

Calcitriol Accelerates Vascular Calcification Irrespective of Vitamin K Status in a Rat Model of Chronic Kidney Disease with Hyperphosphatemia and Secondary Hyperparathyroidism^S

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ABSTRACT

Patients with chronic kidney disease (CKD) have a markedly increased risk for developing cardiovascular disease. Non-traditional risk factors, such as increased phosphate retention, increased serum fibroblast growth factor 23 (FGF-23), and deficiencies in vitamins D and K metabolism, likely play key roles in the development of vascular calcification during CKD progression. Calcitriol [1,25-(OH)₂-D₃] is a key transcriptional regulator of matrix Gla protein, a vitamin K-dependent protein that inhibits vascular calcification. We hypothesized that calcitriol treatment would inhibit the development of vascular calcification and this inhibition would be dependent on vitamin K status in a rat model of CKD. Rats were treated with dietary adenine (0.25%) to induce CKD, with either 0, 20, or 80 ng/kg of calcitriol with low or high dietary vitamin K1 (0.2 or 100 mg/kg) for 7 weeks. Calcitriol at

both lower (20 ng/kg) and moderate (80 ng/kg) doses increased the severity of vascular calcification, and contrary to our hypothesis this was not significantly improved by high dietary vitamin K1. Calcitriol had a dose-dependent effect on: 1) lowering serum parathyroid hormone, 2) increasing serum calcium, and 3) increasing serum FGF-23. Calcitriol treatment significantly increased aortic expression of the calcification genes *Runx2* and *Pit-1*. These data also implicate impaired vitamin D catabolism in CKD, which may contribute to the development of calcitriol toxicity and increased vascular calcification. The present findings demonstrate that in an adenine-induced rat model of CKD calcitriol treatment at doses as low as 20 ng/kg can increase the severity of vascular calcification regardless of vitamin K status.

Introduction

Chronic kidney disease (CKD) affects approximately 13% of the Western population (Coresh et al., 2007; Hill et al., 2016). Patients with CKD have a marked increased risk of developing cardiovascular disease and are more likely to die of cardiovascular disease before ever requiring renal replacement therapy (Andrade and Ignaszewski, 2008). In addition to Framingham risk factors, CKD patients also have many nontraditional risk factors including disorders in calcium and phosphate metabolism, which lead to the development of vascular calcification (VC). VC occurs when calcium-phosphate crystals deposit within the medial layer of the vasculature leading to an increase in vessel stiffness, a decrease in arterial compliance, and an increase in cardiovascular and all-cause mortality (London et al., 2003). Multiple studies have confirmed associations between the extent of VC and mortality in CKD patients (Guérin et al., 2001, 2006, 2008; London et al., 2001, 2003; Safar et al., 2002; Pannier

et al., 2005; Briet et al., 2006). Fibroblast growth factor 23 (FGF-23) is a hormone that regulates phosphate homeostasis and is also elevated in the early stages of CKD. Some studies also suggest that FGF-23 could be an independent risk factor for cardiovascular disease and mortality (Gutiérrez et al., 2008; Faul et al., 2011; Isakova et al., 2011).

Vitamin D also plays a key role in calcium/phosphate homeostasis, and it is well-recognized that vitamin D metabolism becomes impaired with the development of CKD. Circulating calcitriol [1,25-(OH)₂-D₃], the active vitamin D hormone, is derived from 25-OH-D₃ via renal CYP27B1 and regulates calcium and phosphate homeostasis in concert with parathyroid hormone (PTH) and FGF-23 (Fig. 11A) (Crenshaw et al., 2011; Quarles, 2012). Many studies support the finding that, together, a loss of renal CYP27B1 activity (due to inhibition by FGF-23 and loss of renal mass) (Petkovich and Jones, 2011; Nigwekar et al., 2012) and a reduction in the levels of 25-OH-D₃ (Nigwekar et al., 2012) lead to severe calcitriol deficiency. In addition, studies support upregulation of CYP24A1, the enzyme responsible for catabolism of 25-OH-D₃ and 1,25-(OH)₂-D₃; however, the levels of these metabolites appear to be low, suggesting that vitamin D catabolism is dysregulated in CKD (Helvig et al., 2010; Jones et al., 2012). To the best of our knowledge, the impact of calcitriol therapy on the

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ABBREVIATIONS: CKD, chronic kidney disease; FGF-23, fibroblast growth factor 23; MGP, matrix Gla protein; PTH, parathyroid hormone; VC, vascular calcification.

upregulation of CYP24A1 and catabolism of vitamin D substrates has not been studied in a CKD model.

There remains considerable debate regarding the safety of vitamin D treatment in this population. A number of clinical studies have found that treating patients with vitamin D receptor activators, such as calcitriol [1,25-(OH)₂-D₃], versus no treatment provides a survival advantage for early stage CKD and end-stage kidney disease patients (Teng et al., 2003, 2005; Melamed et al., 2006; Tentori et al., 2006; Levin et al., 2008; Naves-Díaz et al., 2008; Wolf et al., 2008; Nigwekar et al., 2012). However, despite these data, treatment of CKD patients with calcitriol is primarily directed at targeting specific PTH levels. This hesitation to treat CKD patients with calcitriol may be a consequence of a number of studies in vitro and in animal models of CKD that suggest calcitriol promotes VC (Inagaki et al., 1995; Haffner et al., 2005; Henley et al., 2005; Wu-Wong et al., 2006b; Terai et al., 2009). However, emerging preclinical data suggest that treatment with lower doses of calcitriol can inhibit VC (Mathew et al., 2008; Lau et al., 2012). One explanation is that calcitriol alters vascular susceptibility to calcification in a dose- and microenvironment-dependent manner, where decreases and increases beyond the optimal therapeutic window promote VC (Razzaque, 2011; Rodriguez et al., 2011).

Calcitriol is a known transcriptional regulator of matrix Gla protein (MGP) in vascular smooth muscle cells (Farzaneh-Far et al., 2001; Proudfoot and Shanahan, 2006), bone cells (Fraser et al., 1988), and kidney (Fu et al., 2008). MGP is a vitamin K-dependent protein and a potent inhibitor of VC (Luo et al., 1997). The γ -carboxylation of five Gla domains by the enzyme γ -glutamyl carboxylase, which requires vitamin K as a cofactor, is critical to the calcium binding function of MGP (Krueger et al., 2009; Holden et al., 2012). Marked MGP upregulation has been demonstrated in vivo in the aortas of animals with experimental CKD (Sweatt et al., 2003; Lomashvili et al., 2011), although the increase is of the inactive form of MGP when there is insufficient vitamin K-mediated carboxylation. In vivo, calcitriol-induced VC is markedly enhanced in vitamin K-deficient states (e.g., warfarin therapy) (Price et al., 2000). Our group has demonstrated that long-term treatment with a vitamin K antagonist, warfarin, is independently associated with greater severity of aortic valve calcification in dialysis patients, and this effect was shown to be amplified in those subjects also taking calcitriol (Holden et al., 2007). Taken together, there may be important interactions between calcitriol and vitamin K status in the development and progression of VC. The data thus far support the hypothesis that calcitriol upregulates the expression of the key calcification inhibitor MGP; however, this requires sufficient vitamin K status to fully carboxylate and thus activate its anticalcification properties. The primary objective of this study was to determine in an animal model of CKD if calcitriol could inhibit VC and if this inhibition was dependent on adequate vitamin K status. The secondary objective was to determine the impact of calcitriol treatment on vitamin D catabolism and phosphate/calcium homeostasis in a rat model of CKD.

Materials and Methods

Experimental Animals. Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) were individually housed in standard polypropylene cages and maintained on a 12-hour light-dark

cycle. The animals used in this experiment were treated in accordance with the Canadian Council on Animal Care ethical guidelines of animal care, handling, and termination.

Treatment Groups. An animal model of CKD was employed, using dietary adenine as described previously (McCabe et al., 2013; Shobeiri et al., 2013) and a modified version of the adenine model described by Price et al. (2006). At the start of the experiments, normal Purina Rat Chow was exchanged with a specially formulated (but nutritionally balanced) diet (Harlan, Teklad, Madison, WI) on which the animals were maintained. The specially formulated diet contained either 0.25% adenine (CKD) or 0% adenine (control) along with 1% phosphate, 1% calcium, 0.2 mg/kg vitamin K1 (low vitamin K1), 1 IU/g vitamin D, and 6% protein. Male Sprague-Dawley rats (beginning at 14 weeks of age) were divided into groups receiving either 0.25% adenine (CKD) or no adenine (control). Serum creatinine levels were measured after 3 weeks and animals were stratified into one of five treatment groups to ensure each group had equivalent kidney dysfunction. For the next 4 weeks, animals were maintained on their CKD diet with either 0, 20, or 80 ng/kg body weight calcitriol maintained on low vitamin K1, or 20 and 80 ng/kg calcitriol with the addition of high dietary vitamin K1, 100 mg/kg (Fig. 1, $n = 8/\text{group}$, $N = 40$). An additional group of eight rats was maintained on a control diet (no adenine). Weights and food intake were monitored on a daily basis, and animals were supplemented with normal chow and/or Nutri-Cal if their weight loss reached 10%. There is no vitamin K or calcitriol in Nutri-Cal. The high vitamin K1 dose (100 mg/kg of diet) was selected based on previous work that demonstrated this dose of K1 blunted the development of VC in an adenine-induced rat model of CKD (McCabe et al., 2013; Kaesler et al., 2014). The low vitamin K dose (0.2 mg/kg) was chosen to create a mild deficiency state while maintaining coagulation and was based on previous work (Carrié et al., 2004; Booth et al., 2008; McCabe et al., 2013; Shobeiri et al., 2013). At euthanasia, approximately 6 ml of blood was drawn using a 22 g hypodermic needle inserted into the left ventricle of the heart while the animal was under isoflurane-induced anesthesia. Blood samples were spun (4°C, 4000 g, 20 minutes) using a BHG Hermle Z320K refrigerated centrifuge (Mandel Scientific Company Inc., Gosheim, Germany). The following tissues were collected, cleaned, and weighed: liver, kidneys, thoracic aorta, abdominal aorta, and various arteries (renal, superior mesenteric, iliac, and carotid). Tissues were collected and snap frozen in liquid nitrogen and stored at -80°C for further analysis. A portion of the kidney, liver, and thoracic aorta was also collected and stored in RNAlater storage solution (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA) and stored at -20°C until further analysis.

Serum Analysis. Serum creatinine levels were measured using QuantiChrom Creatinine Assay Kit (DICT-500) (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Serum phosphate was measured using the malachite green method as described by Heresztyn and Nicholson (2001). Serum calcium was measured using the *O*-cresolphthalein complexone method (Sigma St. Louis, MO) described previously (McCabe et al., 2013). Serum PTH levels were assessed using a rat intact PTH ELISA Kit (Immutopics, Inc. San Clemente, CA) according to the manufacturer's instructions. Serum concentrations of C-terminal FGF-23 were assessed using a Rat FGF-23 (C-term) ELISA Kit (Immutopics, Inc.) according to the manufacturer's instructions.

25-(OH)-D₃, 24,25-(OH)₂-D₃, 1,25-(OH)₂-D₃, and 1,24,25-(OH)₃-D₃. Serum 25-OH-D₃, 24,25-(OH)₂-D₃, 1,25-(OH)₂-D₃, and 1,24,25-(OH)₃-D₃ were measured using liquid chromatography-tandem mass spectrometry on a Waters BEH-C18 column (1.7 μm , 2.1 \times 50) and a Waters Acquity-Xevo-TQ-S (Waters Limited, Mississauga, ON, Canada) in multiple reactions monitoring mode (with a MeOH/H₂O gradient system) as described elsewhere (Kaufmann et al., 2014), with some modifications for 1,25-(OH)₂-D₃ and 1,24,25-(OH)₃-D₃. For measurement of 1,25-(OH)₂-D₃ and 1,24,25-(OH)₃-D₃, 150 μl of serum was equilibrated with 200 pg/ml d6-1,25-(OH)₂-D₃ and 12.5 pg/ml d6-1,24,25-(OH)₃-D₃ internal standard. The sample was incubated with 100 μl

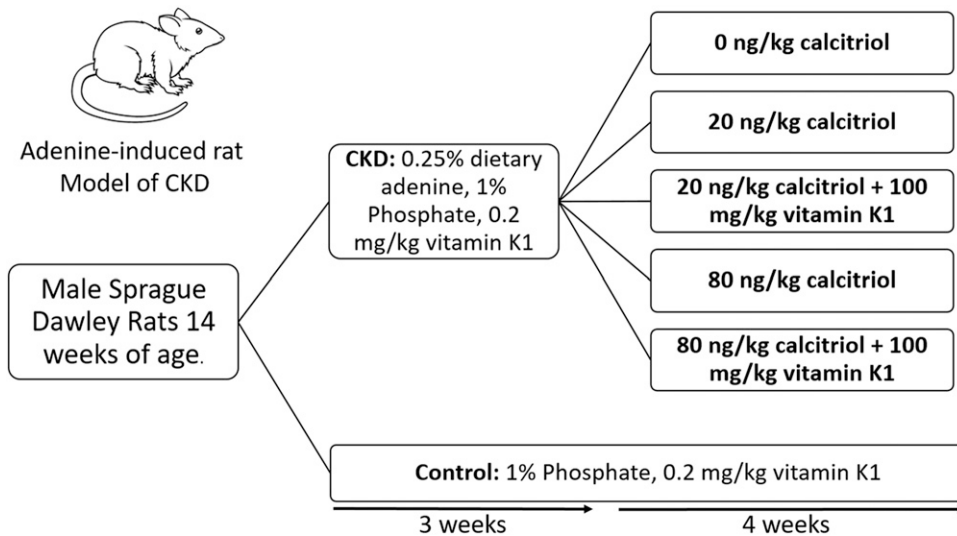


Fig. 1. A graphical description of the experimental outline.

of anti-1,25-(OH)₂D₃ antibody slurry (Immundiagnostik, Bensheim, Germany) for 2 hours at room temperature with orbital shaking at 1200 rpm. The slurry was isolated by vacuum filtration and rinsed with 4X 400 μ l of water, and vitamin D metabolites were eluted with 2X 200 μ l of ethanol. The eluate was dried and derivatized with 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) as previously described (Kaufmann et al., 2014). The sample was redissolved in 50 μ l 50/50 (percentage by volume) methanol/water and 35 μ l was injected into the liquid chromatography–tandem mass spectrometry system as previously described (Kaufmann et al., 2017). The multiple reactions monitoring transitions used for analysis of 1,25-(OH)₂D₃ and 1,24,25-(OH)₃D₃ were m/z 762 > 468 + 762 > 484 and m/z 778 > 468 + 778 > 484, respectively. Quantification was based on a six-point calibrator generated in-house containing 5–300 pg/ml 1,25-(OH)₂D₃ and 1–25 pg/ml 1,24,25-(OH)₃D₃.

Hemodynamic Measurements. Pulse wave velocity was assessed using the foot-to-foot method as described by Essalihi et al. (2003) and modified as described previously (McCabe et al., 2013). Systolic blood pressure, diastolic blood pressure, as well as pulse pressure were calculated in the Chart program using the carotid catheter.

Vessel Calcium and Phosphorus Content. Vessels (thoracic aorta, abdominal aorta, and renal, superior mesenteric, iliac, and carotid arteries) were thawed, weighed, and homogenized in 1N hydrochloric acid for 24 hours at 4°C. The samples were spun, and the calcium content was determined using the *O*-cresolphthalein complexone method (Sigma) as described previously (McCabe et al., 2013) and the phosphate levels were determined using the malachite green method as described by Heresztyn and Nicholson (2001).

Real-Time Polymerase Chain Reaction. At time of sacrifice, sections of kidney, liver, and thoracic aorta were collected and stored in RNAlater storage solution (Life Technologies, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and stored at –20°C until further analysis. Total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen, Inc. Toronto, ON, Canada) and purity and concentration were confirmed using an eukaryote total RNA nano chip on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc. Santa Clara, CA; an instrument run by the department of Pathology, Queen's University). Complimentary DNA was generated using the high-capacity cDNA reverse transcription kit (Life Technologies, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and stored at –20 until further analysis. The primers used for quantitative polymerase chain reaction are shown in Supplemental Table 1. Quantitative polymerase chain reaction was carried out on a CFX96 Real-Time System (Bio-Rad

Laboratories Inc. Montreal, QC, Canada) using SYBR Select Master Mix for CFX according to the manufacturer's instructions.

Von Kossa Method of Visualizing Vascular Calcification. The arteries were fixed in 10X neutral phosphate-buffered saline with 4% paraformaldehyde and embedded in paraffin blocks. Sections (3 to 4 μ m) were stained for calcification using Von Kossa's method (Proudfoot et al., 1998, 2000) as described previously (McCabe et al., 2013). Areas of calcification appeared as dark brown regions in the medial wall of the artery.

Statistical Analysis. To analyze correlations between parameters, stepwise linear regression analysis was performed. The quantitative polymerase chain reaction data are presented as mean \pm S.E.M., all other data are presented as mean \pm S.D. Data were compared using one-way analysis of variance followed by Newman-Keuls post hoc test for >2 groups. Given that the distribution of calcium content within the vessels was not normal, log transformation was performed prior to statistical analysis. Analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

Results

Progression of Adenine-Induced CKD and Serum Biochemistry. CKD animals were treated with 0.25% dietary adenine for a total of 7 weeks. Animals were sampled at baseline and after 3, 5, and 7 weeks for serum creatinine and phosphate (Fig. 2, A and B). Rats were treated with 0, 20, or 80 ng/kg calcitriol with low vitamin K1, and 20 or 80 ng/kg calcitriol with high vitamin K1 (100 mg/kg). Serum creatinine was elevated in all CKD groups compared with control after 3 (3-fold), 5 (7-fold), and 7 (7-fold) weeks (Fig. 2A). Serum phosphate was also elevated at 3 (1.2-fold), 5 (1.8-fold), and 7 (2.1-fold) weeks of CKD compared with control (Fig. 2B). There was no difference in serum creatinine or serum phosphate at any time point within the different CKD treatment groups.

After 7 weeks, CKD rats with 0 ng/kg calcitriol had similar levels of serum calcium to control (Fig. 3A). Serum calcium was significantly elevated with the calcitriol 20 ng/kg (1.1-fold) and 80 ng/kg (1.2 fold) doses (Fig. 3A). Serum phosphate was elevated in all CKD groups compared with control with no differences between calcitriol doses (2.3-fold for all groups) (Fig. 3B). Serum PTH was elevated 10-fold in CKD 0 ng/kg compared with control (Fig. 3C)

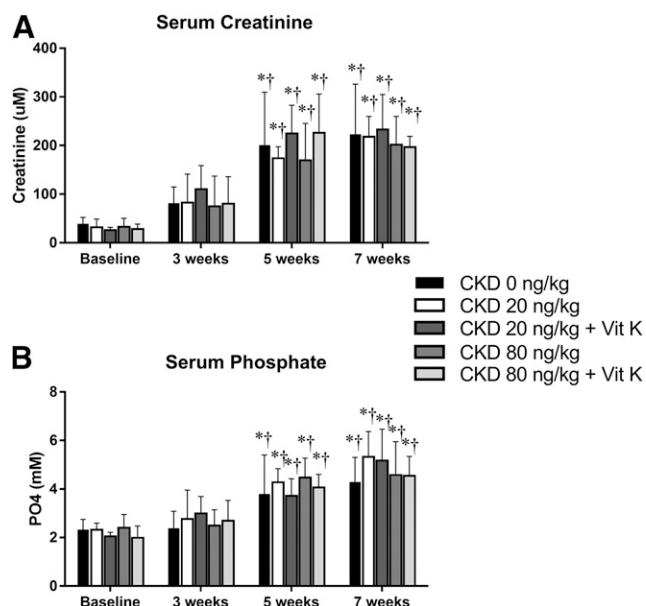


Fig. 2. Time course of serum creatinine (A) and phosphate (B) of rats given a 0.25% adenine diet for a total of 7 weeks to induce CKD. All rats were sampled for serum creatinine (A) and phosphate (B) at baseline and after 3, 5, and 7 weeks. Rats were treated with either 0 or 20 ng/kg calcitriol, or 80 ng/kg calcitriol with or without the addition of high dietary vitamin K (Vit K, 100 mg/kg). The data are shown as mean \pm S.D. ($n = 8$ /group, $*P < 0.05$ vs. baseline, $\dagger P < 0.05$ vs. 3 weeks).

and significantly decreased in a dose-dependent manner in response to calcitriol (Fig. 3C). Serum FGF-23 was elevated 83-fold in CKD 0 ng/kg compared with control (163 ± 115 RU/ml) and was further elevated in a dose-dependent manner in response to calcitriol 20 ng/kg

(288-fold) and 80 ng/kg (859-fold) (Fig. 3D). There was no significant effect of high dietary vitamin K1 on any of these measures.

Vessel Calcification. CKD increased vessel calcification compared with control in the thoracic aorta, abdominal aorta, and the renal, carotid, iliac, and superior mesenteric arteries (Fig. 4). Calcitriol treatment alone at 20 and 80 ng/kg significantly increased vessel calcium content further in all vessels studied except for the superior mesenteric artery (Fig. 4). The addition of high dietary vitamin K1 to the 20 ng/kg calcitriol group returned the vessel calcium content to the levels of the 0 ng/kg calcitriol group in all of the vessels studied except for the superior mesenteric artery (Fig. 4). There was no effect of the addition of high dietary vitamin K1 to the 80 ng/kg calcitriol treatment.

Hemodynamic Consequences of Calcification. Pulse wave velocity was significantly elevated in CKD at both 20 and 80 ng/kg of calcitriol compared with control (Fig. 5A). Pulse pressure was significantly elevated in CKD at 20 ng/kg calcitriol compared with control, and further elevated at 80 ng/kg calcitriol (Fig. 5B). There was no significant effect of the addition of high dietary vitamin K1 to the 20 or 80 ng/kg calcitriol treatment groups.

Effect of Hypercalcemia on Calcification. To determine if the calcitriol-induced hypercalcemia was associated with increased vessel calcification, we compared the vessel calcium levels in animals with and without hypercalcemia ($2.8 \mu\text{M}$ was chosen to reflect hypercalcemia because it was the upper 99% confidence interval of the mean of control serum calcium). There was no difference in vessel calcium levels between animals with or without serum hypercalcemia (Supplemental Fig. 1) and no correlation between serum calcium and vessel calcium (data not shown).

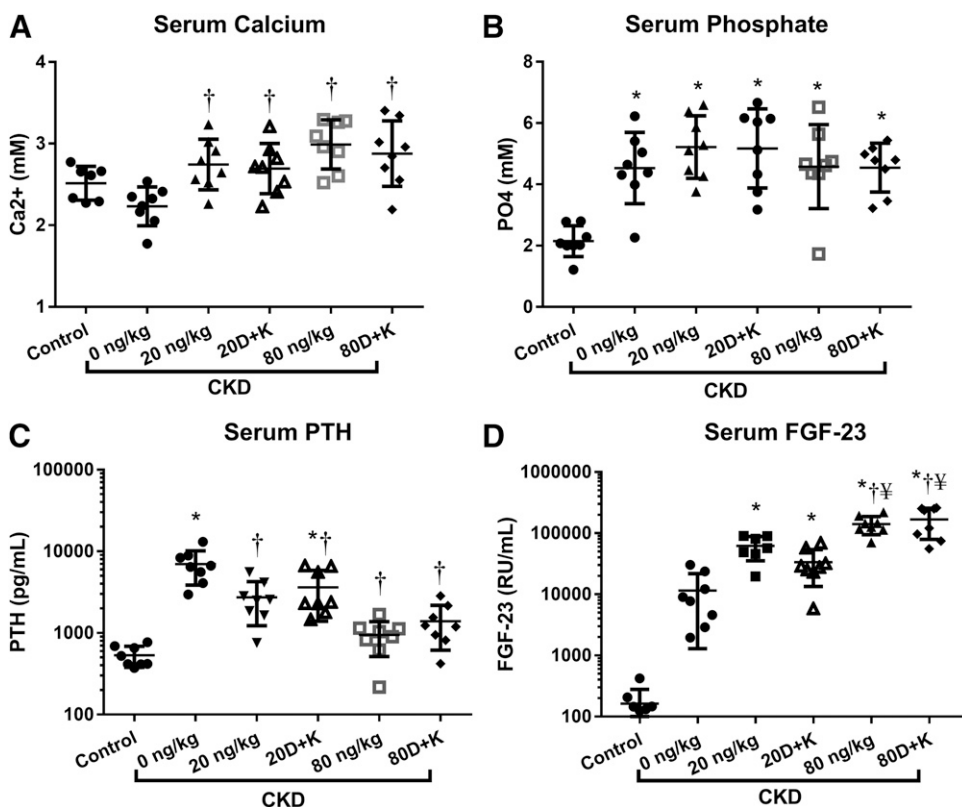


Fig. 3. Rats were given a 0.25% adenine diet for a total of 7 weeks to induce CKD or 0% adenine in control. Rats with CKD were treated with 0, 20, or 80 ng/kg calcitriol, with or without the addition of high dietary vitamin K1 (100 mg/kg, D + K). Results are shown for serum calcium (A), phosphate (B), PTH (C), and FGF-23 (D). The data are shown as mean \pm S.D. ($n = 8$ /group), $*P < 0.05$, vs. control, $\dagger P < 0.05$ vs. CKD 0 ng/kg, $\ddagger P < 0.05$ vs. CKD 20 ng/kg.

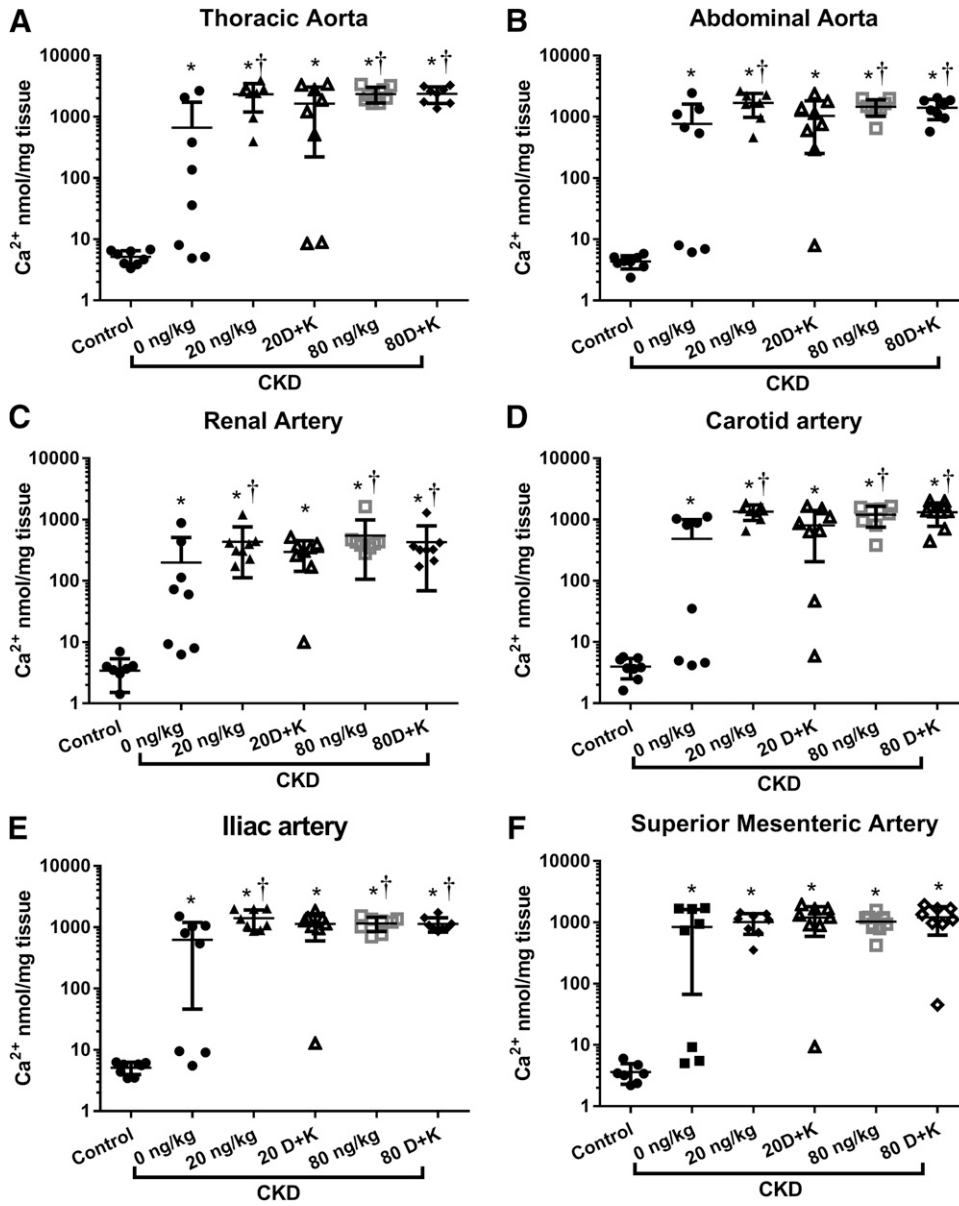


Fig. 4. Vessel calcium (nanomoles per milligram tissue) in thoracic aorta (A), abdominal aorta (B), renal artery (C), carotid artery (D), iliac artery (E), and superior mesenteric artery (F) of rats given a 0.25% adenine diet for a total of 7 weeks to induce CKD and treated with 0, 20, or 80 ng/kg calcitriol, with or without the addition of high dietary vitamin K (100 mg/kg vitamin K1, D + K). The data are shown as mean \pm S.D. ($n = 8$ /group), * $P < 0.05$ vs. control, † $P < 0.05$ vs. CKD 0 ng/kg.

Expression of Calcification Genes. To investigate the mechanism of calcitriol-induced VC we looked at its effect on kidney and thoracic aorta gene expression, since these are

two tissues known to calcify in adenine-induced CKD (Kaesler et al., 2014; McCabe et al., 2013), in control, CKD, and CKD + 80 ng/kg calcitriol animals (Figs. 6 and 7).

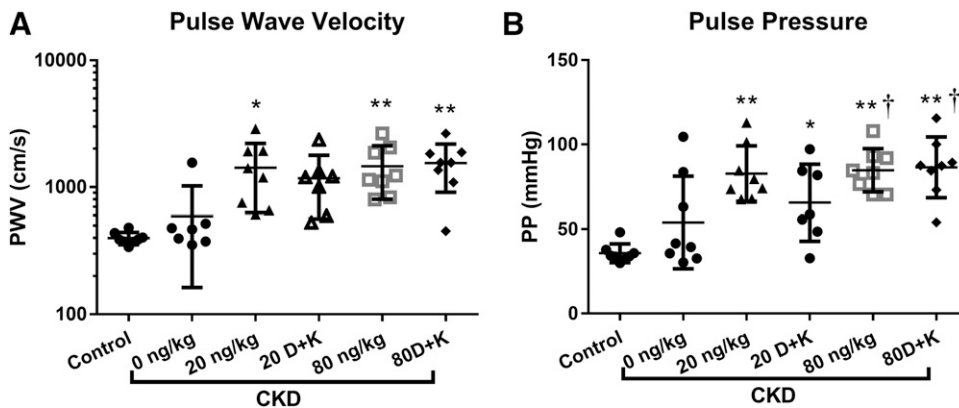


Fig. 5. Rats were given 0.25% dietary adenine for a total of 7 weeks to induce CKD or 0% adenine for control. CKD rats were treated with 0, 20, or 80 ng/kg calcitriol, with or without the addition of high dietary vitamin K (100 mg/kg vitamin K1, D + K). Pulse wave velocity (A) and pulse pressure (B) data are presented here. The data are shown as mean \pm S.D. ($n = 8$ /group), * $P < 0.05$; ** $P < 0.01$ vs. control, † $P < 0.05$ vs. CKD 0 ng/kg.

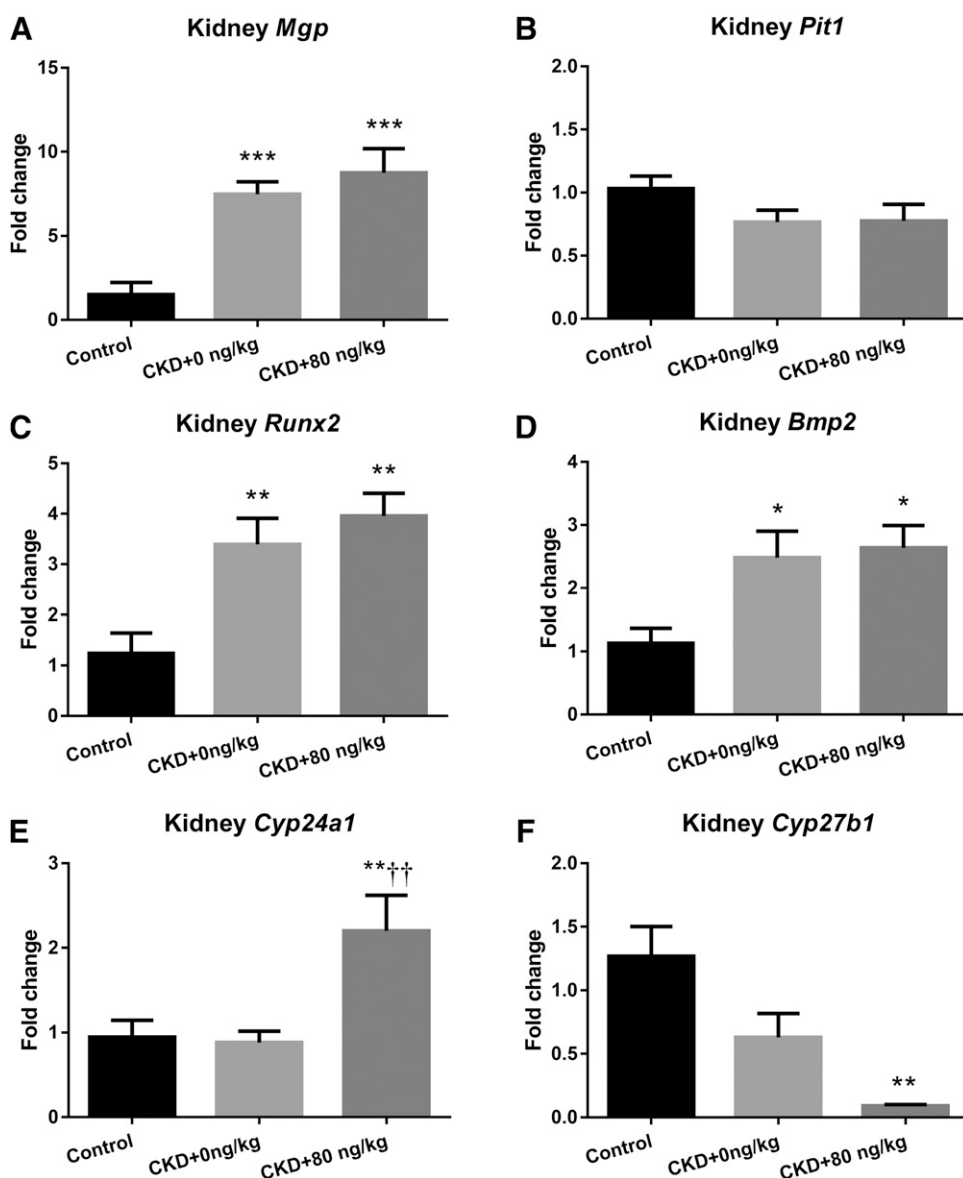


Fig. 6. *Mgp* (A), *Pit1* (B), *Runx2* (C), *Bmp2* (D), *Cyp24a1* (E), and *Cyp27b1* (F) transcripts were assessed by quantitative polymerase chain reaction in the kidney of rats treated with 0% dietary adenine (control), 0.25% dietary adenine (CKD), or 0.25% dietary adenine and 80 ng/kg calcitriol (CKD 80 ng/kg). Values are expressed as fold change vs. control rats normalized by beta-actin. The data are shown as mean \pm S.E.M. ($n = 6-8$ /group). * $P < 0.05$; ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control † $P < 0.01$ vs. CKD.

In CKD and CKD + 80 ng/kg calcitriol the kidney had significantly elevated levels of *Mgp* (7.5- and 8.8-fold, respectively), *Runx2* (3.4- and 4.0-fold, respectively), and *Bmp-2* (2.5- and 2.6-fold, respectively) compared with control (Fig. 6, A, C, and D). There was no significant change in *Pit-1* (Fig. 6B). *Cyp24a1* expression increased 2.2-fold, while *Cyp27b1* expression decreased to 0.1-fold in response to calcitriol treatment (Fig. 6, E and F). Since the liver is not known to calcify in this model of CKD, we looked at liver expression as a negative control. Liver expression of these genes showed no changes in expression (Supplemental Fig. 2). In the thoracic aorta, calcitriol treatment in CKD (80 ng/kg) significantly increased the expression *Pit-1* and *Runx2* by 3-fold compared with control (Fig. 7).

Serum Vitamin D Metabolites. Serum vitamin D metabolites 25-OH-D₃, 24,25-(OH)₂-D₃, 1,25-(OH)₂-D₃, and 1,24,25-(OH)₃-D₃ were measured. The 25-OH-D₃ levels were elevated in CKD 0 ng/kg (70% increase) compared with control (Fig. 8A), and significantly decreased with calcitriol treatment at 20 and 80 ng/kg (Fig. 8A). Serum 24,25-(OH)₂-D₃ was

decreased in CKD 0 ng/kg (40% decrease) compared with control (Fig. 8B) and calcitriol treatment dose dependently further decreased 24,25-(OH)₂-D₃ at 20 and 80 ng/kg (Fig. 8B). 1,25-(OH)₂-D₃ was not detectable in CKD rats, and was increased in a dose-dependent manner by calcitriol treatment back to the level of control rats (Fig. 8C). 1,24,25-(OH)₃-D₃ was not detectable in any of the CKD groups, regardless of calcitriol treatment. The ratio of 25-OH-D₃:24,25-(OH)₂-D₃ was elevated in CKD (approximately 2-fold) compared with control but there was no difference between calcitriol doses (Fig. 8E). There was no effect of vitamin K status on any of these measures. There was a significant linear relationship between serum 25-OH-D₃ and 24,25-(OH)₂-D₃ for all groups (Fig. 8F), with the slope (rate of 25-OH-D₃ metabolism to 24,25-(OH)₂-D₃) for control animals being significantly steeper than the slopes for all CKD groups ($P < 0.05$, Fig. 8F).

Correlations with Serum FGF-23. Overall, as serum FGF-23 levels increased, vessel calcium levels also increased until a plateau was reached (Fig. 9, A-F). There was a significant correlation between FGF-23 and vessel calcium

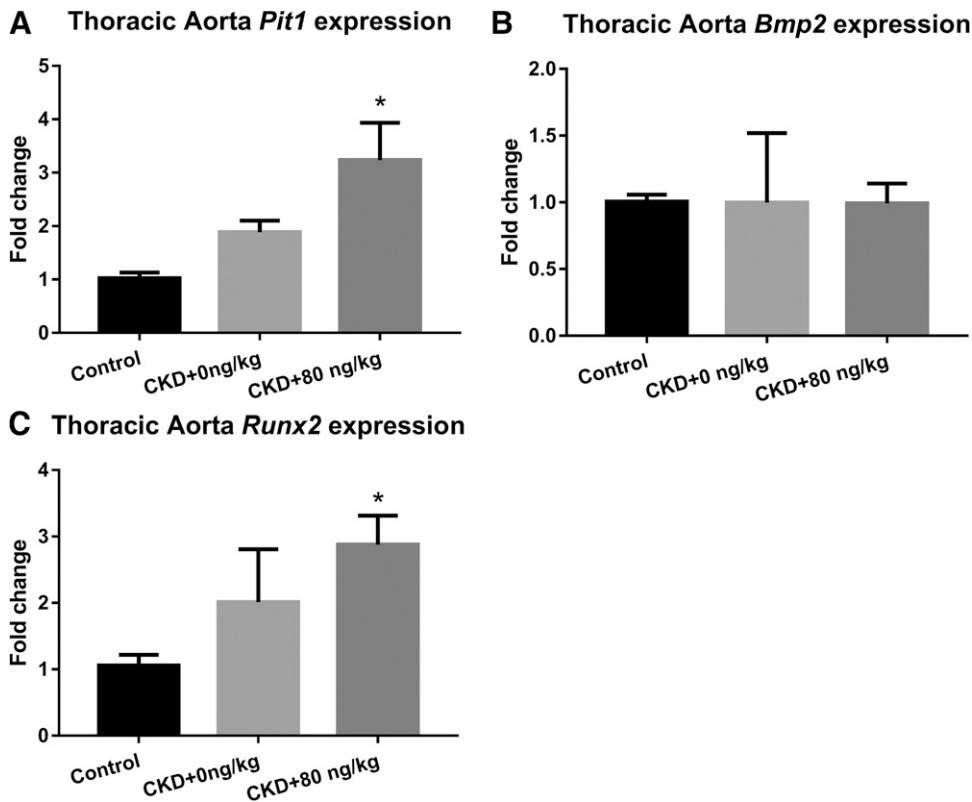


Fig. 7. *Pit1* (A), *Bmp2* (B), and *Runx2* (C) transcripts were assessed by qPCR in the thoracic aorta of rats treated with 0% dietary adenine (control), 0.25% dietary adenine (CKD + 0 ng/kg), or 0.25% dietary adenine and 80 ng/kg calcitriol (CKD + 80 ng/kg). Values are expressed as fold change vs. control rats normalized by beta-actin. The data are shown as mean \pm S.E.M. ($n = 6-8$ /group). * $P < 0.05$ vs. control.

in CKD 0 ng/kg calcitriol ($P < 0.05$) in all vessels studied except for the superior mesenteric artery ($R^2 = 0.46, 0.37, 0.32, 0.25$, and 0.28 for the thoracic aorta, abdominal aorta, carotid, iliac, and renal arteries, respectively) (Fig. 9, A–F). The correlation between FGF-23 and vessel calcium content in the animals treated with 20 and 80 ng/kg of calcitriol was not significant (Fig. 9, A–F). There was also a significant positive correlation between serum FGF-23 and the ratio of 25-OH-D₃: 24,25-(OH)₂-D₃ ($P < 0.05$, $R^2 = 0.39$), suggesting a decrease in 25-OH-D₃ metabolism at higher levels of FGF-23 (Fig. 9G). There was also a significant positive correlation between serum FGF-23 and serum calcium ($P < 0.05$, $R^2 = 0.43$) (Fig. 9H).

Von Kossa Staining. Sections of thoracic and abdominal aorta were stained for VC using the Von Kossa method, in which dark brown staining is indicative of phosphate in the crystals (Fig. 10). Staining confirmed the localization of the calcium-phosphate deposits in the vessel media (Fig. 10). There were some vessels that were positive for Von Kossa staining in the CKD group treated with 0 ng/kg calcitriol; however, in those treated with 20 or 80 ng/kg every vessel stained was Von Kossa positive (Fig. 10). No Von Kossa positive staining was found in any control animal (data not shown).

Discussion

Calcitriol, at both 20 and 80 ng/kg doses, increased the severity of VC and generated more severe hemodynamic consequences in this rat model of adenine-induced CKD. Contrary to our hypothesis, this effect of calcitriol was not significantly offset by high dietary vitamin K. Although MGP

was upregulated in this model, calcitriol treatment was also associated with upregulation of procalcific genes in the thoracic aorta. Calcitriol had the expected dose-dependent therapeutic effect of lowering PTH toward control levels, yet it also resulted in hypercalcemia and a dose-dependent increase in FGF-23 levels, both of which may also contribute directly to VC. Calcitriol treatment restored 1,25-(OH)₂-D₃ in CKD animals to the levels observed in healthy animals; however, its metabolite, 1,24,25-(OH)₃-D₃, was undetectable despite the upregulation of kidney *Cyp24a1* by calcitriol treatment. Similarly, the slope between 25-OH-D₃ and its key metabolite, 24,25-(OH)₂-D₃, was less steep in CKD animals, indicating reduced metabolism. Taken together, these data suggest that despite the upregulation of *Cyp24a1* transcription its enzymatic activity is impaired in this model. We propose that absent substrate catabolism may enhance tissue levels of 1,25-(OH)₂-D₃ and promote off-target effects such as VC. This occurred even at the relatively low dose of calcitriol used in this experiment. The transcription of *Pit-1*, a key vascular smooth muscle phosphate transporter, was significantly upregulated in calcitriol-treated rats, providing a potential signaling pathway that could directly promote VC in concert with the impact of calcitriol on circulating levels of phosphate and calcium (see Fig. 11).

In this study, 20 ng/kg calcitriol significantly increased calcification compared with 0 ng/kg; however, although the addition of high vitamin K1 to 20 ng/kg calcitriol lowered calcification levels such that they were no longer significantly greater than the 0 ng/kg group, it did not provide any further benefit. The lower calcitriol dose was chosen based on the consolidation of evidence from other experimental models (Mizobuchi et al., 2007; Mathew et al., 2008; Koleganova et al.,

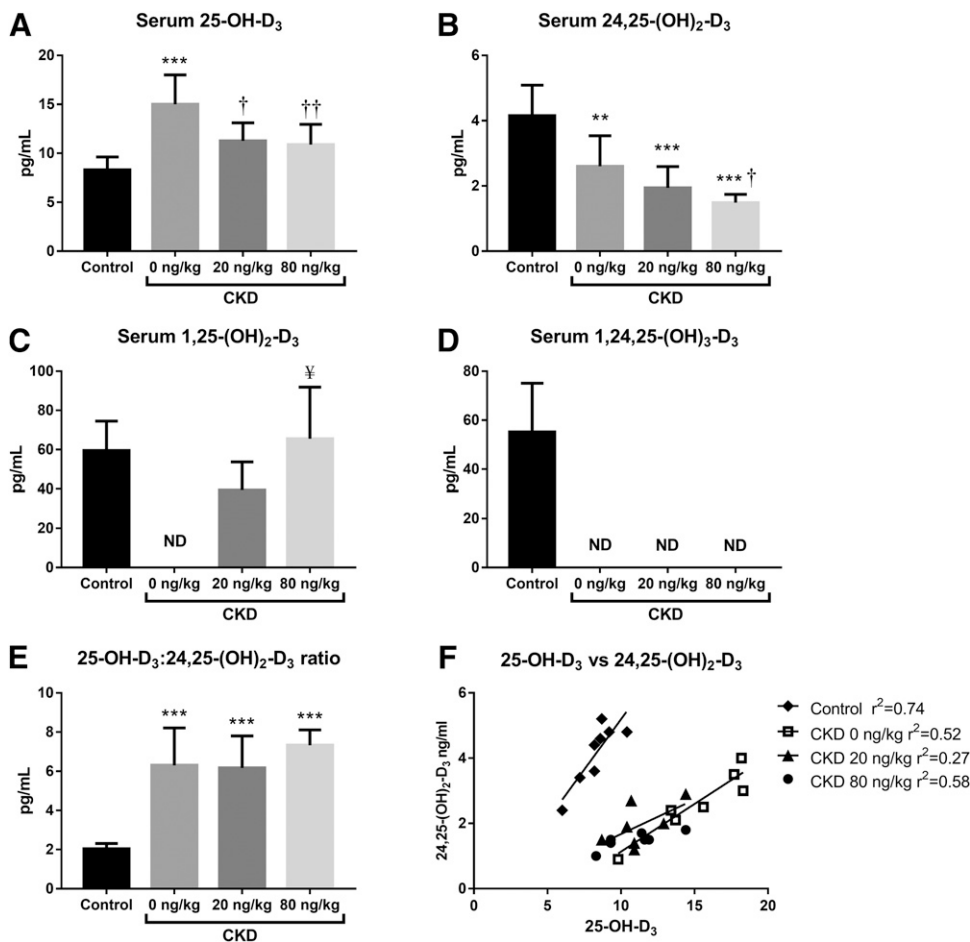


Fig. 8. Rats given a 0.25% adenine diet for a total of 7 weeks to induce CKD or 0% adenine for control. Rats with CKD were treated with 0, 20, or 80 ng/kg calcitriol. Results are shown for serum 25-OH-D₃ (A), 24,25-(OH)₂-D₃ (B), 1,25-(OH)₂-D₃ (C), 1,24,25-(OH)₃-D₃ (D), and the ratio of 25-OH-D₃:24,25-(OH)₂-D₃ (E). Data are presented as mean \pm S.D., $n = 8$ /group; ** $P < 0.01$, *** $P < 0.001$ vs. control; † $P < 0.05$, †† $P < 0.01$ vs. CKD 0 ng/kg; ‡ $P < 0.05$ vs. CKD 20 ng/kg. (F) Linear relationship between 25-OH-D₃ and 24,25-(OH)₂-D₃ ($P < 0.05$, r^2 shown in graph).

2009; Terai et al., 2009; Lau et al., 2012). This 20 ng/kg dose has been shown to increase (Mizobuchi et al., 2007; Koleganova et al., 2009) or decrease (Mathew et al., 2008; Lau et al., 2012) VC in various kidney disease models. Two studies performed in a murine model of CKD showed that a daily dose of 20 ng/kg of calcitriol decreased calcification of atherosclerotic plaques (Mathew et al., 2008) and medial VC (Lau et al., 2012), respectively. In these murine studies, phosphate levels were elevated in CKD approximately 1.5-fold compared with control, and calcitriol treatment significantly decreased serum phosphate nearly back to control levels (Mathew et al., 2008; Lau et al., 2012) and consequently also decreased serum FGF-23 (Lau et al., 2012). However, in our study, serum phosphate was over 2-fold elevated in all CKD groups and a significant increase in serum calcium and FGF-23 was observed in all calcitriol-treated rats. Phosphate and calcium are well-known initiators of VC (Block, 2001; Reynolds et al., 2004; Lau et al., 2010) and FGF-23 levels are associated with increased cardiovascular consequences (Parker et al., 2010; Seiler et al., 2010). It may be that the adenine-induced rat model produced more severe CKD and that the rat model is more susceptible to changes in serum phosphate/calcium in response to a high phosphate diet and/or calcitriol. Dialysis patients treated with calcitriol frequently experience hyperphosphatemia and hypercalcemia; therefore, these changes are similarly observed in humans and have been linked to VC in this population (Goldsmith et al., 1997; Guérin et al., 2001).

To examine whether the increased serum calcium was a key factor linked to the increased VC, we compared the severity of vessel calcification in rats with and without hypercalcemia and found that there was no difference in VC, suggesting that additional factors must be involved. This is consistent with previous research, which has reported that calcitriol can induce calcification in uremic and nonuremic rats without necessarily inducing changes in serum calcium and phosphate (Koleganova et al., 2009).

The transcription of MGP, a key local inhibitor of VC, is regulated by calcitriol. MGP was upregulated in response to the uremic environment but was not further upregulated significantly in response to calcitriol. The upregulation of MGP expression may have been near its maximum response in CKD (7.5-fold increase) and thus was not able to significantly increase any further in response to calcitriol (8.7-fold increase). Our original hypothesis that calcitriol at low doses would inhibit VC in the presence of a high vitamin K diet was based on presumed upregulation of MGP, which we did not observe. Ultimately, the severity of VC is determined by a balance between those factors that promote VC (e.g., hypercalcemia and hyperphosphatemia) and those that inhibit VC (e.g., MGP). Despite overall upregulation of MGP in the CKD rats there was no impact of calcitriol on MGP expression nor was there any interaction between calcitriol and vitamin K status with respect to the calcification outcome. Previous studies in this adenine model have shown that the identical dose of vitamin K attenuated VC (McCabe et al., 2013; Kaesler

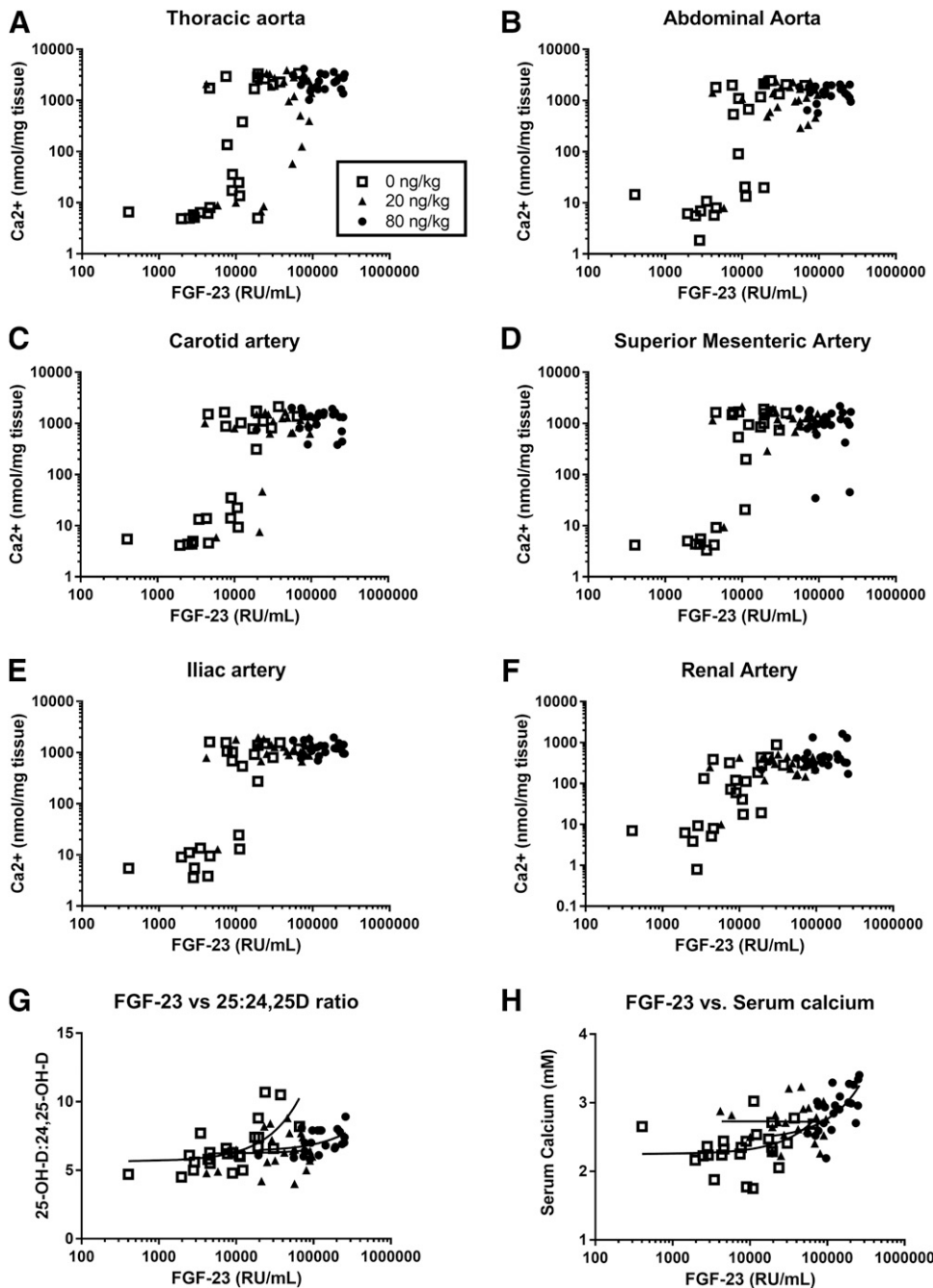


Fig. 9. Correlation between serum FGF-23 on the x-axis and vessel calcium level on the y-axis in the thoracic aorta (A), abdominal aorta (B), carotid (C), superior mesenteric (D), iliac (E), and renal (F) arteries and correlation between serum FGF-23 and the ratio of 25-OH-D:24,25-OH-D (G) and serum calcium (H) in rats treated with 0.25% dietary adenine and either 0 ng/kg (□), 20 ng/kg (▲), or 80 ng/kg (●) of calcitriol; 0 ng/kg correlations are significant in each artery except for the superior mesenteric ($P < 0.05$). $n = 22-24/\text{group}$.

et al., 2014; Zaragatski et al., 2016); however, neither of these studies included calcitriol treatment and levels of serum calcium were not different from control animals. Furthermore, FGF-23 levels were significantly elevated in a dose-dependent manner in response to calcitriol treatment. Taken together, the inhibitory effect of adequate MGP carboxylation was not sufficient to surmount the adverse effects of calcitriol on the vasculature in this model of CKD.

It has been suggested that calcitriol has a direct effect within the vasculature and therefore may have broad implications in cardiovascular health (Jones, 2007). To further examine this, we looked at the expression of some key calcification regulating genes in response to calcitriol treatment. *Runx2*, a marker of osteogenic transformation,

was upregulated in response to calcitriol in the thoracic aorta. There was also a significant impact of calcitriol treatment on the expression of *Pit1* in the thoracic aorta, which was not observed in the kidney. PIT1 is a sodium-dependent phosphate transporter that is primarily responsible for phosphate uptake into vascular smooth muscle cells, an event that initiates the process of VC. These results indicate a direct effect of calcitriol treatment on the regulation of procalcific genes in vascular tissue and support the work of others (Mizobuchi et al., 2007; Koleganova et al., 2009) indicating that there may indeed be a tissue-specific response. Furthermore, a study by Wu-Wong et al. (2006a) looked at the effect of calcitriol on gene expression in human coronary artery smooth muscle cells. They found a total of

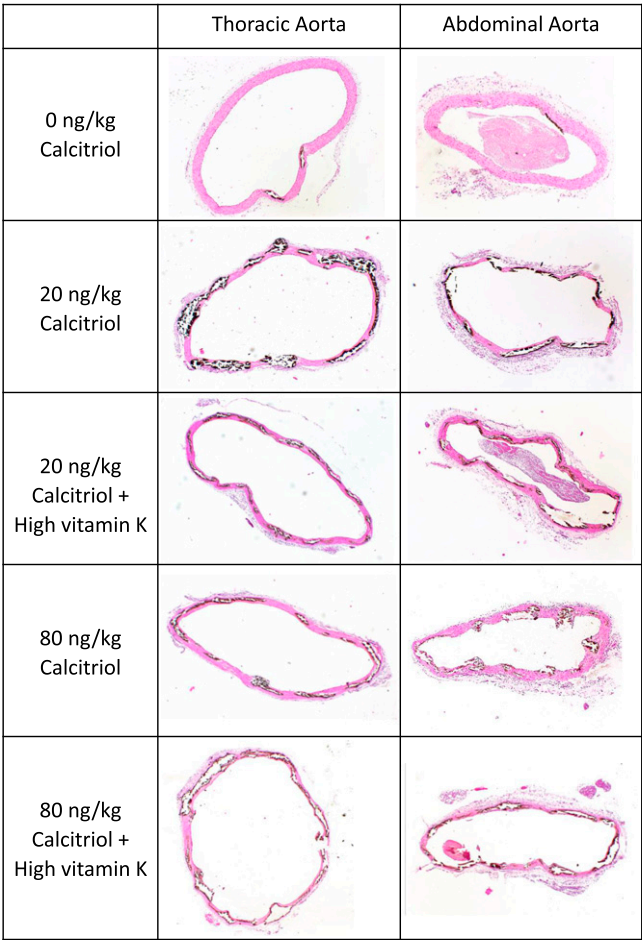


Fig. 10. Von Kossa stained blood vessels from animals treated with 0.25% dietary adenine along with low dietary vitamin K1 (0.2 mg/kg food) or high dietary vitamin K1 (100 mg/kg food) and 0, 20, or 80 ng/kg calcitriol. Positive staining (dark brown areas in vessel) denotes the region of calcification in the thoracic and abdominal aortas. All photographs were taken at the same magnification.

176 target genes of calcitriol; therefore, it is also possible that genes other than those measured here are implicated in development of VC.

CYP24A1 is the major route of catabolism of both calcitriol (1,25-(OH)₂-D₃) and 25-OH-D₃ into 1,24,25-(OH)₃-D₃ and 24,25-(OH)₂-D₃, respectively (Jones et al., 2012). The expression of *Cyp24a1* in the kidney was similar in control and untreated CKD rats but was markedly upregulated in response to calcitriol (Helvig et al., 2010). Despite this, there was a significantly smaller increment in 24,25-(OH)₂-D₃ for a given increment in 25-OH-D₃ in CKD animals compared with controls, suggesting a decrease in 25-OH-D₃ catabolism. Human data, pooled from five large cohort studies, similarly showed that the relationship between 24,25-(OH)₂-D₃ and 25-OH-D₃ was modified by eGFR, in which the slope became significantly less steep as kidney function declined (de Boer et al., 2014). Batacchi et al. (2017) also found that while vitamin D₂ supplementation induces 1,25-(OH)₂-D₃ catabolism in control subjects this response is blunted in individuals with CKD. It was surprising that the 25-OH-D₃ levels were higher in the CKD rats than in control animals since this finding is not typical for humans with CKD. The reasons for the higher levels of 25-OH-D₃ in this experiment

are unknown but could reflect severe abnormalities with 25-OH-D₃ catabolism and, therefore, accumulation in this model. CYP24A1 also metabolizes calcitriol, and despite restored levels of 1,25-(OH)₂-D₃ with calcitriol treatment the metabolite 1,24,25-(OH)₃-D₃ could not be detected in the CKD animals. These data support that CYP24A1 enzymatic function may be severely impaired in CKD. The absence of calcitriol catabolism may be a key factor contributing to calcitriol toxicity, which can be characterized by VC, hypercalcemia, and markedly elevated levels of FGF-23. FGF-23 inhibits activation of vitamin D, and in a negative feedback manner active vitamin D stimulates FGF-23 (Wolf, 2010). There are potentially many off-target effects of FGF-23 that may be detrimental (Jüppner et al., 2010) and studies have shown that an elevated FGF-23 is associated with increased risk of cardiovascular events (Seiler et al., 2010; Kendrick et al., 2011) and mortality (Jean et al., 2009). In our study, there was a positive correlation between FGF-23 and VC in the CKD animals. Although FGF-23 has been implicated in the upregulation of CYP24, the ratio between 25-OH-D₃ and 24,25-(OH)₂-D₃ increased significantly with higher FGF-23 levels in all rats with CKD. Similarly, Dai et al. (2012) reported a correlation between higher FGF-23 levels and reduced 24,25-(OH)₂-D₃ concentrations. Taken together, these data and the work of Dai et al. (2012) do not support FGF-23-mediated catabolism of vitamin D metabolites as has been suggested by others (Hasegawa et al., 2010; Helvig et al., 2010). One possibility is that 24,25-(OH)₂-D₃ is rapidly cleared to calcitroic acid in CKD. Measurement of the enzymatic activity of CYP24A1 within the kidney and further downstream metabolites would help resolve some of these uncertainties.

In summary, human studies indicate that low levels of serum calcitriol are associated with an increase in VC (Doherty et al., 1997; Watson et al., 1997), and patients treated with calcitriol to manage secondary hyperparathyroidism have an increased survival rate (Wu-Wong et al., 2006b; Mathew et al., 2008; Terai et al., 2009; Razzaque, 2011). The ADVANCE study showed that calcimimetics with low-dose vitamin D sterols in dialysis patients was associated with less progression of coronary calcium score than patients taking greater doses of vitamin D sterols alone (Raggi et al., 2011; Ureña-Torres et al., 2013), suggesting that a combination of therapies may have a greater impact. Whether calcitriol treatment accelerates calcification remains controversial. In our experimental animal model of CKD, we found that 20 and 80 ng/kg doses of calcitriol clearly accelerated VC and its hemodynamic consequences and that this effect of calcitriol was not significantly modified by the underlying vitamin K status. Furthermore, these data implicate impaired vitamin D catabolism in CKD, which may contribute to the development of calcitriol toxicity. Further studies assessing CYP24A1 enzyme activity and the full profile of vitamin D metabolites are necessary to help explain these findings. This experiment is an example of the complexities of VC in chronic kidney disease. Phosphate, calcium, PTH, FGF-23, and direct vascular effects of calcitriol, along with other factors not analyzed in this study, including serum Mg²⁺ (ter Braake et al., 2018) and pyrophosphate (Lomashvili et al., 2014), may all independently contribute to VC and represent a complex interplay of factors that need to be

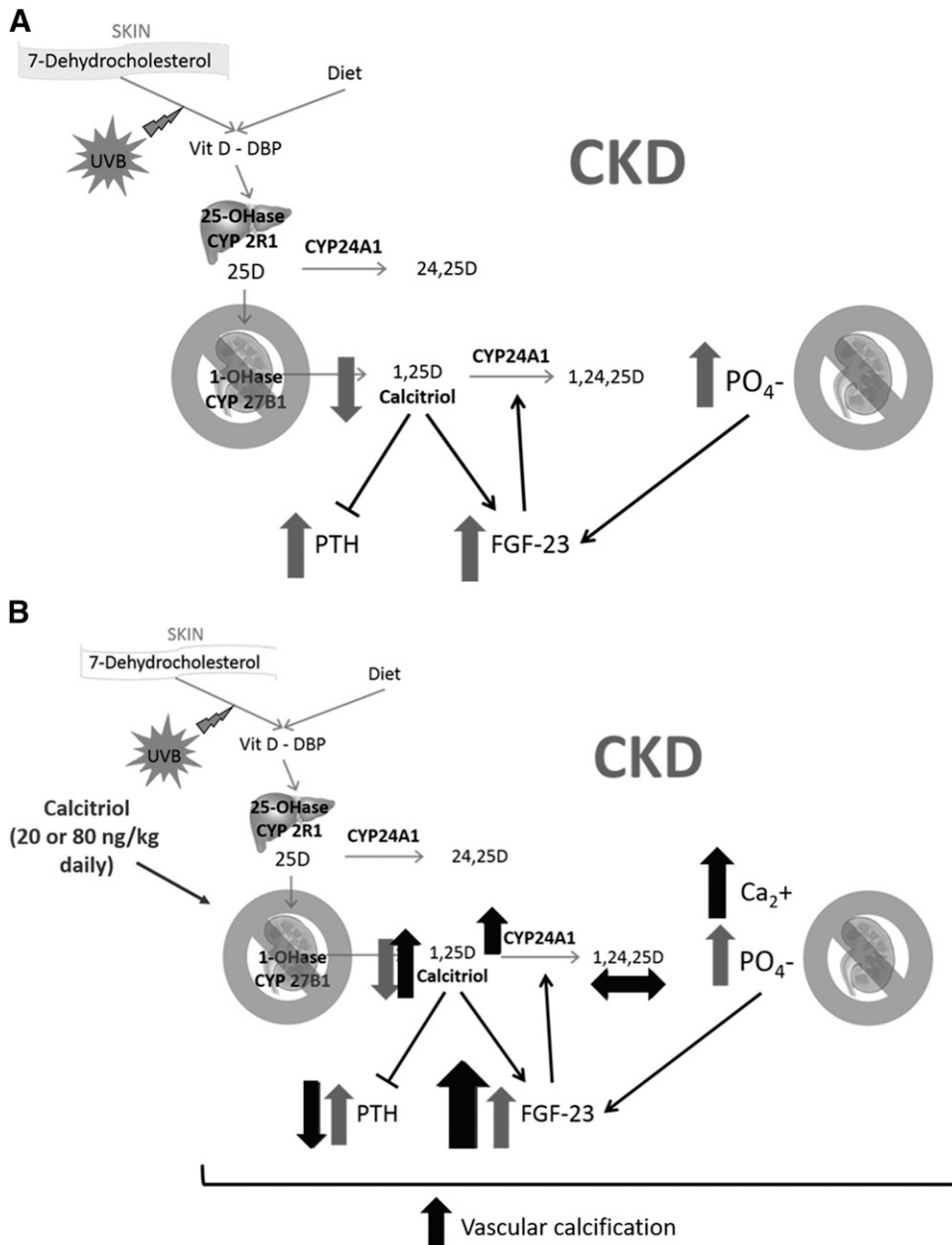


Fig. 11. Calcitriol metabolism in CKD. (A) Vitamin D, acquired either via the diet or via the sun, is hydroxylated to 25-OH-D₃ in the liver by the enzymes CYP2R1 and CYP27A1 and then hydroxylated a second time primarily in the kidneys by CYP27B1 to 1,25-(OH)₂-D₃ (calcitriol), the active form. Calcitriol increases calcium and phosphate reabsorption and resorption in the small intestine and bone, respectively. It also increases FGF-23, a hormone that decreases phosphate reabsorption in the kidney, and in a negative feedback manner upregulates CYP24A1, which is the enzyme that metabolizes calcitriol. Patients with CKD have a deficiency in 1,25-(OH)₂-D₃ (calcitriol) due to loss of renal CYP27B1 with declining renal function and upregulation of the catabolic enzyme CYP24A1 by FGF-23. This decrease in 1,25-(OH)₂-D results in secondary hyperparathyroidism, which activates FGF-23 and, in turn, further inhibits renal CYP27B1. Despite high FGF-23 levels, further decline in renal function leads to a decrease in phosphate excretion and, therefore, an increase in circulating phosphate. The end result is a 1,25-OH-D₃-deficient, high-PTH, high-FGF-23, and hyperphosphatemic state. (B) With calcitriol treatment in our rat model of CKD, levels of circulating calcitriol and PTH were both normalized back to control levels; however, serum FGF-23 was further increased beyond CKD levels, serum Ca²⁺ was elevated, and despite an increase in the expression of CYP24A1 the metabolite 1,24,25-(OH)₃-D₃ was still undetectable. These changes are indicated by the black arrows.

considered in the management of this disease. Further research regarding the optimal therapeutic window of calcitriol is needed and will likely involve a more personalized medicine targeting multiple factors involved.

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Authorship Contributions

Participated in research design: McCabe, Zelt, Kaufmann, Jones, Adams, Holden.

Conducted experiments: McCabe, Zelt, Kaufmann, Laverty, Ward, Barron.

Contributed new reagents or analytic tools: Kaufmann, Jones.

Performed data analysis: McCabe, Zelt, Kaufmann.

Wrote or contributed to the writing of the manuscript: McCabe, Adams, Holden.

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