Androgen therapy does not prevent bone loss and arterial calcifications in male rats with CKD.

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ABSTRACT

Patients suffering from chronic kidney disease (CKD) often experience bone loss and arterial calcifications. It is unclear if hypogonadism contributes to the development of these complications, and whether androgen therapy might prevent them. Male adult rats were randomized into 4 groups. The first group received standard chow (Control), while three other groups were fed a 0.25% adenine/low vitamin K diet (CKD). Two CKD groups were treated with testosterone (T) or dihydrotestosterone (DHT), whereas the control group and one CKD group received vehicle (VEH). CKD animals had 10-fold higher serum creatinine and more than 15-fold higher PTH-levels compared to controls. Serum T levels were more than 2-fold lower in the CKD-VEH group compared to Control-VEH and CKD-T groups. Seminal vesicle weight was reduced by 50% in CKD-VEH animals, and restored by T and DHT. CKD animals showed a low bone mass phenotype with decreased trabecular bone volume fraction and increased cortical porosity, which was not rescued by androgen treatment. Aortic calcification was much more prominent in CKD animals and not unequivocally prevented by androgens. Messenger RNA expression of the androgen receptor-responsive genes *Acta1* and *Col1a1* was reduced by CKD and stimulated by androgen treatment in levator ani muscle, but not in bone or aortic tissue. We conclude that adenine-induced CKD results in the development of hypogonadism in male rats. Androgen therapy is effective in restoring serum T levels and androgen-sensitive organ weights, but does not prevent bone loss or arterial calcifications, at least not in the presence of severe hyperparathyroidism.

INTRODUCTION

Chronic kidney disease (CKD) is a very common disease affecting up to 15% of the general population and its prevalence markedly increases with age (CDC, 2022). Chronic kidney disease–mineral and bone disorder (CKD-MBD) is one of the many complications associated with CKD. It represents a systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following: abnormalities of calcium, phosphate, parathyroid hormone (PTH), or vitamin D metabolism, abnormalities in bone turnover, mineralization, volume, linear growth, or strength, and arterial or other soft-tissue calcification. CKD-MBD accounts at least partly for the excessive burden of fractures and cardiovascular disease in patients with CKD (Moe et al, 2006). The risk of fracture increases with decreasing kidney function. The non-vertebral fracture risk is 4 to 6-fold higher in CKD patients on dialysis compared to age- and sex-matched controls (Rodriguez Garcia et al, 2005). Arterial calcifications are present in more than 60% of dialysis-dependent patients and contribute to the higher cardiovascular risk and mortality in this population (Jankowski et al, 2021; Okuno et al, 2007). The link between bone loss and arterial calcifications is often referred to as the 'calcification paradox' or 'bone-vascular axis'. Many factors are involved in the underlying pathophysiology of this calcification paradox, however the contribution of decreased sex steroid levels to the development and maintenance of bone and vascular complications of CKD and their interconnection remains unclear (Evenepoel et al, 2019; Jørgensen et al, 2021).

Total testosterone (T) levels decline with about 0.8% per year in healthy middle-aged men (Feldman et al, 2002). T levels have been reported to be low in male CKD patients as well, with up to 60% of men undergoing dialysis having low circulating T concentrations (Carrero et al, 2011; Yilmaz et al, 2011). Multiple studies have shown a correlation between circulating sex steroid levels and bone mineral density (BMD) or fracture risk in 'healthy' older men not suffering from CKD. However, the relatively small age-related decline in T levels probably has only minor contribution to the development of osteoporosis and related fractures in ageing men (David et al, 2022). The question arises whether in

men with CKD a possible greater and faster decline in sex steroid levels does imply an increased risk for bone loss and/or fractures, and if T replacement therapy (TRT) could partly overcome these risks. In male kidney transplant recipients, bioavailable T levels were positively associated with BMD at the lumbar spine (Jørgensen et al, 2018). One interventional study did not show beneficial effects of 6-months transdermal TRT on BMD in male patients with end-stage renal disease, though therapy was also not successful in increasing T levels and BMD was only a secondary end-point in this study (Brockenbrough et al, 2006). Likewise, low T levels have been associated with arterial calcifications, cardiovascular risk and mortality both in the general population and in men with CKD (Travison et al, 2016; Yilmaz et al, 2011). Although the connection between TRT and cardiovascular risk remains a controversial topic, adequately treating hypogonadal men achieving mid-normal range levels of T does not seem to increase cardiovascular risk or mortality (Gagliano-Jucá & Basaria, 2019; Kelly & Jones, 2014). Taken together, these findings suggest a possible link between androgens and the bone-vascular axis in men with CKD.

We hypothesized that androgen deficiency contributes to the development of bone and vascular complications in CKD, and that androgen replacement therapy may partly rescue the CKD-MBD phenotype. We used an established CKD rat model that develops bone loss and arterial calcifications simultaneously, as this model allowed us to study the effect of therapeutic interventions on both these complications (Neven et al, 2015). Androgen replacement was started early, focusing on the prevention of bone and vascular complications. To differentiate between androgen receptor (AR)-mediated and estrogen receptor (ER)-mediated androgen effects, we included a treatment group with T (which can be aromatized into estrogens) and a second treatment group with the non-aromatizable androgen dihydrotestosterone (DHT).

MATERIALS AND METHODS

Animals

52 Wistar Han rats (Charles River Laboratories) were divided into 4 experimental groups: control+VEH, CKD+VEH, CKD+T and CKD-+DHT. Mean body weight at the start of the experiment was 329.00 +/- 11.09 grams (±12 weeks of age). Rats were maintained either on standard chow diet (7 mg/kg vitamin K, 1% Ca, 0.7% P, 1 IU/g vitamin D, and 19% protein) (SSNIFF Spezialdiäten, Soest, Germany) or CKD diet (0.25% adenine, 0.2 mg/kg vitamin K, 1% Ca, 1% P, 1 IU/g vitamin D, and 6% protein) (SSNIFF Spezialdiäten, Soest, Germany) (Neven et al, 2015). After 2 weeks on the diet, rats were subcutaneously implanted either an empty silastic stick (VEH), or a silastic stick filled with T (3 cm - 69µg/day release) or DHT (6 cm - 180 µg/day release) in the dorsal region under isoflurane anesthesia, as previously described (Vandenput et al, 2002; Vanderschueren et al, 1992). After surgery rats received analgesia with meloxicam 1mg/kg (Metacam, Boehring Ingelheim, Ingelheim am Rhein, Germany) once daily during 3 days. Rats were placed in metabolic cages for 24 hours every 2 weeks for collection of urine and faeces, and blood was collected every 2 weeks via the tail vein. Rats were euthanized after 10 weeks on the diet after anesthesia with sodium pentobarbital (Dolethal, Vetoquinol, Lure CEDEX, France) 60 mg/kg via intraperitoneal injection followed by cardiac puncture. Three rats died prematurely at 8-9 weeks on the diet (1 from the CKD+VEH and 2 from the CKD+T group). Three additional animals were excluded from the final analysis because of damage of the silastic stick at euthanasia (1 CKD+T and 2 CKD+DHT animals). 4 additional untreated control rats were sacrificed at 10 weeks of age for *in vitro* aortic vessel experiment. Rats were housed per 2 in conventional facilities at 20 °C with 12-hour light/dark cycle and *ad libitum* access to food and water. The animal experiments were conducted in accordance with the KU Leuven guidelines for animal experimentation and approved by the KU Leuven ethical committee (P174/2019).

Biochemistry

Serum creatinine, urea, calcium and phosphate levels were analyzed by DxC 700 AU clinical chemistry platform (Beckman Coulter, Brea, CA, USA) every 2 weeks. Other biochemistry was determined at timepoint of euthanasia. Serum intact PTH (Immutopics, San Clemente, CA, USA) and FGF23 (Kainos Laboratories, Tokyo, Japan) levels were determined by ELISA. T levels were analyzed via LC-MS/MS (Antonio et al, 2018). Luteinizing hormone (LH) levels were determined by ultra-sensitive ELISA (Steyn et al, 2013).

Micro-computed tomography (micro-CT)

L5 vertebral bodies and right tibiae were scanned *ex vivo* using a Skyscan 1272 microCT (Bruker, Kontich, Belgium) with 9 µm pixel size, 1 mm Al filter, 80 kV, 125 µA and 360° angular rotation at 0.2° steps. Images were reconstructed with the NRecon software (Bruker) and morphometric parameters were calculated using CTAn (Bruker). Parameters are reported according to the ASBMR guidelines (Bouxsein et al, 2010) and include cortical thickness (mm), cortical porosity (%), trabecular bone volume fraction (BV/TV, %), trabecular thickness (mm), trabecular separation (mm), and trabecular number (1/mm).

Evaluation of vascular calcification

The distal part of the thoracic aorta (1 cm) was fixed in paraformaldehyde 2% overnight at 4°C, embedded in paraffin, sectioned at 4 µm and subsequently stained with hematoxylin and eosin (H&E) and Von Kossa. Images were captured using TissueFAXS 7.0 (Tissuegnostics GmbH, Vienna, Austria). Quantification of the Von Kossa-positive stained surface (% calcified tissue/non-calcified tissue of the vessel ring) was performed using Histoquest software 7.0 (Tissuegnostics GmbH, Vienna, Austria). For each animal, 3 sections at 3 different levels (9 in total) were analyzed. Quantification of the calcium load in proximal part of the thoracic aorta was performed by decalcifying in hydroxychloride 0.1 M during 24h and analyzing the calcium concentration in the supernatant using DxC 700 AU clinical chemistry platform (Beckman Coulter, Brea, CA, USA); data were corrected for the wet tissue weight.

A distinction was made between mild to no calcifications and severe calcifications based on a visually apparent 'on-off' phenomenon (>0.11 mg/g wet tissue). For the *in vitro* experiments, thoracic aortae were isolated from 10-week-old control rats, stripped from adventitial tissue and washed with Dulbecco's Phosphate Buffered Saline (DPBS 1x, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the aorta was cut into 1-2 mm vessel rings which were cultured in Medium 199 (M2154) (Sigma-Aldrich, Darmstadt, Germany) supplemented with 1% penicillin (10.000 U/mL)-streptomycin (10.000 µg/mL) (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific) at 37°C with 5% CO_2 for 7 days with change of culture medium every 2 days. The induction of calcification was obtained by increasing phosphate (Pi) concentration in the medium during the 7 days of culture through addition of $Na_2H_2PO_4$/$NaHPO_4$ (pH 7.4) to a final concentration of 1.5 mmol/L (procalcifying medium) (Akiyoshi et al, 2016) in presence of either vehicle (ethanol) or R1881 (methyltrienolone, a very potent AR-ligand (Bonne & Raynaud, 1975)) at a concentration of 1nM. After culture, rings were washed with DPBS, decalcified in hydroxychloride 0.1 M for 24h, and calcium concentration in the supernatant was analyzed by o-cresolphthalein complexone method (Thermo Fisher Scientific) (Shroff et al, 2008) corrected for the wet tissue weight. We processed three technical replicates per animal for each condition.

Real-time quantitative PCR

Aorta, right femur and levator ani muscle collected at euthanasia were snap-frozen in liquid nitrogen and stored at -80°C until further processing. The bone marrow fraction of the femur was removed by centrifugation. Total RNA was extracted from tissues using RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was synthesized from 1 µg RNA using the FastGene Scriptase II kit (NIPPON Genetics Europe, Dueren, Germany) and random hexamer primers. The PCR reactions were performed using Fast SYBR Green Master Mix and the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized for *Actb* and *Gapdh* housekeeping genes and expressed relative to the control group ($2^{-\Delta\Delta Ct}$method). The following

primer sequences were used: *Actb* (5'-CATTGCTGACAGGATGCAGAAGG-3' ; 5'-TGCTGGAAGGTGGACA GTGAGG-3'), *Gapdh* (5'-TCTTGTGCAGTGCCAGCCTC-3' ; 5'-TGAAGGGGTCGTTGATGGCAA-3'), *Ar* (5'-AA GAGCTGCGGAAGGGAAAC-3' ; 5'-ACATTTCCGGAGACGACACGA-3'); *Acta1* (5'-GAACCCCAAAGCTAACC GGG-3' ; 5'-ATCCAACACGATGCCGGTG-3'); *Col1a1* (5'-GCATGGCCAAGAAGACATCCC-3' ; 5'-CATAGCAC GCCATCGCACAC-3') and *Fkbp5* (5'-TAACTTGGGCGACCCTCACC-3'; 5'-ACTTCTGGCTCGGAACCCTG-3'). All primers were designed to hybridize to different exons, and generation of single correct amplicons was checked by melting curve dissociation.

Statistical analysis

Data are represented as mean +/-SD and median [range] for parametric and non-parametric data, respectively. Normality was tested by Shapiro-Wilk test. Parametric data were analyzed using one-way ANOVA followed by Tukey multiple comparison test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's multiple comparison test was applied. Longitudinal comparative analysis of creatinine levels was performed using two-way ANOVA followed by Tukey multiple comparison test. Differences in proportions were determined by Fisher's exact test with Benjamini-Hochberg correction for multiple testing. Pearson correlation was used to investigate associations between aortic calcium content and androgen-related outcomes. Two tailed $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using GraphPad Prism v9.3.1 (GraphPad, La Jolla, CA, USA) and R Statistical Software v4.2.2.

RESULTS

Adenine diet results in development of CKD and severe hyperparathyroidism

After 2 weeks of the dietary intervention, animals from all CKD groups had elevated serum creatinine levels compared to control animals (**Figure 1A**). In the weeks thereafter, serum creatinine levels further increased in all CKD groups without differences between androgen-treated and vehicle-treated CKD rats (**Figure 1A**). After 10 weeks, serum creatinine levels were more than 10-fold higher in the CKD

groups compared to control animals (**Figure 1B**). Similarly, serum urea levels were 3 times higher in CKD animals compared to controls at sacrifice (**Figure 1C**). Histological analysis showed altered morphology of the kidney with induction of fibrosis, tubular atrophy, inflammation and brown adenine deposits in CKD animals (**Supplemental figure S1A**). As expected, CKD animals developed severe hyperparathyroidism as evidenced by increased PTH and phosphate, and decreased calcium levels, without differences between the different CKD-groups (**Table 1**).

CKD-induced hypogonadism can be successfully treated with androgen replacement therapy

Serum T levels were significantly decreased in rats with CKD, but were restored upon treatment with T (**Table 1**). LH levels were significantly lower in CKD animals compared to controls (**Table 1**). The weight of androgen-sensitive organs (seminal vesicles, levator ani muscle, ventral prostate and cowper glands) was significantly lower in vehicle-treated CKD animals compared to controls. Seminal vesicle and levator ani muscle weight were 2 and 3-times lower in CKD versus controls, respectively. Treatment with androgens, both T and DHT, was able to restore these weights (**Figure 2A**). The atrophy of these androgen-sensitive organs was further confirmed by macroscopic analysis (**Figure 2B)**. In contrast to kidney, testis morphology analyzed on H&E staining was unaltered by the adenine diet (**Supplemental figure S1B**).

Androgen treatment does not rescue trabecular bone loss and increased cortical porosity in CKD rats

Micro-CT analysis showed that trabecular bone volume fraction (BV/TV) in L5 vertebral body was unchanged in CKD animals compared to controls (**Figure 3A**). However, trabecular architecture was altered as the number of trabeculae was decreased whereas trabeculae were thicker resulting in increased trabecular separation. In the proximal tibia, trabecular BV/TV was decreased in CKD animals compared to controls, with a manifest decrease in the number of trabeculae, associated with an increase in thickness (**Figure 3B**). Cortical thickness at the diaphysis of the tibia showed a trend to increase in CKD animals, although not significantly, whereas cortical porosity was highly increased in

CKD animals compared to controls (**Figure 3C**). Androgen treatment did not influence vertebral or tibial bone phenotype.

CKD results in development of aortic calcifications which are not unequivocally prevented by androgen therapy

In vivo aortic calcium content was higher in CKD animals compared to control animals (**Figure 4A**). Median aortic calcium content in the CKD+VEH, CKD+T and CKD+DHT group was 0.46 mg/g, 0.07 mg/g and 0.06 mg/g respectively. When distinguishing between no/mild calcifications and severe calcifications (>0.11 mg/g wet tissue), the proportion of severely affected animals tended to be lower in the androgen-treated than in the vehicle-treated CKD animals (CKD+VEH 61.5% vs. CKD+T 18.2% vs. CKD+DHT 16.7%), though this was not significant after correction for multiple testing, and androgen treatment could not clearly prevent calcification in the CKD animals (**Figure 4B**). Over half of the CKD animals showed increased percentage of calcified tissue area in the aorta compared to controls, although not statistically significant for the entire group (**Figure 4C**). There was no correlation between aortic calcium content and serum T levels, seminal vesicle weight, and levator ani muscle weight in the CKD animals (**Figure 4E**). Representative images of a non-calcified control aorta and calcified CKD aorta (with typical calcification in the tunica media of the vessel wall) are shown in **Figure 4F**. The potent AR-agonist R1881 could not prevent development of calcification of aortic vessel rings upon stimulation with procalcifying medium *in vitro* (**Figure 4D**).

Androgen receptor gene expression and response

Messenger RNA expression of the AR gene was investigated in levator ani muscle, bone and aortic tissue (**Figure 5A**). *Ar* transcript levels were decreased in the CKD+DHT group in levator ani muscle and in the CKD+VEH and CKD+T groups in femur compared to controls. No differences in AR expression were observed in aorta between the different groups. To test whether CKD changed androgen responsiveness in the different tissues, gene expression of downstream targets of the AR was determined. Actin alpha 1 (*Acta1* gene) expression was decreased in levator ani muscle in CKD

compared to controls (**Figure 5B**). Treatment with DHT increased expression of *Acta1* compared to vehicle-treated CKD rats. No significant difference in *Acta1* expression was observed in in femur or aortic tissue. Similarly, collagen type 1 alpha chain (*Col1a1* gene) expression was decreased in non-treated CKD animals compared to controls in levator ani muscle, and therapy with DHT increased expression compared to vehicle-treated CKD group (**Figure 5C**). Expression of *Col1a1* in bone and aorta did not differ among the groups. Finally, FKBP prolyl isomerase 5 (*Fkbp5* gene) expression tended to be increased in CKD animals compared to controls in levator ani muscle, and treatment with DHT further increased this expression. In aortic tissue *Fkbp5* was also increased in CKD animals compared to controls, but therapy with DHT did not further increase its expression. No differences in *Fkbp5* expression were seen between the different groups at the level of the femur (**Figure 5D**).

DISCUSSION

The key finding of the present study is that androgen replacement therapy restores CKD-induced male hypogonadism, but fails to rescue the bone and vascular phenotype, at least in the presence of severe hyperparathyroidism.

Previous studies have shown the presence of male hypogonadism in experimental CKD rodent models. In a subtotal nephrectomy model of uremia, lower T levels and lower weight of androgen-sensitive organs were observed (Handelsman et al, 1985b). Adachi *et al.* demonstrated low T levels in both a model of renal failure induced by 5/6 nephrectomy and adenine-induced CKD in male rats (Adachi & Nakada, 1999). The results of the present study do not only confirm that CKD is a state of hypogonadism, but also demonstrate that this condition can be reverted by androgen supplementation. Moreover, we confirm that experimental uremia results in decreased LH levels. It has been previously shown that hypogonadism after subtotal nephrectomy is principally due to aberrant hypothalamic regulation of pituitary LH secretion and decreased LH pulse frequency (Dong & Handelsman, 1991; Handelsman et al, 1985a). Of note, we exclude direct testicular toxicity by adenine as contributing factor as testes morphology is unaltered by the diet (Adachi et al, 1998).

Male rats seem to be more susceptible to develop CKD under adenine diet compared to female rats, and both total T levels and BMD at the lumbar spine further decline with increasing dietary adenine concentrations and thereby decreasing kidney function (Ogirima et al, 2006). The bone phenotype as assessed by histomorphometry of the present adenine 0.25%/low vitamin K diet has been well described by Neven *et al* (Neven et al, 2015). A typical hyperparathyroid bone disease with high turnover was observed. This is compatible with our microCT findings showing high cortical porosity and loss of trabecular bone volume fraction in the tibia of the CKD rats compared to controls. In this model there is a positive correlation between different bone parameters and aortic calcification, making it an appropriate model to study bone and vascular complications in CKD simultaneously and evaluate possible effects of interventions on this bone-vascular axis.

Detrimental effects of sex steroid deficiency for development and maintenance of male bone are well established. Both global AR-knockout mice, as well as bone cell specific AR-knockout mouse models show reduced bone mass (Almeida et al, 2017). Castration, surgically or chemically, leads to rapid bone loss as well, mainly characterized by a loss in trabecular number without major influence on trabecular thickness and by a decrease in cortical thickness (Khalil et al, 2020; Kim et al, 2020). Additionally, androgen replacement therapy, either with T or DHT, has been shown to be able to prevent this bone loss in different rodent models (Khalil et al, 2020; Vanderschueren et al, 1992). The rat model of CKD used in the present study represents also a model of androgen deficiency. However, in this particular model androgen replacement therapy, although resulting in T levels and seminal vesicle weights comparable to controls, is not able to rescue the CKD-induced bone loss which is characterized by a loss in trabecular number, but increase in trabecular thickness and high cortical porosity. It is tempting to speculate that the pronounced secondary hyperparathyroidism in this model is overwhelming, masking any androgen-related effect on the bone. Alternatively, the androgen deficiency may not have been severe enough to result in sex steroid-induced bone loss (David et al, 2022). In this way, therapy with T and DHT may not have resulted in positive effects on bone in this particular CKD rat model.

Literature data on the effects of sex steroids on the development of vascular calcifications is much more scarce and conflicting (Woodward et al, 2021; Zhang et al, 2019). Androgen treatment with T and DHT in eugonadal male and female mice increased vascular calcification in apolipoprotein E-null mice (McRobb et al, 2009). *In vitro* studies in murine vascular smooth muscle cells (VSMCs) by Zhu *et al.* showed increased calcification upon treatment with androgens T and DHT, which was no longer present after deleting the AR in the VSMCs (Zhu et al, 2016). Others suggest the involvement of the AR in macrophages in induction of VSMC calcification (Pang et al, 2020). In contrast, Son *et al.* showed inhibitory effects of T and DHT on induction of calcification in human VSMCs *in vitro* which were reverted by treatment with an AR-blocker (Son et al, 2010). Moreover, ginsenoside Rb1 served as a selective AR-modulator inhibiting VSMC calcification (Nanao-Hamai et al, 2019). In human coronary arteries the AR is expressed in all arterial wall layers but most abundantly in the medial layer (Liu et al, 2005). Interestingly, it is in this medial layer of the vessel wall, mainly consisting of VSMCs, where the typical vascular calcifications in CKD are observed, which is also the case in the model we present here. We started androgen therapy early, before development of bone and vascular complications, as intervention with androgens at a later timepoint, could have resulted in an irreversible phenotype, which has especially been shown for arterial calcifications (Wu et al, 2013). Treatment with T and DHT tended to decrease the proportion of CKD animals severely affected by vascular calcifications. However, an 'on-off' phenomenon seemed to be present, with some of the treated animals still displaying a pronounced vascular phenotype similar as the non-treated animals. There was no correlation between aortic calcium content and circulating T levels or androgen-sensitive organ weights in the CKD rats. We hence conclude that androgen therapy does not unequivocally prevent arterial calcification in this male CKD rat model. Additionally, we could not prevent development of calcification of rat aortic rings *in vitro* with the strong AR-agonist R1881. Next to direct effects of sex steroids and their receptors on VSMCs, also indirect effects may play an important role, e.g. via endothelial cells or circulating hormonal factors (Woodward et al, 2021). Future *in vivo* studies should

investigate specific androgen actions on vascular calcification to further disentangle these local and systemic effects.

We confirm the AR mRNA expression in bone and aortic tissue. *Ar* expression in aorta was not different in control rats compared to CKD rats, despite the observed differences in calcification. Expression of downstream target genes of AR signaling served as readout for tissue responsiveness to androgen treatment. These genes (*Acta1, Col1a1, Fkbp5*) have previously shown to be androgen-regulated both in muscle and bone (Otto-Duessel et al, 2012). Expression of *Acta1* and *Col1a1* was lower in levator ani muscle of non-treated CKD animals and treatment with DHT was able to at least partly restore these levels compared to controls, showing responsiveness of these genes to androgen treatment. This androgen responsiveness was not observed in bone and aortic tissue. Finally, *Fkbp5* was increased in levator ani muscle and aorta of CKD animals compared to controls, unlike in bone. Therapy with DHT further increased the expression in levator ani muscle, but not in aorta. These findings confirm that levator ani muscle is a very sensitive readout for androgen activity, and might suggest that the bone and aortic tissue in this CKD model are less responsive to androgens (Dubois et al, 2014). Whether this resistance is mediated by the severe hyperparathyroidism and resulting high PTH-levels is subject for further study.

Our study has several strengths. We are the first study to investigate effects of androgen replacement therapy on the bone-vascular axis in male CKD. We confirm that CKD induces hypogonadism in male rats and we are able to successfully treat the androgen deficiency. This rat model is an ideal model to be used in future studies to address other relevant questions linked to both CKD and androgen deficiency, such as anemia and erectile dysfunction. There are however also some limitations. First, this CKD model is a model of advanced CKD with a very severe secondary hyperparathyroidism which may mask androgen effects on bone and vasculature. Future studies should investigate androgen replacement therapy in alternative CKD models with less pronounced hyperparathyroidism or treat

this hyperparathyroidism either pharmacologically or surgically. Second, adult rats continue to grow during ageing without closure of the epiphyseal growth plates, which is different from humans.

In conclusion, androgen replacement therapy restores male hypogonadism in CKD, but fails to rescue the bone and vascular phenotype, at least in the presence of severe hyperparathyroidism. Whether TRT confers skeletal and vascular benefits in CKD animals (and patients) with well-controlled hyperparathyroidism remains to be studied.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR'S CONTRIBUTIONS

KD, VD, DV, PE, FC and BD conceptualized the study. KD, DS and LD performed the experimental work. KM executed the histological stainings. KD performed data analysis. KD wrote the first draft of the manuscript with assistance of BD, PE, FC and DV. All authors reviewed and edited the manuscript before submission.

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FIGURES

Figure 1: Adenine diet results in decreased kidney function, secondary hyperparathyroidism and low T levels. **A** Longitudinal change in serum creatinine levels. **B** Levels of serum creatinine at sacrifice. **C** Levels of serum urea at sacrifice. Significant difference between Control+VEH vs. CKD+VEH: **p<0.01, ***p<0.001, ****p<0.0001; Control+VEH vs. CKD+T: $^{\$\$}$p<0.01, $^{\$\$\$\$}$p<0.0001; Control+VEH vs. CKD+DHT: $^{°°}$p<0.01, $^{°°°}$p<0.001, $^{°°°°}$p<0.0001. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone. n = 10-13/group. Data represented as mean +/- SD. Two-way ANOVA followed by Tukey multiple comparison test in panel a. Kruskal-Wallis test followed by Dunn's multiple comparison in panel b.

Figure 2: Androgen therapy is effective in reverting CKD-induced hypogonadism. A Androgen-sensitive organ weights (seminal vesicles, levator ani muscle, ventral prostate, cowper glands). **B** Representative images of androgen-sensitive organs. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone. n = 10-13/group. Data represented as mean +/-SD. One-way ANOVA followed by Tukey multiple comparison or Kruskal-Wallis test followed by Dunn's multiple comparison where appropriate.

Figure 3: Androgen therapy does not influence bone loss in CKD animals. A Trabecular bone parameters vertebral body lumbar 5. **B** Trabecular bone parameters proximal metaphysis tibia. **C** Cortical bone parameters diaphysis tibia. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone, BV/TV = bone volume fraction. n = 10-13/group. Data represented as mean +/-SD. One-way ANOVA followed by Tukey multiple comparison or Kruskal-Wallis test followed by Dunn's multiple comparison where appropriate.

Figure 4: Androgen therapy does not rescue aortic calcification in CKD rats. **A** *In vivo* aortic calcium content. **B** Stratification of aortic calcification into no/mild and severe calcification **(**>0.11 mg/g wet tissue calcium content). **C** *In vivo* aortic calcification measured as % surface Von Kossa staining. **D** *In vitro* calcification of aortic rings of untreated control rats upon procalcifying medium (1.5 mM phosphate) during 7 days of culture with or without androgen treatment (1 nM R1881). **E** Correlation between aortic calcium content and serum T levels (left), seminal vesicle weight (middle), and levator ani muscle weight (right) in CKD animals. **F** H&E (left panels) and Von Kossa (right panels) staining of thoracic aorta from control+VEH (upper panels) and CKD+VEH animal (lower panels). Magnification 1x scale bar 200 µm; magnification 20x scale bar 20 µm. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone, EtOH = ethanol, R1881 =

methyltrienolone, Procalcif = procalcifying medium with 1.5mM phosphate, H&E = hematoxylin and eosin. Panel A-C: n = 10-3/group. Data represented as median +/- interquartile range. Kruskal-Wallis test followed by Dunn's multiple comparison. Differences in proportions were determined by Fisher's exact test with Benjamini-Hochberg correction for multiple testing. Panel D: n = 4/condition. Data represented as mean +/-SD. Kruskal-Wallis test followed by Dunn's multiple comparison.

Figure 5: Androgen receptor responsive genes in bone and aorta are not increased by androgen therapy in CKD rats. Relative mRNA expression levels of *Ar* (**A**), *Acta1* (**B**), *Col1a1* (**C**) and *Fkbp5* (**D**) in levator ani muscle, femur and aorta. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone, *Ar* = encoding androgen receptor, *Acta1* = encoding actin alpha 1, *Col1a1* = encoding collagen type 1 alpha chain, *Fkbp5* = encoding FKBP prolyl isomerase 5. n = 8-13/group. Data represented as mean +/-SD. One-way ANOVA followed by Tukey multiple comparison or Kruskal-Wallis test followed by Dunn's multiple comparison where appropriate. Data normalized to control levels.

Supplemental figure 1: Adenine diet induces severe alterations in kidney but not testis morphology. Kidneys and testes were fixed in paraformaldehyde 2% and Bouin's solution respectively overnight at 4°C, embedded in paraffin, sectioned at 4 µm and subsequently stained with H&E. Images were captured using TissueFAXS 7.0 (Tissuegnostics GmbH, Vienna, Austria). **A** Representative H&E staining of kidney from control+VEH animal (upper panels) and CKD+VEH animal (lower panels). **B** Representative H&E staining of testis from control+VEH animal (upper panels) and CKD+VEH animal (lower panels). From left to right: magnification 5x scale bar 100 µm; magnification 10x scale bar 50 µm; magnification 20x scale bar 20 µm. VEH = vehicle, CKD = chronic kidney disease; H&E = hematoxylin and eosin.

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TABLES

Table 1: biochemistry

	Control + VEH	CKD + VEH	CKD + T	CKD + DHT	p value
Calcium (mg/dL)	10.2[9.5-10.6]	6.9[5.5-8.4]*	6.5[5.2-9.8]$^{\$\$}$	5.7[4.4-10.6]$^{\circ\circ\circ\circ}$	<0.0001
Phosphate (mg/dL)	6.6 [5.0-10.3]	16.8 [12.5-20.6]*	18.6 [15.4-22.4]$^{\$\$\$\$}$	19.1 [8.5-26.1]$^{\circ\circ\circ\circ}$	<0.0001
PTH (pg/mL)	235.5 +/-177.4	4832.0 +/-1971.0****	3521.0 +/-1271.0$^{\$\$\$\$}$	3473.0 +/-1665.0$^{\circ\circ\circ\circ}$	<0.0001
FGF23 (pg/mL)	408.4 [260.4-801.7]	5078.0 [2481.0-168000.0]****	3695.0 [1445.0-73375.0]$^{\$\$}$	4733.0 [1590.0-12904.0]$^{\circ\circ}$	<0.0001
T (ng/dL)	140.3 +/-70.3	62.78 +/-69.1* §	138.4 +/-49.6	ND	0.0098
LH (ng/mL)	1.0 [0.4-1.9]	0.3 [0.1-1.1]	ND	ND	0.0002

Biochemistry at euthanasia. n = 10-13/group. Data are represented as mean +/-SD and median [range] for parametric and non-parametric data, respectively. One-way ANOVA followed by Tukey multiple comparison or Kruskal-Wallis test followed by Dunn's multiple comparison where appropriate. Significant difference between Control+VEH vs. CKD+VEH: *p<0.05, **p<0.01, ****p<0.0001; Control+VEH vs. CKD+T: $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001, $^{\$\$\$\$}$p<0.0001; Control+VEH vs. CKD+DHT: $^{\circ\circ}$p<0.01, $^{\circ\circ\circ\circ}$p<0.0001; CKD+VEH vs. CKD+T: §p<0.05; CKD vs. CKD+DHT: $^{+}$p<0.05. Difference in LH levels between Control + VEH and CKD + VEH were determined by Mann-Whitney test. VEH = vehicle, CKD = chronic kidney disease, T = testosterone, DHT = dihydrotestosterone, PTH = parathyroid hormone, FGF23 = fibroblast growth factor 23, LH = luteinizing hormone, ND = not determined.

584 **FIGURES**

Figure 1

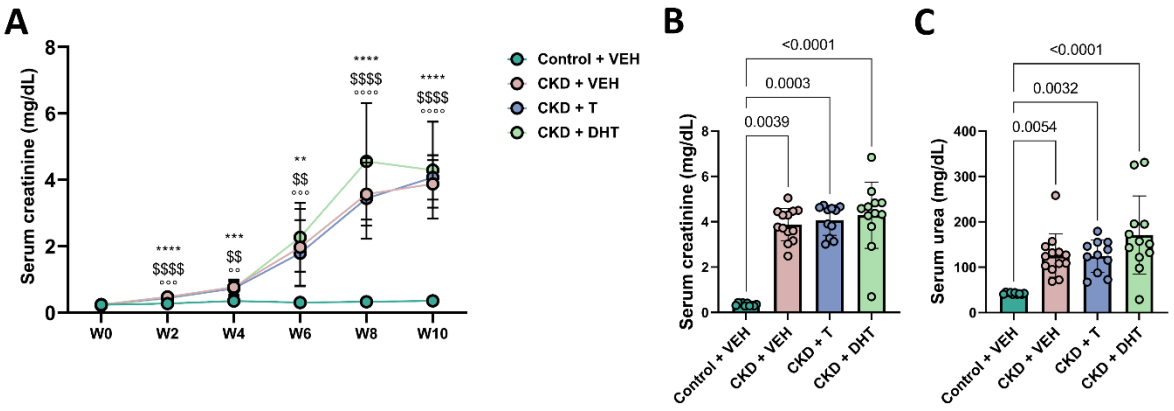

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Figure 2

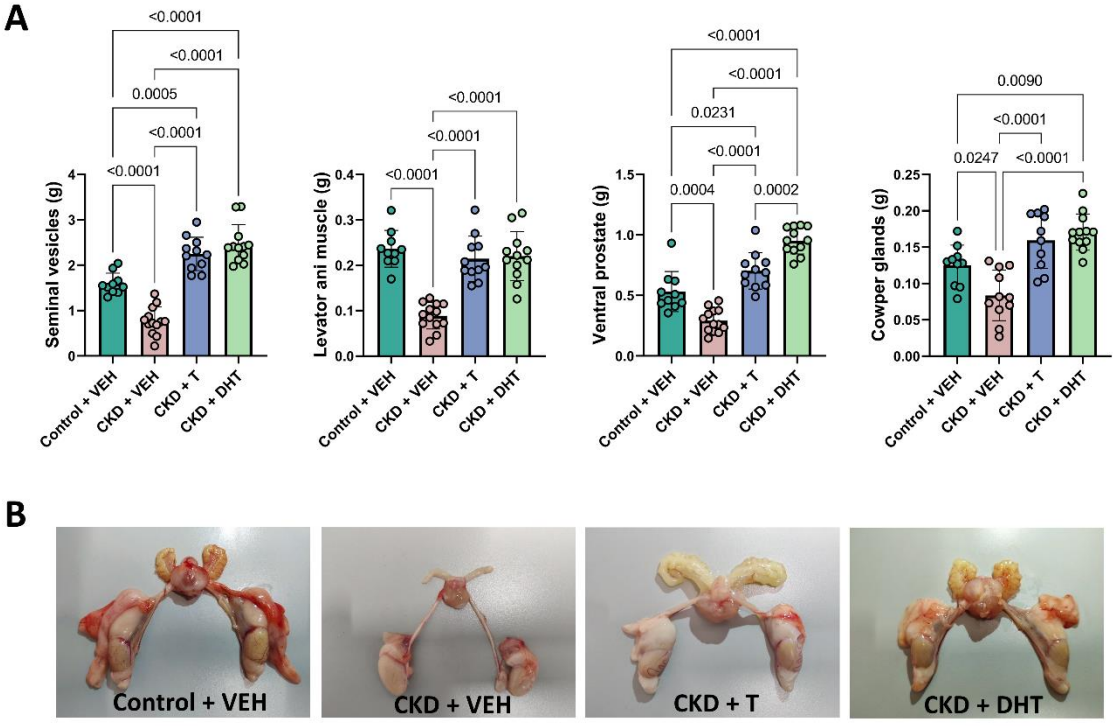

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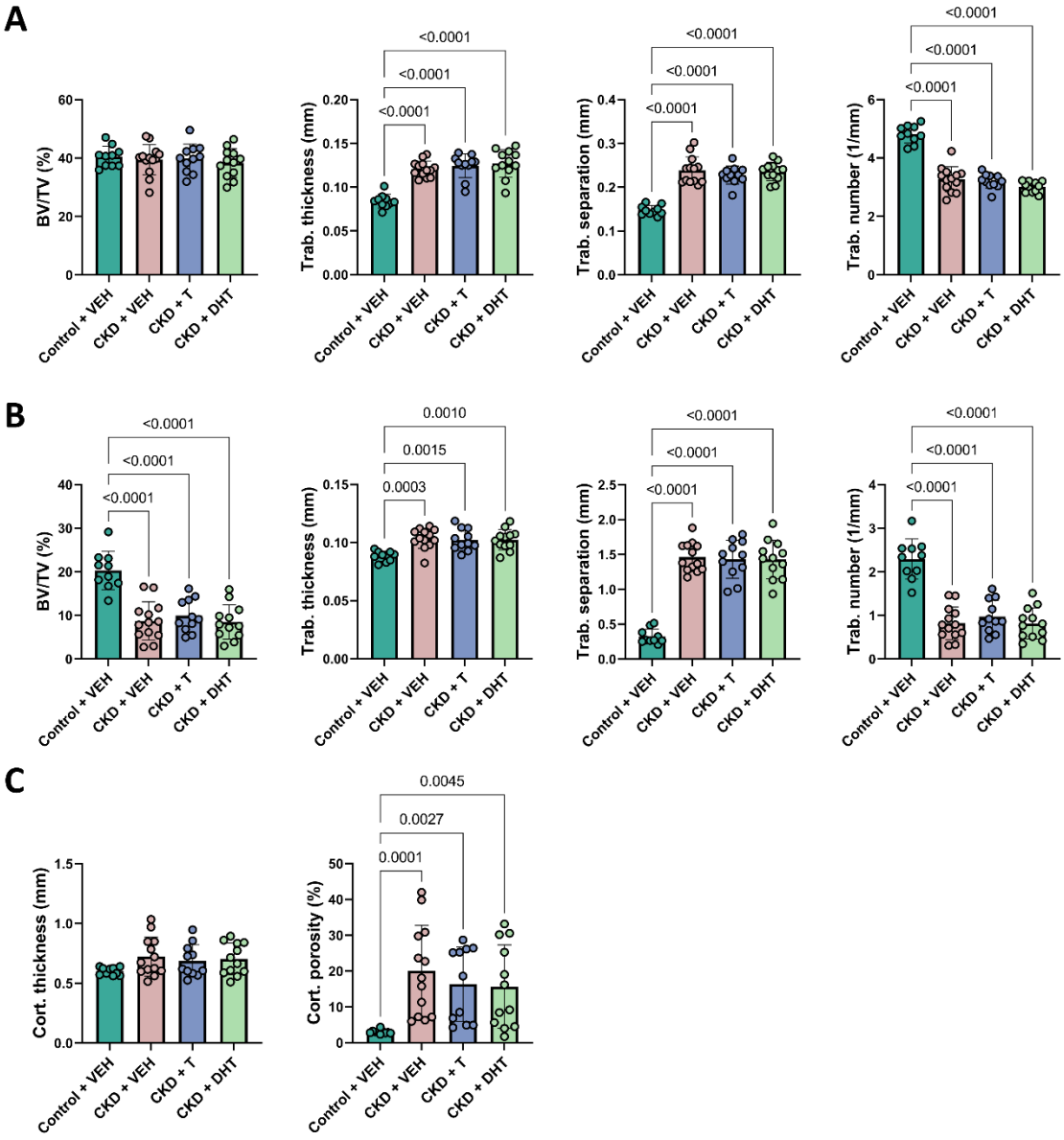
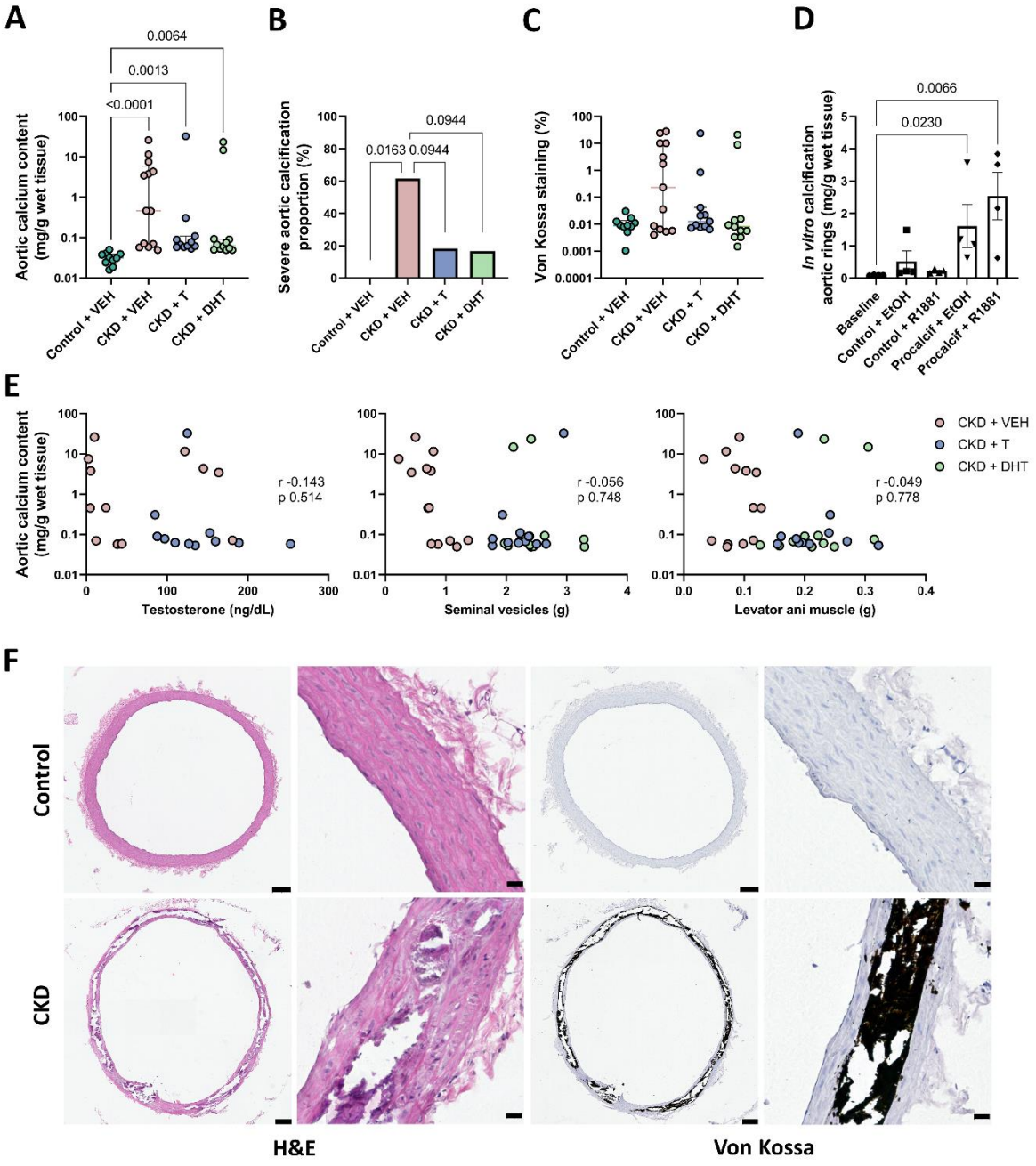
Figure 3


A
BV/TV (%)
Trab. thickness (mm)
Trab. separation (mm)
Trab. number (1/mm)
<0.0001
Control + VEH
CKD + VEH
CKD + T
CKD + DHT
B
BV/TV (%)
Trab. thickness (mm)
Trab. separation (mm)
Trab. number (1/mm)
0.0010
0.0015
0.0003
<0.0001
C
Cort. thickness (mm)
Cort. porosity (%)
0.0045
0.0027
0.0001

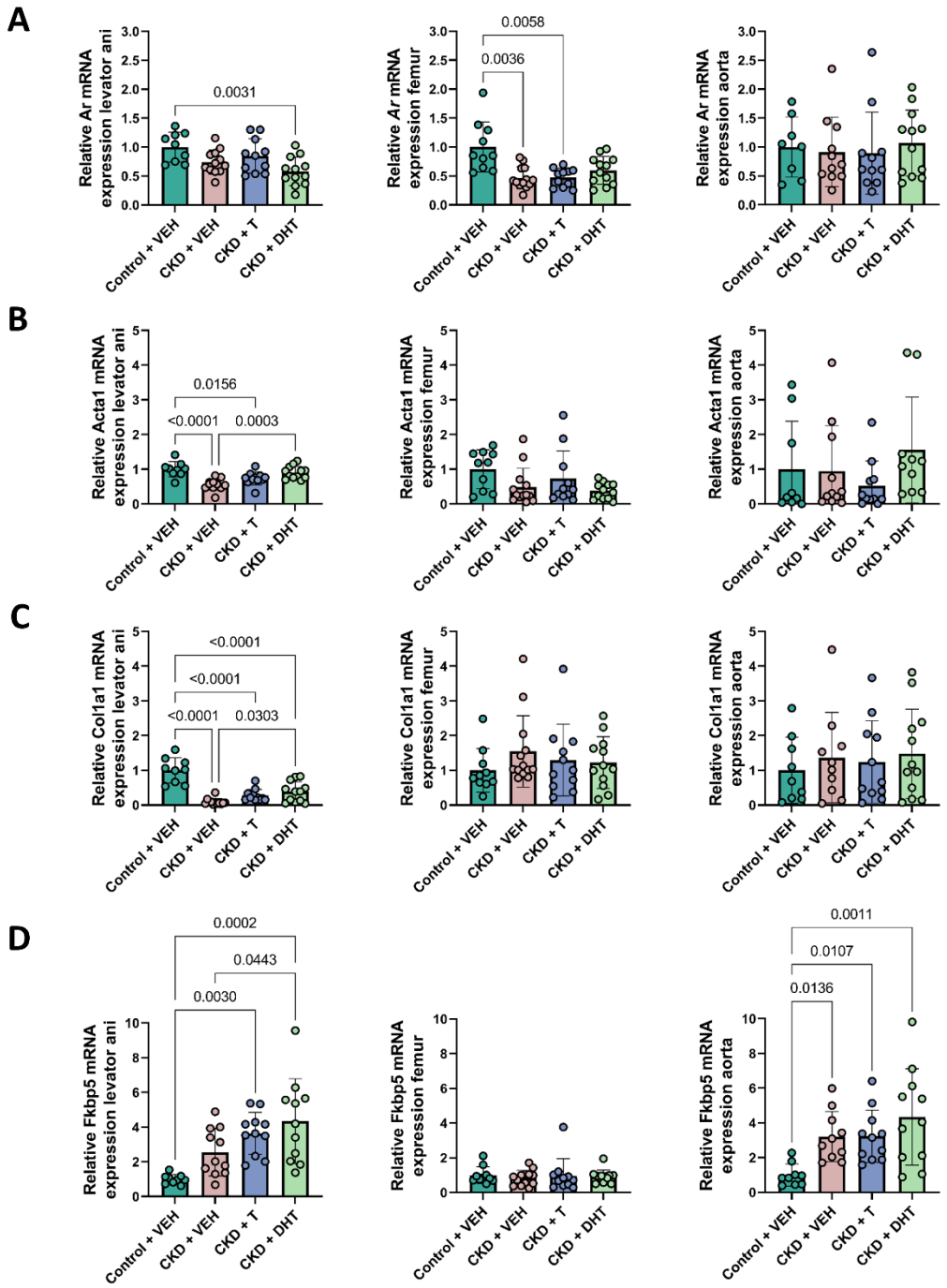
587

Figure 4


A
B
C
D
E
F
Aortic calcium content (mg/g wet tissue)
Severe aortic calcification proportion (%)
Von Kossa staining (%)
In vitro calcification aortic rings (mg/g wet tissue)
0.0064
0.0013
<0.0001
0.0944
0.0163
0.0066
0.0230
Control + VEH
CKD + VEH
CKD + T
CKD + DHT
Baseline
Control + EtOH
Control + R1881
Procalcif + EtOH
Procalcif + R1881
r -0.143
p 0.514
r -0.056
p 0.748
r -0.049
p 0.778
Testosterone (ng/dL)
Seminal vesicles (g)
Levator ani muscle (g)
Control
CKD
H&E
Von Kossa

588

Figure 5


A
Relative Ar mRNA expression levator ani
0.0031
Relative Ar mRNA expression femur
0.0058
0.0036
Relative Ar mRNA expression aorta
B
Relative Acta1 mRNA expression levator ani
0.0156
<0.0001
0.0003
Relative Acta1 mRNA expression femur
Relative Acta1 mRNA expression aorta
C
Relative Col1a1 mRNA expression levator ani
<0.0001
<0.0001
<0.0001
0.0303
Relative Col1a1 mRNA expression femur
Relative Col1a1 mRNA expression aorta
D
Relative Fkbp5 mRNA expression levator ani
0.0002
0.0443
0.0030
Relative Fkbp5 mRNA expression femur
Relative Fkbp5 mRNA expression aorta
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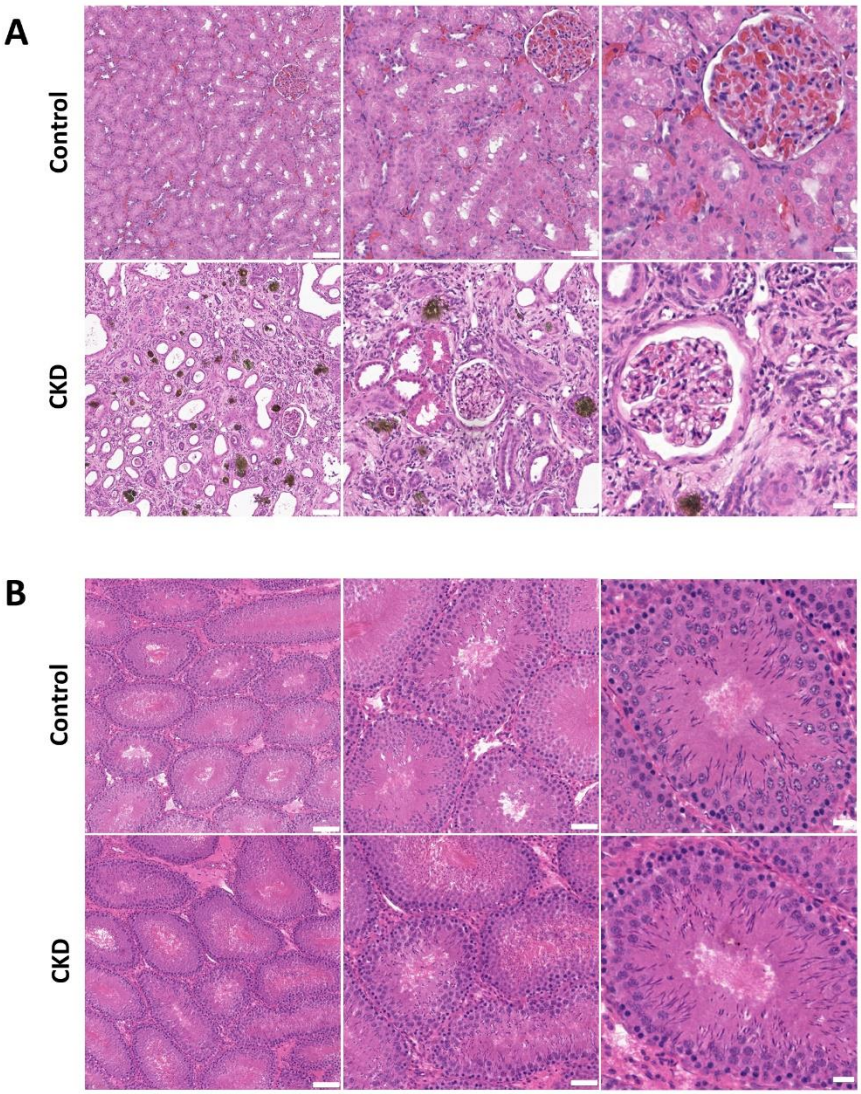
0.0107
0.0136
Control + VEH
CKD + VEH
CKD + T
CKD + DHT

589

Supplemental figure 1



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