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ABSTRACT

Renal calcium and phosphate handling is an important contributor to mineral homeostasis and bone health and the androgen receptor (AR) is highly expressed in the kidney. We investigated the short term effects of androgen deprivation on renal calcium and phosphate reabsorption, independent of their effects on bone. Two weeks following orchidectomy (ORX) of adult mice, bone loss occurred along with hypercalciuria, which was similarly prevented by testosterone and dihydrotestosterone supplementation. Treatment with bisphosphonates prior to ORX also inhibited hypercalciuria, indicating that the calcium flux originated from the bone. Renal calcium and phosphate transporter expression was increased post-ORX, independent of bisphosphonates. Furthermore, androgen deprivation appeared to stimulate local synthesis of $1,25(OH)_2D_3$. When bisphosphonate-treated mice were fed a low calcium diet, bone resorption was no longer blocked and secondary hyperparathyroidism developed, which was more pronounced in ORX mice than sham-operated mice. In conclusion, this study shows that androgen deprivation increased renal calcium and phosphate transporter expression, independent of bone, and underlines the importance of adequate intestinal calcium supply in circumstances of androgen deprivation and bisphosphonate treatment.

Key words: bone, calcium, phosphate, testosterone, orchidectomy

1. INTRODUCTION

Recent studies suggested that sex steroids might play a role in the regulation of renal calcium and phosphate handling. Similar to calciophosphotropic hormones, sex hormones may not only influence calcium and phosphate handling in the bone but also in other organs such as the kidneys. Urinary calcium excretion has been shown to be higher in males than females, in both humans and mice (1,2). In addition, male mice exhibit lower expression levels of renal calcium transporters (2). In rats, orchidectomy (ORX) increased urinary calcium excretion, which was inhibited by testosterone (T) replacement (3). In contrast, decreased urinary calcium excretion was reported 2 weeks after ORX in mice, accompanied by an increased expression of renal calcium transporters (2). The available data on the effects of androgens on renal phosphate handling are limited and contradictory as well. Men treated with GnRH analogs exhibited increased serum phosphate levels as well as increased renal phosphate reabsorption (4,5). In contrast, ORX in male rats had no influence on serum phosphate or urinary phosphate excretion (6). Kidney stones, with hypercalciuria as major risk factor, are 2 to 3 times more frequent in males compared to females, and males with low T have lower odds of kidney stones (7,8). Mice lacking renal androgen receptor (AR) were shown to exhibit less calcium oxalate crystal formation (9).

T is the main circulating androgen in men. It exerts its effects via the AR as T or, after conversion by the 5α-reductase, as dihydrotestosterone (DHT). In addition, T, but not DHT, can be aromatized into estradiol and bind to the estrogen receptor (ER). Hence, in tissues T can act via the AR as T or DHT, or via the ER as estradiol (10). In previous studies, we and others have shown that the well-established bone-sparing action of androgens is not entirely explained by direct effects on bone cells (11–14). Androgen action may at least partly be explained by its action on the kidneys. Renal AR expression is high, but the exact location of AR expression in the kidney is still debated, with expression reported along the entire nephron as well as in the glomerulus (15). The role of androgens, however, remains unresolved despite gender differences and hormonal dependence of typical kidney diseases such as nephrolithiasis, chronic kidney disease (incidence and progression) and predisposition for renal injury (16,17).

Serum calcium and phosphate levels are tightly regulated between narrow ranges (15). The kidney but also the intestine and the bone represent exchange routes for calcium and phosphate. As such, the kidneys are major regulators of mineral homeostasis, as illustrated by the profound dysregulation of mineral metabolism during chronic kidney disease (18). Moreover, the role of the kidney becomes more dominant when intestinal calcium or phosphate absorption is suboptimal or when bone turnover is low (19,20). In the kidney, urinary calcium is mainly reabsorbed via passive paracellular transport in the proximal tubulus (PT) and the thick ascending limb of the loop of Henle (TAL), and partially by claudins which are epithelial tight junction proteins expressed along the entire nephron. Several claudins, including claudin-2, -12, -16 and -19, form channels to transport calcium from the tubular fluid into the circulation (21–24). The fine-tuning of renal calcium reabsorption, however, is believed to occur in the distal tubulus (DT) where calcium is taken up transcellularly via the transient receptor potential cation channel subfamily V member 5 (TRPV5) channel, transported by calbindin-D9K (CaBP9K) and calbindin-D28K (CaBP28K) to the basolateral membrane and exits the cell to the circulation via the sodium/calcium exchanger (NCX1) and plasma membrane calcium ATPase (PMCA) (25). The calcium-sensing receptor (CaSR) is expressed throughout the nephron with the highest expression in the TAL (26). When serum calcium increases, the CaSR will promote renal calcium

excretion through interaction with the claudin network and by altering potassium transport, hereby influencing the transepithelial potential difference and providing a driving force for the excretion of calcium (26,27). By contrast, urinary phosphate is reabsorbed mainly in the PT. Although much less is known about the transporters involved, several apical membrane phosphate transporters have been identified, including sodium-phosphate cotransporter 2c (NaPi-2c) and sodium-dependent phosphate transporter 1 and 2 (PiT1 and PiT2) (15).

The kidney is also the main source of $1,25(OH)_2D_3$ synthesis, which takes place in the PT. CYP27B1, predominantly expressed in the PT, is able to convert $25(OH)D_3$ to $1,25(OH)_2D_3$. CYP24A1 limits the amount of $1,25(OH)_2D_3$ when circulating $1,25(OH)_2D_3$ is elevated by catalyzing the conversion of $1,25(OH)_2D_3$ into 24-hydroxylated products targeted for excretion or by producing $24,25(OH)_2D_3$, thus decreasing the pool of $25(OH)D_3$ available for 1-hydroxylation. In the PT, VDR action mainly limits CYP27B1 activity, whereas in the DT it stimulates active transcellular calcium transport, in particular via CaBP9K/28K and to a lesser extent TRPV5 (25).
In conclusion, current knowledge on the effects of androgens on renal calcium and phosphate handling is based on few and contradicting data. In addition, available findings could be confounded by effects of androgens on bone. Therefore, the aim of this study was to investigate the acute effects of androgen deprivation on renal calcium and phosphate handling in adult male mice, and this in the presence or absence of a bisphosphonate treatment.

2. MATERIALS AND METHODS

2.1. Animals

Male C57BL/6J mice (Charles River, Saint-Germain-Nuelles, France) were housed in a light and temperature-controlled room with *ad libitum* access to drinking water and standard chow (1% calcium, 0.7% phosphate, Ssniff, Soest, Germany), unless stated otherwise. Nembutal (i.p. 100 mg/kg, Ceva Santé Animale, Libourne, France) followed by cardiac puncture was used for euthanasia. All animal procedures were approved by the KU Leuven animal ethics committee (P042/2014). For all experimental setups, male C57BL/6J mice were randomly allocated into different groups (n=12 per group) at 18 weeks of age to undergo a SHAM operation or a bilateral ORX under isoflurane anesthesia (3% induction, 2% maintenance), after 1 week of acclimatization. ORX was used as a model for primary, organic hypogonadism with acute and complete androgen deprivation (28).

Experimental setup A (fig 1A): After the operation, implants of medical-grade silicone tubing (Silclear, Degania Medical, Degania, Israel) sealed with medical adhesive silicone (Silastic, Biesterfeld, Germany) were implanted in the nuchal region, either empty (vehicle, VEH) or filled with T or DHT (SHAM+VEH, ORX+VEH, ORX+T, ORX+DHT, n=12 per group).

Experimental setup B (fig 1B): One week before the operation, mice were given vehicle (PBS, i.p., ThermoFisher Scientific, Massachusetts, USA) or risedronate injections (20 µg/kg, i.p., Merck, Darmstadt, Germany) every 4 days (SHAM+VEH, SHAM+RIS, ORX+VEH, ORX+RIS).

Experimental setup C (fig 1C): One week before the operation, all mice started receiving risedronate injections every 4 days. Mice were also started on a normal calcium diet (NCD, 1%) or a low calcium diet (LCD, 0.02%) (SHAM+RIS+NCD, SHAM+RIS+LCD, ORX+RIS+NCD, ORX+RIS+LCD).

For all setups, 1 and 2 weeks after the operation, serum samples were taken via the submandibular vein and cardiac puncture respectively, and mice were put in metabolic cages for 24 h urine collections. Mice were euthanized and kidneys, femurs and vertebrae were taken out for further processing and analyses.

2.2. Serum and urine analyses

Calcium and phosphate levels in serum and urine were analyzed by SYNCHRON Clinical Systems (Beckman Coulter). Osteocalcin levels were assessed by radioimmunoassay as previously described (29). Serum $1,25(OH)_2D_3$ was measured via LC-MS/MS. Serum PTH (Immutopics International, California, USA) and FGF23 (Kainos Laboratories Inc., Tokyo, Japan) levels were determined by ELISA.

2.3. Gene expression analysis

Total RNA of tissues was extracted with TRIzol (ThermoFisher Scientific) followed by phenol/chloroform purification. cDNA was synthesized using reverse transcriptase SuperScript II RT (ThermoFisher Scientific) and qRT-PCR was performed. Gene expression was normalized for hypoxanthine-guanine phosphoribosyltransferase (Hprt) and expressed relative to the control group ($2^{-\Delta\Delta CT}$ method). Details on the primers used are provided in supplementary table 1. A TaqMan assay for NaPi-2a was purchased from Thermofisher Scientific.

2.4. Bone structure

Micro-computed tomography (µCT) analysis of the L5 vertebrae was performed *ex vivo* using the high resolution SkyScan 1172 system (50 kV, 200 µA, 0.5 mm Al filter). Serial tomographs, reconstructed from raw data with the cone-beam reconstruction software (NRecon, V1.7.0.4; Skyscan), were used to compute trabecular parameters.

2.5. Bone calcium content

Femur dry weight was measured after overnight incubation at 100°C, followed by 8 hours at 500°C for ashing. Ashes were dissolved in 1M HCl and diluted 1/100 in water for calcium measurements with the SYNCHRON Clinical Systems (Beckman Coulter). Results were expressed relative to the dry weight and as total calcium weight.

2.6. Immunohistochemistry

Immunofluorescence co-stainings were carried out with antibodies directed against the AR and renal markers (podocin, megalin, uromodulin, trpv5) on kidneys of adult, male C57BL/6J mice. Antigen retrieval was performed in a 0.01M citrate solution using a pressure cooker. Antigen detection was performed using a Tyramide signal amplification (Perkin Elmer) system, followed by nuclear counterstaining with DAPI (4',6-diamidino-2-phenyl-indole dihydrochloride). Samples incubated without primary antibody were used as negative controls. Images were captured using a slide scanner microscope Axio Scan.Z1 (Zeiss). Details of antibodies and dilutions are provided in supplementary table 2.

2.7. Statistical analyses

Values are expressed as mean±SEM. Statistical significance between groups ($p<0.05$) was determined by One-Way (data from experimental setup A) or Two-Way (data from experimental setup B and C)

ANOVA, followed by Bonferroni's test for multiple comparisons. All analyses were performed using GraphPad Prism (version 6.07, La Jolla California USA).

RESULTS

3.1. Androgen deprivation induces hypercalciuria and early bone loss

In order to determine whether and how androgens influence renal calcium and phosphate handling, adult male mice were orchidectomized and treated with T, DHT, or vehicle (fig 1A). Serum and urinary calcium and phosphate levels after 1 and 2 weeks were compared with those of SHAM-operated mice treated with vehicle.

Seminal vesicle weight, the most androgen-sensitive organ and measured to verify efficacy of ORX, was decreased 5 fold after ORX. Dietary calcium and phosphate intake was similar between the groups, as well as urinary volumes and renal function assessed by serum cystatin C levels (table 1). Serum calcium and phosphate levels were not different between ORX and SHAM mice (table 1). Urinary phosphate excretion was not affected either, while an increase in urinary calcium excretion was observed compared to SHAM (1.5 fold in week 1 and 2). This effect was abolished by both T or DHT replacement.

Table 1. Effect of orchidectomy (ORX) on calcium and phosphate balance and general parameters

Parameter	SHAM + VEH	ORX + VEH	ORX + T	ORX + DHT
BW (g) Week 2	28.67 ± 0.53	27.34 ± 0.43	29.26 ± 0.31 [b]	28.22 ± 0.42
Seminal vesicle weight (g/100g BW) Week 2	1.11 ± 0.03	0.21 ± 0.01 [a]	1.44 ± 0.04 [ab]	1.37 ± 0.05 [ab]
Urinary volume (mL) Week 1 Week 2	 1.55 ± 0.15 1.65 ± 0.15	 1.24 ± 0.11 1.31 ± 0.10	 1.79 ± 0.23 1.99 ± 0.24	 1.49 ± 0.15 1.7 ± 0.19
Serum Cystatine C (ng/mL) week 2	556.70 ± 36.19	525.90 ± 22.49	ND	ND
Calcium intake (mg/100g BW/24h)	139.59 ± 10.47	137.95 ± 3.22	136.26 ± 6.94	135.27 ± 3.78
Phosphate intake (mg/100g BW/24h)	92.31 ± 6.59	90.21 ± 3.11	93.26 ± 4.78	91.04 ± 2.81
Serum calcium (mg/dL) Week 1 Week 2	 9.44 ± 0.58 7.57 ± 0.11	 9.23 ± 0.14 7.83 ± 0.09	 8.95 ± 0.23 7.78 ± 0.17	 9.32 ± 0.12 7.97 ± 0.13
Serum phosphate (mg/dL) Week 1 Week 2	 7.76 ± 0.28 10.09 ± 0.51	 7.29 ± 0.24 9.86 ± 0.53	 7.51 ± 0.20 9.04 ± 0.60	 7.83 ± 0.30 8.86 ± 0.47
Urinary calcium (mg/dL) Week 1 Week 2	 5.40 ± 0.72 5.94 ± 0.39	 8.25 ± 0.70 [a] 8.97 ± 0.65 [a]	 4.61 ± 0.41 [b] 6.25 ± 0.32 [b]	 5.63 ± 0.51 [b]

				6.81 ± 0.29 [b]
Urinary phosphate (mg/dL)	112.00 ± 11.32	124.20 ± 21.33	116.30 ± 14.28	130.40 ± 15.36
Week 1	121.10 ± 14.7	168.00 ± 20.42	136.90 ± 18.92	107.50 ± 11.22
Week 2				

SHAM+VEH = sham-operated mice treated with vehicle; ORX+VEH = orchidectomized mice treated with vehicle; ORX+T = orchidectomized mice treated with T; ORX+DHT = orchidectomized mice treated with DHT; BW = body weight; ND = not done.

Data are presented as mean ± SEM. One-Way ANOVA, n = 12 per group, [a] P<0.05 vs. SHAM+VEH; [b] P<0.05 vs. ORX+VEH.

Since sex steroid deficiency induces early bone loss, which could interfere with the obtained results, trabecular bone was analyzed using microCT. Bone loss was observed already 2 weeks after ORX, as evidenced by a 15% decrease in bone mass with an 11% decrease in trabecular number, and a 4% increased trabecular separation. This bone loss was inhibited by T or DHT replacement (fig 2A). Increased bone turnover in ORX mice was confirmed by elevated serum osteocalcin levels (fig 2A).

3.2. Bisphosphonates inhibit bone-loss induced calciuria in circumstances of sufficient dietary calcium

As ORX induces early bone loss, mice were, prior to ORX or SHAM, treated with the bisphosphonate risedronate (versus vehicle) to inhibit bone resorption (fig 1B). MicroCT analysis confirmed that bone loss after ORX was prevented by risedronate (fig 2B). To confirm that the ORX-associated bone loss was induced by increased resorption and not by a mineralization defect, femurs were ashed and analyzed for calcium content. Calcium per dry weight was not affected by ORX, while total calcium levels decreased with 14%. This decrease was prevented by risedronate. Furthermore, risedronate prevented the increase of serum osteocalcin levels (fig 2B) and inhibited the ORX-induced hypercalciuria (fig 3A), indicating that the increased renal calcium loss originated from the bone. Phosphaturia remained unaffected.

Regular chow for mice contains higher amounts of calcium than the recommended dietary intake. This high dietary calcium potentially affects renal calcium and phosphate handling. To minimize interference and study the effects of ORX independent of both bone and dietary calcium, mice were given either a normal calcium diet (NCD) or a low calcium diet (LCD). All the animals were SHAM or ORX-operated and treated with risedronate (fig 1C). MicroCT analysis showed that while risedronate efficiently inhibited ORX-induced bone loss under the regular diet, this was no longer the case under the LCD. The LCD decreased bone mass with 13% and 8% in SHAM and ORX mice, respectively (fig 2C). Femoral calcium/dry weight was not altered. LCD, however, did decrease total calcium levels with 15% in SHAM-operated mice and 14% in ORX mice (fig 2C). In addition, serum osteocalcin was significantly increased in ORX+RIS+LCD mice (fig 2C). Calciuria was not affected under LCD in circumstances of bisphosphonate treatment, but urinary phosphate excretion was 2.5 fold increased (fig 3B). The LCD did not influence intestinal calcium absorption, as assessed by 24h fecal calcium content corrected for dietary calcium intake (data not shown).

3.3. Androgen deprivation increases the expression of renal calcium and phosphate transporters

Next, we investigated the effects of ORX on the expression of renal transporters involved in calcium and phosphate reabsorption after 2 weeks. As shown in figure 4A, renal mRNA expression of *claudin-2, -12,* (located in the PT) *-16* and *-19* (located in the TAL) increased after ORX. The calcium transporters located in the DT (*Trpv5*, *Cabp9k, Cabp28k, Ncx1* and *Pmca*) showed higher expression after ORX as well. Renal expression of *CaSR* was 2.1 fold increased in ORX mice. Also for the renal phosphate transporters *NaPi-2c*, *Pit1* and *Pit2* increased expression was observed, while *NaPi-2a* expression was not altered (fig 5A). Of note, although kidney weight decreased following ORX, relative cortical and medullary area was not different between SHAM and ORX mice (data not shown), thereby making it unlikely to affect the expression of these transporters. Following supplementation with T or DHT the increased expression of renal calcium and phosphate transporters was no longer observed.

Following treatment with risedronate, enhanced expression of renal calcium transporters persisted. An additional increase was even observed for the DT-related renal calcium transporters *Trpv5*, *Cabp9k,* and *Cabp28k* after ORX (fig 4B). *CaSR* expression was lower in risedronate-treated versus vehicle-treated ORX mice. Expression of renal claudins (fig 4B) and phosphate transporters (fig 5B) was similarly increased in vehicle-treated and risedronate-treated mice.

Finally, the LCD induced an additional increase in the calcium transporters *claudin-19, Trpv5*, *Cabp9k,* and *Pmca* in ORX mice (fig 4C). The diet had no effect on *CaSR* or phosphate transporter expression (fig 5C).

3.4. The renal androgen receptor expression is mainly located in the proximal tubulus

To understand androgen-AR action in the kidney, including renal calcium and phosphate handling, it is essential to know where the AR is expressed. We performed immunofluorescence co-stainings for the AR with specific markers of different renal substructures. As shown in figure 6, the AR is located predominantly in the PT (nuclear expression, whereas megalin is located at the apical border), without expression in the TAL or DT.

3.5. Androgen deprivation increases renal vitamin D metabolism

ORX increased renal mRNA expression of the vitamin D receptor (*Vdr*), as well as *Cyp27b1* and *Cyp24a1* (fig 7A). This increase was absent in case of T or DHT supplementation. When mice were treated with risedronate, an additional increase in *Cyp27b1* expression was observed after ORX (fig 7B). Finally, in circumstances of low dietary calcium a 10 fold increased expression of *Cyp27b1* was observed in the ORX+LCD group compared to the control group (fig 7C). Serum analyses indicated secondary hyperparathyroidism under the low calcium diet, with increased PTH and $1,25(OH)_2D_3$ levels, most pronounced for the ORX mice (fig 8B).

3. DISCUSSION

We show that androgen deprivation by orchidectomy in adult mice acutely increased the expression of renal calcium and phosphate transporters and local vitamin D metabolism independent of bone in cicumstances of sufficient dietary calcium intake.

The observation of increased calciuria post-ORX is in agreement with other studies (3,6). Hsu *et al.* however reported reduced urinary calcium excretion in mice 2 weeks post-ORX, assuming that this was a too short period to cause significant bone changes (2). Yet we show significant bone loss by microCT, calcium content in ashed bone and increased serum bone turnover markers. The finding of early bone loss following ORX, due to a well-established imbalance between bone resorption and formation, is in line with previous findings of our group (30). In order to circumvent this potentially confounding impact on bone homeostasis in the study of renal effects of androgen modulation, we suppressed bone resorption with a bisphosphonate prior to ORX and showed that hypercalciuria was prevented, indicating that the calcium flux originated from the bone.

Dietary calcium intake in mice is high due to regular mouse chow containing high calcium levels (31), which contrasts with the often low dietary calcium intake in – especially elderly and frail- humans. Hence, we investigated whether the changes we observed could be confirmed in circumstances of low dietary calcium intake. Surprisingly, the combination of bisphosphonate treatment and very low calcium diet did not decrease serum or urinary calcium levels after ORX, while phosphaturia increased. However, when mice were fed a low calcium diet, bisphosphonate treatment was no longer able to fully block bone resorption. Most likely this was due to the secondary hyperparathyroidism, as shown by high serum levels of PTH and $1,25(OH)_2D_3$. This calciotropic response was particularly pronounced in ORX mice and reflected by the high phosphaturia as well. These acute changes probably reflect a compensatory renal action to maintain serum calcium and phosphate levels in circumstances of androgen deprivation. This finding is clinically relevant, as men treated with androgen deprivation therapy and at increased risk for secondary osteoporosis often have a low dietary calcium and vitamin D intake (32–34). Treatment with bisphosphonates might thus be less effective under circumstances of combined hypogonadism and low dietary calcium (35). Very few data are available with respect to the effects of androgens on renal phosphate handling. Similar to our study, serum and urinary phosphate levels were unaffected in male rats, 3 months after ORX (6).

Expression of renal calcium and phosphate transporters, involved in both paracellular and transcellular reabsorption along the nephron, was increased after androgen deprivation. This finding persisted after inhibition of bone resorption by bisphosphonate treatment, indicating that the effects on the renal calcium and phosphate transporter expression are independent of the effects on bone. The calcium transporters that are expressed at the level of the DT even exhibited an additional increase after bisphosphonate treatment. This does not appear to be a direct effect on transporter expression, as risedronate did not alter expression in sham-operated mice. The increased expression of renal *CaSR*, promoting calciuria, after ORX could be secondary to the calcium flux from bone to serum thereby preventing transient hypercalcemia. This is supported by the finding of decreased *CaSR* expression after bisphosphonate treatment. T or DHT suppressed calcium and phosphate transporter expression to a similar extent, suggesting that androgens inhibit renal calcium and phosphate transporters exclusively via the AR. Similar to our experiments, others showed increased expression of renal DT calcium transporters following ORX in mice as well (2). To our knowledge, no other data are available on the effects of androgens on renal PT phosphate transporters. Mice fed a low calcium diet exhibited a further increase of renal calcium transporters *Cldn19*, *Trpv5*, *Cabp9k* and *Pmca*. *CaSR* expression remained elevated as well, probably explaining the absence of expected hypocalcuria which is usually observed in circumstances of low intestinal calcium supply. The physiological role of the increase in renal calcium and phosphate transporters, including CaSR, shortly after androgen deprivation is unclear. In order to investigate the role of the renal AR in renal calcium and phosphate handling, and

whether the absence of renal AR in its turn influences bone, the development of a mouse model with kidney-specific knockout of the AR would be desirable.

The mechanism of the unexpected androgen/AR-mediated modulation of calcium and phosphate transporters in different parts of the nephron remains speculative. Renal $1,25(OH)_2D_3$/VDR action, however, might mediate the increased calcium and phosphate transporter expression during androgen deprivation. First, we observed increased renal expression of markers of vitamin D metabolism post-ORX, independent of bisphosphonate treatment. Our data regarding vitamin D metabolism are in line with renal transcriptome analyses, showing reduced *Cyp24a1* mRNA 12 hours after T treatment of ORX male mice. *Cyp27b1* was also decreased after 3 days of T treatment (36). Second, we have shown that the AR is dominantly expressed in the PT, where $25(OH)D_3$ is taken up by endocytosis and where the synthesis of $1,25(OH)_2D_3$ takes place (25). Third, renal VDR action is also known to be present in the DT where it regulates active transcellular calcium transport. We have shown that the vitamin D-regulated calcium transporters *Trpv5*, *Cabp9k,* and *Cabp28k* were increased in ORX mice, independent of bisphosphonate treatment. Thus, increased synthesis of $1,25(OH)_2D_3$ in circumstances of low androgens at the level of the PT could increase active phosphate reabsorption at the level of the PT but also active calcium reabsorption at the level of the DT.

Our study underlines the complex and dynamic interplay between the kidney, bone and intestines with respect to calcium and phosphate homeostasis. In contrast to other studies, our experimental setup took into account the confounding effect of early bone loss by androgen deprivation as well as the role of the dietary calcium content. Similar to earlier studies, androgen deprivation induced an increase of major renal calcium transporters in the DT, mediated via the AR and persisting after modulation of bone resorption and calcium intake. Moreover, as schematically summarized in figure 9, we extended this observation to more renal (transcellular as well as paracellular) calcium and phosphate transporters which are also expressed in the PT, which appears to be the primary target site of androgens with dominant localization of the AR. As androgens decrease renal calcium and phosphate transporter expression, it is unlikely that the kidney plays a role in the bone-sparing effect of androgens. However, the overall striking impact of androgens on renal calcium and phosphate transporters appears to be a highly conserved AR-dependent effect, which may play a role in the pathophysiology of kidney diseases with a sex (hormone) difference in prevalence, such as nephrolithiasis and chronic kidney disease. The main limitation of this study is the lack of a mechanistic pathway. We explored short-term effects of androgen deprivation in mice with a normal kidney function. Future studies with long-term androgen deprivation and with models of nephrolithiasis and renal insufficiency are of course of high interest.

In conclusion, this study shows that androgens modulate renal calcium and phospate transporters via the AR and independent from bone. This effect on the kidney is probably not clinically relevant in hypogonadal osteoporosis but could play a role in prevalent kidney diseases. Finally, we show that adequate intestinal calcium supply is pivotal in combined circumstances of androgen deprivation and bisphosphonate treatment.

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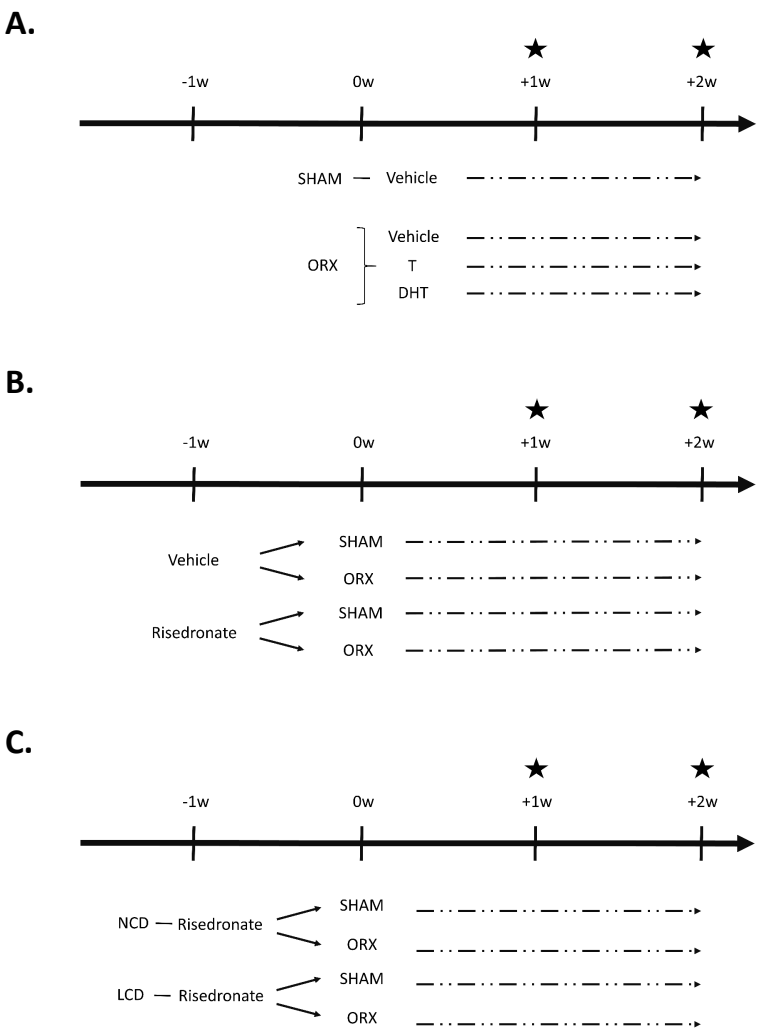

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437 **Figure 1. Schematic overview of the experimental setups. A.** Orchidectomy (ORX) and androgen
438 replacement (T or DHT) **B.** ORX preceded by bisphosphonate (risedronate) treatment **C.** ORX preceded
439 by bisphosphonate treatment and low calcium diet. Asterixes indicate the timing of metabolic cage
440 housing for 24-hour urine collections and blood sampling.

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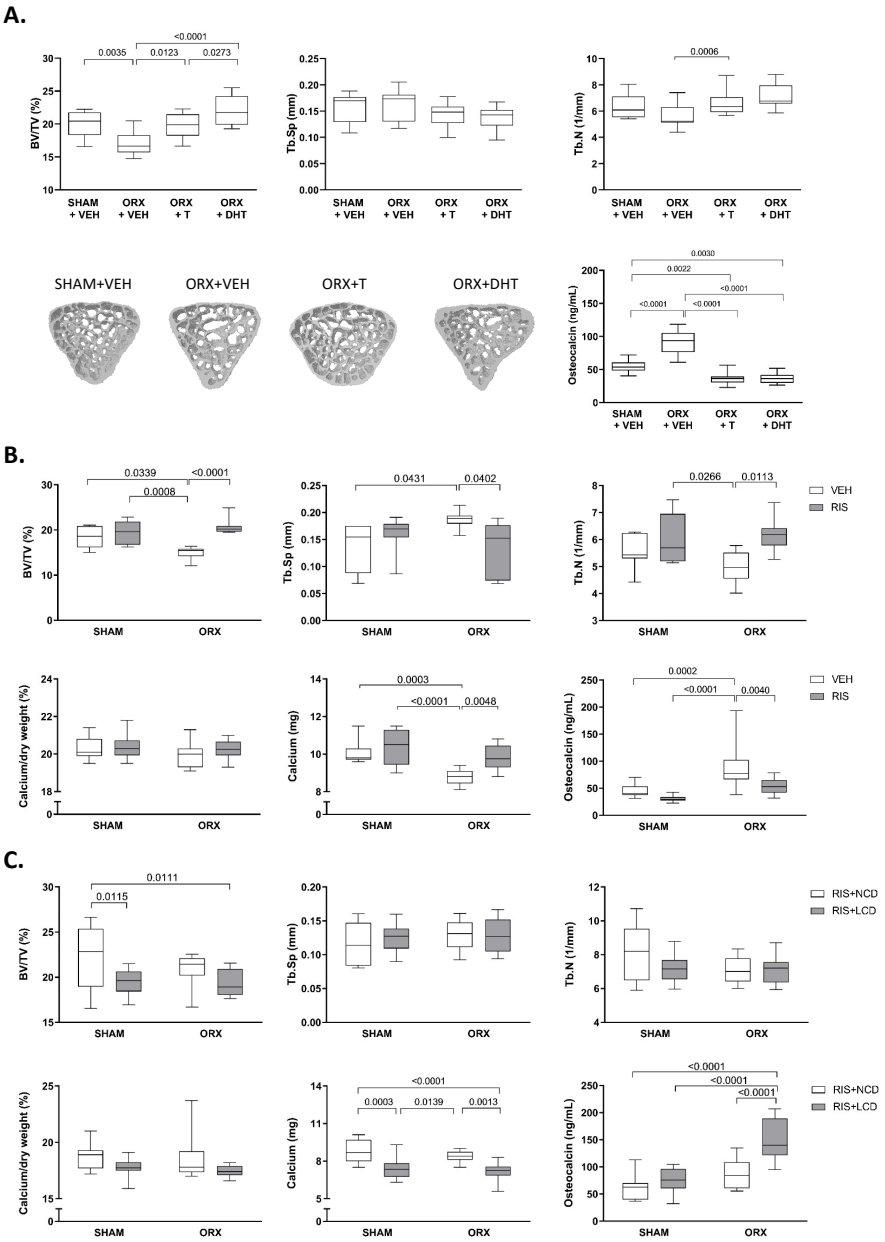

Figure 2. Early effects of androgen deprivation on bone. A. Effect of orchidectomy (ORX) and androgen replacement on trabecular bone parameters in the L5 vertebrae (top), 3D reconstructions of the vertebral body and serum osteocalcin (bottom) **B.** Effect of ORX and risedronate on trabecular bone parameters in the L5 vertebrae (top), femoral calcium content and serum osteocalcin (bottom) **C.** Effect of ORX, risedronate, and dietary calcium on trabecular bone parameters in the L5 vertebrae (top), femoral calcium content and serum osteocalcin (bottom). BV/TV = bone mass; Tb. N = trabecular number; Tb. Sp = trabecular separation. SHAM+VEH: sham-operated mice treated with vehicle; ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized mice treated with T; ORX+DHT: orchidectomized mice treated with DHT. VEH: vehicle; RIS: risedronate; NCD: normal calcium diet; LCD: low calcium diet. Data are presented as mean ± SEM. One-Way ANOVA (A) and Two-Way ANOVA (B-C) with Bonferroni's test for multiple comparisons, n = 12 per group.

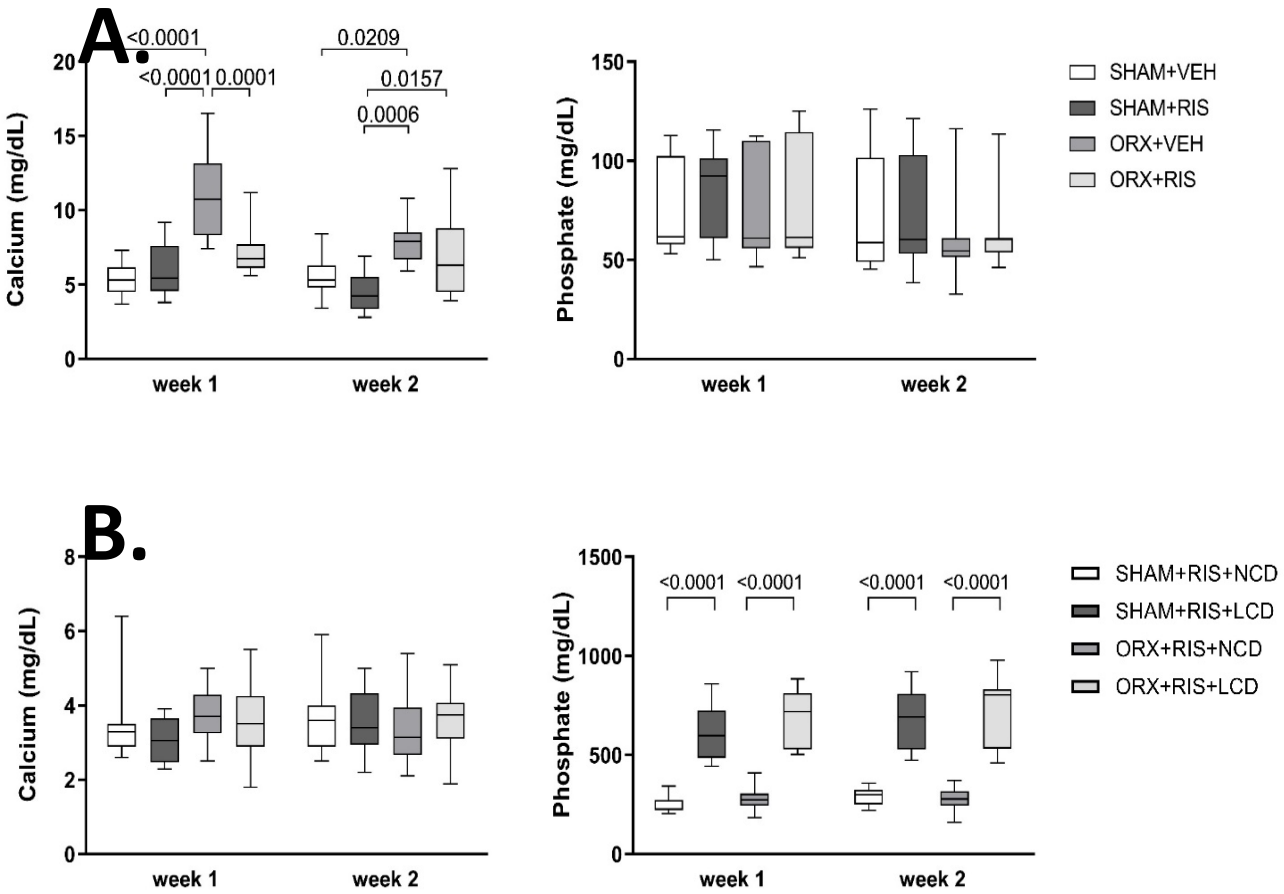

Figure 3. Effects of bisphosphonate treatment and low dietary calcium on urinary calcium and phosphate excretion after androgen deprivation. A. Effect of orchidectomy (ORX) and risedronate **B.** Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle; SHAM+RIS: sham-operated mice treated with risedronate; ORX+VEH: orchidectomized mice treated with vehicle; ORX+RIS: orchidectomized mice treated with risedronate. SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are presented as mean ± SEM. Two-Way ANOVA with Bonferroni's test for multiple comparisons, n = 12 per group.

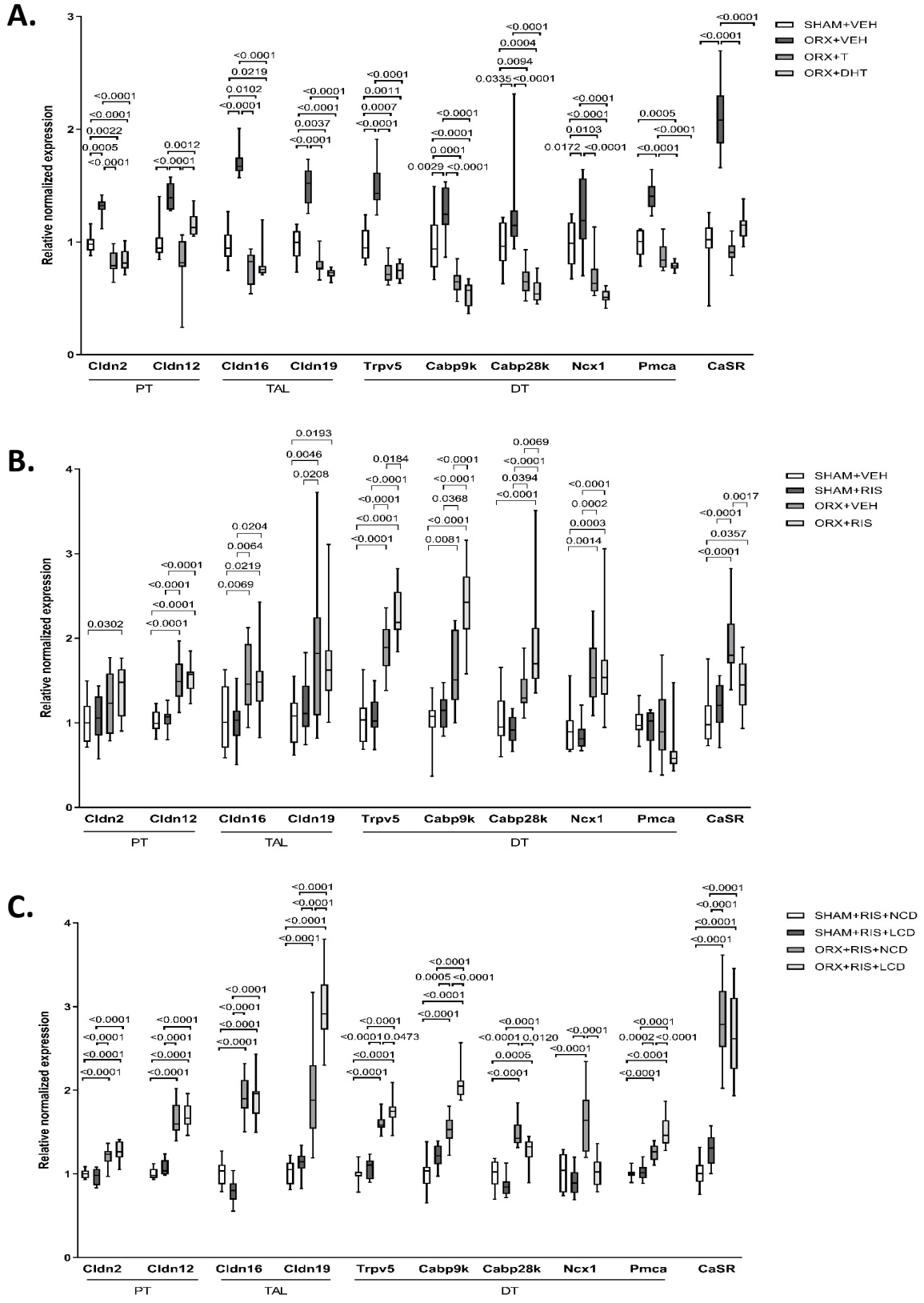

Figure 4. Effects of androgen deprivation on renal mRNA expression of calcium transporters. A. Effect of orchidectomy (ORX) and androgen replacement **B.** Effect of ORX and risedronate **C.** Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle; ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized mice treated with T; ORX+DHT:

orchidectomized mice treated with DHT; SHAM+RIS: sham-operated mice treated with risedronate; ORX+RIS: orchidectomized mice treated with risedronate; SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are presented as mean ± SEM. One-Way ANOVA (A) and Two-Way ANOVA (B-C) with Bonferroni's test for multiple comparisons, n = 12 per group.

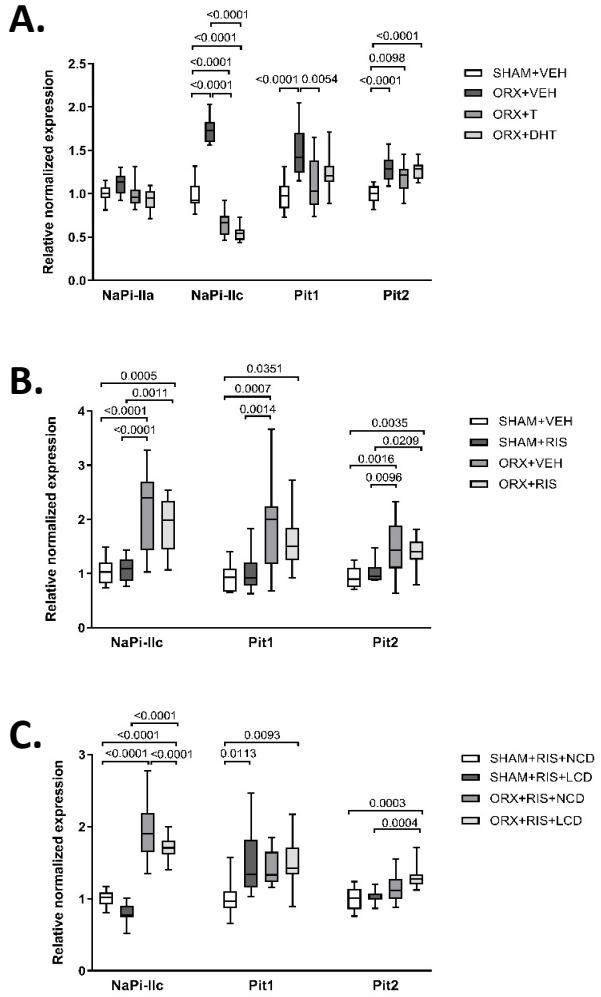

Figure 5. Effects of androgen deprivation on renal mRNA expression of phosphate transporters. A. Effect of orchidectomy (ORX) and androgen replacement **B.** Effect of ORX and risedronate **C.** Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle; ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized mice treated with T; ORX+DHT: orchidectomized mice treated with DHT; SHAM+RIS: sham-operated mice treated with risedronate; ORX+RIS: orchidectomized mice treated with risedronate; SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are presented as mean ± SEM. One-Way ANOVA (A) and Two-Way ANOVA (B-C) with Bonferroni's test for multiple comparisons, n = 12 per group.

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Figure 6. AR localization in the kidney. Immunofluorescence co-stainings of the AR (red) and different kidney markers (green), with nuclei in blue. Markers from top to bottom: podocin (glomerulus); megalin (PT); uromodulin (TAL); Trpv5 (DT). Scale bar = 50 µm.

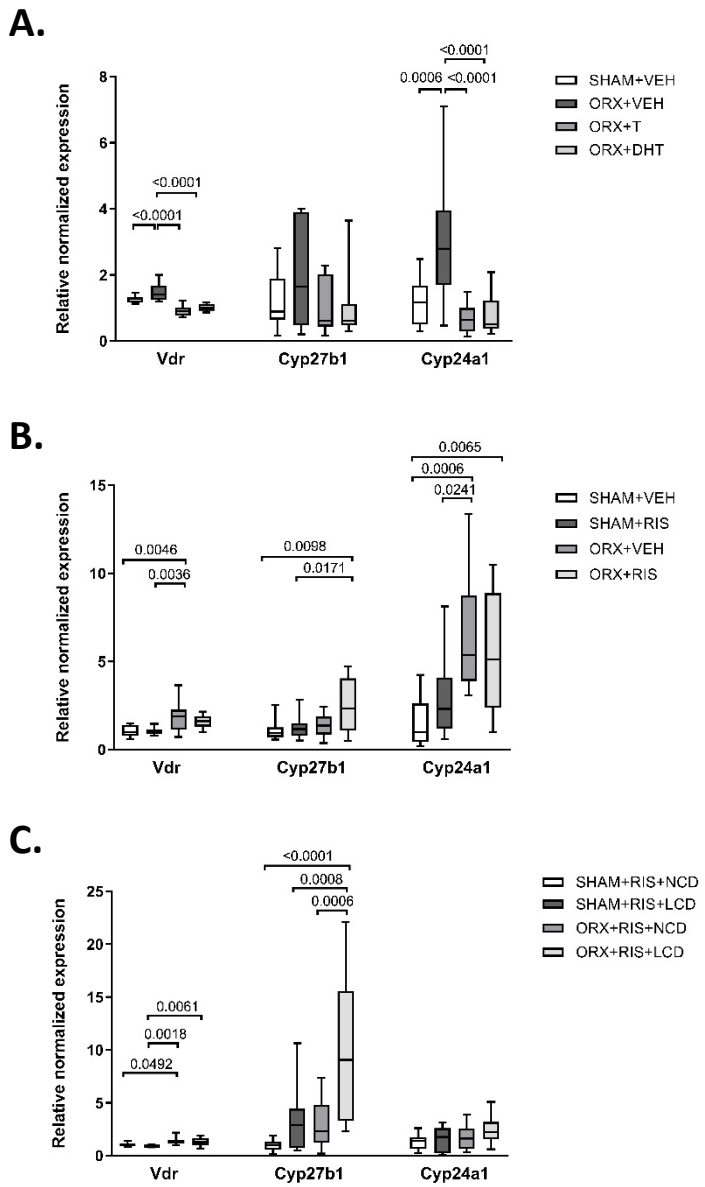

Figure 7. Effects of androgen deprivation on renal mRNA expression of markers of vitamin D metabolism. A. Effect of orchidectomy (ORX) and androgen replacement **B.** Effect of ORX and risedronate **C.** Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle; ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized mice treated with T; ORX+DHT: orchidectomized mice treated with DHT; SHAM+RIS: sham-operated mice treated with risedronate; ORX+RIS: orchidectomized mice treated with risedronate; SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are

509 presented as mean ± SEM. One-Way ANOVA (A) and Two-Way ANOVA (B-C) with Bonferroni's test for
510 multiple comparisons, n = 12 per group.

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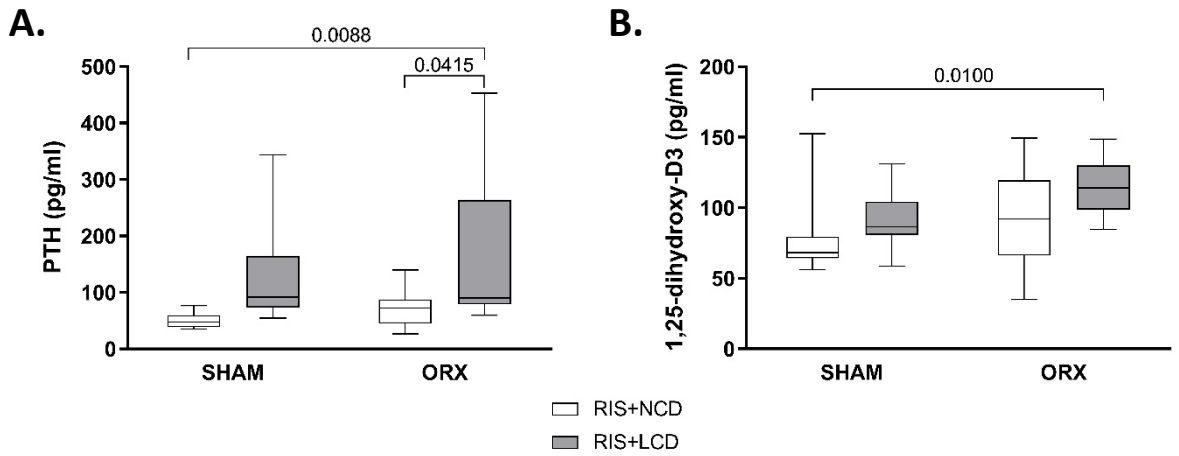

Figure 8. Effect of low calcium diet on calciophosphotropic hormones. A. Serum PTH **B.** Serum 1,25$(OH)_2D_3$. SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are presented as mean ± SEM. Two-Way ANOVA with Bonferroni's test for multiple comparisons, n = 12 per group.

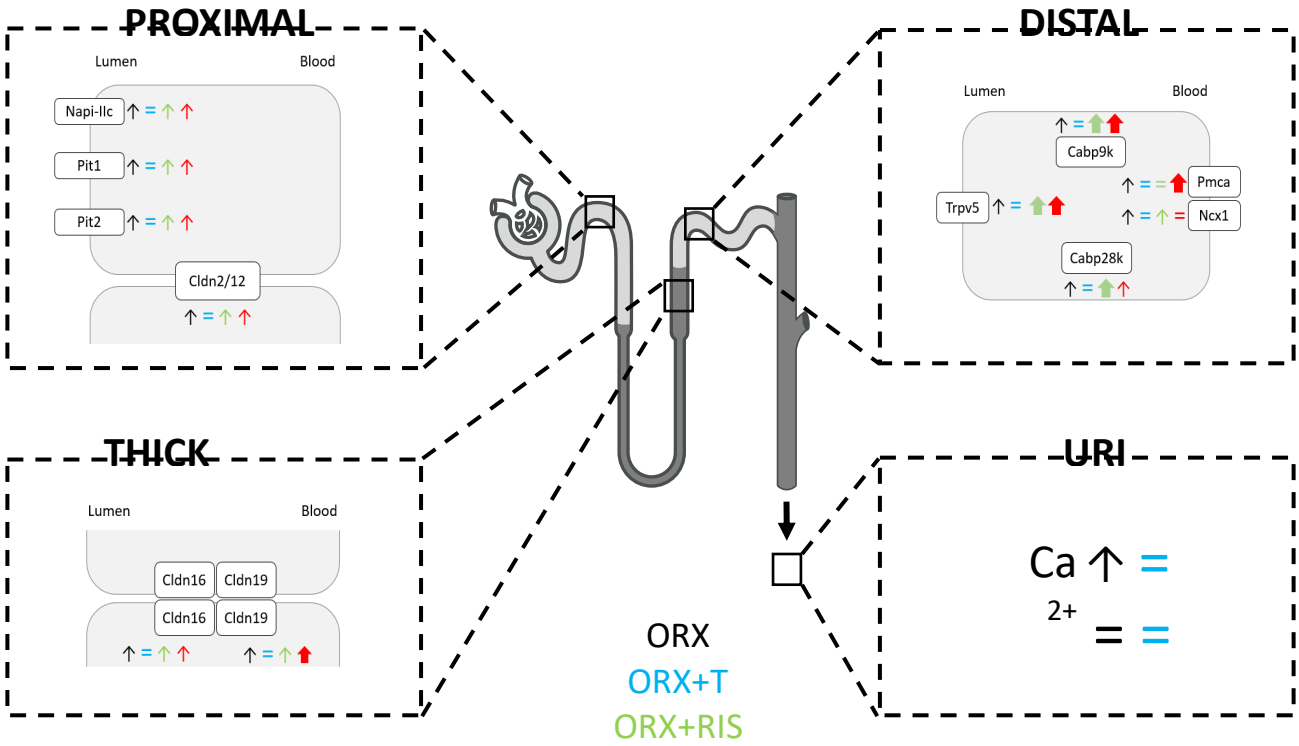

Figure 9. Summary of the effects of short term androgen deprivation on renal calcium and phosphate handling in adult male mice. ORX (black arrows) induced hypercalciuria and increased expression of renal calcium and phosphate transporters, which was inhibited by T and DHT replacement (blue) to a similar extent, indicative for an AR-mediated effect. Treatment with bisphosphonates (green) prior to ORX prevented hypercalciuria, confirming bone as the origin for the urinary calcium loss. However, increased expression of renal calcium and phosphate transporters persisted or even increased, indicating bone-independent effects. In bisphosphonate-treated orchidectomized mice fed a low calcium diet (red), an additional increase in distal renal calcium transporters was seen which was accompanied by secondary hyperparathyroidism, most probably explaining the pronounced hyperphosphaturia.

SUPPLEMENTAL DATA

Supplementary table 1. Primers used for gene expression analysis

Hprt	Forward	TTATCAGACTGAAGAGCTACTGTAATGATC
	Reverse	TTACCAGTGTCAATTATATCTTCAACAATC
	Probe	TGAGAGATCATCTCCACCAATAACTTTTATGTCCC
Trpv5	Forward	CGTTGGTTCTTACGGGTTGAAC
	Reverse	GTTTGGAGAACCACAGAGCCTCTA
	Probe	TGTTTCTCAGATAGCTGCTCTTGTACTTCCTCTTTGT
Cabp9k	Forward	CCTGCAGAAATGAAGAGCATTTT
	Reverse	CTCCATCGCCATTCTTATCCA
	Probe	CAAAAATATGCAGCCAAGGAAGGCGA
Cabp28k	Forward	AACTGACAGAGATGGCCAGGTTA
	Reverse	TGAACTCTTTCCCACACATTTTGAT
	Probe	ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA
Ncx1	Forward	TCCCTACAAAACTATTGAAGGCACA
	Reverse	TTTCTCATACTCCTCGTCATCGATT
	Probe	ACCTTGACTGATATTGTTTTGACTATTTCATCATTCTGGA
Pmca	Forward	CGCCATCTTCTGCACCATT
	Reverse	CAGCCATTGCTCTATTGAAAGTTC
	Probe	CAGCTGAAAGGCTTCCCGCCAAA
Cldn2	Forward	GCTGCCCAGGCCATGAT
	Reverse	GCTCGAGAATCCTGGCAGAA
	Probe	TGCATCTCATGCCCACCACAGAGATAAT
Cldn12	Forward	GCAGTGACTGCCTGATGTACGA
	Reverse	ACATTCCAATCAGGCAGAGTAGC
	Probe	CCTGCGTGTCCTCCAGTTTGCCC
Cldn16	Forward	CCATGTGTCCCTTCCCAACA
	Reverse	GTGGCCACGATCAAAAACCC
Cldn19	Forward	ACTGCTGCCAGAGAACCTGT
	Reverse	AACCCTGGCCTTTACACACC
Napi-2c	Forward	CAG GAA TCT CCG GTT CCA TTC
	Reverse	TCA GTT GGT CAG CGT TCT TC
Pit1	Forward	GCTGCTTCACGAGTGGGTAG
	Reverse	ACGCAAGTTCATCCAAAGGAA
Pit2	Forward	GATTGTCGCCTCCTGGTTTAT
	Reverse	GGAACAGGGTCCTCCTTAGTA
Vdr	Forward	CAGCACATTATCGCCATCCT
	Reverse	GGTTCCATCATGTCCAGTGAG
Cyp24a1	Forward	CCATTCACAACTCGGACCCT
	Reverse	AAGACTGTTCCTTTGGGTAGC
Cyp27b1	Forward	CCAATATGGTCTGGCAGCTTT
	Reverse	CATTCTTCACCATCCGCCGTTA

538 **Supplementary table 2. Antibodies used for immunofluorescence study**

539

Protein	Antibody	Dilution
AR	Spring Bioscience M4070	1/3000
Podocin	Abcam ab93650	1/2000
Megalin	Santa Cruz Biotechnology sc-515750	1/3000
Uromodulin	R&D systems MAB5175	1/3000
Trpv5	Novus Biologicals NB100-93520	1/2000

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