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Title

Calcitriol accelerates vascular calcification irrespective of vitamin K status in a rat model of CKD with hyperphosphatemia and secondary hyperparathyroidism

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Running title
Calcitriol and vitamin K treatment in a model of CKD

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ABSTRACT

Patients with chronic kidney disease have a markedly increased risk for developing cardiovascular disease. Non-traditional risk factors, such as increased phosphate retention, increased serum FGF-23, and deficiencies in vitamins D and K metabolism, likely play key roles in the development of vascular calcification during CKD progression. Calcitriol ($1,25\text{-}(OH)_2\text{-}D_3$) is a key transcriptional regulator of Matrix Gla protein (MGP), 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: (i) lowering serum PTH, (ii) increasing serum calcium and (iii) increasing serum FGF-23. Calcitriol treatment significantly increased aortic expression of the calcification genes *Runx2* and *Pit-1*. This data also implicates 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.

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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 for developing cardiovascular disease (CVD) and they are more likely to die of CVD before ever requiring renal replacement therapy (Andrade and Ignaszewki, 2008). In addition to Framingham risk factors, CKD patients also have many non-traditional risk factors including disorders in calcium and phosphate metabolism which leads 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 extent of VC and mortality in CKD patients (Guerin *et al.*, 2001; London *et al.*, 2001, 2003; Safar *et al.*, 2002; Pannier *et al.*, 2005; Briet *et al.*, 2006; Guérin *et al.*, 2006, 2008). 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 ($1,25\text{-}(OH)_2\text{-}D_3$), the active vitamin D hormone, is derived from $25\text{-}OH\text{-}D_3$ via renal CYP27B1 and regulates calcium and phosphate homeostasis in concert with parathyroid hormone (PTH) and FGF-23 (Figure 11A) (Crenshaw *et al.*, 2011; Quarles, 2012). Many studies support 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)) as well as a reduction in the

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levels of 25-OH-D_3 (Nigwekar *et al.*, 2012) that, together, lead to severe calcitriol deficiency. In addition, studies support up-regulation of CYP24A1, the enzyme responsible for catabolism of 25-OH-D_3 and 1,25-$(OH)_2$-D_3, yet 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). The impact of calcitriol therapy on the up-regulation of CYP24A1 and catabolism of vitamin D substrates has not been studied in a CKD model to the best of our knowledge.

There remains considerable debate about 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-$(OH)_2$-D_3), versus no treatment provides a survival advantage for early-stage CKD and end-stage kidney disease (ESKD) 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 that calcitriol promotes VC (Inagaki *et al.*, 1995; Haffner *et al.*, 2005; Henley *et al.*, 2005; Wu-Wong, Noonan, *et al.*, 2006; Terai *et al.*, 2009). However, emerging pre-clinical data suggests 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 (VSMCs) (Farzaneh-Far *et al.*, 2001; Proudfoot and Shanahan, 2006), bone

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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 (GGCX), which requires vitamin K as a co-factor, is critical to the calcium binding function of MGP (Krueger *et al.*, 2009; Holden *et al.*, 2012). Marked MGP up-regulation 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 supports the hypothesis that calcitriol up-regulates the expression of the key calcification inhibitor MGP but this requires sufficient vitamin K status to fully carboxylate and thus activate its anti-calcification 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.

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MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec) were individually housed in standard polypropylene cages and maintained on a 12 h light-dark cycle. The animals used in this experiment were treated in accordance with Canadian Council on Animal Care ethical guidelines of animal care, handling, and termination.

Treatment groups

An animal model of CKD using dietary adenine was used as described previously (McCabe *et al.*, 2013; Shobeiri *et al.*, 2013), a modified version of the adenine model described by Price *et al* (Price *et al.*, 2006). At the start of the experiments, the normal Purina Rat Chow was exchanged with a specially formulated (but nutritionally balanced) diet (Harlan, Teklad, Madison, WI) on which the animals were maintained. 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), 1IU/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 5 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 ng/kg, 20 ng/kg, or 80 ng/kg calcitriol maintained on low vitamin K1, or 20 and 80 ng/kg calcitriol with the addition of high dietary vitamin K1, 100 mg/kg (Figure 1, n=8/group, N=40). An additional group of 8 rats were 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

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if their weight loss reached 10%. There is no vitamin K or calcitriol in Nutri-Cal. The high vitamin K1 dose (100 mg/kg) was selected based on previous work that demonstrated that this dose of K1 blunted the development of vascular calcification 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 (Carrie *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 22g hypodermic needle inserted into the left ventricle of the heart while the animal was under isoflurane induced anesthesia. Blood samples were spun (4°C, 4000g, 20 min) 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, Waltham, MA, USA) 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, USA) as per manufacturer's instructions. Serum phosphate was measured using the malachite green method as described by Heresztyn and Nicholson (Heresztyn and Nicholson, 2001). Serum calcium was measured using the O-cresolphthalein complexone method (Sigma) described previously (McCabe *et al.*, 2013). Serum PTH levels were assessed using a rat intact PTH ELISA kit (Immutopics, Inc. San Clemente, CA, USA) as per manufacturer's instructions. Serum concentrations of C-terminal FGF-23 were assessed

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using a 'Rat FGF-23 (C-term) ELISA kit' (Immutopics, Inc. San Clemente, CA, USA) as per manufacturer's instructions.

25-OH-D_3, 24,25-$(OH)_2$-D_3, 1,25-$(OH)_2$-D_3, and 1,24,25-$(OH)_3$-D_3

Serum 25-OH-D_3, 24,25-$(OH)_2$-D_3, 1,25-$(OH)_2$-D_3, and 1,24,25-$(OH)_3$-D_3 were measured using LC-MSMS on a Waters BEH-C18 column (1.7 micron, 2.1x50), and a Waters Acquity-Xevo-TQ-S in MRM mode (with a MeOH/H_2O gradient system) as described elsewhere (Kaufmann *et al.*, 2014) with some modifcations for 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$. For measurement of 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$, 150 uL of serum was equilibrated with 200 pg/mL d6-1,25-$(OH)_2D_3$ and 12.5 pg/mL d6-1,24,25-$(OH)_3D_3$ internal standard. The sample was incubated with 100 uL of anti-1,25-$(OH)_2D_3$ antibody slurry (Immundiagnostik) for 2h at room temperature with orbital shaking at 1200 RPM. The slurry was isolated by vacuum filtration and rinsed with 4X400 uL of water, and vitamin D metabolites were eluted with 2X200 uL of ethanol. The eluate was dried and derivatized with DMEQ-TAD as previously described (Kaufmann *et al.*, 2014). The sample was re-dissolved in 50 uL 50/50 (% by vol.) Methanol/Water and 35 uL was injected into the LC-MS/MS system as previously described (Kaufmann *et al.*, 2017). MRM transitions used for analysis of 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ were m/z 762->468 + 762-> 484, and m/z 778->468 + 778-> 484. Quantification was based on a 6-point calibrator generated in-house containing 5-300 pg/mL 1,25-$(OH)_2D_3$ and 1-25 pg/mL 1,24,25-$(OH)_3D_3$.

Hemodynamic measurements

Pulse wave velocity was assessed using the foot-to-foot method as described by Essalihi *et al.* (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 Chart program using the carotid catheter.

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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 (HCL) for 24 h 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 using the malachite green method as described by Heresztyn and Nicholson (Heresztyn and Nicholson, 2001).

Real-Time PCR

At time of sacrifice a section of kidney, liver, and thoracic aorta were collected and stored in RNAlater storage solution (Life Technologies, Thermo Fisher Scientific inc. Waltham, MA, USA) as per manufacturer's instructions and stored at -20˚C until further analysis. Total RNA was extracted using RNeasy Plus Universal kit (Qiagen) and purity and concentration was confirmed using an Eukaryote total RNA nano chip on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc. 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. Waltham, MA, USA) as per manufacturer's instructions and stored at -20 until further analysis. Primers used for qPCR are shown in supplemental table 1. qPCR was carried out on a CFX96 Real-Time System (Bio-Rad Laboratories Inc.) using SYBR Select Master Mix for CFX as per 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 (3-4μm) were stained for calcification with Von Kossa's

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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. qPCR data is presented as mean ± SEM, all other data is presented as mean ± SD. Data were compared using a one-way analysis of variance (ANOVA) 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 Graph Pad Prism v.5.

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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, 3, 5, and 7 weeks for serum creatinine and phosphate (Figure 2 A,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 to control after 3 (3 fold), 5 (7 fold), and 7 weeks (7 fold, Figure 2A). Serum phosphate was also elevated at 3 (1.2 fold), 5 (1.8 fold), and 7 (2.1 fold) weeks of CKD compared to control (Figure 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 (Figure 3A). Serum calcium was significantly elevated with calcitriol doses 20 ng/kg (1.1 fold) and 80 ng/kg (1.2 fold, Figure 3A). Serum phosphate was elevated in all CKD groups compared to control with no differences between calcitriol doses (2.3 fold for all groups, Figure 3B). Serum PTH was elevated 10 fold in CKD 0 ng/kg compared to control (Figure 3C) and significantly decreased in a dose dependent manner in response to calcitriol (Figure 3C). Serum FGF-23 was elevated 83 fold in CKD 0 ng/kg compared to 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, Figure 3D). There was no significant effect of high dietary vitamin K1 on any of these measures.

Vessel calcification

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CKD increased vessel calcification compared to control in the thoracic aorta, abdominal aorta, and the renal, carotid, iliac, and superior mesenteric arteries (Figure 4 A-F). Calcitriol treatment alone at 20 ng/kg and 80 ng/kg significantly increased vessel calcium content further in all vessels studied except for the superior mesenteric artery (Figure 4 A-F). 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 the vessels studied except for the superior mesenteric artery (Figure 4 A-F). 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 (PWV) was significantly elevated in CKD at both 20 ng/kg and 80 ng/kg of calcitriol compared to control (Figure 5A). Pulse pressure (PP) was significantly elevated in CKD at 20 ng/kg calcitriol compared to control, and further elevated at 80 ng/kg calcitriol (Figure 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 uM 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 Figure 1) and no correlation between serum calcium and vessel calcium (data not shown).

Expression of Calcification Genes

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To investigate the mechanism of calcitriol-induced vascular calcification we looked at its effect on kidney and thoracic aorta gene expression, as these are two tissues known to calcify in adenine-induced CKD (McCabe *et al.* 2013; Kaesler *et al.* 2013), in control, CKD, and CKD + 80 ng/kg calcitriol animals (Figure 6 and 7). 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 to control (Figure 6 A,C,D). There was no significant change in *Pit-1* (Figure 6B). *Cyp24a1* expression increased 2.2 fold while *Cyp27b1* expression decreased to 0.1 fold in response to calcitriol treatment (Figure 6E,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 figure 2).

In the thoracic aorta, calcitriol treatment in CKD (80 ng/kg) significantly increased the expression *Pit-1* and *Runx2* by 3 fold compared to control (Figure 7).

Serum Vitamin D Metabolites

Serum vitamin D metabolites 25-OH-D_3, 24,25-$(OH)_2$-D_3, 1,25-$(OH)_2$-D_3, and 1,24,25-$(OH)_3$-D_3 were measured. The 25-OH-D_3 levels were elevated in CKD 0 ng/kg (70% increase) compared to control (Figure 8A), and significantly decreased with calcitriol treatment at 20 ng/kg and 80 ng/kg (Figure 8A). Serum 24,25-$(OH)_2$-D_3 was decreased in CKD 0 ng/kg (40% decrease) compared to control (Figure 8B) and calcitriol treatment dose-dependently further decreased 24,25-$(OH)_2$-D_3 at 20 ng/kg and 80 ng/kg (Figure 8B). 1,25-$(OH)_2$-D_3 was not detectable in CKD rats, and was increased in a dose dependent manner by calcitriol treatment back to the level of control rats (Figure 8C). 1,24,25-$(OH)_3$-D_3 was not detectable in any of the CKD groups, regardless of calcitriol treatment. The ratio of 25-OH-D_3: 24,25-$(OH)_2$-D_3 was

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elevated in CKD (approximately 2 fold) compared to control but there was no difference between calcitriol doses (Figure 8E). There was no effect of vitamin K status on any of these measures. There was a significant linear relationship between serum 25-OH-D_3 and 24,25-$(OH)_2$-D_3 for all the groups (Figure 8F) with the slope (rate of 25-OH-D_3 metabolism to 24,25-$(OH)_2$-D_3) for control animals being significantly steeper than the slopes for all CKD groups ($p<0.05$, Figure 8F).

Correlations with Serum FGF-23

Overall, as serum FGF-23 levels increase, vessel calcium levels also increase until a plateau was reached (Figure 9 A-F). There was a significant correlation between FGF-23 and vessel calcium in CKD 0 ng/kg calcitriol ($p<0.05$) in all vessels studied except 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, Figure 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 (Figure 9 A-F).

There is also a significant positive correlation between serum FGF-23 and the ratio of 25-OH-D_3:24,25-$(OH)_2$-D_3 ($p<0.05$, R^2=0.39) suggesting a decrease in 25-OH-D_3 metabolism at higher levels of FGF-23 (Figure 9G). There is also a significant positive correlation between serum FGF-23 and serum calcium ($p<0.05$, R^2=0.43, Figure 9H).

Von Kossa Staining

Sections of thoracic and abdominal aorta were stained for vascular calcification using the Von Kossa method, in which dark brown staining is indicative of phosphate in the crystals (Figure 10). Staining confirmed the localization of the calcium-phosphate deposits in the vessel

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media (Figure 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 ng/kg or 80 ng/kg, every vessel stained was Von Kossa positive (Figure 10). No Von Kossa positive staining was found in any control animal (data not shown).

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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 off-set by high dietary vitamin K. Although MGP was up-regulated in this model, calcitriol treatment was also associated with up-regulation of pro-calcific genes in the thoracic aorta. Calcitriol had the expected dose-dependent therapeutic effect of lowering PTH towards 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-$(OH)_2$-D_3 in CKD animals to the levels observed in healthy animals however its metabolite, 1,24,25-$(OH)_3$-D_3, was undetectable despite the up-regulation of kidney *Cyp24a1* by calcitriol treatment. Similarly, the slope between 25-OH-D_3 and its key metabolite, 24,25-$(OH)_2$-D_3 was less steep in CKD animals, indicating reduced metabolism. Taken together, these data suggest that, despite the up-regulation of *Cyp24a1* transcription, its enzymatic activity is impaired in this model. We propose that absent substrate catabolism may enhance tissue levels of 1,25-$(OH)_2$-D_3 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 up-regulated in calcitriol treated rats providing a potential signalling pathway that could directly promote VC in concert with the impact of calcitriol on circulating levels of phosphate and calcium.

In this study, the 20ng/kg calcitriol dose significantly increased calcification compared to 0ng/kg, and although the addition of high vitamin K1 to the 20ng/kg calcitriol lowered calcification levels back down so that they were no longer significantly greater then the 0ng/kg

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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.*, 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 20ng/kg of calcitriol decreased calcification of atherosclerotic plaques (Mathew *et al.*, 2008) and medial vascular calcification (Lau *et al.*, 2012) respectively. In these murine studies, phosphate levels were elevated in CKD approximately 1.5 fold compared to control, calcitriol treatment significantly decreased serum phosphate nearly back to control levels (Mathew *et al.*, 2008; Lau *et al.*, 2012) and consequently, in the Lau *et al*. paper, 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; Guerin *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.

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This is consistent with previous research which has reported that calcitriol can induce calcification in uremic and non-uremic 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 up-regulation of MGP expression may have been near its maximum response in CKD (7.5 fold increase) and 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 up-regulation 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, 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 *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. Further, 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

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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 VSMCs, an event which initiates the process of VC. These results indicate a direct effect of calcitriol treatment on the regulation of pro-calcific genes in vascular tissue and support the work of others (Mizobuchi *et al.*, 2007; Koleganova *et al.*, 2009) indicating that there may indeed be tissue specific response. Further, a study by Wu-Wong *et al*. looked at the effect of calcitriol on gene expression in human coronary artery smooth muscle cells (Wu-Wong, Nakane, *et al.*, 2006). They found a total of 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-$(OH)_2$-D_3) and 25-OH-D_3 into 1,24,25-$(OH)_3$-D_3 and 24,25-$(OH)_2$-D_3 respectively (Jones *et al.*, 2012). The expression of *Cyp24a1* in the kidney was similar in control and un-treated CKD rats but was markedly up-regulated in response to calcitriol (Helvig *et al.*, 2010). Despite this, there was a significantly smaller increment in 24,25-$(OH)_2$-D_3 for a given increment in 25-OH-D_3 in CKD animals compared to controls suggesting a decrease in 25-OH-D_3 catabolism. Human data, pooled from five large cohort studies, similarly showed that the relationship between 24,25-$(OH)_2$-D_3 with 25-OH-D_3 was modified by eGFR in which, as kidney function declined, the slope became significantly less steep (de Boer *et al.*, 2014). Batacchi *et al*. also found that while vitamin D_2 supplementation induces 1,25-$(OH)_2$-D_3 catabolism in control subjects, this response is blunted in individuals with CKD (Batacchi *et al.*, 2017). It was surprising that the 25-OH-D_3 levels were higher in the CKD rats then control animals as this finding is not typical of humans with CKD.

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The reasons for the higher levels of 25-OH-D_3 in this experiment are unknown but could reflect severe abnormalities with 25-OH-D_3 catabolism, and therefore accumulation, in this model. CYP24A1 also metabolizes calcitriol and, despite restored levels of 1,25-$(OH)_2$-D_3 with calcitriol treatment, the metabolite 1,24,25-$(OH)_3$-D_3 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 that 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 up-regulation of CYP24, the ratio between 25-OH-D_3 and 24,25-$(OH)_2$-D_3 increased significantly with higher FGF-23 levels in all rats with CKD. Similarly, Dai *et al.* reported a correlation between higher FGF-23 levels and reduced 24,25-$(OH)_2$-D_3 concentrations (Dai *et al.*, 2012). Taken together, these data and the work of Dai *et al* do not support an 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-$(OH)_2$-D_3 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,

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to manage secondary hyperparathyroidism, have an increased survival (Wu-Wong, Noonan, *et al.*, 2006; 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. Further, this data implicates 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 Mg^{2+} (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 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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Footnotes:

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Legends for Figures:

Figure 1: A graphical description of the experimental outline.

Figure 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, 3 weeks, 5 weeks, and 7 weeks. Rats were treated with either 0 ng/kg, 20 ng/kg calcitriol, or 80 ng/kg calcitriol with or without the addition of high dietary vitamin K (Vit K, 100mg/kg). The data are shown as mean ± SD (n=8/group, *p<0.05 vs baseline, †p<0.05 vs. 3 weeks).

Figure 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 either 0 ng/kg, 20 ng/kg, 80 ng/kg calcitriol, with or without the addition of high dietary vitamin K1 (100mg/kg, D+K). Serum calcium (A), phosphate (B), PTH (C), and FGF-23 (D). The data are shown as mean ± SD (n=8/group), *p<0.05, vs. control, † p<0.05 vs. CKD 0 ng/kg, ¥p<0.05 vs. CKD 20ng/kg.

Figure 4: Vessel calcium (nmol/mg 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 either 0 ng/kg, 20 ng/kg, 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 ± SD (n=8/group), *p<0.05 vs. control, † p<0.05 vs.CKD 0 ng/kg.

Figure 5: Rats were given a 0.25% dietary adenine for a total of 7 weeks to induce CKD or 0% adenine for control. CKD rats were treated with either 0 ng/kg, 20 ng/kg, or 80 ng/kg calcitriol, with or without the addition of high dietary vitamin K (100 mg/kg vitamin K1, D+K). Pulse

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wave velocity (A) and pulse pressure (B) data are presented here. The data are shown as mean ± SD (n=8/group), *p<0.05, **p<0.01 vs. control, † p<0.05 vs. CKD 0 ng/kg.

Figure 6: Mgp (A), Pit1 (B), Runx2 (C), Bmp2 (D), Cyp24a1 (E), and Cyp27b1 (F) transcripts were assessed by qPCR 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 ± SEM (n=6-8/group). *p<0.05, ** p<0.01 vs. control, †† p<0.01 vs. CKD.

Figure 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+0ng/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 ± SEM (n=6-8/group). *p<0.05 vs. control.

Figure 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 either 0 ng/kg, 20 ng/kg, or 80 ng/kg calcitriol. Serum 25-OH-D3 (A), 24,25-OH2-D3 (B), 1,25-OH2-D3 (C), 1,24,25-OH3-D3 (D) and the ratio of 25-OH-D:24,25-OH-D (E), Data presented as mean ± SD, n=8/group, *p<0.05, **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 shows the linear relationship between 25-OH-D and 24,25-OH-D (p<0.05, r2 shown on graph).

Figure 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

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(red), 20 ng/kg (green), or 80 ng/kg (black) of calcitriol. 0 ng/kg correlations are significant in each artery except for the superior messenteric ($p<0.05$). n=22-24/group.

Figure 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 ng/kg, 20 ng/kg or 80 ng/kg calcitriol. Positive staining, dark brown areas in vessel, denotes the region of calcification in the thoracic and abdominal aortas. All photos were taken at the same magnification.

Figure 11: **Calcitriol metabolism in CKD**. A) Vitamin D, acquired either via the diet or via the sun, is hydroxylated to 25-OH-D_3 in the liver by the enzymes CYP2R1 and CYP27A1 and then hydroxylated a second time primarily in the kidneys by CYP27B1 to 1,25-$(OH)_2$-D_3 (calcitriol), the active form. Calcitriol increases calcium and phosphate reabsorption and resorption in the small intestine and bone respectively. It also increases fibroblast growth factor 23 (FGF-23), a hormone which decreases phosphate reabsorption in the kidney and, in a negative feedback manner, up-regulates CYP24A1 which is the enzyme that metabolizes calcitriol[12,16]. Patients with CKD have a deficiency in 1,25-$(OH)_2$-D_3 (calcitriol) due to a loss of renal CYP27B1 with declining renal function and an up-regulation of the catabolic enzyme CYP24A1 by FGF-23[12,14,16]. This decrease in 1,25-$(OH)_2$-D results in secondary hyperparathyroidism which activates FGF-23 which, 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-D_3 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

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increase in the expression CYP24A1 the metabolite 1,24,25-$(OH)_3$-D_3 was still undetectable. These changes are indicated by the black arrows.

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Figures:

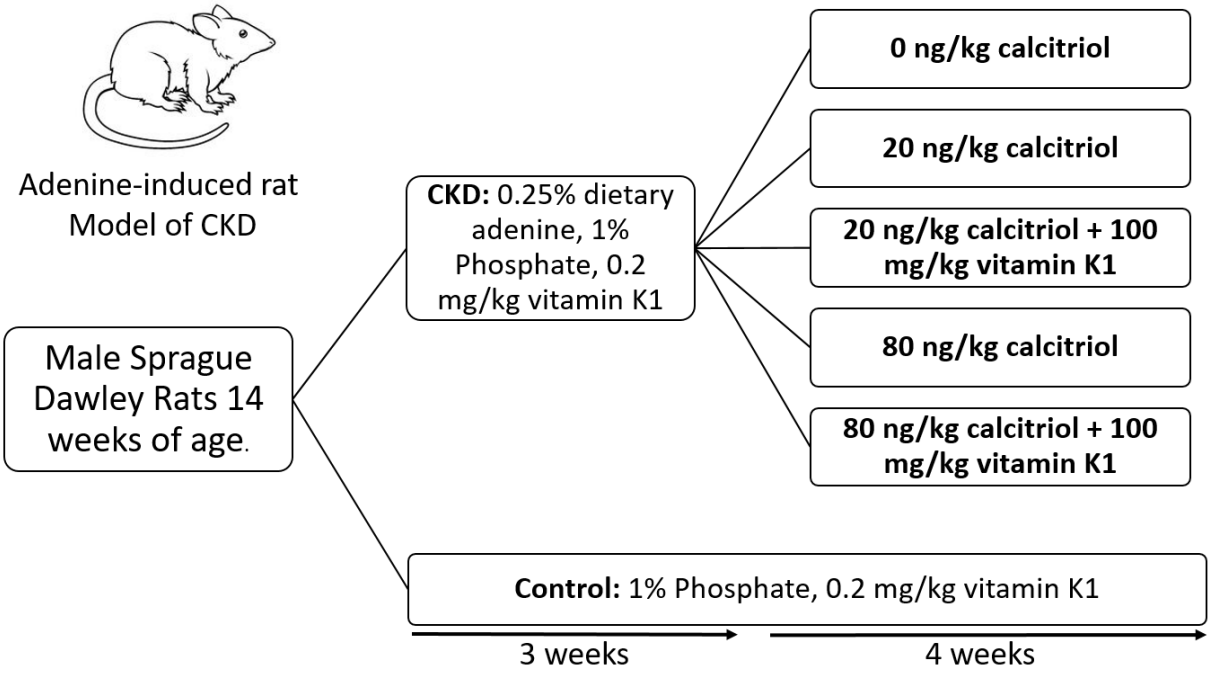

Figure 1:

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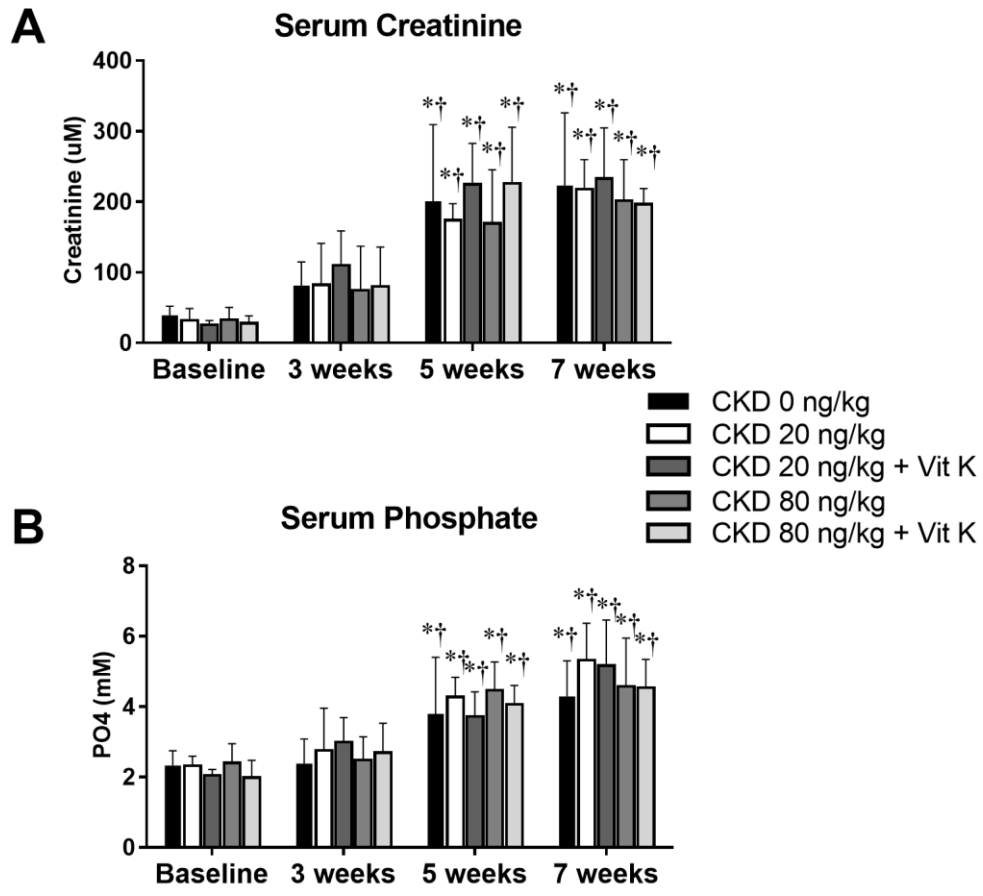

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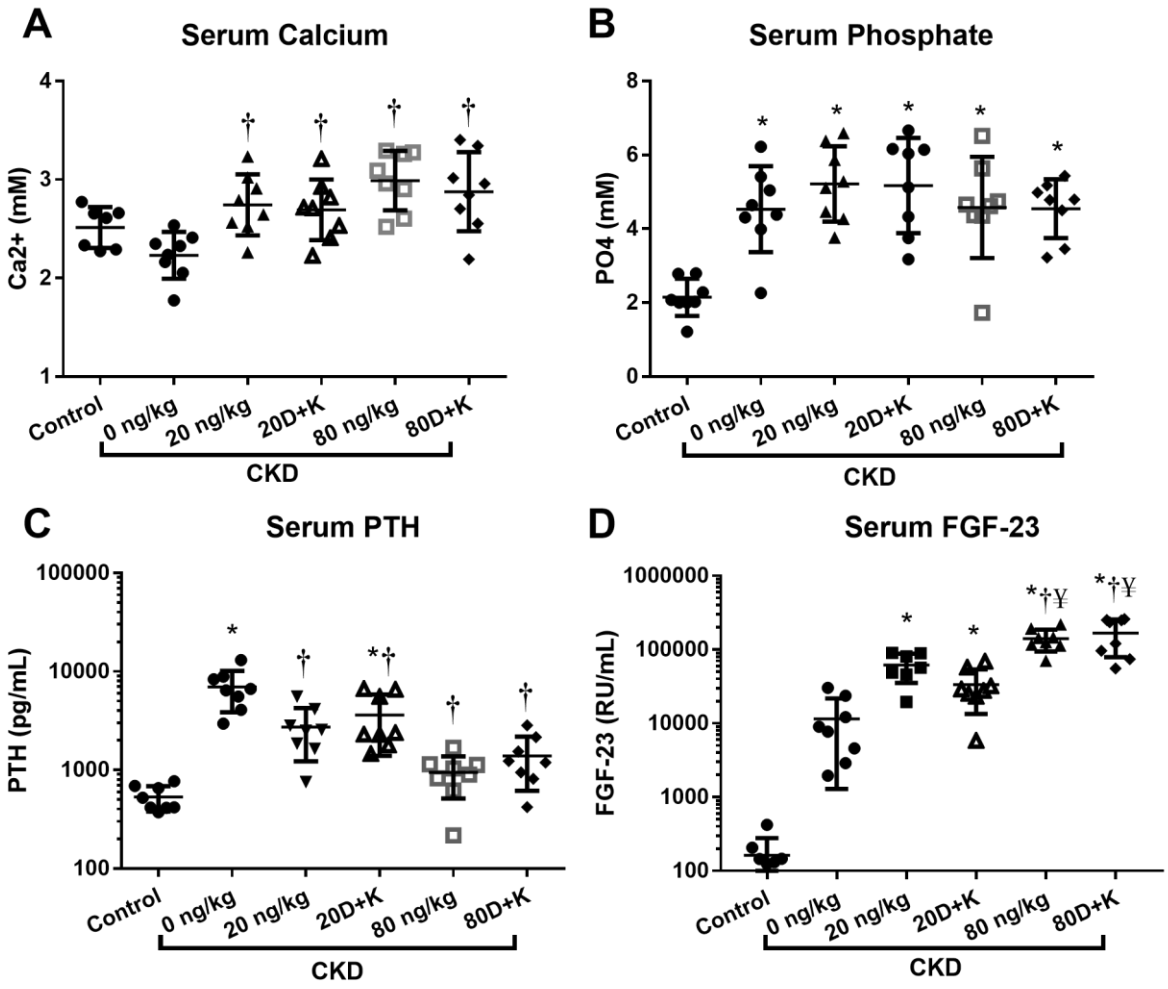

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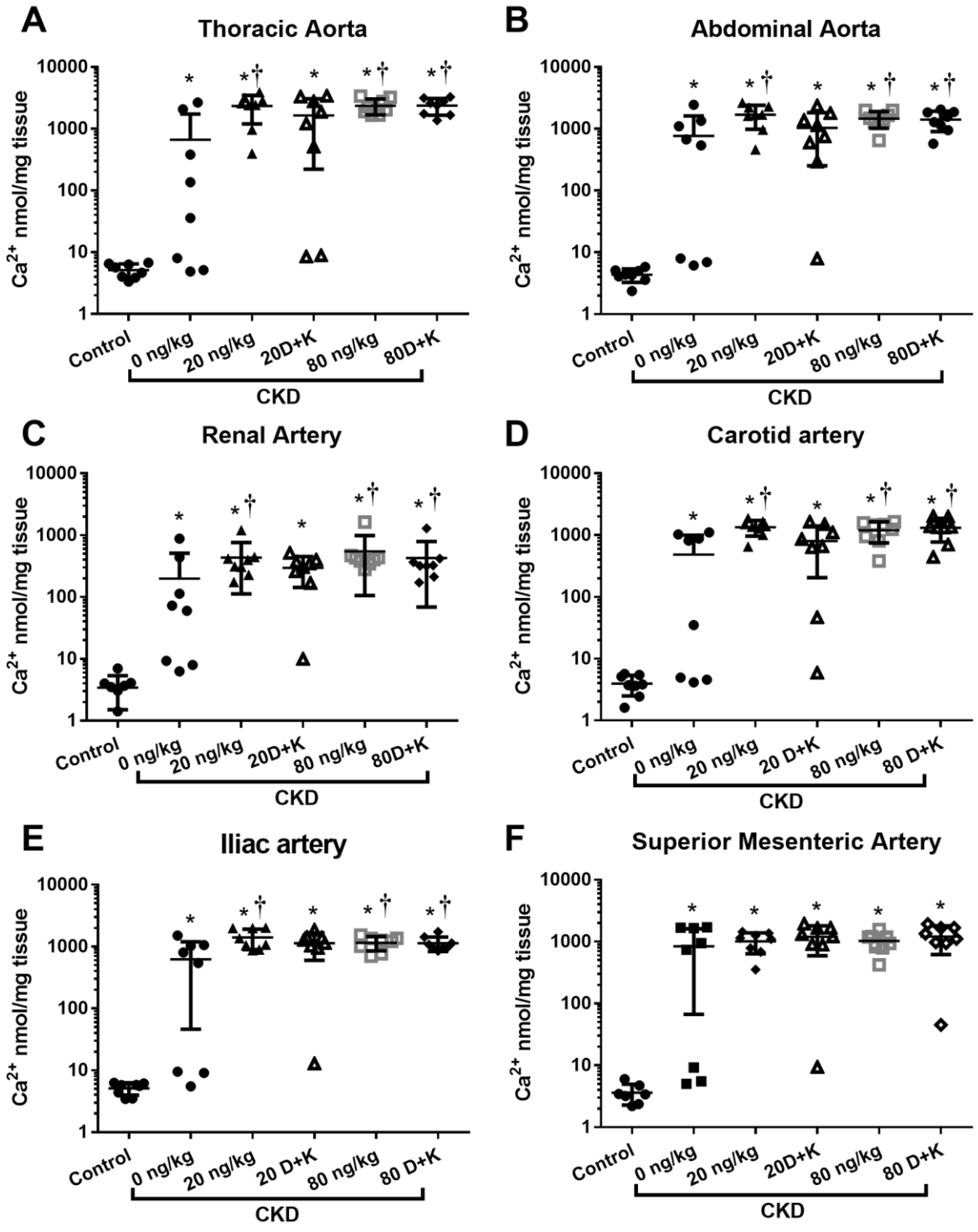

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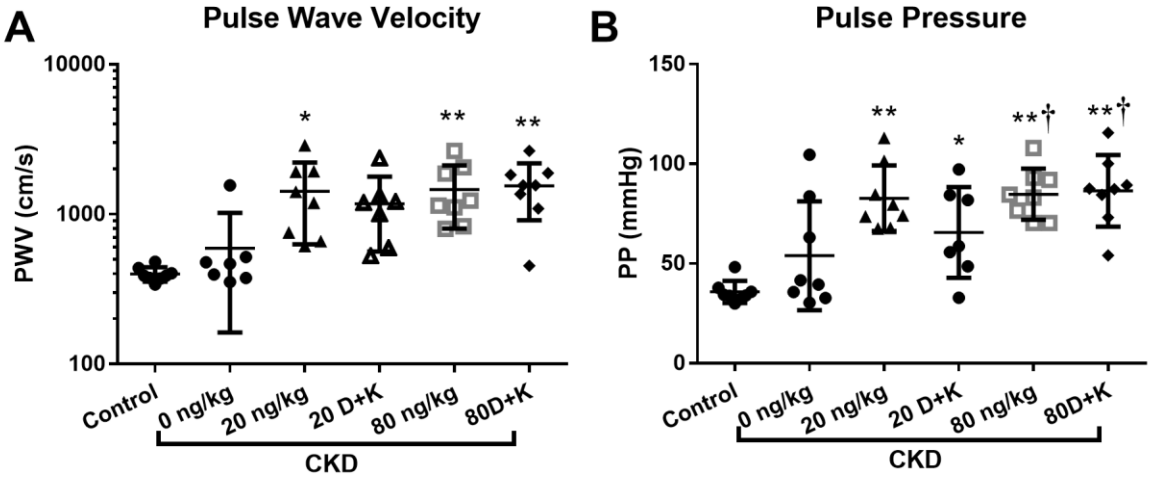

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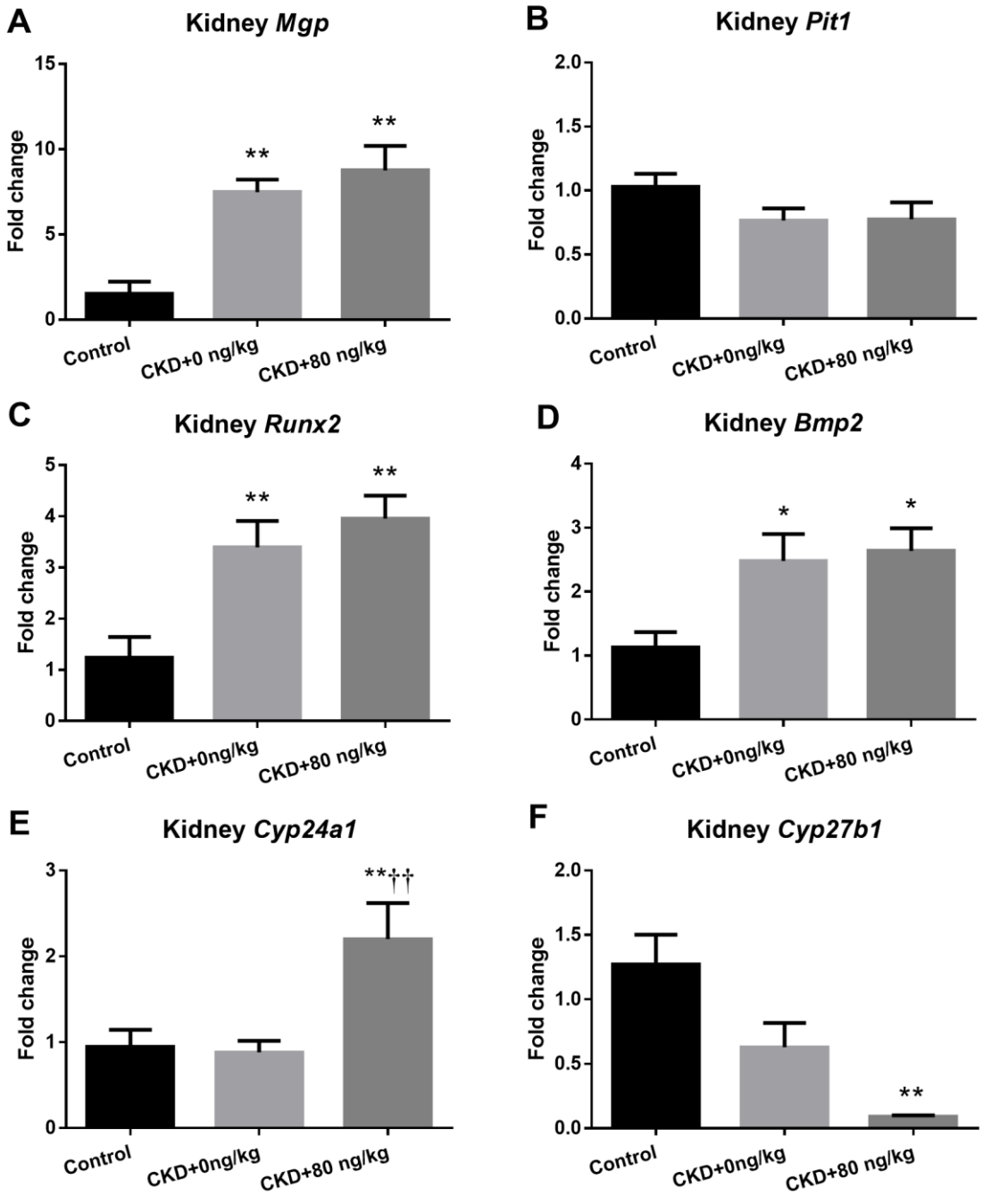

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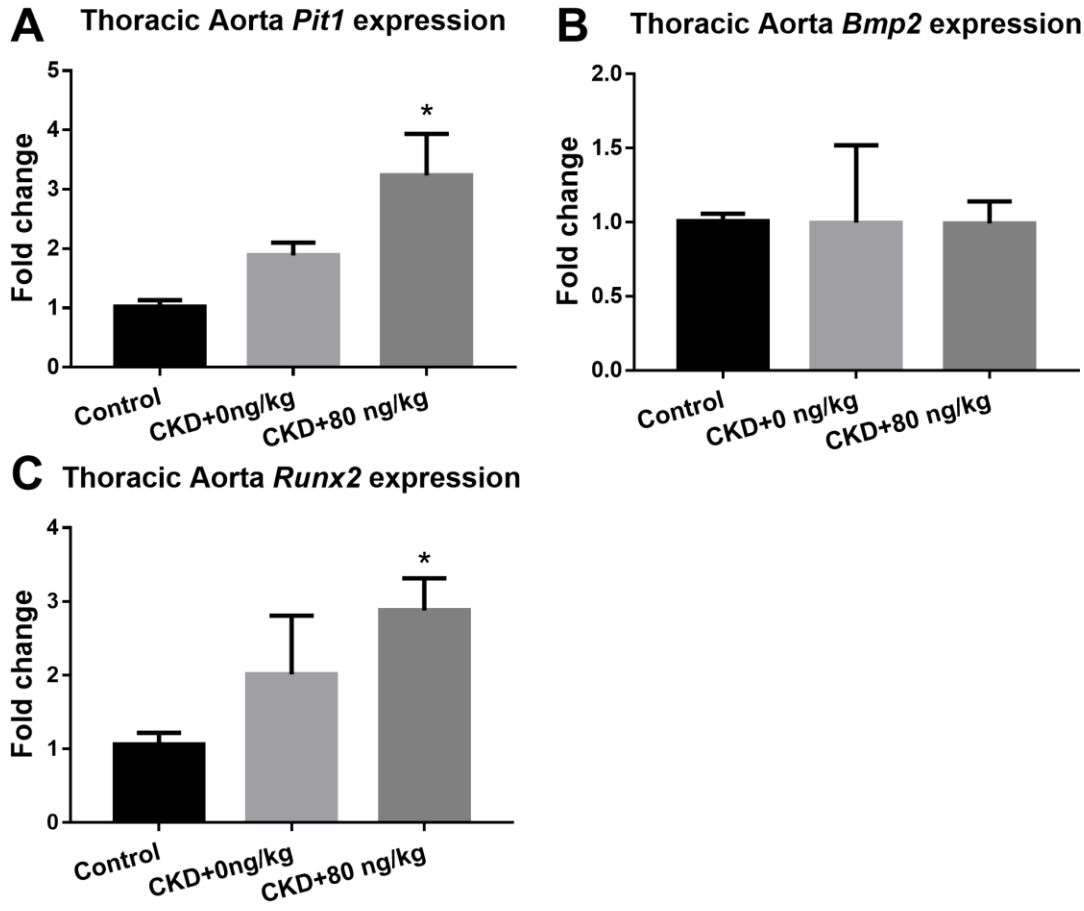

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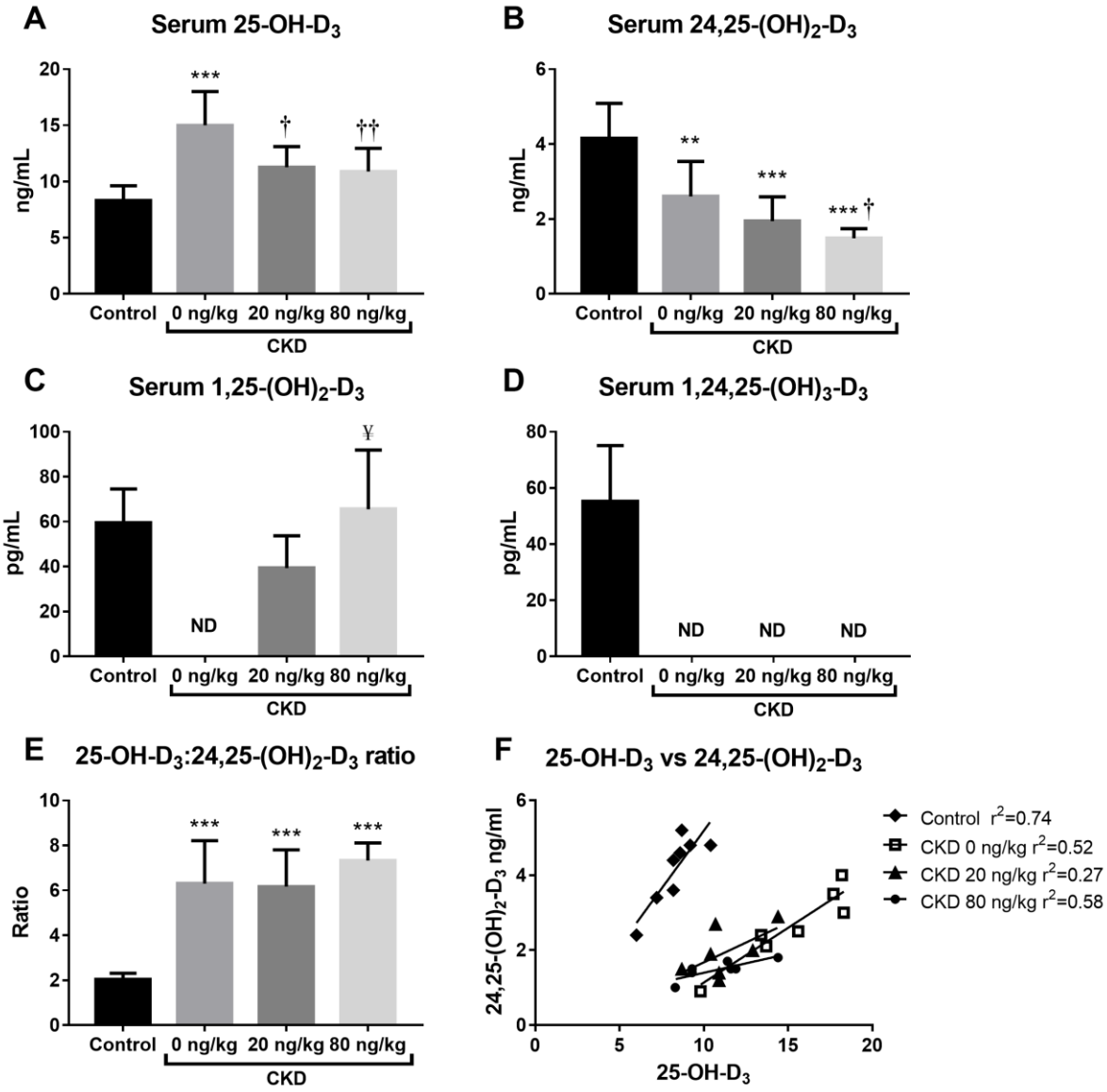

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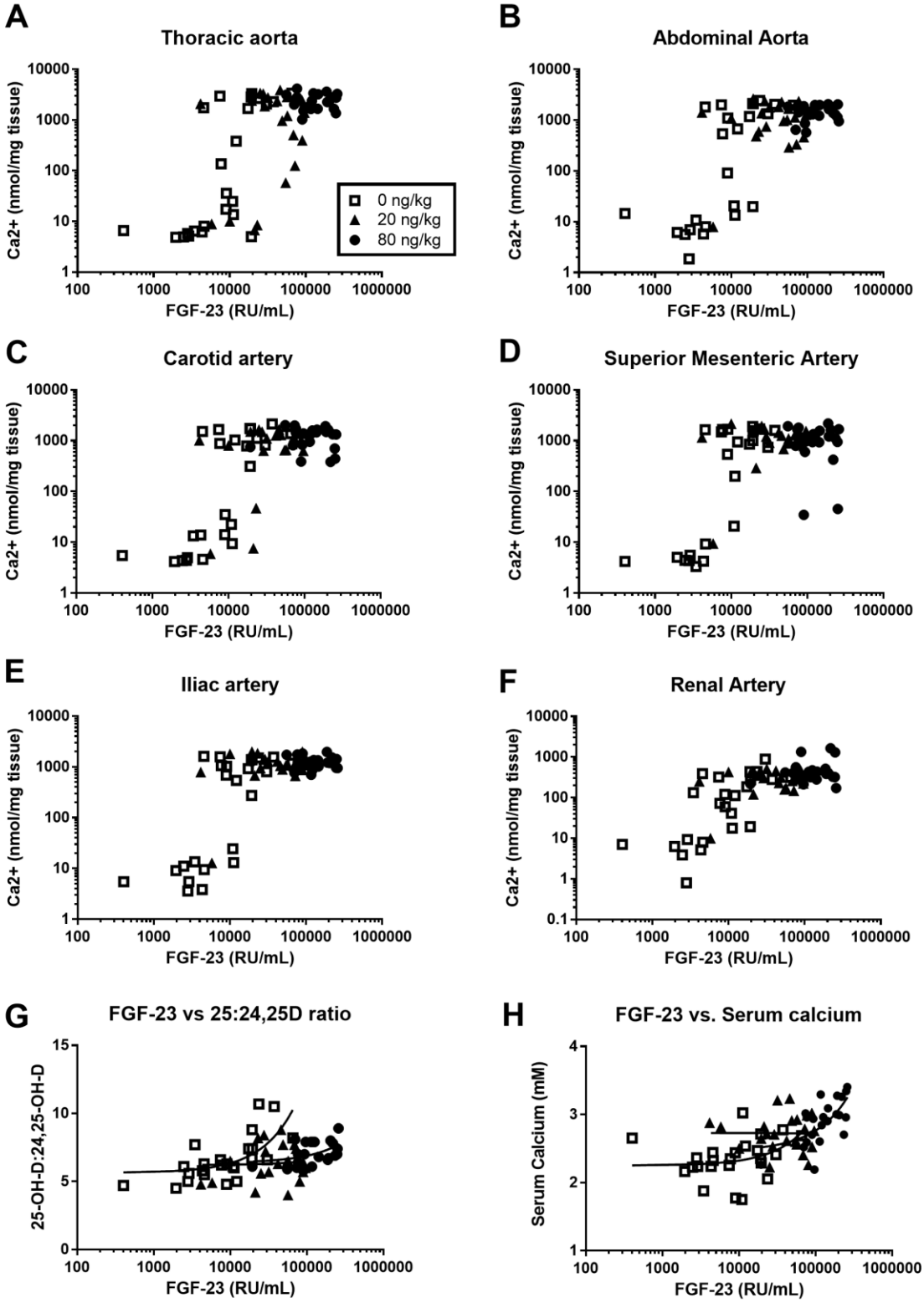

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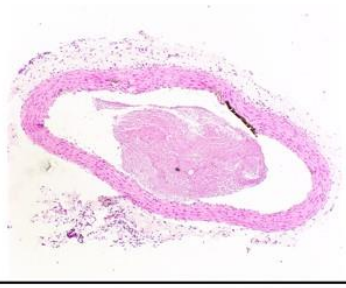
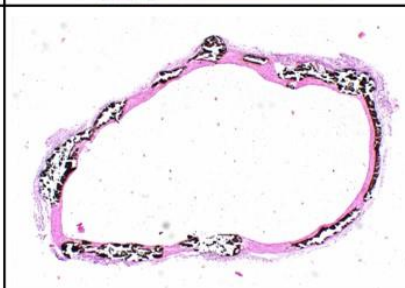
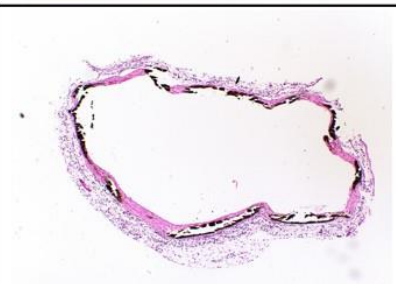
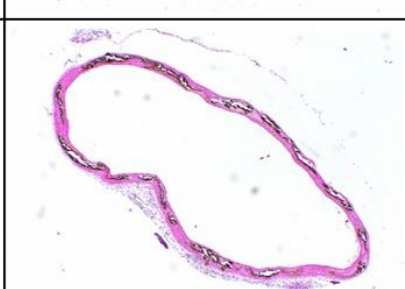
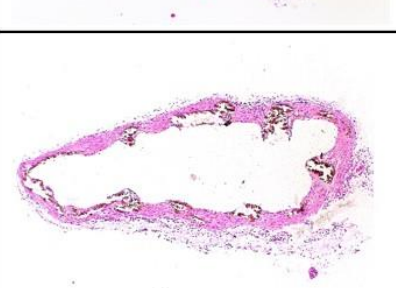
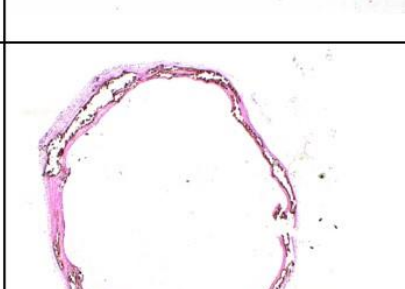
	Thoracic Aorta	Abdominal Aorta
0 ng/kg Calcitriol		
20 ng/kg Calcitriol		
20 ng/kg Calcitriol + High vitamin K		
80 ng/kg Calcitriol		
80 ng/kg Calcitriol + High vitamin K		

Figure 10

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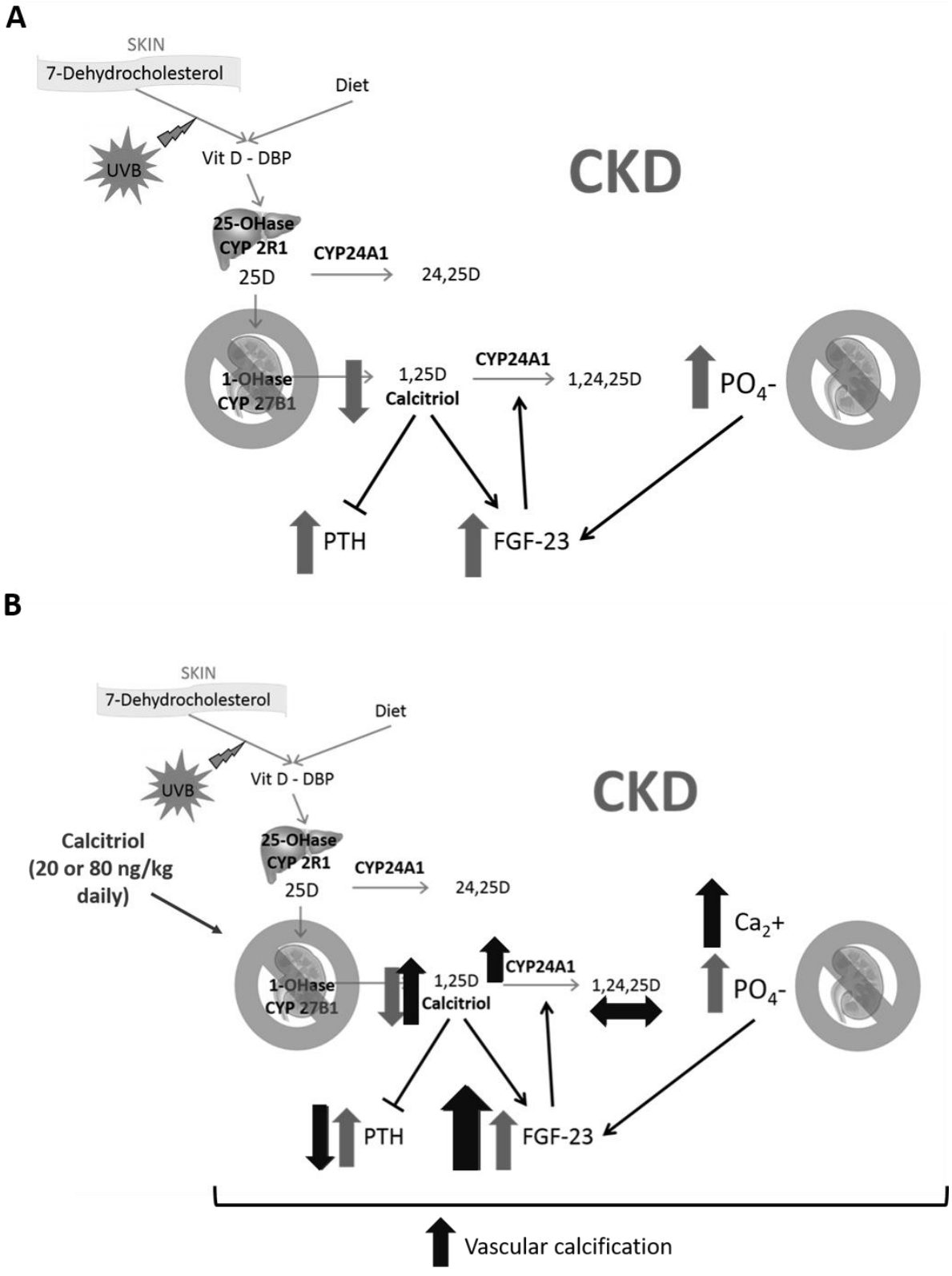

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Figure 11: **Calcitriol metabolism in CKD**.

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