glands from UnTx were also stimulated throughout the PSTM time course with significantly elevated PTH secretion compared to 2 hours, the baseline of the PSTM. Glands retrieved from CT-Ab treated animals were significantly suppressed by 1 hour in the PIM ($p<0.001$) while glands were retrieved from CT-Grad animals were suppressed later after 2 hours ($p<0.05$). Glands from both calcitriol-treated groups were also significantly elevated after 2 hours in the PSTM media (Fig. 3-9).

Both CTx groups resulted in decreased basal secretion of PTH *in vitro* compared to UnTx animals ($p<0.05$). Despite differences in individual treatment profiles there were no differences in the maximum % suppression or maximum % stimulation achieved between the treatment groups (Fig 3-10).

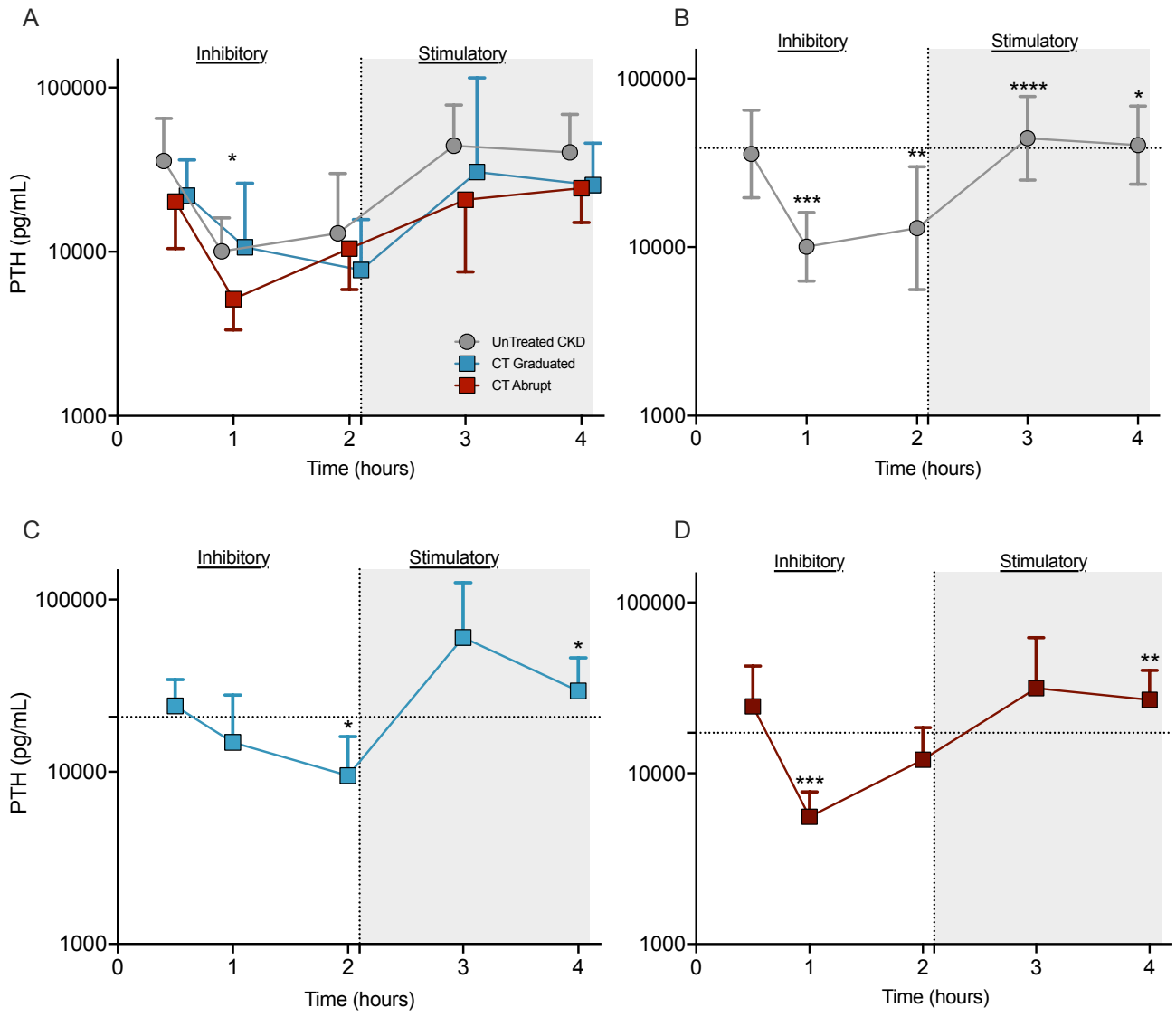

Figure 3-9 In vitro PTH secretion profile from single parathyroid glands

A) all treatment groups (N=27), **B)** untreated chronic kidney disease (UnTx CKD) (N=10), **C)** calcitriol-graduated (CT-Grad) (N=8) and **D)** calcitriol-abrupt (CT-Ab) (N=9). Data represented as mean +/- SD. The horizontal dotted line represents the median value at 0.5 hour. The vertical line shows the transition from the inhibitory to stimulatory condition (grey box). A) Comparisons between treatment groups were made at each time point using an ordinary one-way ANOVA with post-hoc Tukey's multiple comparisons test. B-D) Values at in the inhibitory phase were compared to 0.5 hour using two-way ANOVA with Geisser-Greenhouse corrections and Dunnett's test for multiple comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Values at in the stimulatory phase were compared to 2 hours using two-way ANOVA with Geisser-Greenhouse corrections and Dunnett's test for multiple comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Stars denote comparisons between untreated CKD and CT-Ab (+ $p<0.05$) and differences between timepoints within groups.

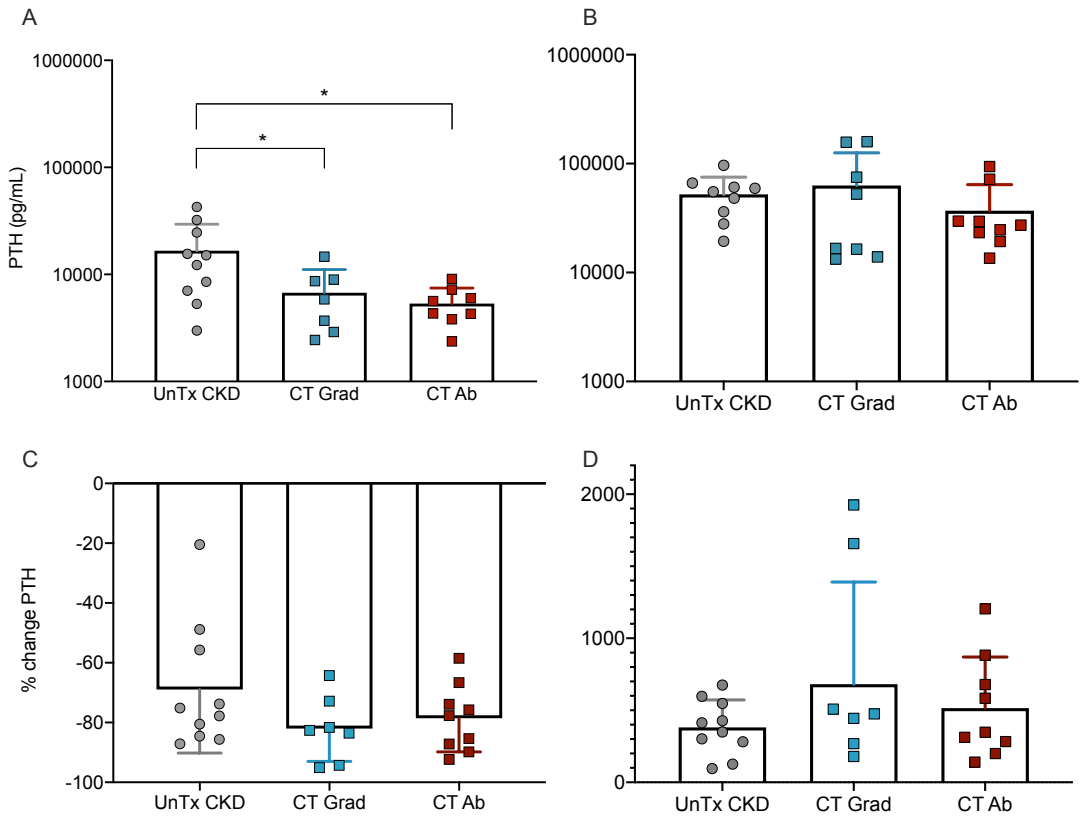

Figure 3-10 In vitro secretion of PTH in response to inhibitory and stimulatory media

A) Minimum Secretion of parathyroid hormone (PTH) and **B)** Maximum Secretion of PTH **C)** Maximum % Suppression and **D)** Maximum % Stimulation. Data represented as mean +/- SD. A) Minimum PTH secretion value for each gland was obtained from the inhibitory condition (PIM). B) Maximum PTH secretion value for each gland was obtained from the stimulatory condition (PSTM). C) Max % suppression was calculated as a % change between the maximum PTH secreted in stimulatory PO_4 to the minimum PTH secreted with inhibitory Ca. D) Maximum % stimulation was calculated as a % change between the minimum PTH secreted in inhibitory calcium to the maximum PTH secreted in stimulatory phosphate. Raw values and % changes compared between untreated CKD, calcitriol graduated, and calcitriol abrupt using ordinary one-way ANOVA with Tukey's multiple comparisons test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

3.5 Discussion

There were several major findings in this collaborative study including (i) regardless of the calcitriol treatment protocol, there was a dramatic attenuation of the calcitriol mediated suppression of PTH only on HPD, as occurred in Chapter 2, and yet (ii) chronic calcitriol treatment *in vivo* did not result in a statistically detectable difference in PTG responsiveness to alterations in Ca and PO_4 *in vitro* although the basal release of PTH was suppressed, and (iii) circulating levels of FGF-23 were markedly elevated in the CTx groups, most particularly, on a relative basis in rats still on a LPD. The latter finding of increased FGF-23 in the CT-Grad by half an order of magnitude compared to the CT-Ab or the UnTx group at 4 weeks of treatment indicates that despite the circulating levels of calcitriol being similar to baseline levels the target of exogenously administered calcitriol actions appears to include off-target FGF-23 producing locations more so than does the endogenous source of circulating calcitriol.

The major focus of my research was the *in vitro* responsiveness of the PTGs following chronic *in vivo* calcitriol administration. As indicated, PTGs were placed in different concentrations of Ca and PO_4 *in vitro* to characterize the differences in tissue responsiveness to inhibitory Ca or stimulatory PO_4 media. The data from both CTx groups show that although the PTH release profile was initially reduced, the PTGs retained the ability to be inhibited following exposure to high Ca[56,137] and stimulated upon exposure to high PO_4[94]. This indicated that the CTx had not modified a basic property of the PTG to respond to changes in mineral concentrations in the microenvironment, particularly with respect to the stimulation by PO_4. That is, the findings did not find any significant differences between the maximum % suppression to high Ca or maximum % stimulation with high PO_4 despite differences in basal PTH release. However, the decreased basal PTH release profile in the CTx groups does suggest there might be some residual action of calcitriol just not regarding responsiveness to Ca and PO_4. Whether the persistent suppression of PTH *in*

vitro results from ongoing direct VDR binding will need to be investigated using receptor antagonists.

Many studies have found an association between the effectiveness of CTx for SHPT and an increased number of oxyphil versus chief cells in the respective glands.[140,141] Activation of CaSR increased apoptosis of chief cells, which have a high density of VDR and CaSR, *in vitro*.[142] Instead of oxyphil cells, rats contain a PTG cell-type called water clear cells[143,144], which similar to oxyphil cells, are derived from overstimulation of chief cells.[145,146] It is possible that similar to humans, overstimulation of CaSR by calcitriol may increase the prevalence of water-clear cells which contain reduced VDR and CaSR. The CaSR has been determined to also be involved in phosphate sensing as phosphate acts as an allosteric inhibitor of the receptor.[57]

Preliminary correlations were done to determine whether the maximum % suppression to high Ca or maximum % stimulation with high PO_4 of each gland was associated with the animal's *in vivo* levels of circulating Ca, PO_4, PTH, FGF-23, and vascular tissue mineral burden (data not shown). However, maximum % suppression and % stimulation was determined to not correlate with circulating Ca and FGF-23 or tissue Ca and PO_4 levels. Circulating *in vivo* PO_4 was found to be negatively correlated with the maximum % suppression and positively correlated to maximum % stimulation while *in vivo* PTH was determined to be positively correlated to maximum % stimulation. Although these were preliminary analysis further studies will need to be done to determine whether this means that circulating PO_4 and PTH are both mechanistically linked to PTG responsiveness. This finding is somewhat contrary to what has been determined previously, where elevated PO_4 and PTH were associated with greater SHPT severity and reduced gland response to Ca or PO_4.[77, 78, 147]

Calcitriol-mediated suppression of PTH was fully attenuated after only one week of HPD. However, by the second week of HPD, we observed that a subset of animals in the calcitriol group had regained some calcitriol mediated PTH suppression. If there is a correlation between the *in vivo*

and *in vitro* response, then this would mean that the effects of calcitriol and HPD may directly impact the expression of receptors within the PTG and that response to calcitriol is correlated with abnormal regulation of Ca and PO_4 [57,75, 77,78, 129, 137, 148]. The results of these kind of studies could help to inform current management of SHPT with vitamin D analogues as well as highlight the importance of the PTG's role in dysregulation of mineral homeostasis in the CKD population.

A limitation of this research is that PTG placed in organ culture suffer a progressive loss of function over time. This means that the % maximum stimulation achieved in PSTM is an underestimate of the response of the glands to high PO_4. In addition, a media condition which replicated the concentrations of Ca and PO_4 of the rats *in vivo* was not used in this protocol. If longer gland viability can be achieved, then adding a baseline phase that recapitulated the *in vivo* conditions could improve the characterization of inhibitory actions of low PO_4 and high Ca. A further limitation was that the study design did not allow for distinguishing whether the effects on the gland were calcitriol-mediated or a result of HPD and SHPT on the gland's responsiveness *in vitro.* Future studies involving a chronic LPD group would provide very useful context to the mechanisms involved.

HPD has been an established stimulus for the development of SHPT and PTG hyperplasia that results in pan reduction of receptors within the gland[75]. SHPT has been determined to be associated with a right-ward shift in the PTH-Ca sensing curve and an increase in the minimum secretion of PTH, changes that are indicative of abnormal Ca sensing[137]. However, most of the glands were still able to achieve the 50% PTH suppression as a result of high Ca. Therefore, in the future, different concentrations of Ca may need to be implemented in order to appropriately distinguish differences in calcium sensing between calcitriol treated and untreated glands.

This results from this study provided insight into the lack of impact of administering calcitriol early versus later in an experimental CKD model. In fact, the concept of replacing calcitriol as it declines during the progression of CKD was undermined by the off-target impact on

FGF-23 secretion and because it did not cause regression of SHPT or maintain the gland's response to calcitriol on HPD. Given that these findings differ from studies which determined that treatment with calcitriol regressed PTG hyperplasia thereby preventing pan reductions of VDR and CaSR further confirmatory work is needed.[135,148] The advancement of the *in vitro* PTG studies was found to be very revealing in terms of segregating response to minerals from the actions of calcitriol. Further investigations looking at changes in the morphology of the gland through characterization of different PTG cell types and expression of VDR and CaSR between treatment groups will be necessary to better characterize the mechanisms involved more directly. Finally, the approach taken in this experimental protocol has pointed the way to *in vitro* studies using human parathyroid tissue to elucidate fundamental mechanism controlling of PTH release.

Chapter 4

Calcitriol treatment causes persistent alteration of parathyroid gland responsiveness *in vitro*

4.1 Abstract

The PTG is a major regulator of mineral homeostasis. Alterations to the PTG can cause abnormal Ca and PO_4 handling which has been linked to the development of VC, the active deposition of hydroxyapatite crystals into the wall of the vasculature. Calcitriol is a mainstay therapy for management of SHPT, a major consequence of dysregulated mineral homeostasis in CKD. However, many animal studies have shown that therapeutic doses of calcitriol further exacerbate the development of VC, indicating a calcitriol-mediated mechanism of VC induction.

The aim of this study was to determine whether persistent CTx in the calcitriol induced VC model (CTx model), a robust model of VC in non-CKD animals, impacts the gland's response to alterations in Ca and PO_4. Rats (N=24) were stratified into 4 groups that were sacrificed at 4 days CTx, 8 days CTx, 8 days CTx + 7 days off treatment (OT), and 8 days CTx + 14 days OT. At sacrifice, PTGs were excised and placed in PTH-Inhibitory Media (PIM; DMEM with 2.4 mM Ca/1 mM, PO_4) for 2 hours followed by PTH-Stimulating Media (PSTM; DMEM with 0.6 mM Ca, 4 mM PO_4) for 2 hours to assess parathyroid hormone (PTH) responsiveness to inhibition with exposure to a hypercalcemic environment and stimulation with exposure to a hyperphosphatemic environment. Daily treatment with high dose calcitriol reduced PTG responsiveness. Glands obtained from 4 days of CTx had measurable PTH levels *in vitro* despite having circulating PTH below the lower assay limit of detection. After the OT period, glands were still unable to respond to the mineral stimuli, indicating that calcitriol may have directly and permanently altered the PTG resulting in abnormal PO_4 and Ca handling. The results of this study suggest that caution should be used with higher dose calcitriol therapy, that is typically used in patients with calcitriol resistance. The risk of developing VC and unwanted PTG modification suggests that a different approach should be used for treating SHPT in the CKD population such as providing a precursor which can facilitate appropriate and feedback controlled local calcitriol production and mediate PTG sensitivity to Ca and PO_4.

4.2 Introduction

VC as a manifestation of perturbed mineral balance, is associated with aging, diabetes, kidney dysfunction, and worsened patient outcomes.[11-13,] Current limited understanding of the pathophysiology of VC makes the development of effective therapeutic strategies a significant clinical challenge. Recent evidence suggests that traditional risk factors for cardiovascular disease, identified by the Framingham risk factor score, fail to account for VC that is observed clinically in the CKD population.[8,10] Therefore, more complex underlying processes involving physiochemical changes to mineral balance, vascular remodeling and perturbed hormonal responses are likely to contribute to VC.[10,13]

Calcitriol is the active form of vitamin-D, normally made in the kidney through 1-α-hydroxylase mediated conversion of the precursor calcifediol.[68] Calcitriol has multiple important functions in the body, especially in regard to regulation of mineral homeostasis.[39] Deficiency in vitamin D can result in biochemical disturbances, reduced bone mineralization, and increased risk of bone fracture.[149] Insufficiency of vitamin D as a level of calcifediol of less than 30ng/mL is a hallmark of CKD, and worsens as the disease progresses.[83,129] Calcitriol is a recommended therapy for CKD patients in stages 3-5 of CKD suffering from severe SHPT (greater than 600pg/mL).[83] However, many animal studies have shown that both vitamin D deficiency and excess, *via* CTx, can lead to VC in experimental CKD.[108,129,150] This indicates that calcitriol, like high dietary PO_4,[22] can be an independent driver of VC and thus an understanding of physiological mechanisms are critical for appropriate dosing.

The PTG is a major regulator of mineral homeostasis. Abnormal mineral regulation by the PTG heavily alters the setpoint of Ca and PO_4 such as in SHPT.[57,137] Therefore, manipulations in the morphology of the PTG through modification of the gland's receptors or cell types will alter the gland's ability to respond to circulating Ca and PO_4 optimally. Previous studies have found an

association between treatment with calcitriol or other Ca mimetics and prevalence of nodular hyperplasia, a condition which results in pan reduction of PTG receptors.[140,143]

Induction of calcification using pharmacological doses of calcitriol is very reproducible. Animal studies using this model have consistently observed increased tissue deposition of Ca and PO_4 as hydroxyapatite.[85,150] Our lab has generated a similar model where a dose of 0.5 μg/kg/day calcitriol for 8 days is sufficient to induce the development of VC in rats with normal kidney function. This is in contrast to uremic rat models in which development of VC is associated with high levels of PO_4 and PTH instead of high circulating levels of Ca and vitamin D in the CTx model.[128] In the CTx model, PTH is fully suppressed while in the CKD model PTH can become markedly elevated especially on HPD. The two models are similar in terms of elevated FGF-23 and similar tissue Ca and PO_4 deposition across the vasculature. In this study, we sought to determine whether persistent treatment with calcitriol impacts the parathyroid gland's ability to respond to alterations in Ca and PO_4. The findings of this study will also enable us to better understand the relationship between development of VC and PTG function potentially helping to inform more effective dosing strategies for vitamin D analogue therapy in CKD.

4.3 Methods

Experimental Animals

All animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by Queen's Animal Care Committee. Male Sprague-Dawley rats (N=24, 14 weeks old, 400-450g): (Hilltop Lab Animals Inc. PA, USA) were acclimated for a week prior to experimental start, individually housed, and maintained on a 12-hour light/dark cycle for the duration of the study. Two days prior to interventions, animals were transferred from *ad libitum* standard rat chow to restrictive feeding, 20g/day of formulated diet containing 0.75% PO_4, 1% Ca,

0.05% magnesium, 0.2mg/kg vitamin K, 1IU/g cholecalciferol, and 6% protein (Harlan Teklad, WI, USA, TD160324). Water was provided *ad libitum* for the duration of the study. Body weight and food intake were monitored for the duration of the study. Animals were supplemented with high calorie supplement (Boost®, Clear H_2O, PA, USA) when they reached 10% body weight loss or ate no food the previous day.

To generate VC, animals were injected subcutaneously with a calcitriol solution (0.5μg/kg bodyweight) daily for 8 days. Animals were stratified into four experimental groups by baseline bodyweight, and circulating Ca, PO_4, and parathyroid hormone (PTH). Group stratification was performed to control for potential confounds by baseline differences in circulating mineral and hormone levels and allow for the detection of differences between groups (Table 4-1). Group 1 (N=6) was sacrificed at experimental day 4, following 4 doses of CT (Day 4). Group 2 (N=6) was sacrificed at experimental day 8, following 8 doses of CT (Day 8). Group 3 (N=6) was sacrificed at experimental day 15, following 8 doses of CT and 7 days off treatment (OT) (Day 15). Group 4 (N=6) was sacrificed at experimental day 22, following 8 doses of CT and 14 days OT (Day 22) (Fig 4-1). Three healthy non-CKD animals were used as controls and were also placed on the 0.75% PO_4 until the same age as the Day 22 treatment animals.

Table 4-1 Experimental Group Stratification

	Group 1 (N=6)	Group 2 (N=6)	Group 3 (N=6)	Group 4 (N=6)
	4 days CTx	8 days CTx	8 days CTx + 7 days OT	8 days CTx + 14 days OT
Ca (mM)	2.11±0.11	2.11±0.11	2.14±0.13	2.15±0.05
PO_4 (mM)	2.33±0.29	2.36±0.14	2.56±0.60	2.57±0.39
PTH (pg/mL)	593.2±546.6	589.1±421.4	590.6±378.0	595.4±694.8
Body Weight (kg)	0.415±0.007	0.419±0.011	0.420±0.006	0.408±0.011

Values presented as Mean±SD. Animals were stratified prior to treatment into four groups Group 1 (4 daily doses of calcitriol treatment, group 2 (8 daily doses of calcitriol treatment), group 3 (8 daily doses of calcitriol treatment and 7 days off-treatment (OT)), and group 4 (8 daily doses of calcitriol treatment and 14 days OT). Animals were stratified based on circulating calcium (Ca), phosphate (PO_4), parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23). No significant differences between treatment groups at baseline in any stratification variable as assessed by one-way ANOVA with *post hoc* Tukey's multiple comparisons test between groups.

Blood Sample Preparation

Circulating Ca, PO_4, PTH, and FGF-23 were measured at 10 time points (days 1, 4, 8, 9, 11, 13, 15, 18, 20, and 22) for comparison with baseline values (Fig 4-1). These values were chosen in order to assess how CT affected circulating minerals and hormones with treatment and determine whether changes in biomarkers, and VC can be recovered OT. Blood was sampled from the saphenous vein prior to dosing at various timepoints throughout the study beginning at baseline (Day 0) to map changes in biochemical profile. Sampling was done with a 22G needle and microcapillary blood collection tubes (3 whole blood, 5 heparinized plasma). Whole blood was left at room temperature for at least 45 minutes and heparinized whole blood was collected on ice prior to centrifugation to generate serum and plasma, respectively. Samples were centrifuged at 9,000 RPM for 20 minutes at 4°C. Calcium, PO_4, PTH, and FGF-23 were measured from each sample collection timepoint.

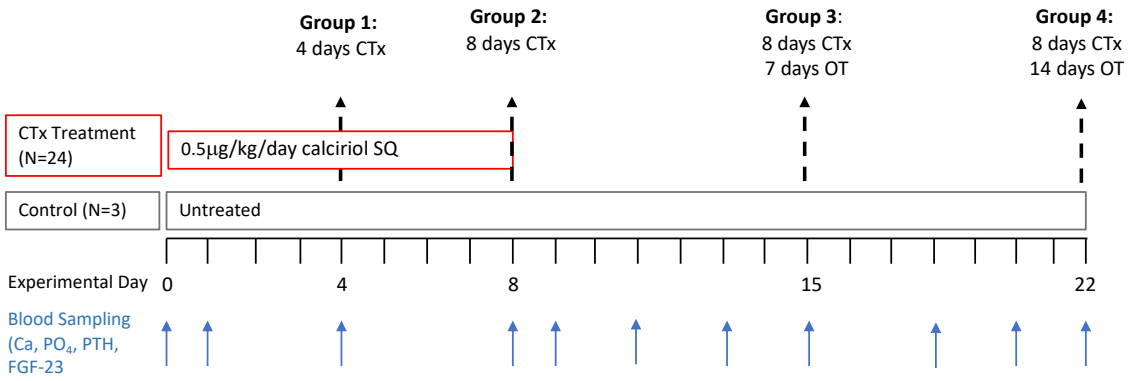

Figure 4-1 Experimental design for the calcitriol induced vascular calcification model

Animals were stratified into their respective 4 sacrifice groups (indicated by the top labels) at baseline based on circulating minerals (calcium and phosphate) and hormones (parathyroid hormone and fibroblast growth factor 23). An additional group of healthy controls (N=3) were untreated and sacrificed at day 22. The experiment ran for 22 days. On the first 8 days, animals (N=24) received 0.5µg/kg/day of calcitriol and left for 14 days off-treatment days. The blue lines below the timeline indicates the timepoints selected for blood sampling.

Calcitriol Solution:

Lyophilized calcitriol was reconstituted in saline. Each mL contained 0.5 ug calcitriol, 4 mg polysorbate-20, 1.5 mg sodium chloride, 1.11mg sodium edetate, 8.89 mg ascorbic acid, and sodium mono- and di-basic phosphate (all reagents from Sigma-Aldrich Co., ON, CAN) as buffers. Solution was sterile filtered and brought to a final pH of 7.4.

Sacrifice Protocol:

Animals were sacrificed at experimental day 4, following 4 doses of CT (Day 4). Group 2 (N=6) was sacrificed at experimental day 8, following 8 doses of CT (Day 8). Group 3 (N=6) was sacrificed at experimental day 15, following 8 doses of CT and 7 days OT (Day 15). Group 4 (N=6) was sacrificed at experimental day 22, following 8 doses of CT and 14 days OT (Day 22) in accordance with differences in circulating FGF-23 levels. The initial design of the study was created for assessing whether there was a correlation between circulating FGF-23 levels and development of VC in CKD and healthy animals. Animals were anesthetized with inhaled isoflurane (5% isoflurane, 2% O_2). Animals were sacrificed via cardiac puncture and exsanguination. Various somatic tissues were collected (34) including: arteries, veins, kidney, liver, pancreas, lung, and bone. Tissues were patted dry, weighed, and placed in 1N HCl (50uL/mg tissue) for one week at room temperature to facilitate demineralization and for measurements of tissue Ca and PO_4 concentrations.

Incubation Protocol:

PTGs from the experimental animals were excised immediately after animals were sacrificed. DMEM F-12 (ThermoFisher Co, ON, CAN) pH 7.7 at 37°C, 5% CO_2 for a total of 4 hours. Glands were placed in two conditions of DMEM media for 2 hours each; 1. a PTH inhibiting

condition (2.4mM Ca and 1mM PO_4) (PIM), and 2. a PTH stimulating condition (0.6mM Ca, 4mM PO_4) (PSTM). The concentrations of Ca and PO_4 were assessed prior to the start of the experiment using spectrophotometric assays for Ca and PO_4. The cell plate was plated as shown in Fig. 4-2 with 200uL of media per well. Media was collected every 30 minutes for 4 hours. These samples were stored at -80°C for PTH measurements.

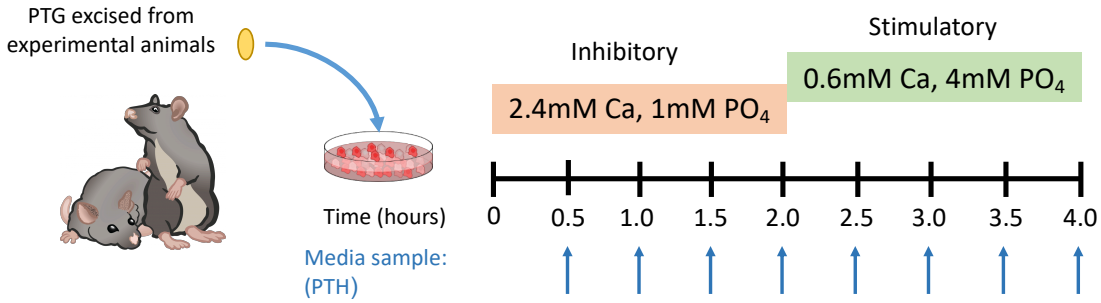

Figure 4-2 Parathyroid gland culture protocol

Glands were excised from healthy control (N=3), non-CKD animals (N=24) treated with calcitriol (0.5µg/kg/day). The orange section (2.4mM Ca, 1mM PO_4), represents the inhibitory phase (PIM), the green section, phase 2 (0.6mM Ca, 4mM PO_4), represents the stimulatory phase (PSTM), post excision. The blue arrows represent 30-minute samples which were taken for assessment of PTG responsiveness, over a period of 4 hours.

Biochemical blood, tissue, and media measurements:

Ca and PO_4 were measured spectrophotometrically (SynergyHT Microplate Reader; BioTek Instruments, Winooski, VT) from serum samples. Ca was measured using the o-cresolphthalein complexone method, a procedure in which Ca forms a purple complex with the o-cresolphthalein colour reagent (Sigma-Aldrich Co., ON, CAN) that is measured spectrophotometrically at 540nm. PO_4 was measured using the malachite green (Sigma-Aldrich Co., ON, CAN) method[21]. A green complex is formed between ammonium molybdate, malachite green, and free PO_4 which can be measured spectrophotometrically at 650nm. Tissue Ca and PO_4 were measured per mg wet weight tissue from acid using the same methods with pH-matched standards. Rat intact PTH, intact FGF-23, and secretions of PTH from PTG cultures were measured from plasma using ELISAs (Immunotopics Inc., CA, USA) following the method provided by the manufacturer.

Statistical Analyses:

Longitudinal measurements in Ca, PO_4, PTH, and FGF-23 at each experimental timepoint will be compared to baseline. Differences in circulating Ca, PO_4, PTH, and FGF-23 between the measured timepoints within the weeks of the experiment are compared to baseline values using two-way ANOVA with Geisser-Greenhouse corrections and post-hoc Dunnet's multiple comparisons test to assess changes in circulating biomarkers over time. Analyses were separated in two parts 1. calcitriol treatment where comparisons were made between days 1, 4 and 8 with baseline and 2. post-treatment where comparisons were made between day 8 and days 11, 13, 15, 18, 20, and 22.

Tissue Ca and PO_4 between treatment groups (healthy control, day 4, day 8, day 15, and day 22) will be presented as individual data points and organized by tissue type. Differences in

tissue minerals will be assessed using ordinary one-way ANOVA and *post-hoc* Tukey's multiple comparisons test.

Comparisons between each timepoint in the PTG culture protocol within treatment groups and between treatment groups will be made using mixed effects model with Geissers-Greenhouse correction and *post-hoc* Dunnett's multiple comparisons test for 0.5 hour to 2 hours to assess the effects of inhibitory Ca, and 2 hours to 4 hours the effects of stimulatory PO_4 on PTG responsiveness *in vitro*. Comparisons of maximum and minimum secretion, maximum % stimulation, and minimum % suppression between treatment groups will be made using ordinary one-way ANOVA with *post-hoc* Tukey's multiple comparisons test. The maximum secretion and minimum secretion of PTH was used to assess PTG responsiveness due to their frequency in pharmacological studies to determine the effects of a drug after its administration. The maximum and minimum values are the most relevant ones to use in this time course due to the limitations of PTG gland viability in the study which may impact PTH secretion over time. Maximum % suppression and maximum % stimulation was calculated as a percent change between the maximum PTH achieved in the PSTM and the minimum PTH secreted in PIM or vice versa respectively.

4.4 Results

Biochemical Metabolite Profile

Administration of subcutaneous calcitriol (0.5μg/kg) daily caused perturbations in circulating minerals and hormones which normalized after 14 weeks OT (Fig 4-3). Ca rose progressively with CTx, which maximized at a level ~50% greater than baseline (2.13 ± 0.10 mM) at day 8 (3.28 ± 0.16 mM, $p<0.0001$). Ca levels then decreased throughout the OT period, but still remained significantly above baseline levels (~10%) even at 14 days OT (2.32 ± 0.08mM, $p<0.001$) (Fig. 4-3 A). PO_4 increased transiently by ~30% at one day following the first dose of calcitriol, then decreased until day 8 of treatment. After stopping CTx, PO_4 levels returned towards baseline

levels after 3 days and remained in the normal range until the end of the experiment (Fig. 4-3 B). To assess hormone dysregulation, intact PTH (Fig. 4-3 C) and FGF-23 (Fig 4-3D) were measured. During CTx PTH was suppressed below the lower level of detectability of the assay (<7.5 pg/mL) by 2 days until the end of the administration period and returned to at least baseline levels between 4 and 6 days after stopping. FGF-23 levels were significantly elevated by day 4 of CTx (4659.1 ± 985.81 pg/mL, $p<0.0001$) and increased to a maximum value ~327x baseline by day 8 (24142.2 ± 6904.62 pg/mL, $p<0.0001$). FGF-23 levels decreased throughout the OT period but remained significantly elevated compared to baseline (Fig 4-3D).

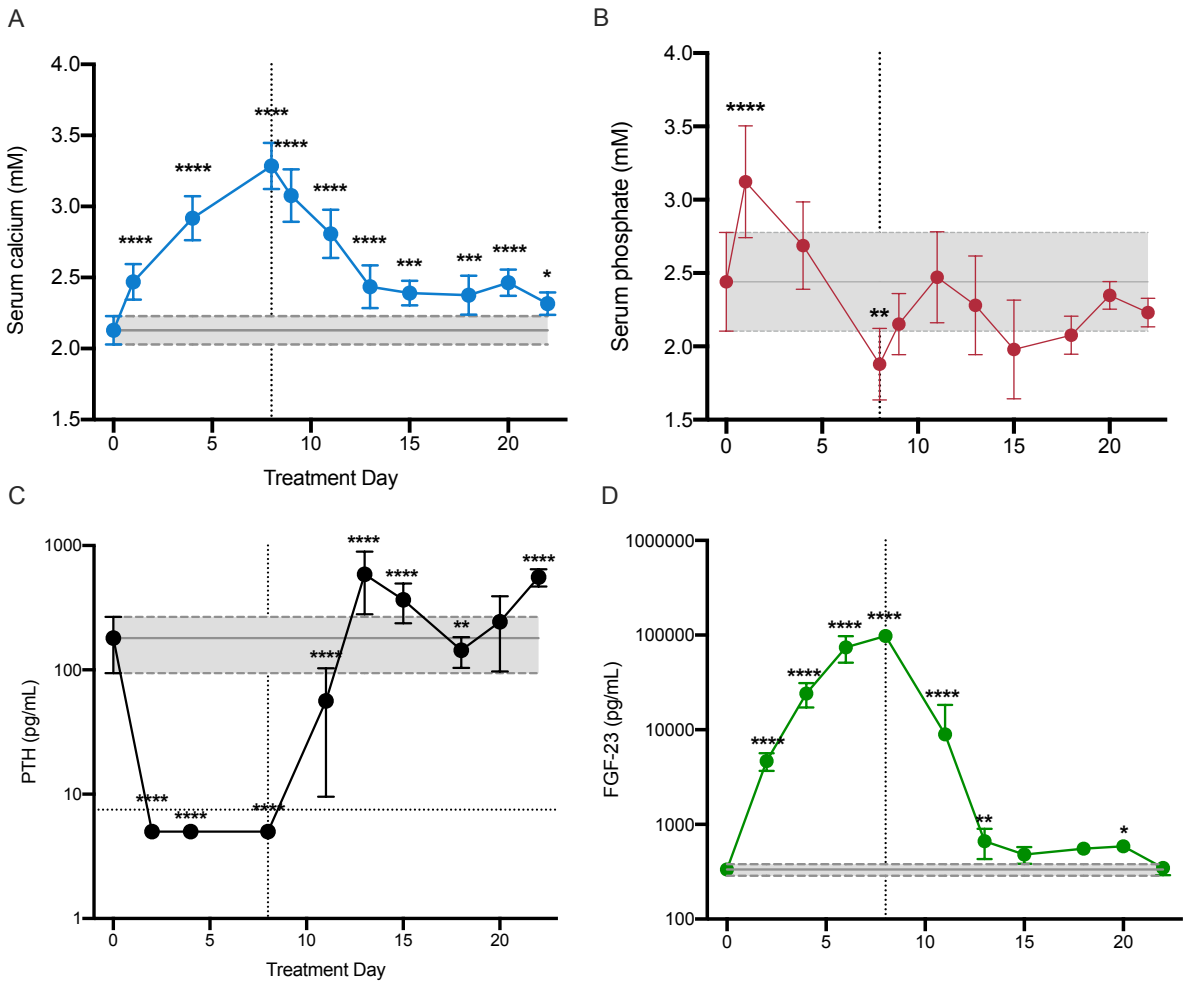

Figure 4-3 Longitudinal measurements of circulating minerals and hormones

A) calcium, **B)** phosphate, **C)** parathyroid hormone (PTH), and **D)** fibroblast growth factor 23 (FGF-23). Data represented as mean +/- SD. The grey box on each graph represents the values of the circulating minerals, and hormones in the CTx model rats (N=24) prior to treatment with calcitriol (0.5µg/kg/day). Difference between the measured timepoints within the weeks of the experiment are compared to baseline values using two-way ANOVA with Geisser-Greenhouse corrections and post-hoc Dunnet's multiple comparisons test. Analyses were separated in two parts 1. calcitriol treatment where comparisons were made between days 1, 4 and 8 with baseline and 2. post-treatment where comparisons were made between day 8 and days 11, 13, 15, 18, 20, and 22. Analyses for PTH and FGF-23 were done on log transformed data. Stars denote differences between timepoints compared to week 0 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Persistent calcitriol-induced vascular calcification

To assess VC across the vascular tree, tissue Ca (Fig 4-4 A) and PO_4 (Fig 4-4 B) content were measured in central (thoracic and abdominal aortae) and peripheral (carotid, iliac, renal, superior mesenteric, celiac, and pudendal arteries) blood vessels. Animals in the Day 4 group showed significant elevations in central and peripheral vessel Ca (325.38 ± 303.54 nmol/mg tissue, $p<0.0001$) and PO_4 content (267.34 ± 218.07 nmol/mg tissue, $p<0.0001$) compared to control (Ca= 8.40 ± 3.68 nmol/mg tissue, PO_4= 20.56 ± 8.35 nmol/mg tissue). This was further elevated by 8 days (Ca= 411.29 ± 183.36 nmol/mg tissue, $p<0.05$; PO_4= 308.97 ± 128.30 nmol/mg tissue, $p<0.05$) of CTx. These mineral levels persisted in animals 14 days post-treatment with no significant differences in tissue Ca between groups that received 8 days of CTx indicating a similar overall VC profile. However, 14 days OT (day 22) (345.84 ± 51.31 nmol/mg tissue, $p<0.05$) had slightly higher tissue PO_4 levels than 7 days OT (day 15) (298.69 ± 70.31 nmol/mg tissue).

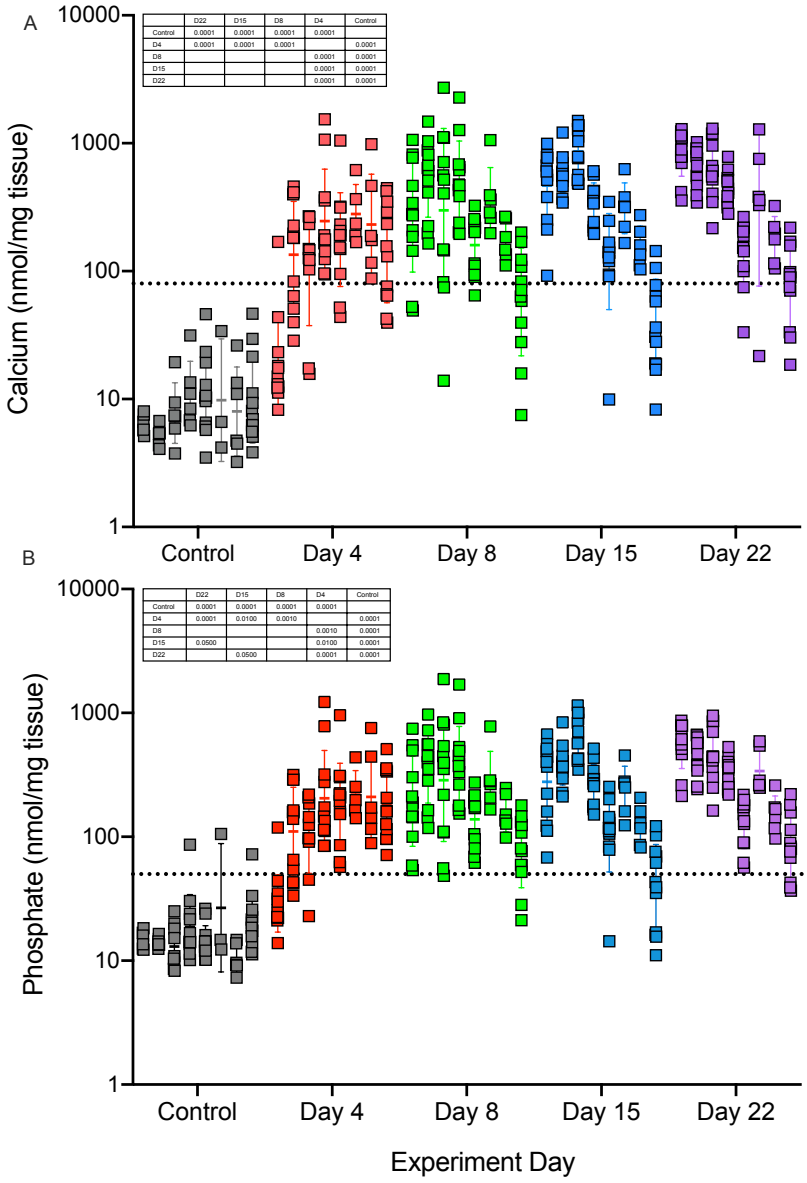

Figure 4-4 Time course of vascular calcification of peripheral blood vessels

A) Tissue calcium and **B)** phosphate levels in central and peripheral blood vessels in calcitriol treated rats (N=27). Each column represents a blood vessel, left to right: thoracic aorta, abdominal aorta, carotid artery, iliac artery, renal artery, superior mesenteric artery, celiac artery, and pudendal artery. The grey box indicates vessels which do not have histologically visible vascular calcification *via* von Kossa staining (80 nmol/mg Ca and 50 nmol/mg PO_4). One-way ANOVA on log-transformed data with post hoc Tukey's multiple comparisons test between experimental groups (4 days of calcitriol treatment (CTx), 8 days CTx, 8 days CTx and 7 days off treatment (OT) (day 15), and 8 days CTx and 14 days OT (day 22)) and healthy control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Ex vivo PTG Profile

PTH secreted from glands collected from all treatment groups were not significantly suppressed in the PIM condition or significantly elevated in the PSTM media. Despite a lack of significant trend, a suppression in PIM and spike in PTH secretion in the PSTM was still observed for the healthy controls. PTH levels from Day 8 glands were significantly suppressed throughout the experiment compared to controls. (Fig 4-5)

PTH levels *in vivo* of animals within the Day 4 treatment group were lowered to a concentration below the level of detection of the assay with CTx. However, this decrease was not observed *in vitro*, which was contrary to what was found for glands taken from rats treated for 8 days of calcitriol. PTH *in vitro* was not fully suppressed in glands taken from animals sacrificed at Day 15 and Day 22. Overall, the minimum PTH secreted by the glands was lower in all the calcitriol treated glands compared to glands from healthy controls (6 glands). Despite a greater minimum secretion of PTH in all treatment groups compared to controls, there were no statistically significant difference in the maximum % suppression and % stimulation between treatment groups. (Fig 4-6)

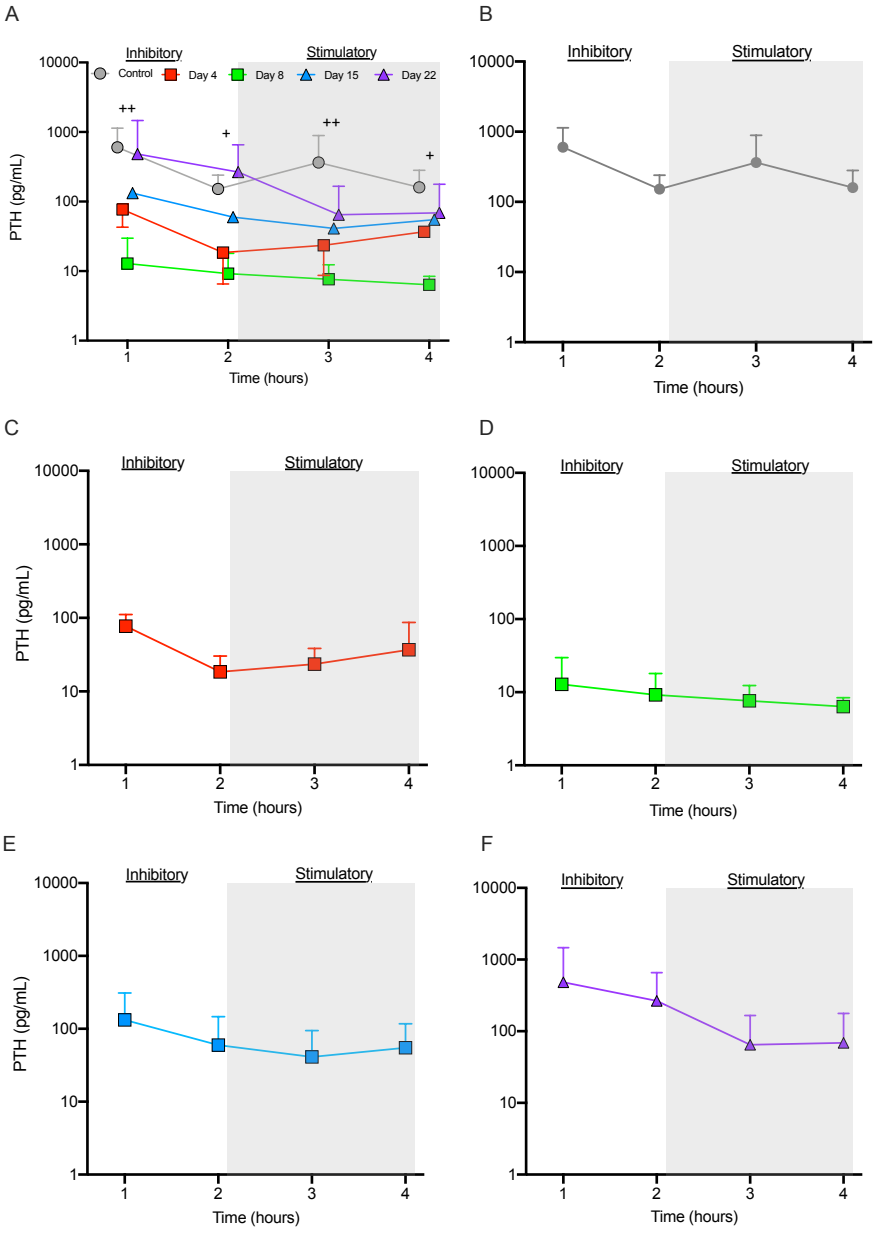

Figure 4-5 *In vitro* PTH secretion profile from single parathyroid glands

A) all treatment groups (N=27), **B)** healthy control (N=3), **C)** Day 4 (N=6) **D)** Day 8 (N=6) **E)** Day 15 (N=6) and **F)** Day 22 (N=6). Data represented as mean +/- SD. Each gland went over two phases: PIM (white) 2.4mM Ca, 1mM PO_4; PSTM (dark grey) 0.6mM Ca, 4mM PO_4. A) Differences between treatment groups at each time point were compared using mixed effects model with Geissers-Greenhouse correction and *post-hoc* Tukey's test for multiple comparisons. B-F) Values within PIM were compared to 1 hour (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$) and values within PSTM were compared to 2 hours (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

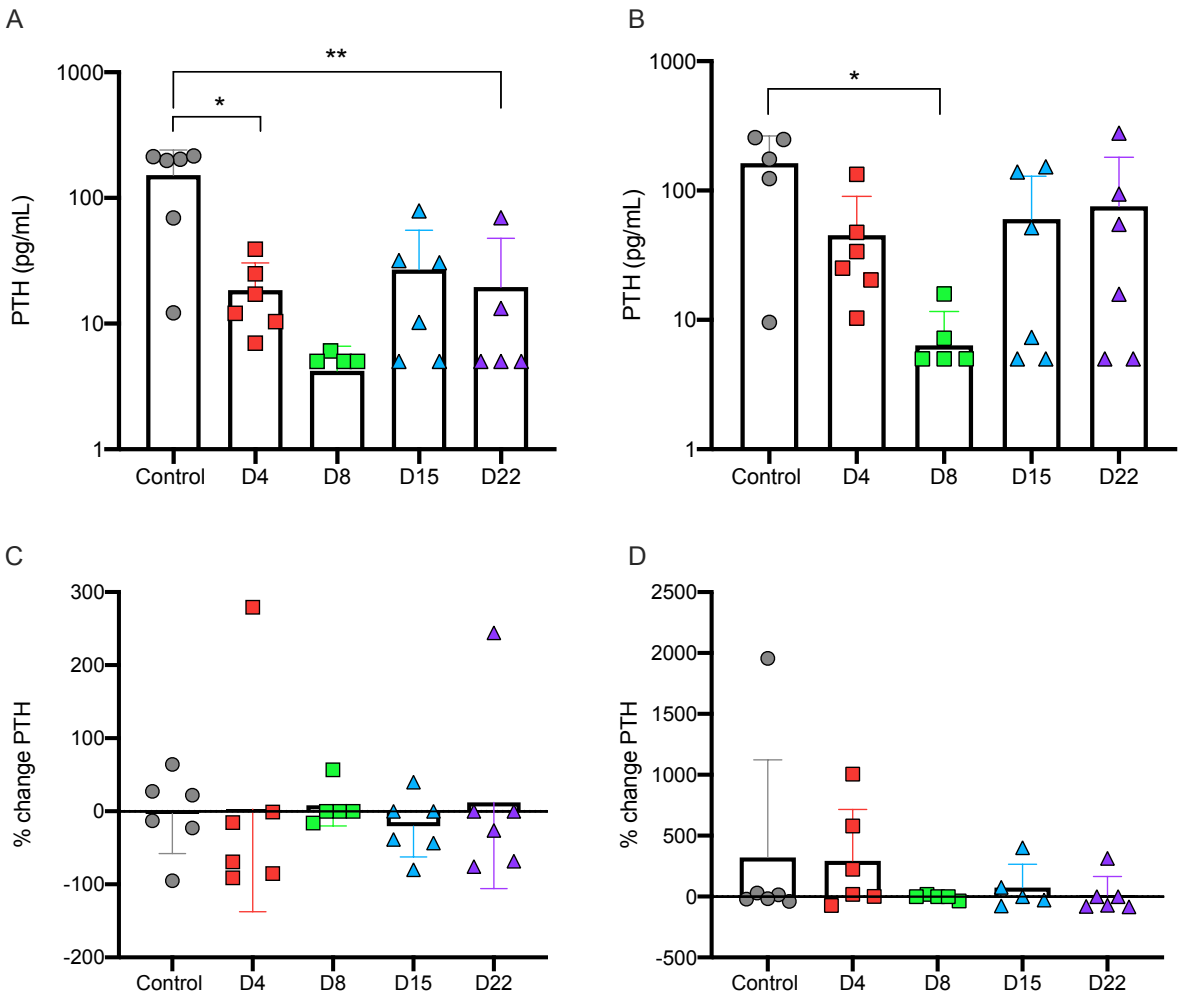

Figure 4-6 *In vitro* secretion of PTH in response to inhibitory and stimulatory media

A) Minimum Secretion of parathyroid hormone (PTH) and **B)** Maximum PTH Secretion **C)** Maximum % Suppression and **D)** Maximum % Stimulation. Data represented as mean +/- SD. A) Minimum PTH secretion value for each gland was obtained from the inhibitory condition (PIM) (timepoints 0.5 hours to 2 hours). B) Maximum PTH secretion value for each gland was obtained from the stimulatory condition (PSTM) (timepoints 2hr to 4hr). C) Maximum % suppression was calculated as a % change between the maximum PTH secreted in PSTM to the minimum PTH secreted obtained in the PIM. D) Maximum % stimulation was calculated as a % change between the minimum PTH secreted in inhibitory calcium to the maximum PTH secreted in stimulatory phosphate. Raw values and % relative changes compared between control, Day 4, 8, 15, and 22 were done using ordinary one-way ANOVA with Tukey's multiple comparisons test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

4.5 Discussion

In this collaborative study, we used a CTx model to determine whether persistent treatment with high dose calcitriol impacted the PTG's ability to respond to alterations in Ca and PO_4. Our study is the first study to assess PTG function in this model. Sprague Dawley rats (n=24) were dosed with calcitriol (0.5ug/kg) for 4 or 8 days daily to induce VC and then went OT for 2 weeks to determine whether mineral homeostasis can be recovered, and severity of VC can be regressed. Treatment with CT for 8 days daily caused significant elevations in Ca and FGF-23 and significant suppression of PTH. Circulating PO_4 was only elevated on day 1 but remained otherwise at similar levels of PO_4 to baseline, prior to CTx. This spike of PO_4 is likely attributed to suppression of PTH that results in loss of acute PO_4 regulation which is later taken over by FGF-23 and increased calcitriol mediated bone resorption. In addition, circulating PO_4 declined by day 8 of the experiment, suggesting increased uptake of PO_4 and accrual into the vasculature. Calcitriol has been determined to independently promote the development of VC *via* stimulation of free Ca and PO_4 levels and/or stimulation of calcification promoters.[84,85, 108]

Calcium and FGF-23 levels decreased throughout the OT period but still remained significantly elevated compared to baseline. PTH levels also increased in the OT period and, similar to Ca and FGF-23, was significantly elevated from baseline. These results indicate that the setpoint of Ca may have been altered in these animals resulting in a rightward shift of the PTH-Ca curve whereby higher Ca levels are required to induce suppression of PTH[137]. This finding is similar to a study where daily doses of CT in healthy men showed altered Ca metabolism and increased osteoblast activity[152]. If the animals were left to recover for a longer period of time (3 weeks) then the circulating biomarkers may return to baseline levels similar to what was found in humans.[152,153]

Animals were sacrificed after 4 and 8 days of calcitriol treatment and 7 and 14 days after stopping the daily 8-day CTx. The finding that high doses of CT can promote persistent suppression

of PTH and decreased response to inhibitory Ca and stimulating PO_4 *in vitro* even after treatment is stopped suggests that persistent changes are occurring [89,92]. Interestingly, this effect seems to be time dependent since PTGs from animals treated for only 4 days were less impacted *in vitro*. Whether the persistent *in vitro* results from changes in receptors density or metabolism within the vitamin D pathway (e.g. local expression of 24-hydroxylase) will require further study.

In the previous chapter, PTGs taken from rats with experimental CKD were able to respond *in vitro* to both inhibitory Ca and stimulatory PO_4. Vitamin D deficiency is a well-known stimulus for PTH production and proliferation[83,136] Development of parathyroid hyperplasia in CKD is known to cause reductions in receptors in the PTG but does not cause complete loss of response to either CT or circulating minerals[75]. Studies have shown contrasting views on whether SHPT affects sensitivity of Ca,[77,78,137] while effects of SHPT on sensitivity to PO_4 is still under investigation[57]. Studies have shown that calcitriol treatment at high doses increases apoptosis in PTG hyperplasia.[141] In fact, treatment causes a reduced number of chief cells in human PTGs which contain the majority of CaSR and vitamin D receptor in the gland.[140,148]

Future studies will have to repeat the PTG culture protocol on healthy non-CKD animals. The current number which we have completed (n=3) are not enough to fully characterize the response of normal PTGs to alterations in Ca and PO_4 in our study. Therefore, we are lacking an appropriate comparator for the glands in the calcitriol-induced CTx model. We were able to obtain both the left and right PTGs from our healthy controls but observed a fairly significant difference in gland responsiveness between each side. This indicates that we will also have to consider the differences in the left and right PTG when interpreting results of immunohistochemistry studies.

Future immunohistochemistry and gene transcriptomics will allow us to characterize effects on the PTG structure and function by quantifying the expression in PTGs of VDR, CaSR, and enzymes involved in vitamin D metabolism such as 1-α-hydroxylase and 24-hydroxylase.

Our study captured the effects of circulating biomarkers, vascular calcification, and parathyroid gland function within two-time courses, 1. a 0.5μg/kg/day calcitriol for the first 8 days of the experiment and 2. an off-treatment period for two weeks. This allowed us to see whether the effects induced by the high calcitriol dose could be recovered OT. We determined that daily CTx persistently impacted the PTG *in vitro* resulting in reduced response even post-CTx for two weeks. The findings of this study cautions vitamin D overdose in healthy individuals, and even more so in the CKD population, where it is common to increase calcitriol treatment with the development of calcitriol resistance. Based on these findings, alternatives to calcitriol should be explored for treating SHPT in the CKD population.

Chapter 5

General Discussion and Future Directions

The research in this thesis will contribute to our understanding of the development of calcitriol resistance and the effects of stimuli such as Ca, PO_4, and calcitriol on the PTG. The findings will also add to the growing evidence on the negative impact of HPD and calcitriol CTx on the PTG's response profiles and ability to optimally regulate mineral homeostasis. The results of this thesis will hopefully help to guide improvements on the current management strategies and highlight the importance of restrictive phosphate diets for treatment of SHPT.

The findings in both chapter 2 and chapter 3 showed that HPD attenuates calcitriol mediated suppression of PTH. Based on previous findings indicating the role of HPD in stimulating PTG proliferation and PTH secretion [94,120], it was suspected that decreased response to calcitriol therapy was in part a result of developing nodular parathyroid hyperplasia and downregulation of CaSR and VDR. In chapter 3, we were unable to detect a difference in glandular response to alterations in Ca and PO_4 between the UnTx CKD, and both dosing strategies, abrupt (20ng/kg/day from 4-9 weeks of the experiment) and graduated (2-20ng/kg/day from 1-4 weeks of the experiment and 20ng/kg/day from 4-9 weeks of the experiment). The response profiles of calcitriol treated animals were more split in the CT-Grad, meaning that there were responders and non-responders to CTx and/or manipulations in mineral concentrations. Alterations in response profiles within the calcitriol groups suggest that persistent changes to the gland which affected receptor profile or cell type had occurred. In chapter 4, we determined that persistent treatment with calcitriol even in healthy animals affected PTG response to Ca and PO_4. The results found in chapter 4 also suggest caution in the common strategy of increasing calcitriol doses to overcome calcitriol resistance as this method can have persistent effects on PTG regulation of mineral homeostasis.

Significant strengths of chapter 2 and 3 were the investigation of calcitriol efficacy throughout advancing CKD severity and with differing PO_4 diets. This allowed us to better characterize the onset of calcitriol resistance and assess the effectiveness of different calcitriol dosing strategies. The untreated and treated time courses in chapter 2 enabled normalization of calcitriol's effects on Ca, PO_4, PTH, and FGF-23 to the daily rhythm of the circulating biomarkers. The time course also allowed us to determine whether the daily rhythm of the biomarkers was also affected on HPD and with CKD duration and severity. The rigorous time course of chapter 3 helped gain insight on the role of CTx early and late in an experimental CKD model. Specifically, roles of different CTx strategies *in vivo* and on PTG responsiveness *in vitro*. However, further investigations are required to see if different calcitriol dosing strategies impact the morphology of the gland. Mainly through alteration of PTG cell types (transition from chief to water-clear cells) and expression of VDR and CaSR between treatment groups. Current literature has only classified rat parathyroid cell type without direct comparisons to cell types in human PTGs.[142-146] Therefore, the use of the rat model and its applicability to humans still needs to be justified. Finally, chapter 4 allowed us to assess the role of calcitriol both on and OT. The results found in this chapter showed reduced PTG responsiveness to Ca and PO_4 after 4 days of consecutive CTx. This attenuated response persisted even after treatment was stopped for 2 weeks and suggests the use of other vitamin D analogues to prevent calcitriol-mediated changes to the PTG.

In order to elucidate the differences in gland response profiles, preliminary immunohistochemistry was done for CaSR, VDR, CYP27B1 (production of calcitriol), and CYP24A1 (metabolism of calcitriol) to determine the impact of HPD and calcitriol on PTG receptors and/or local vitamin-D metabolism. Both the CaSR and VDR are crucial receptors which mediate response to calcitriol therapy. Calcitriol acts directly on the VDR to induce PTH suppression but also acts indirectly on the CaSR *via* increased circulating Ca with treatment.[53,55,62] The CaSR has also been determined to be involved in PO_4 sensing, a mechanism as PO_4 acts as an

allosteric inhibitor of the CaSR[57].Therefore, effects on the CaSR will impact regulation of both Ca and PO_4.[53-55,, 57] Local production of vitamin-D in the PTG is important for regulating the negative feedback loop of PTH.[61,73,74] Many studies have determined that expression of CYP27B1 and CYP24A1 in the PTG is altered by its surrounding environment and stimuli. Increase in CYP27B1 expression has been associated with high circulating PO_4 and vitamin-D deficiency, major contributors to the development of SHPT.[88-91] Increases in CYP24A1 expression have also been associated with treatment with calcitriol, a mechanism that has been proposed to occur to prevent excess activation of the VDR. [137,151]

Finally, future studies will strive to improve the PTG culture protocol *via* the addition of an initial physiologically similar Ca and PO_4 condition and a high Ca and high PO_4 exposure arm. The addition of an initial 30-minute media which is similar to the levels of Ca and PO_4 *in vivo* will allow us to better assess the Ca mediated suppression of PTH and allow the gland to stabilize and recover more readily after excision. The current protocol which we used was a direct PTH suppression condition due to the lack of literature surrounding the PTG viability in culture. The addition of the high Ca and high PO_4 condition will allow us to distinguish the contribution of either Ca or PO_4 on the PTG's regulation and compliment future clinical and animal studies in the lab. Future studies in the lab will further elucidate how HPD, associated with a high PO_4 and low Ca ratio *in vivo*, induces changes in the PTG cells which generate consequences that alter the structure and functions of bone and blood vessels. We propose that the phenotypic shift of the PTG cells towards a transitioned cell that promotes hypersecretion of PTH and increased systemic PO_4 and Ca exposure will not occur if calcium is normalized to the elevated PO_4. Our optimized immunohistochemistry will be done together with gene transcriptomics to evaluate the role of PTG sensitivity on CKD vascular and bone outcomes, correlations which were not determined in the experiments done in this thesis.

Research including the use of both male and female rats has been highly recommended for studies of disease models in order to provide optimal care for both sexes. However, only male rats were used throughout the studies. It is recognized that there are differences between sexes regarding the prevalence and progression of CKD[155,156], incidence of cardiovascular disease[157] and differences in mineral homeostasis[158]. Assessing these differences was not feasible because the protocols we were performing were preliminary. Chapter 2 was the first time that we gave a dose of 160ng/kg biweekly to animals and chapter 3 and 4 were the first time the PTG culture protocol was carried out in experimental CKD and in the CTx model.

Overall, the research in this thesis focused on the PTG as a central mediator of mineral homeostasis. A key technological advancement was that these studies generated a viable PTG culture model for assessing the physiological function of PTGs in both rat and human models in future studies. Finally, the studies revealed that different treatments *in vivo* either through dietary manipulations or with vitamin D analogues can affect the basic responsiveness of the PTG and its sensitivity to Ca and PO_4 both *in vivo* and *in vitro*.

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Appendix

Teklad Custom Diet

TD.150555

6% Protein Diet (1% Ca, 0.5% P, 0.2 mg/kg Vit K), Y

Formula	g/Kg
Casein, "Vitamin-Free" Test	67.0
L-Cystine	1.0
Corn Starch	446.2
Maltodextrin	132.0
Sucrose	176.2336
Corn Oil	70.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, monobasic, monohydrate	12.0
Calcium Carbonate	7.7
Niacin	0.03
Calcium Pantothenate	0.016
Pyridoxine HCl	0.007
Thiamin (81%)	0.006
Riboflavin	0.006
Folic Acid	0.002
Biotin	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.025
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.15
Vitamin A Palmitate (500,000 IU/g)	0.008
Vitamin D_3, cholecalciferol (500,000 IU/g)	0.002
Vitamin K_1, phylloquinone	0.0002
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014
Yellow Food Color	0.1

Footnote

6% protein diet in a series of diets with 1% calcium, varying levels of phosphate, and 0.2 mg/kg diet added vitamin K. Ethanol extracted (VFT) casein and corn oil used to minimize background vitamin K. Color coded yellow. Part of series TD.150555-150560.

Key Features

+ **Purified Diet**
+ **Adjusted Minerals Ca & P**
+ **Reduced Vitamin K**
+ **Color Coded Yellow**

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	6.1	6.6
CHO	71.1	76.5
Fat	7.0	16.9
Kcal/g	**3.7**	

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Key Planning Information

+ *Products are made fresh to order*
+ Store product at 4°C or lower
+ Use within 6 months (applicable to most diets)
+ Box labeled with product name, manufacturing date, and lot number
+ Replace diet at minimum once per week
  More frequent replacement may be advised
+ Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

+ 1/2" Pellet or Powder (free flowing)
+ Minimum order 3 Kg
+ Irradiation not advised
  · Contact a nutritionist for recommendations

Options (Fees Will Apply)

+ Rush order (pending availability)
+ Irradiation (see Product Specific Information)
+ Vacuum packaging (1 and 2 Kg)

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+ **askanutritionist@envigo.com**

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+ **(608) 277·2066** *facsimile*

09/24/15

Teklad Custom Diet

TD.150558

6% Protein, 0.25% Adenine Diet (1% Ca, 0.5% P), G

Formula	g/Kg
Casein, "Vitamin-Free" Test	67.0
L-Cystine	1.0
Corn Starch	443.7
Maltodextrin	132.0
Sucrose	176.2336
Corn Oil	70.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, monobasic, monohydrate	12.0
Calcium Carbonate	7.7
Niacin	0.03
Calcium Pantothenate	0.016
Pyridoxine HCl	0.007
Thiamin (81%)	0.006
Riboflavin	0.006
Folic Acid	0.002
Biotin	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.025
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.15
Vitamin A Palmitate (500,000 IU/g)	0.008
Vitamin D_3, cholecalciferol (500,000 IU/g)	0.002
Vitamin K_1, phylloquinone	0.0002
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014
Adenine	2.5
Green Food Color	0.1

Footnote

6% protein & 0.25% adenine diet in a series of diets with 1% calcium, different levels of phosphorus, and 0.2 mg/kg diet added vitamin K. Ethanol extracted (VFT) casein and corn oil used to minimize background vitamin K. Color coded green. Part of series TD.150555-TD.150560.

Key Features

+ **Purified Diet**
+ **0.25% Adenine**
+ **Calcium and Phosphate Adjusted**
+ **Reduced Vit K**

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	6.1	6.6
CHO	70.9	76.4
Fat	7.0	17.0
Kcal/g	**3.7**	

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Key Planning Information

+ *Products are made fresh to order*
+ Store product at 4°C or lower
+ Use within 6 months (applicable to most diets)
+ Box labeled with product name, manufacturing date, and lot number
+ Replace diet at minimum once per week
  More frequent replacement may be advised
+ Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

+ 1/2" Pellet or Powder (free flowing)
+ Minimum order 3 Kg
+ Irradiation not advised
  · Contact a nutritionist for recommendations

Options (Fees Will Apply)

+ Rush order (pending availability)
+ Irradiation (see Product Specific Information)
+ Vacuum packaging (1 and 2 Kg)

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09/24/15

Teklad Custom Diet

TD.160324

6% Protein Diet (1% Ca, 0.75% P, 0.2 mg/kg Vit K, B)

Formula	g/Kg
Casein, "Vitamin-Free" Test	67.0
L-Cystine	1.0
Corn Starch	440.0
Maltodextrin	132.0
Sucrose	176.2336
Corn Oil	70.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, monobasic, monohydrate	22.2
Calcium Carbonate	3.7
Niacin	0.03
Calcium Pantothenate	0.016
Pyridoxine HCl	0.007
Thiamin (81%)	0.006
Riboflavin	0.006
Folic Acid	0.002
Biotin	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.025
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.15
Vitamin A Palmitate (500,000 IU/g)	0.008
Vitamin D_3, cholecalciferol (500,000 IU/g)	0.002
Vitamin K_1, phylloquinone	0.0002
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014
Blue Food Color	0.1

Footnote

A 6% protein diet in a series of diets modified from TD.08670. It contains ~1% calcium, 0.75% phosphorus, and 0.2 mg/kg diet added vitamin K. Ethanol extracted (VFT) casein and corn oil used to minimize background vitamin K. Color coded blue.

Key Features

+ **Purified Diet**
+ **Adjusted Minerals Ca & P**
+ **Reduced Vitamin K**
+ **Color Coded Blue**

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	6.1	6.6
CHO	70.5	76.3
Fat	7.0	17.0
Kcal/g	**3.7**	

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Key Planning Information

+ *Products are made fresh to order*
+ Store product at 4°C or lower
+ Use within 6 months (applicable to most diets)
+ Box labeled with product name, manufacturing date, and lot number
+ Replace diet at minimum once per week
  More frequent replacement may be advised
+ Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

+ 1/2" Pellet or Powder (free flowing)
+ Minimum order 3 Kg
+ Irradiation not advised
  · Contact a nutritionist for recommendations

Options (Fees Will Apply)

+ Rush order (pending availability)
+ Irradiation (see Product Specific Information)
+ Vacuum packaging (1 and 2 Kg)

Speak With A Nutritionist

+ **(800) 483·5523**
+ **askanutritionist@envigo.com**

Contact Us

Obtain Pricing · Check Order Status

+ **teklad@envigo.com**
+ **(800) 483·5523**

International Inquiry *(Outside USA or Canada)*

+ **askanutritionist@envigo.com**

Place Your Order (USA & Canada)

Please Choose One

+ **www.envigo.com/teklad-orders**
+ **tekladorders@envigo.com**
+ **(800) 483·5523**
+ **(608) 277·2066** *facsimile*

RDX 05/02/16