



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Androgen action on renal calcium and phosphate handling: Effects of bisphosphonate treatment and low calcium diet

Citation for published version:

Khalil, R, Simitsidellis, I, Kim, NR, Jardi, F, Schollaert, D, Deboel, L, Saunders, P, Carmeliet, G, Claessens, F, Vanderschueren, D & Decallonne, B 2020, 'Androgen action on renal calcium and phosphate handling: Effects of bisphosphonate treatment and low calcium diet', *Molecular and Cellular Endocrinology*, vol. 514, pp. 110891. <https://doi.org/10.1016/j.mce.2020.110891>

Digital Object Identifier (DOI):

[10.1016/j.mce.2020.110891](https://doi.org/10.1016/j.mce.2020.110891)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Molecular and Cellular Endocrinology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 **Androgen action on renal calcium and phosphate handling: effects of**
2 **bisphosphonate treatment and low calcium diet**

3 Rougin Khalil¹, Ioannis Simitsidellis², Na Ri Kim¹, Ferran Jardi¹, Dieter Schollaert¹, Ludo Deboel¹, Philippa
4 Saunders², Geert Carmeliet¹, Frank Claessens³, Dirk Vanderschueren¹, Brigitte Decallonne¹

5 ¹Clinical and Experimental Endocrinology, Department of Chronic Diseases, Metabolism and Aging, KU
6 Leuven

7 ²Centre for Inflammation Research, Queen's Medical Research Institute, The University of Edinburgh

8 ³Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven

9
10 Correspondence:

11 Brigitte Decallonne

12 Laboratory of Clinical and Experimental Endocrinology

13 Herestraat 49, box 902, 3000 Leuven, Belgium

14 brigitte.decallonne@kuleuven.be

15 +32 16 34 69 94

16
17 Financial support:

18 KU Leuven [grant GOA/15/017] and the Research Foundation Flanders (FWO) [grant GOD2217N]

19 This manuscript contains supplemental data.

20
21 **ABSTRACT**

22 Renal calcium and phosphate handling is an important contributor to mineral homeostasis and bone
23 health and the androgen receptor (AR) is highly expressed in the kidney. We investigated the short
24 term effects of androgen deprivation on renal calcium and phosphate reabsorption, independent of
25 their effects on bone. Two weeks following orchidectomy (ORX) of adult mice, bone loss occurred along
26 with hypercalciuria, which was similarly prevented by testosterone and dihydrotestosterone
27 supplementation. Treatment with bisphosphonates prior to ORX also inhibited hypercalciuria,
28 indicating that the calcium flux originated from the bone. Renal calcium and phosphate transporter
29 expression was increased post-ORX, independent of bisphosphonates. Furthermore, androgen
30 deprivation appeared to stimulate local synthesis of 1,25(OH)₂D₃. When bisphosphonate-treated mice
31 were fed a low calcium diet, bone resorption was no longer blocked and secondary
32 hyperparathyroidism developed, which was more pronounced in ORX mice than sham-operated mice.
33 In conclusion, this study shows that androgen deprivation increased renal calcium and phosphate
34 transporter expression, independent of bone, and underlines the importance of adequate intestinal
35 calcium supply in circumstances of androgen deprivation and bisphosphonate treatment.

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

37

38 1. INTRODUCTION

39 Recent studies suggested that sex steroids might play a role in the regulation of renal calcium and
40 phosphate handling. Similar to calciophosphotropic hormones, sex hormones may not only influence
41 calcium and phosphate handling in the bone but also in other organs such as the kidneys. Urinary
42 calcium excretion has been shown to be higher in males than females, in both humans and mice (1,2).
43 In addition, male mice exhibit lower expression levels of renal calcium transporters (2). In rats,
44 orchidectomy (ORX) increased urinary calcium excretion, which was inhibited by testosterone (T)
45 replacement (3). In contrast, decreased urinary calcium excretion was reported 2 weeks after ORX in
46 mice, accompanied by an increased expression of renal calcium transporters (2). The available data on
47 the effects of androgens on renal phosphate handling are limited and contradictory as well. Men
48 treated with GnRH analogs exhibited increased serum phosphate levels as well as increased renal
49 phosphate reabsorption (4,5). In contrast, ORX in male rats had no influence on serum phosphate or
50 urinary phosphate excretion (6). Kidney stones, with hypercalciuria as major risk factor, are 2 to 3 times
51 more frequent in males compared to females, and males with low T have lower odds of kidney stones
52 (7,8). Mice lacking renal androgen receptor (AR) were shown to exhibit less calcium oxalate crystal
53 formation (9).

54 T is the main circulating androgen in men. It exerts its effects via the AR as T or, after conversion by
55 the 5 α -reductase, as dihydrotestosterone (DHT). In addition, T, but not DHT, can be aromatized into
56 estradiol and bind to the estrogen receptor (ER). Hence, in tissues T can act via the AR as T or DHT, or
57 via the ER as estradiol (10). In previous studies, we and others have shown that the well-established
58 bone-sparing action of androgens is not entirely explained by direct effects on bone cells (11–14).
59 Androgen action may at least partly be explained by its action on the kidneys. Renal AR expression is
60 high, but the exact location of AR expression in the kidney is still debated, with expression reported
61 along the entire nephron as well as in the glomerulus (15). The role of androgens, however, remains
62 unresolved despite gender differences and hormonal dependence of typical kidney diseases such as
63 nephrolithiasis, chronic kidney disease (incidence and progression) and predisposition for renal injury
64 (16,17).

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

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

87 The kidney is also the main source of $1,25(\text{OH})_2\text{D}_3$ synthesis, which takes place in the PT. CYP27B1,
88 predominantly expressed in the PT, is able to convert $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$. CYP24A1 limits the
89 amount of $1,25(\text{OH})_2\text{D}_3$ when circulating $1,25(\text{OH})_2\text{D}_3$ is elevated by catalyzing the conversion of
90 $1,25(\text{OH})_2\text{D}_3$ into 24-hydroxylated products targeted for excretion or by producing $24,25(\text{OH})_2\text{D}_3$, thus
91 decreasing the pool of $25(\text{OH})\text{D}_3$ available for 1-hydroxylation. In the PT, VDR action mainly limits
92 CYP27B1 activity, whereas in the DT it stimulates active transcellular calcium transport, in particular
93 via CaBP9K/28K and to a lesser extent TRPV5 (25).

94 In conclusion, current knowledge on the effects of androgens on renal calcium and phosphate handling
95 is based on few and contradicting data. In addition, available findings could be confounded by effects
96 of androgens on bone. Therefore, the aim of this study was to investigate the acute effects of androgen
97 deprivation on renal calcium and phosphate handling in adult male mice, and this in the presence or
98 absence of a bisphosphonate treatment.

99

100 **2. MATERIALS AND METHODS**

101 **2.1. Animals**

102 Male C57BL/6J mice (Charles River, Saint-Germain-Nuelles, France) were housed in a light and
103 temperature-controlled room with *ad libitum* access to drinking water and standard chow (1% calcium,
104 0.7% phosphate, Ssniff, Soest, Germany), unless stated otherwise. Nembutal (i.p. 100 mg/kg, Ceva
105 Santé Animale, Libourne, France) followed by cardiac puncture was used for euthanasia. All animal
106 procedures were approved by the KU Leuven animal ethics committee (P042/2014). For all
107 experimental setups, male C57BL/6J mice were randomly allocated into different groups (n=12 per
108 group) at 18 weeks of age to undergo a SHAM operation or a bilateral ORX under isoflurane anesthesia
109 (3% induction, 2% maintenance), after 1 week of acclimatization. ORX was used as a model for primary,
110 organic hypogonadism with acute and complete androgen deprivation (28).

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

115 Experimental setup B (fig 1B): One week before the operation, mice were given vehicle (PBS, i.p.,
116 ThermoFisher Scientific, Massachusetts, USA) or risedronate injections (20 $\mu\text{g}/\text{kg}$, i.p., Merck,
117 Darmstadt, Germany) every 4 days (SHAM+VEH, SHAM+RIS, ORX+VEH, ORX+RIS).

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

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

125 **2.2. Serum and urine analyses**

126 Calcium and phosphate levels in serum and urine were analyzed by SYNCHRON Clinical Systems
127 (Beckman Coulter). Osteocalcin levels were assessed by radioimmunoassay as previously described
128 (29). Serum 1,25(OH)₂D₃ was measured via LC-MS/MS. Serum PTH (Immutopics International,
129 California, USA) and FGF23 (Kainos Laboratories Inc., Tokyo, Japan) levels were determined by ELISA.

130 **2.3. Gene expression analysis**

131 Total RNA of tissues was extracted with TRIzol (ThermoFisher Scientific) followed by
132 phenol/chloroform purification. cDNA was synthesized using reverse transcriptase SuperScript II RT
133 (ThermoFisher Scientific) and qRT-PCR was performed. Gene expression was normalized for
134 hypoxanthine-guanine phosphoribosyltransferase (Hprt) and expressed relative to the control group
135 ($2^{-\Delta\Delta CT}$ method). Details on the primers used are provided in supplementary table 1. A TaqMan assay
136 for NaPi-2a was purchased from ThermoFisher Scientific.

137 **2.4. Bone structure**

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

142 **2.5. Bone calcium content**

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

147 **2.6. Immunohistochemistry**

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

156 **2.7. Statistical analyses**

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

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

161

162 **RESULTS**

163 **3.1. Androgen deprivation induces hypercalciuria and early bone loss**

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

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

175 **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	1.55 ± 0.15	1.24 ± 0.11	1.79 ± 0.23	1.49 ± 0.15
Week 2	1.65 ± 0.15	1.31 ± 0.10	1.99 ± 0.24	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	9.44 ± 0.58	9.23 ± 0.14	8.95 ± 0.23	9.32 ± 0.12
Week 2	7.57 ± 0.11	7.83 ± 0.09	7.78 ± 0.17	7.97 ± 0.13
Serum phosphate (mg/dL) Week 1	7.76 ± 0.28	7.29 ± 0.24	7.51 ± 0.20	7.83 ± 0.30
Week 2	10.09 ± 0.51	9.86 ± 0.53	9.04 ± 0.60	8.86 ± 0.47
Urinary calcium (mg/dL) Week 1	5.40 ± 0.72	8.25 ± 0.70 ^a	4.61 ± 0.41 ^b	5.63 ± 0.51
Week 2	5.94 ± 0.39	8.97 ± 0.65 ^a	6.25 ± 0.32 ^b	_b

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

176

177 SHAM+VEH = sham-operated mice treated with vehicle; ORX+VEH = orchidectomized mice treated with vehicle;

178 ORX+T = orchidectomized mice treated with T; ORX+DHT = orchidectomized mice treated with DHT; BW = body

179 weight; ND = not done.

180 Data are presented as mean ± SEM. One-Way ANOVA, n = 12 per group, ^aP<0.05 vs. SHAM+VEH; ^bP<0.05 vs.

181 ORX+VEH.

182 Since sex steroid deficiency induces early bone loss, which could interfere with the obtained results,

183 trabecular bone was analyzed using microCT. Bone loss was observed already 2 weeks after ORX, as

184 evidenced by a 15% decrease in bone mass with an 11% decrease in trabecular number, and a 4%

185 increased trabecular separation. This bone loss was inhibited by T or DHT replacement (fig 2A).

186 Increased bone turnover in ORX mice was confirmed by elevated serum osteocalcin levels (fig 2A).

187 **3.2. Bisphosphonates inhibit bone-loss induced calciuria in circumstances of sufficient dietary** 188 **calcium**

189 As ORX induces early bone loss, mice were, prior to ORX or SHAM, treated with the bisphosphonate

190 risedronate (versus vehicle) to inhibit bone resorption (fig 1B). MicroCT analysis confirmed that bone

191 loss after ORX was prevented by risedronate (fig 2B). To confirm that the ORX-associated bone loss

192 was induced by increased resorption and not by a mineralization defect, femurs were ashed and

193 analyzed for calcium content. Calcium per dry weight was not affected by ORX, while total calcium

194 levels decreased with 14%. This decrease was prevented by risedronate. Furthermore, risedronate

195 prevented the increase of serum osteocalcin levels (fig 2B) and inhibited the ORX-induced

196 hypercalciuria (fig 3A), indicating that the increased renal calcium loss originated from the bone.

197 Phosphaturia remained unaffected.

198 Regular chow for mice contains higher amounts of calcium than the recommended dietary intake. This

199 high dietary calcium potentially affects renal calcium and phosphate handling. To minimize

200 interference and study the effects of ORX independent of both bone and dietary calcium, mice were

201 given either a normal calcium diet (NCD) or a low calcium diet (LCD). All the animals were SHAM or

202 ORX-operated and treated with risedronate (fig 1C). MicroCT analysis showed that while risedronate

203 efficiently inhibited ORX-induced bone loss under the regular diet, this was no longer the case under

204 the LCD. The LCD decreased bone mass with 13% and 8% in SHAM and ORX mice, respectively (fig 2C).

205 Femoral calcium/dry weight was not altered. LCD, however, did decrease total calcium levels with 15%

206 in SHAM-operated mice and 14% in ORX mice (fig 2C). In addition, serum osteocalcin was significantly

207 increased in ORX+RIS+LCD mice (fig 2C). Calciuria was not affected under LCD in circumstances of

208 bisphosphonate treatment, but urinary phosphate excretion was 2.5 fold increased (fig 3B). The LCD

209 did not influence intestinal calcium absorption, as assessed by 24h fecal calcium content corrected for

210 dietary calcium intake (data not shown).

211 **3.3. Androgen deprivation increases the expression of renal calcium and phosphate transporters**

212 Next, we investigated the effects of ORX on the expression of renal transporters involved in calcium
213 and phosphate reabsorption after 2 weeks. As shown in figure 4A, renal mRNA expression of *claudin-*
214 *2*, *-12*, (located in the PT) *-16* and *-19* (located in the TAL) increased after ORX. The calcium transporters
215 located in the DT (*Trpv5*, *Cabp9k*, *Cabp28k*, *Ncx1* and *Pmca*) showed higher expression after ORX as
216 well. Renal expression of *CaSR* was 2.1 fold increased in ORX mice. Also for the renal phosphate
217 transporters *NaPi-2c*, *Pit1* and *Pit2* increased expression was observed, while *NaPi-2a* expression was
218 not altered (fig 5A). Of note, although kidney weight decreased following ORX, relative cortical and
219 medullary area was not different between SHAM and ORX mice (data not shown), thereby making it
220 unlikely to affect the expression of these transporters. Following supplementation with T or DHT the
221 increased expression of renal calcium and phosphate transporters was no longer observed.

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

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

230 **3.4. The renal androgen receptor expression is mainly located in the proximal tubulus**

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

236 **3.5. Androgen deprivation increases renal vitamin D metabolism**

237 ORX increased renal mRNA expression of the vitamin D receptor (*Vdr*), as well as *Cyp27b1* and *Cyp24a1*
238 (fig 7A). This increase was absent in case of T or DHT supplementation. When mice were treated with
239 risedronate, an additional increase in *Cyp27b1* expression was observed after ORX (fig 7B). Finally, in
240 circumstances of low dietary calcium a 10 fold increased expression of *Cyp27b1* was observed in the
241 ORX+LCD group compared to the control group (fig 7C). Serum analyses indicated secondary
242 hyperparathyroidism under the low calcium diet, with increased PTH and 1,25(OH)₂D₃ levels, most
243 pronounced for the ORX mice (fig 8B).

244

245 **3. DISCUSSION**

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

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

258 Dietary calcium intake in mice is high due to regular mouse chow containing high calcium levels (31),
259 which contrasts with the often low dietary calcium intake in – especially elderly and frail- humans.
260 Hence, we investigated whether the changes we observed could be confirmed in circumstances of low
261 dietary calcium intake. Surprisingly, the combination of bisphosphonate treatment and very low
262 calcium diet did not decrease serum or urinary calcium levels after ORX, while phosphaturia increased.
263 However, when mice were fed a low calcium diet, bisphosphonate treatment was no longer able to
264 fully block bone resorption. Most likely this was due to the secondary hyperparathyroidism, as shown
265 by high serum levels of PTH and 1,25(OH)₂D₃. This calciotropic response was particularly pronounced
266 in ORX mice and reflected by the high phosphaturia as well. These acute changes probably reflect a
267 compensatory renal action to maintain serum calcium and phosphate levels in circumstances of
268 androgen deprivation. This finding is clinically relevant, as men treated with androgen deprivation
269 therapy and at increased risk for secondary osteoporosis often have a low dietary calcium and vitamin
270 D intake (32–34). Treatment with bisphosphonates might thus be less effective under circumstances
271 of combined hypogonadism and low dietary calcium (35). Very few data are available with respect to
272 the effects of androgens on renal phosphate handling. Similar to our study, serum and urinary
273 phosphate levels were unaffected in male rats, 3 months after ORX (6).

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

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

295 The mechanism of the unexpected androgen/AR-mediated modulation of calcium and phosphate
296 transporters in different parts of the nephron remains speculative. Renal $1,25(\text{OH})_2\text{D}_3/\text{VDR}$ action,
297 however, might mediate the increased calcium and phosphate transporter expression during androgen
298 deprivation. First, we observed increased renal expression of markers of vitamin D metabolism post-
299 ORX, independent of bisphosphonate treatment. Our data regarding vitamin D metabolism are in line
300 with renal transcriptome analyses, showing reduced *Cyp24a1* mRNA 12 hours after T treatment of ORX
301 male mice. *Cyp27b1* was also decreased after 3 days of T treatment (36). Second, we have shown that
302 the AR is dominantly expressed in the PT, where $25(\text{OH})\text{D}_3$ is taken up by endocytosis and where the
303 synthesis of $1,25(\text{OH})_2\text{D}_3$ takes place (25). Third, renal VDR action is also known to be present in the DT
304 where it regulates active transcellular calcium transport. We have shown that the vitamin D-regulated
305 calcium transporters *Trpv5*, *Cabp9k*, and *Cabp28k* were increased in ORX mice, independent of
306 bisphosphonate treatment. Thus, increased synthesis of $1,25(\text{OH})_2\text{D}_3$ in circumstances of low
307 androgens at the level of the PT could increase active phosphate reabsorption at the level of the PT
308 but also active calcium reabsorption at the level of the DT.

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

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

331 **ACKNOWLEDGEMENTS**

332 The authors would like to thank L. Verlinden for her advice and help with the interpretation of the
333 results, I. Jans for the measurements of $1,25(\text{OH})_2\text{D}_3$, and G. Molenberghs from the Interuniversity
334 Institute for Biostatistics and Statistical Bioinformatics for his assistance with statistical analyses. This

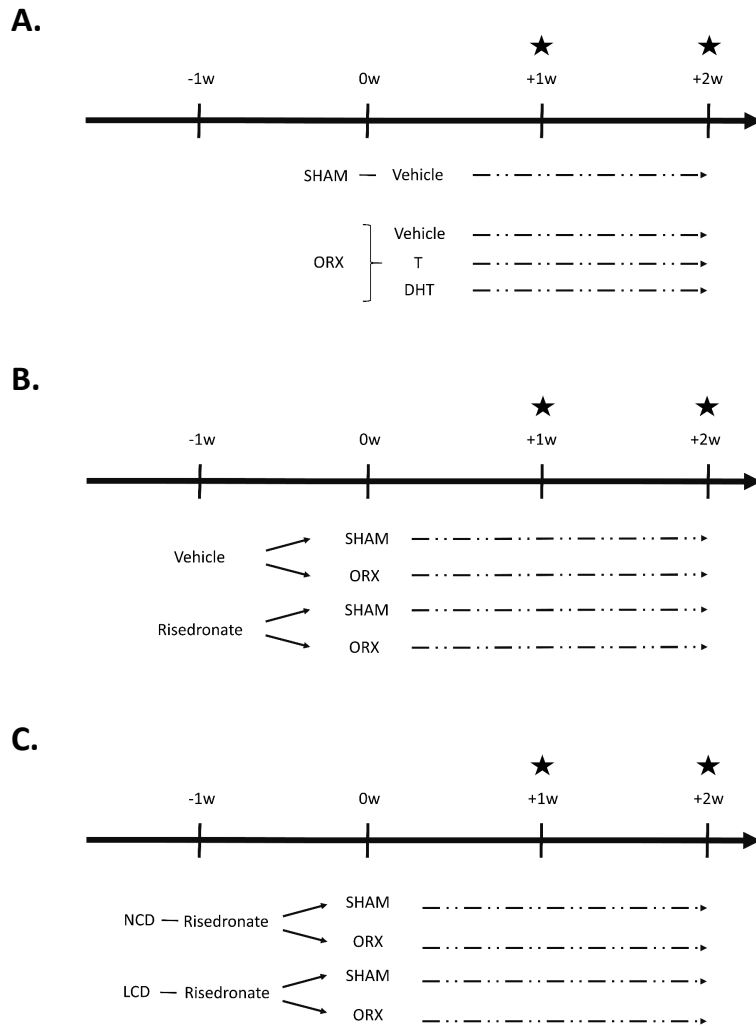
335 work was supported by KU Leuven [grant GOA/15/017] and the Research Foundation Flanders (FWO)
336 [grant G099518N]. The authors have nothing to disclose.

337 REFERENCES

- 338 1. Morgan B, Robertson WG. The urinary excretion of calcium. An analysis of the distribution of
339 values in relation to sex, age and calcium deprivation. *Clin Orthop Relat Res.* 1974
340 Jun;(101):254–67.
- 341 2. Hsu YJ, Dimke H, Schoeber JP, Hsu S-C, Lin S-H, Chu P, et al. Testosterone increases urinary
342 calcium excretion and inhibits expression of renal calcium transport proteins. *Kidney Int.*
343 2010;77(7):601–8.
- 344 3. Lin PH, Jian CY, Chou JC, Chen CW, Chen CC, Soong C, et al. Induction of renal senescence marker
345 protein-30 (SMP30) expression by testosterone and its contribution to urinary calcium
346 absorption in male rats. *Sci Rep.* 2016;6(6):32085.
- 347 4. Burnett-Bowie SAM, Mendoza N, Leder BZ. Effects of gonadal steroid withdrawal on serum
348 phosphate and FGF-23 levels in men. *Bone.* 2007;40(4):913–8.
- 349 5. Maillefert JF, Sibilia J, Michel F, Saussine C, Javier RM, Tavernier C. Bone mineral density in men
350 treated with synthetic gonadotropin-releasing hormone agonists for prostatic carcinoma. *J*
351 *Urol.* 1999;161(4):1219–22.
- 352 6. Gaumet-Meunier N, Coxam V, Robins S, Pastoureau P, Pointillart A, Davicco MJ, et al. Gonadal
353 steroids and bone metabolism in young castrated male rats. *Calcif Tissue Int.* 2000;66(6):470–
354 5.
- 355 7. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC. Time trends in reported
356 prevalence of kidney stones in the United States: 1976–1994. *Kidney Int.* 2003 May;63(5):1817–
357 23.
- 358 8. Yucel E, DeSantis S, Smith MA, Lopez DS. Association between low-testosterone and kidney
359 stones in US men: The national health and nutrition examination survey 2011–2012. *Prev Med*
360 *reports.* 2018 Jun;10:248–53.
- 361 9. Liang L, Li L, Tian J, Lee SO, Dang Q, Huang C-K, et al. Androgen receptor enhances kidney stone-
362 CaOx crystal formation via modulation of oxalate biosynthesis & oxidative stress. *Mol*
363 *Endocrinol.* 2014 Aug;28(8):1291–303.
- 364 10. Laurent M, Sinnesael M, Vanderschueren D, Antonio L, Classens F, Dubois V, et al. Androgens
365 and estrogens in skeletal sexual dimorphism. *Asian J Androl.* 2014;16(2):213.
- 366 11. Venken K, De Gendt K, Boonen S, Ophoff J, Bouillon R, Swinnen J V, et al. Relative Impact of
367 Androgen and Estrogen Receptor Activation in the Effects of Androgens on Trabecular and
368 Cortical Bone in Growing Male Mice: A Study in the Androgen Receptor Knockout Mouse Model.
369 *J Bone Miner Res.* 2006 Jan 3;21(4):576–85.
- 370 12. Callewaert F, Venken K, Ophoff J, De Gendt K, Torcasio A, van Lenthe GH, et al. Differential
371 regulation of bone and body composition in male mice with combined inactivation of androgen
372 and estrogen receptor-alpha. *FASEB J Off Publ Fed Am Soc Exp Biol.* 2009 Jan;23(1):232–40.

- 373 13. Sinnesael M, Claessens F, Laurent M, Dubois V, Boonen S, Deboel L, et al. Androgen receptor
374 (AR) in osteocytes is important for the maintenance of male skeletal integrity: Evidence from
375 targeted AR disruption in mouse osteocytes. *J Bone Miner Res.* 2012;27(12):2535–43.
- 376 14. Sinnesael M, Jardi F, Deboel L, Laurent MR, Dubois V, Zajac JD, et al. The androgen receptor has
377 no direct antiresorptive actions in mouse osteoclasts. *Mol Cell Endocrinol.* 2015;411:198–206.
- 378 15. Khalil R, Kim NR, Jardi F, Vanderschueren D, Claessens F, Decallonne B. Sex steroids and the
379 kidney: role in renal calcium and phosphate handling. *Mol Cell Endocrinol.* 2018 Apr;465:61–
380 72.
- 381 16. Carrero JJ. Gender differences in chronic kidney disease: underpinnings and therapeutic
382 implications. *Kidney Blood Press Res.* 2010;33(5):383–92.
- 383 17. Brar A, Markell M. Impact of gender and gender disparities in patients with kidney disease. *Curr
384 Opin Nephrol Hypertens.* 2019 Mar;28(2):178–82.
- 385 18. Gracioli FG, Neves KR, Barreto F, Barreto D V, Dos Reis LM, Canziani ME, et al. The complexity
386 of chronic kidney disease-mineral and bone disorder across stages of chronic kidney disease.
387 *Kidney Int.* 2017 Jun;91(6):1436–46.
- 388 19. Centeno V, de Barboza GD, Marchionatti A, Rodriguez V, Tolosa de Talamoni N. Molecular
389 mechanisms triggered by low-calcium diets. *Nutr Res Rev.* 2009 Dec;22(2):163–74.
- 390 20. Ohnishi R, Segawa H, Ohmoto T, Sasaki S, Hanazaki A, Mori A, et al. Effect of dietary components
391 on renal inorganic phosphate (Pi) excretion induced by a Pi-depleted diet. *J Med Invest.*
392 2014;61(1–2):162–70.
- 393 21. Jeon US. Kidney and calcium homeostasis. Vol. 6, *Electrolyte and Blood Pressure.* 2008. p. 68–
394 76.
- 395 22. Hou J, Renigunta A, Gomes AS, Hou M, Paul DL, Waldegger S, et al. Claudin-16 and claudin-19
396 interaction is required for their assembly into tight junctions and for renal reabsorption of
397 magnesium. *Proc Natl Acad Sci.* 2009 Sep;106(36):15350–5.
- 398 23. Muto S, Hata M, Taniguchi J, Tsuruoka S, Moriwaki K, Saitou M, et al. Claudin-2-deficient mice
399 are defective in the leaky and cation-selective paracellular permeability properties of renal
400 proximal tubules. *Proc Natl Acad Sci.* 2010 Apr;107(17):8011–6.
- 401 24. Moor MB, Bonny O. Ways of calcium reabsorption in the kidney. *Am J Physiol Ren Physiol.*
402 2016;ajprenal 00273 2015.
- 403 25. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular
404 Mechanism of Action, and Pleiotropic Effects. *Physiol Rev.* 2016 Jan;96(1):365–408.
- 405 26. Riccardi D, Valenti G. Localization and function of the renal calcium-sensing receptor. *Nat Rev
406 Nephrol.* 2016 Jul;12(7):414–25.
- 407 27. Loupy A, Ramakrishnan SK, Wootla B, Chambrey R, de la Faille R, Bourgeois S, et al. PTH-
408 independent regulation of blood calcium concentration by the calcium-sensing receptor. *J Clin
409 Invest.* 2012 Sep;122(9):3355–67.

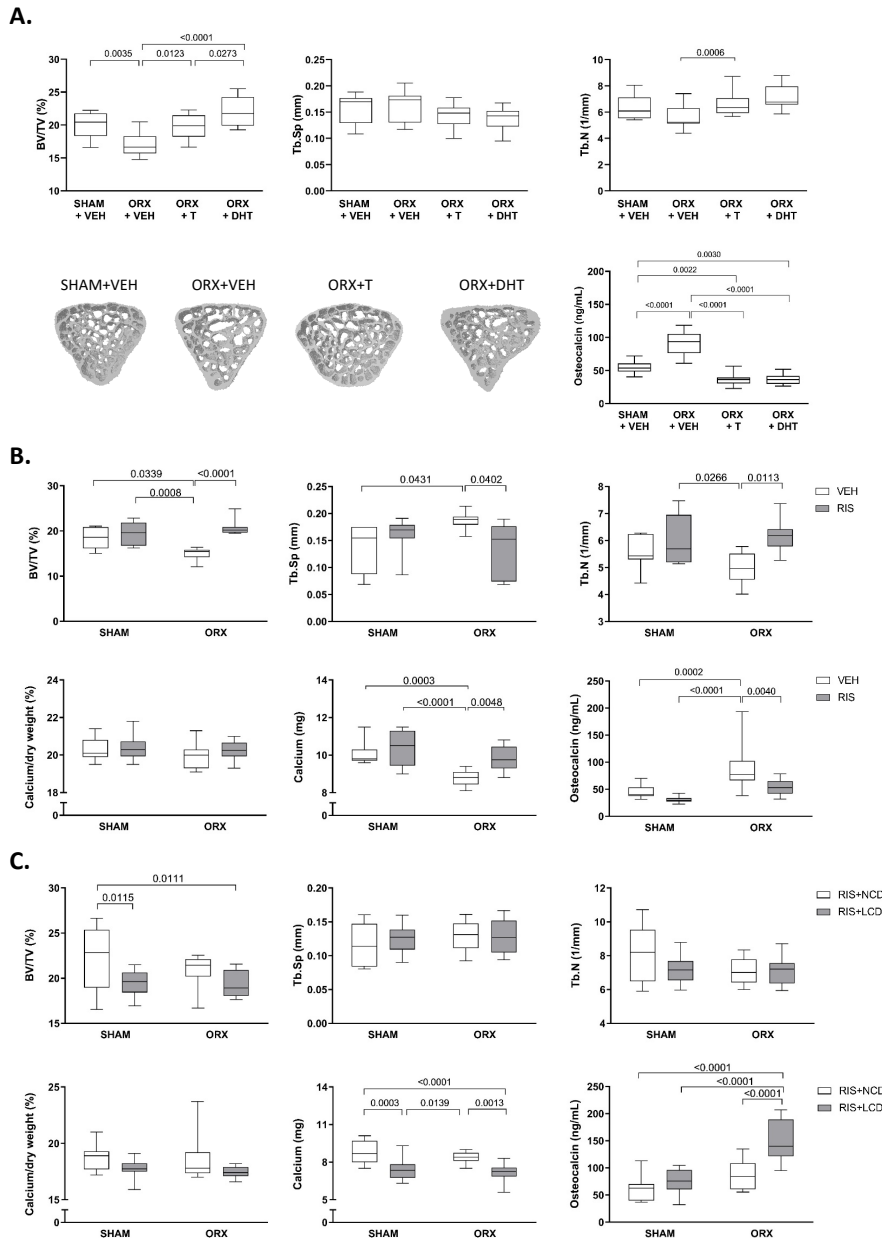
- 410 28. Grossmann M, Matsumoto AM. A Perspective on Middle-Aged and Older Men With Functional
411 Hypogonadism: Focus on Holistic Management. *J Clin Endocrinol Metab.* 2017
412 Mar;102(3):1067–75.
- 413 29. Verhaeghe J, Van Herck E, Van Bree R, Van Assche FA, Bouillon R. Osteocalcin during the
414 reproductive cycle in normal and diabetic rats. *J Endocrinol.* 1989 Jan;120(1):143–51.
- 415 30. Sinnesael M, Laurent MR, Jardi F, Dubois V, Deboel L, Delisser P, et al. Androgens inhibit the
416 osteogenic response to mechanical loading in adult male mice. *Endocrinology.* 2015
417 Apr;156(4):1343–53.
- 418 31. National Research Council (US) Subcommittee on Laboratory Animal Nutrition. Nutrient
419 Requirements of Laboratory Animals,. *Nutrient Requirements of Laboratory Animals: Fourth*
420 *Revised Edition, 1995.* 1995.
- 421 32. Varsavsky M, Reyes-Garcia R, Cortes-Berdonces M, Garcia-Martin A, Rozas-Moreno P, Munoz-
422 Torres M. Serum 25 OH vitamin D concentrations and calcium intake are low in patients with
423 prostate cancer. *Endocrinol Nutr.* 2011 Nov;58(9):487–91.
- 424 33. Greenspan SL, Coates P, Sereika SM, Nelson JB, Trump DL, Resnick NM. Bone loss after initiation
425 of androgen deprivation therapy in patients with prostate cancer. *J Clin Endocrinol Metab.* 2005
426 Dec;90(12):6410–7.
- 427 34. Davison BJ, Wiens K, Cushing M. Promoting calcium and vitamin D intake to reduce the risk of
428 osteoporosis in men on androgen deprivation therapy for recurrent prostate cancer. *Support*
429 *care cancer Off J Multinatl Assoc Support Care Cancer.* 2012 Oct;20(10):2287–94.
- 430 35. Adler RA. Management of osteoporosis in men on androgen deprivation therapy. *Maturitas.*
431 2011 Feb;68(2):143–7.
- 432 36. Pihlajamaa P, Sahu B, Lyly L, Aittomäki V, Hautaniemi S, Jänne OA. Tissue-specific pioneer
433 factors associate with androgen receptor cistromes and transcription programs. *EMBO J.*
434 2014;33(4):312–26.
- 435



436

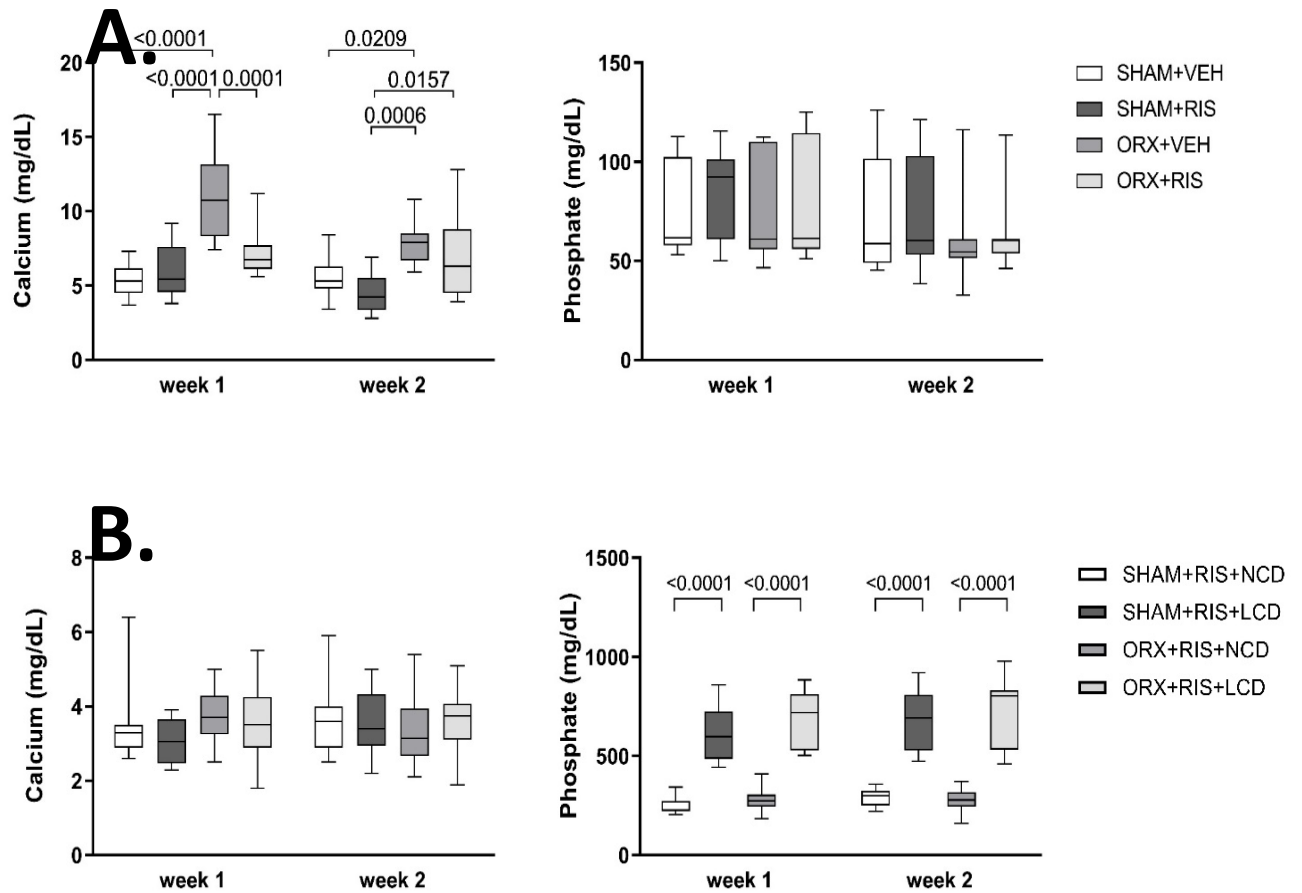
437 **Figure 1. Schematic overview of the experimental setups. A.** Orchidectomy (ORX) and androgen
 438 replacement (T or DHT) **B.** ORX preceded by bisphosphonate (risedronate) treatment **C.** ORX preceded
 439 by bisphosphonate treatment and low calcium diet. Asterixes indicate the timing of metabolic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

441



442

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



454

455

Figure 3. Effects of bisphosphonate treatment and low dietary calcium on urinary calcium and phosphate excretion after androgen deprivation. A.

456

Effect of orchidectomy (ORX) and risedronate B.

457

Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle;

458

SHAM+RIS: sham-operated mice treated with risedronate; ORX+VEH: orchidectomized mice treated

459

with vehicle; ORX+RIS: orchidectomized mice treated with risedronate. SHAM+RIS+NCD: sham-

460

operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-

461

operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized

462

mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice

463

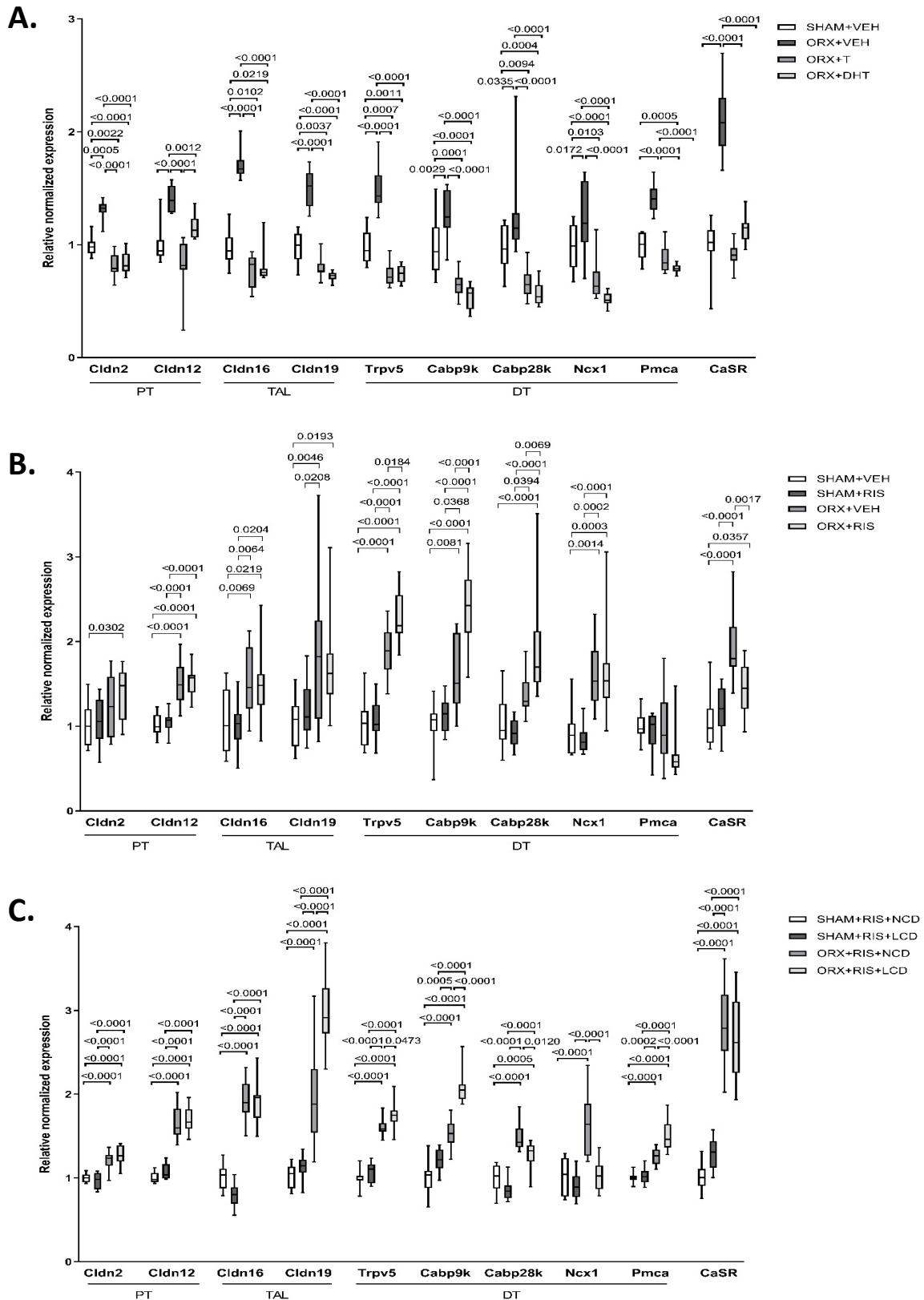
treated with risedronate and fed a low calcium diet. Data are presented as mean \pm SEM. Two-Way

464

ANOVA with Bonferroni's test for multiple comparisons, n = 12 per group.

465

465

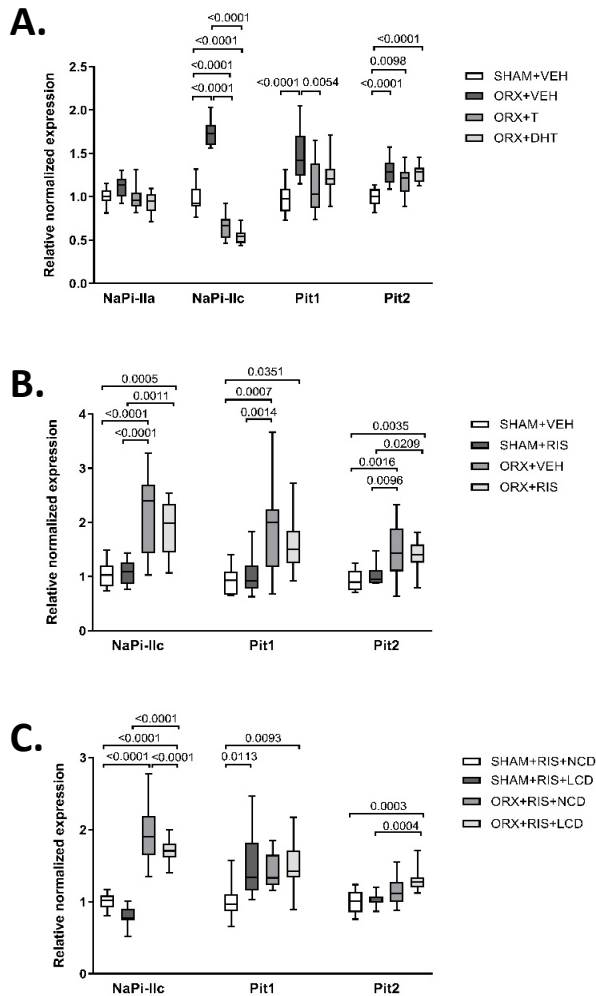


466

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

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

478



479

480

481 **Figure 5. Effects of androgen deprivation on renal mRNA expression of phosphate transporters. A.**

482 Effect of orchidectomy (ORX) and androgen replacement **B.** Effect of ORX and risedronate **C.** Effect of

483 ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice treated with vehicle;

484 ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized mice treated with T;

485 ORX+DHT: orchidectomized mice treated with DHT; SHAM+RIS: sham-operated mice treated with

486 risedronate; ORX+RIS: orchidectomized mice treated with risedronate; SHAM+RIS+NCD: sham-

487 operated mice treated with risedronate and fed a normal calcium diet; SHAM+RIS+LCD: sham-

488 operated mice treated with risedronate and fed a low calcium diet; ORX+RIS+NCD: orchidectomized

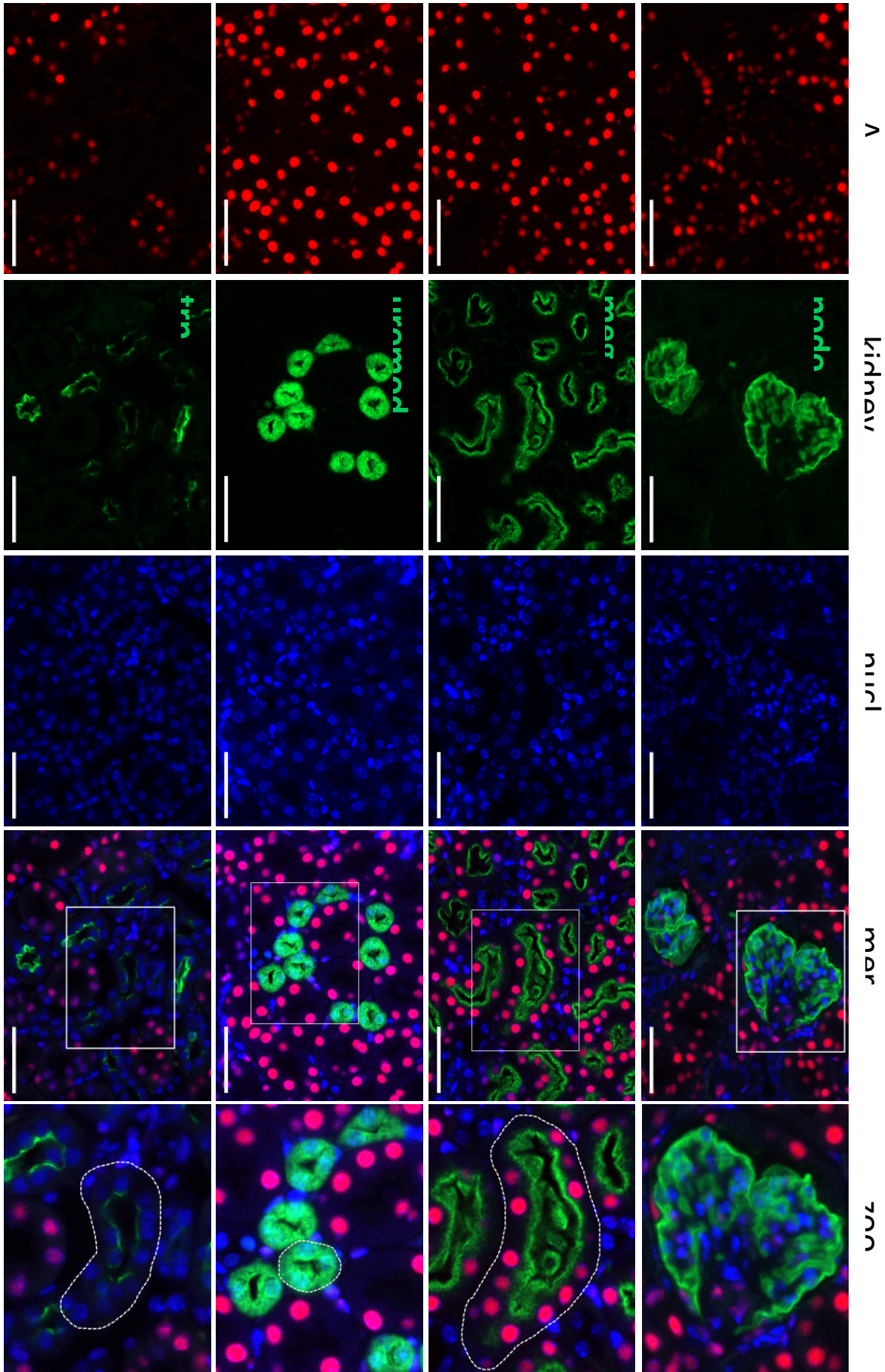
489 mice treated with risedronate and fed a normal calcium diet; ORX+RIS+LCD: orchidectomized mice

490 treated with risedronate and fed a low calcium diet. Data are presented as mean \pm SEM. One-Way

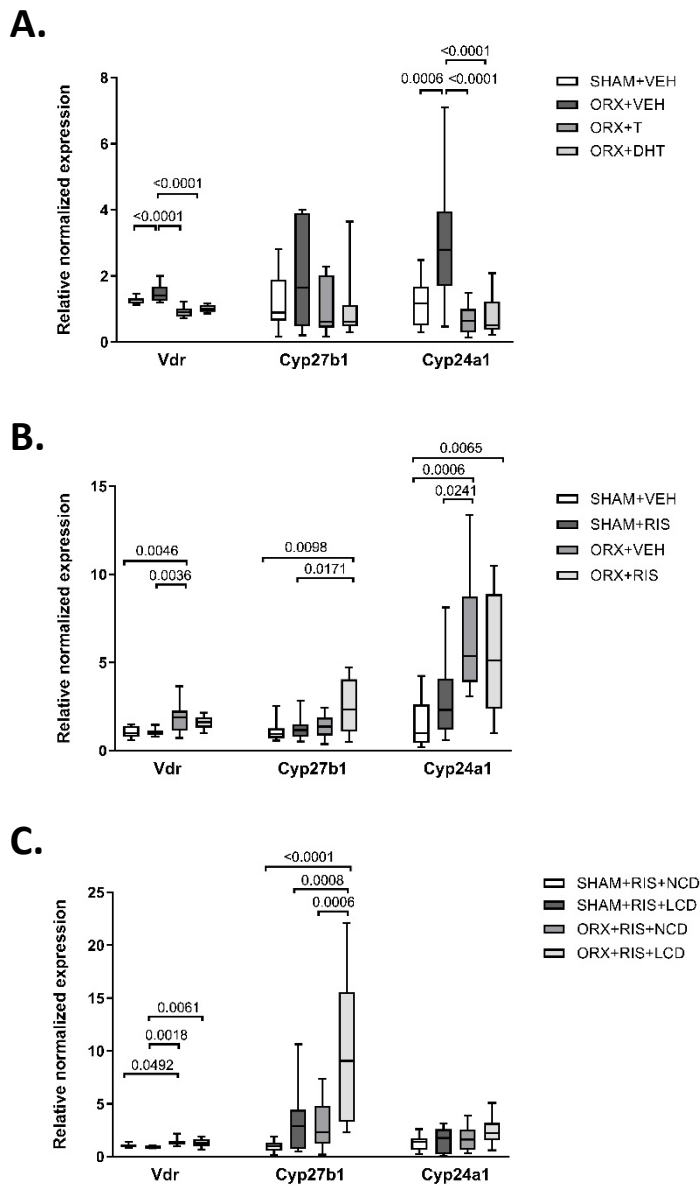
491 ANOVA (A) and Two-Way ANOVA (B-C) with Bonferroni's test for multiple comparisons, n = 12 per

492 group.

493



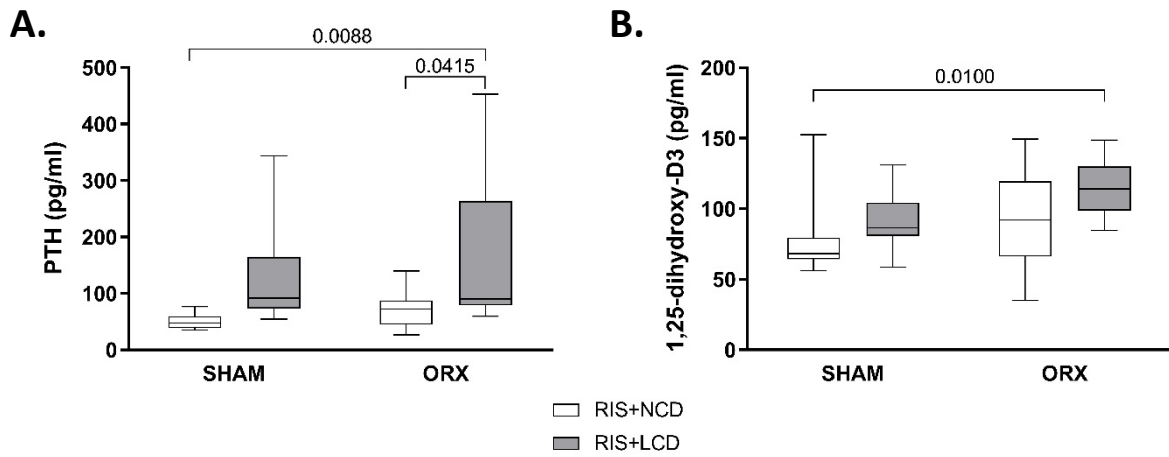
495 **Figure 6. AR localization in the kidney.** Immunofluorescence co-stainings of the AR (red) and different
 496 kidney markers (green), with nuclei in blue. Markers from top to bottom: podocin (glomerulus);
 497 megalin (PT); uromodulin (TAL); Trpv5 (DT). Scale bar = 50 μ m.



498
 499 **Figure 7. Effects of androgen deprivation on renal mRNA expression of markers of vitamin D**
 500 **metabolism.** **A.** Effect of orchidectomy (ORX) and androgen replacement **B.** Effect of ORX and
 501 risedronate **C.** Effect of ORX, risedronate, and dietary calcium. SHAM+VEH: sham-operated mice
 502 treated with vehicle; ORX+VEH: orchidectomized mice treated with vehicle; ORX+T: orchidectomized
 503 mice treated with T; ORX+DHT: orchidectomized mice treated with DHT; SHAM+RIS: sham-operated
 504 mice treated with risedronate; ORX+RIS: orchidectomized mice treated with risedronate;
 505 SHAM+RIS+NCD: sham-operated mice treated with risedronate and fed a normal calcium diet;
 506 SHAM+RIS+LCD: sham-operated mice treated with risedronate and fed a low calcium diet;
 507 ORX+RIS+NCD: orchidectomized mice treated with risedronate and fed a normal calcium diet;
 508 ORX+RIS+LCD: orchidectomized mice treated with risedronate and fed a low calcium diet. Data are

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

511



513

514

515

516

517

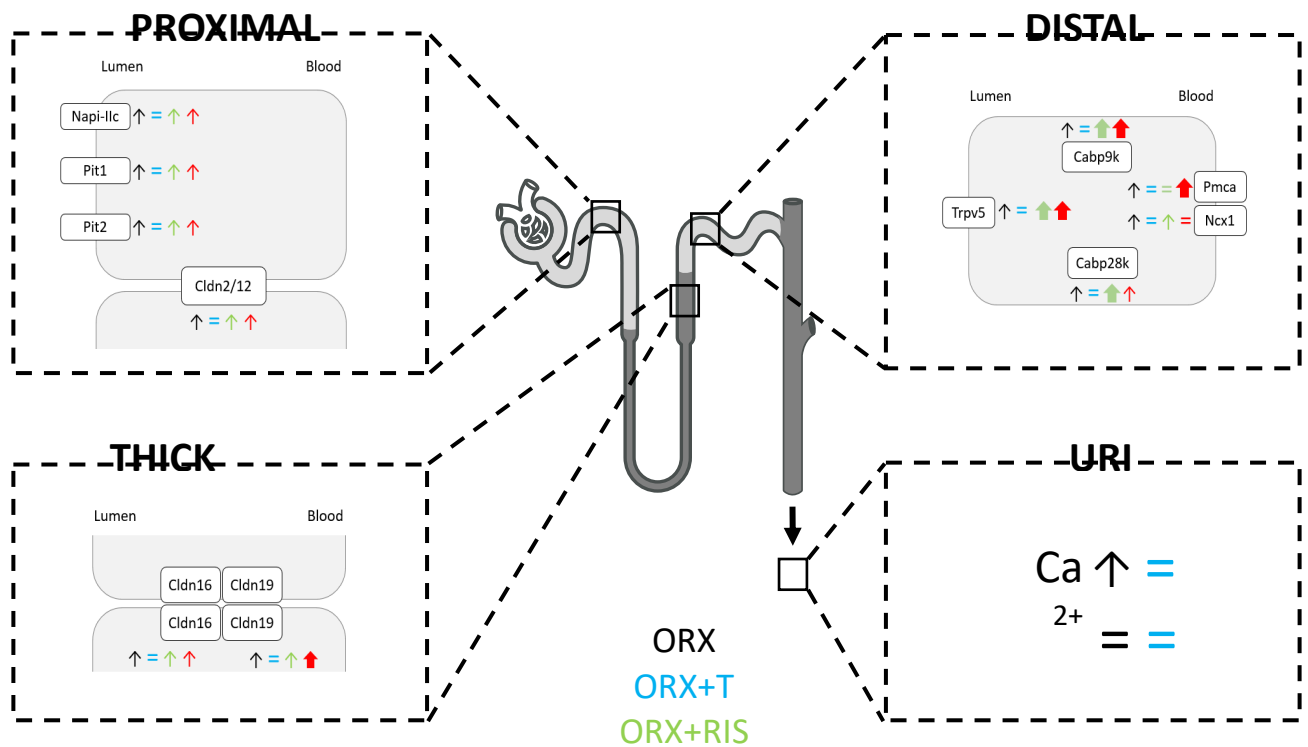
518

519

520

521

Figure 8. Effect of low calcium diet on calciophosphotropic hormones. A. Serum PTH **B.** Serum 1,25(OH)₂D₃. 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.



523

524

525

526

527

528

529

530

531

532

533

534

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.

534

535 SUPPLEMENTAL DATA

536 Supplementary table 1. Primers used for gene expression analysis

Hprt	Forward	TTATCAGACTGAAGAGCTACTGTAATGATC
	Reverse	TTACCAGTGTCAATTATATCTTCAACAATC
	Probe	TGAGAGATCATCTCCACCAATAACTTTTATGTCCC
Trpv5	Forward	CGTTGGTTCTTACGGGTTGAAC
	Reverse	GTTTGGAGAACCACAGAGCCTCTA
	Probe	TGTTTCTCAGATAGCTGCTCTTGTACTTCTCTTTGT
Cabp9k	Forward	CCTGCAGAAATGAAGAGCATTTT
	Reverse	CTCCATCGCCATTCTTATCCA
	Probe	CAAAAATATGCAGCCAAGGAAGGCGA
Cabp28k	Forward	AACTGACAGAGATGGCCAGGTTA
	Reverse	TGAACTCTTCCCACACATTTTGAT
	Probe	ACCAGTGCAGGAAAATTCCTTCTTAAATTCCA
Ncx1	Forward	TCCCTACAAAATATTGAAGGCACA
	Reverse	TTTCTCATACTCCTCGTCATCGATT
	Probe	ACCTTGACTGATATTGTTTTGACTATTTTCATCATTCTGGA
Pmca	Forward	CGCCATCTTCTGCACCATT
	Reverse	CAGCCATTGCTCTATTGAAAGTTC
	Probe	CAGCTGAAAGGCTTCCCGCCAAA
Cldn2	Forward	GCTGCCAGGCCATGAT
	Reverse	GCTCGAGAATCCTGGCAGAA
	Probe	TGCATCTCATGCCACCACAGAGATAAT
Cldn12	Forward	GCAGTGACTGCCTGATGTACGA
	Reverse	ACATTCGAATCAGGCAGAGTAGC
	Probe	CCTGCGTGTCTCCAGTTTGCCC
Cldn16	Forward	CCATGTGTCCCTTCCCAACA
	Reverse	GTGGCCACGATCAAAAACCC
Cldn19	Forward	ACTGCTGCCAGAGAACCTGT
	Reverse	AACCCTGGCCTTACACACC
Napi-2c	Forward	CAG GAA TCT CCG GTT CCA TTC
	Reverse	TCA GTT GGT CAG CGT TCT TC
Pit1	Forward	GCTGCTTACAGAGTGGGTAG
	Reverse	ACGCAAGTTCATCCAAAGGAA
Pit2	Forward	GATTGTGCGCTCCTGGTTTAT
	Reverse	GGAACAGGGTCTCCTTAGTA
Vdr	Forward	CAGCACATTATCGCCATCCT
	Reverse	GGTTCCATCATGTCCAGTGAG
Cyp24a1	Forward	CCATTCACAACCTCGGACCCT
	Reverse	AAGACTGTTCTTTGGGTAGC
Cyp27b1	Forward	CCAATATGGTCTGGCAGCTTT
	Reverse	CATTCTTACCATCCGCCGTTA

537

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

540

541