

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37

**Ankylosis homologue (ANKH) controls extracellular citrate and pyrophosphate homeostasis and affects bone mechanical performance**

Flora Szeri<sup>1</sup>, Stefan Lundkvist<sup>1</sup>, Sylvia Donnelly<sup>1</sup>, Udo F.H. Engelke<sup>2</sup>, Kyu Rhee<sup>3</sup>, Charlene Williams<sup>4</sup>, John P. Sundberg<sup>5</sup>, Ron A. Wevers<sup>2</sup>, Ryan E. Tomlinson<sup>6</sup>, Robert Jansen<sup>3</sup> and Koen van de Wetering<sup>1\*</sup>

<sup>1</sup>Department of Dermatology and Cutaneous Biology, Jefferson Institute of Molecular Medicine and PXE International Center of Excellence in Research and Clinical Care, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia (PA), USA

<sup>2</sup>Translational Metabolic Laboratory, Department Laboratory Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands.

<sup>3</sup>Division of Infectious Diseases, Department of Medicine, Weill Cornell Medicine, New York (NY), USA.

<sup>4</sup>Cooper Medical School of Rowan University, Glassboro (NJ), USA

<sup>5</sup>The Jackson Laboratory, Bar Harbor (ME), USA

<sup>6</sup>Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia (PA), USA

**\*Corresponding author:**

Koen van de Wetering, Department of Dermatology and Cutaneous Biology, Jefferson Institute of Molecular Medicine and PXE International Center of Excellence in Research and Clinical Care, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia (PA), USA. [Koen.vandeWetering@jefferson.edu](mailto:Koen.vandeWetering@jefferson.edu). Tel: +1(215)503-5701

**Keywords:**

extracellular citrate, extracellular pyrophosphate, ATP release, ANKH/ANK, ectopic mineralization, bone

## 38 **Abstract**

39 The membrane protein Ankylosis homologue (ANKH, mouse orthologue: ANK) prevents  
40 mineralization of joint-space and articular cartilage. The accepted view is that ANKH mediates  
41 cellular release of inorganic pyrophosphate (PPi), a strong physiological inhibitor of  
42 mineralization. Using global metabolite profiling, we identified citrate as the most prominent  
43 metabolite leaving HEK293 cells in an ANKH-dependent manner. Although PPi levels were  
44 increased in culture medium of HEK293-ANKH cells, PPi was formed extracellularly after release  
45 of ATP and other nucleoside triphosphates. *Ank<sup>ank/ank</sup>* mice, which lack functional ANK, had  
46 substantially reduced concentrations of citrate in plasma and urine, while citrate was undetectable  
47 in urine of a human patient lacking functional ANKH. Bone hydroxyapatite of *Ank<sup>ank/ank</sup>* mice also  
48 contained markedly reduced levels of citrate and PPi and displayed diminished strength.  
49 Together, our data show that ANKH is a crucial factor in extracellular citrate and PPi homeostasis  
50 that is essential for normal bone development.

51

## 52 **Introduction**

53 Physiological mineralization is essential for normal development of vertebrates, but must  
54 be restricted to specific sites of the body. Vertebrates have evolved mechanisms to allow  
55 regulated mineralization in for instance bones and teeth, but prevent mineralization of soft  
56 connective tissues<sup>1,2</sup>. The molecular details of the mechanism in vertebrates that restrict  
57 mineralization to specific sites of the body are incompletely characterized, however.

58 The *ANKH/Ank* (human/mouse) gene encodes a multi-span transmembrane protein  
59 involved in the prevention of pathological mineralization of cartilage and synovial fluid<sup>3,4</sup>.  
60 *ANKH/Ank*, has a wide tissue distribution, which high levels of expression found in osteoblasts,  
61 prostate, skeletal muscle, brain and the cardiovascular system<sup>1,5,6</sup>. A naturally occurring mouse  
62 mutant, progressive ankylosis (*Ank<sup>ank/ank</sup>*), presents early in life with progressive ankylosis of the  
63 spine and other joints, restricting mobility and critically limiting lifespan<sup>1</sup>. Biallelic loss-of-function

64 mutations in the human orthologue of *Ank*, *Ank homolog (ANKH)*, underlie some forms of  
65 craniometaphyseal dysplasia (CMD), which also presents with progressive ankylosis, mainly  
66 affecting the spine and the joints of hands and feet <sup>7</sup>. In 2000, Ho *et al.* showed that medium of  
67 *Ank<sup>ank/ank</sup>* fibroblasts contained reduced concentrations of the physiological mineralization inhibitor  
68 inorganic pyrophosphate (PPi), leading to the now prevailing view that ANKH/ANK was the  
69 transport of PPi into the extracellular environment <sup>1,8</sup>. An important source of extracellular PPi is  
70 ATP, which is extracellularly converted into AMP and PPi by membrane-bound ecto-nucleotidase  
71 pyrophosphatase/phosphodiesterase 1 (ENPP1) <sup>9</sup>. We have previously shown that ATP release  
72 mediated by the hepatic membrane protein ATP-Binding Cassette subfamily C member 6  
73 (ABCC6) is responsible for 60-70% of all PPi present in plasma <sup>10,11</sup>.

74 Here we tested if release of ATP also underlies most of the PPi found in the extracellular  
75 milieu of ANKH-containing cells. Moreover, we applied global metabolite profiling <sup>12</sup> on medium  
76 of HEK293-ANKH cells to gain a comprehensive overview of metabolites extruded by cells in an  
77 ANKH-dependent manner. Our results provide new and unexpected insights into the substrate  
78 spectrum and anti-mineralization properties of ANKH and also show that ANKH has functions  
79 beyond inhibition of pathological mineralization as it is, for instance, essential for  
80 normal bone development.

81

## 82 **Results**

83

84 **HEK293-ANKH cells release ATP into the extracellular environment.** To study the function of  
85 ANKH *in vitro*, we first generated several HEK293 cell lines overproducing wild type ANKH  
86 (ANKH<sup>wt</sup>) and ANKH<sup>L244S</sup>, a pathogenic loss-of-function mutant which still routes normally to the  
87 plasma membrane <sup>7</sup>. As shown in Fig. 1A, endogenous ANKH was not detectable in parental  
88 HEK293 cells by immunoblot analysis, whereas high levels of ANKH protein were found in cells  
89 overexpressing ANKH<sup>wt</sup>. The loss-of-function ANKH<sup>L244S</sup> mutant was also abundantly expressed,

90 and a clone producing levels of the mutant protein higher than those detected in the HEK293-  
91 *ANKH<sup>wt</sup>* cells was used for further analysis (Fig. 1A). First, we measured PPi levels in the medium  
92 of these cells over a 24-h time period and showed that PPi accumulated at higher levels in medium  
93 of HEK293-*ANKH<sup>wt</sup>* cells than in medium of HEK293-*ANKH<sup>L244S</sup>* or control HEK293 cells (Fig. 1B),  
94 confirming earlier reports that demonstrated the involvement of ANKH in extracellular PPi  
95 homeostasis <sup>1</sup>. We have previously shown that ENPP1 produced by HEK293 cells converts  
96 extracellular ATP into AMP and PPi <sup>10</sup>. Consequently, to determine what part of the PPi found in  
97 medium of *ANKH<sup>wt</sup>* cells might be derived from extracellular ATP, converted by ENPP1 into AMP  
98 and PPi, AMP concentrations were quantified in the culture medium. As shown in Fig. 1C, a clear  
99 time-dependent increase in AMP concentrations was detected in medium of HEK293-*ANKH<sup>wt</sup>*  
100 cells, while medium of untransfected HEK293 parental cells or cells producing the loss-of-function  
101 *ANKH<sup>L244S</sup>* mutant contained only very little AMP. PPi and AMP concentrations in medium of  
102 *ANKH<sup>wt</sup>* cells were within the same range (1-2  $\mu$ M after 12 hours, compare panels B and C of Fig.  
103 1) and the ratio of PPi to AMP was very similar to that previously reported for HEK293 cells  
104 overproducing ABCC6, a plasma membrane protein involved in the release of ATP <sup>10</sup>. We attribute  
105 the somewhat lower abundance of AMP than PPi to further metabolism of AMP and the generation  
106 of PPi from other nucleoside triphosphates (NTPs) also released into the culture medium via  
107 ANKH (see below). A luciferase-based real-time ATP efflux assay was also carried out and  
108 confirmed that ANKH is involved in cellular ATP release (Figure 1D). Only HEK293-*ANKH<sup>wt</sup>* cells  
109 showed robust ATP efflux, whereas release from HEK293-*ANKH<sup>L244S</sup>* cells was indistinguishable  
110 from untransfected parental HEK293 cells in these assays. Collectively, these data indicate that  
111 HEK293-*ANKH<sup>wt</sup>* cells release ATP, which is subsequently extracellularly converted into AMP and  
112 PPi.

113

114 **Culture medium of HEK293-*ANKH<sup>wt</sup>* cells contains large amounts of nucleoside**  
115 **monophosphates (NMPs).** In addition to ATP, ENPP1 can convert various other nucleoside

116 triphosphates (NTPs) into their respective nucleoside monophosphate (NMP) and PPI. Our  
117 previous work has shown that ENPP1 activity in HEK293 cells is high<sup>10</sup>. We therefore used liquid  
118 chromatography/mass spectrometry (LC/MS)-based global metabolite profiling to determine if  
119 ANKH also provides a pathway for release of other NTPs. Substantially elevated levels of AMP,  
120 CMP, GMP and UMP were detected in the culture medium of HEK293-*ANKH*<sup>wt</sup> cells compared to  
121 untransfected parental and HEK293-*ANKH*<sup>L244S</sup> cells (Fig. 2 A-D), For AMP and UMP differences  
122 between untransfected and HEK293-*ANKH*<sup>wt</sup> cells reached statistical significance. These results  
123 support the hypothesis that ANKH provides a previously unanticipated pathway for cellular NTP  
124 release. Based on the levels of PPI, AMP and other NMPs detected in the culture medium, we  
125 estimate that cellular NTP release underlies at least 70% of the ANKH-dependent accumulation  
126 of PPI in the culture medium (for calculation see materials and methods section) of the PPI  
127 detected in medium of the HEK293-*ANKH*<sup>wt</sup> cells.

128

129 **HEK293-ANKH cells release the TCA cycle intermediates citrate, succinate, and malate into**  
130 **the culture medium.** The global metabolite profiling experiments also revealed that the calcium  
131 chelator citrate specifically accumulated in the culture medium of HEK293-*ANKH*<sup>wt</sup> cells (Fig. 3A).  
132 Because global metabolite profiling experiments only provide relative metabolite levels, we also  
133 quantified citrate levels by LC/MS in 24-hour medium samples and found that approximately 1  
134 mM citrate (2.5  $\mu$ mol/24 hrs) was present in medium of HEK293-*ANKH*<sup>wt</sup> cells, while it was almost  
135 undetectable in medium of HEK293 control and HEK293-*ANK*<sup>L244S</sup> cells. To put this in perspective,  
136 the same medium samples of HEK293-*ANKH*<sup>wt</sup> cells contained about 4  $\mu$ M PPI (Fig. 1B),  
137 equivalent to the release of approximately 10 nmoles of NTPs. Thus, the amount of citrate  
138 released by the HEK293-*ANKH*<sup>wt</sup> cells was at least 2 orders of magnitude higher than the amount  
139 of NTPs. Other metabolites found to be selectively elevated in medium of HEK293-*ANKH*<sup>wt</sup> cells  
140 were malate (Fig. 3B) and succinate (Fig. 3C), although absolute levels increases relative to  
141 control cells were clearly less than those found for citrate. Using an independent enzymatic assay,

142 citrate levels in culture medium were also followed over time and as shown in Fig. 3D, these  
143 experiments confirmed that citrate was present at approximately 1.1 mM in the 24-hour culture  
144 medium samples of the *ANKH<sup>wt</sup>* cells, comparable to the concentration determined by LC/MS.  
145 Collectively these data show that ANKH is involved in the cellular release of large amounts of  
146 citrate.

147

#### 148 **ANK affects PPI incorporation into bone**

149 About 70% of the PPI found in plasma depends on ABCC6 activity <sup>11</sup>, indicating that the  
150 contribution of ANKH/ANK to plasma PPI homeostasis is relatively minor. Consequently, instead  
151 of contributing to central PPI homeostasis in plasma, we hypothesized that ANKH/ANK is  
152 important in local PPI homeostasis. Osteoblasts express ANKH/ANK at relatively high levels <sup>5</sup> and  
153 the hydroxyapatite of bone contains substantial amounts of PPI <sup>13</sup>. To determine if ANK has a role  
154 in incorporation of PPI in bone, we quantified PPI in tibiae and femora of wild type, *Ank<sup>ank/ank</sup>*, and  
155 mice heterozygous for *ank*. As shown in Fig. 4 PPI constituted about 0.1% (weight/weight) of bone  
156 tissue in wild type mice, whereas in *Ank<sup>ank/ank</sup>* mice the amount of PPI associated with bone was  
157 reduced by approximately 75%. Moreover, in mice heterozygous for *ank*, PPI levels were also  
158 moderately (by approximately 25%), but significantly reduced. These data show that ANK is a  
159 crucial factor in PPI homeostasis in the local environment of bone tissue.

160

161 **ANKH affects citrate disposition *in vivo*.** Plasma contains substantial amounts of citrate <sup>14</sup>. We  
162 therefore determined the effect of a complete inactivation of ANK in mice on plasma citrate  
163 concentrations and as shown in Fig. 5A, found that approximately 75% of citrate in plasma  
164 depended on ANK. Because citrate is also one of the most abundant organic anions in urine <sup>15</sup>,  
165 we measure citrate excretion in *Ank<sup>ank/ank</sup>* mice. As shown in Fig. 5B, the *ank* mutant mice excreted  
166 approximately 40% less citrate via their urine than their wild type litter mates. The availability of  
167 an NMR spectrum of urine of a 19-year-old female CMD patient carrying biallelic homozygous

168 inactivating mutations in *ANKH* (*ANKH<sup>L244S</sup>*), previously described by Morava *et al.* <sup>7</sup> made it  
169 possible to carry out an analysis of citrate levels. Citric acid was not detected in urine of this CMD  
170 patient (Fig. 5C, upper panel). The lower panel of Fig. 5C shows the typical citrate resonance in  
171 urine of a representative age-matched control, which contained 370  $\mu\text{mol}$  citrate/mmol creatinine.  
172 It is interesting to note that the succinate resonance is visible in the NMR spectrum of control  
173 urine, while its concentration is clearly much lower in urine of the CMD patient (Fig. 5E). These  
174 data suggest that ANKH impacts the *in vivo* disposition of succinate and especially citrate in both,  
175 humans and mice.

176 Like PPI, citrate is also one of the major organic compounds present in bone and also strongly  
177 associates with hydroxyapatite <sup>16</sup>. With 90% of the body's citrate content present in bone, this  
178 tissue is thought to play a central role in extracellular citrate homeostasis <sup>17</sup>. Therefore, we  
179 determined if bone citrate levels depend on ANK. These experiments revealed that femora and  
180 tibiae of *Ank<sup>ank/ank</sup>* mice contained approximately 50% less citrate than the same bones of wild  
181 type mice (Fig. 5D,E). Moreover, bones of mice heterozygous for *ank* also contained less citrate,  
182 which in the case of tibia was significantly lower than in wild type mice (Fig. 5D). Together these  
183 data attest to the major impact of ANK on citrate homeostasis of bone.

184

185 **Material properties of bone tissue of *Ank<sup>ank/ank</sup>* mice are altered.** We next explored the  
186 consequences of the absence of ANK activity on bone physiology, by characterizing geometry  
187 and density of femurs harvested from *Ank<sup>ank/ank</sup>*, wild type and mice heterozygous for *ank* by  
188 microCT. At 3 months of age, most of the bone parameters, including bone area (Fig. 6A),  
189 tissue mineral density (TMD, Fig. 6B), and cortical thickness (Fig. 6C), were not significantly  
190 different between wild type and *Ank<sup>ank/ank</sup>* mice. However, significant differences in cortical bone  
191 properties between *Ank<sup>ank/ank</sup>* and wild type mice were detected for bone area fraction (-12.1%),  
192 cortical bone perimeter (+9.8%), and cross-sectional geometry as indexed by eccentricity (-  
193 9.4%). Next, the structural and material properties of the bone were determined by standard

194 three-point bending. Plotting ultimate bending moment against section modulus (Fig. 6G)  
195 yielded linear relationships for each genotype ( $r^2 = 0.84$  wild type, 0.73 HET, 0.67 *Ank<sup>ank/ank</sup>*) that  
196 did not significantly differ in slope ( $p = 0.88$ ). However, we observed that femurs from *Ank<sup>ank/ank</sup>*  
197 mice required significantly less force per equivalent area of bone to break, as demonstrated by  
198 a significant difference in regression intercept ( $p = 0.0170$ ). Taken together, our results indicate  
199 that the geometry of femora of *Ank<sup>ank/ank</sup>* mice is altered and that these femora have diminished  
200 whole bone strength per equivalent amount of bone, results that are consistent with published  
201 data showing citrate deposition in bone affects hydroxyapatite nanostructure and strength <sup>16</sup>.

202

## 203 Discussion

204

205 Ectopic mineralization – the deposition of hydroxyapatite in soft connective tissues – can be a  
206 sequela of a number of clinical conditions, including aging, cancer, diabetes, chronic kidney  
207 disease and genetic disorders. With no effective treatment currently available, ectopic  
208 mineralization is associated with significant morbidity and mortality <sup>18</sup>.

209 ANKH/ANK is known for its important role in the prevention of pathological mineralization of joints,  
210 and its absence results in severe, progressive, ankylosis in both, humans and mice. It was  
211 previously thought that the main function of ANKH/ANK lies in regulation of extracellular PPI  
212 homeostasis, but here we identified a new and previously unanticipated function of ANKH/ANK:  
213 regulation of extracellular citrate concentrations. Although citrate has long been known to be a  
214 major compound in plasma, urine and bone, the mechanism used by cells to extrude citrate has  
215 been elusive. Our current data firmly link a specific protein, ANKH/ANK to extracellular citrate  
216 disposition *in vivo*. Notably, our results are in line with previous GWAS studies describing a  
217 correlation between plasma citrate levels and certain *ANKH* variants in humans <sup>19</sup> and an  
218 association found in cows between intronic *ANKH* variants and milk citrate concentrations <sup>20</sup>.



219 Extracellular citrate is present in many tissues and body fluids where it serves diverse and, in  
220 some cases, unknown functions<sup>14</sup>. In human plasma citrate levels are substantial (100-300  $\mu$ M),  
221 but its function is unclear<sup>21</sup>. Various cell types express citrate uptake transporters and it has been  
222 proposed that plasma citrate provides an additional energy source for cells under hypoglycemic  
223 conditions<sup>14</sup>. Alternatively, citrate is a powerful anticoagulant and might prevent pathological  
224 blood clotting.

225 Via glomerular filtration, plasma citrate ends up in urine, where it contributes to the prevention  
226 kidney stone formation<sup>22</sup>. Whereas urine of the *Ank<sup>ank/ank</sup>* mice still contained substantial amounts  
227 of citrate, that of the human CMD patient lacking functional ANKH was virtually devoid of citrate.  
228 This difference might be partly explained by dietary differences: Citrate has a high bioavailability  
229 of 80-90%<sup>23</sup> and is present in standard rodent food. Possibly, the human CMD patient had a diet  
230 that was low in citrate, whereas part of the citrate detected in plasma of *Ank<sup>ank/ank</sup>* mice comes  
231 from dietary sources.

232 Most of the body's citrate, over 90%, is present in bone tissue, where it stabilizes hydroxyapatite  
233<sup>16</sup>. Our results show that about 50% of bone citrate depends on ANK activity, in line with the  
234 high expression of *Ank* in osteoblasts<sup>5</sup>. The altered material properties of *Ank<sup>ank/ank</sup>* bones, *i.e.*  
235 the altered relationship between ultimate moment and section modulus, nicely fits the described  
236 role of citrate in stabilizing hydroxyapatite. The altered eccentricity and perimeter of *Ank<sup>ank/ank</sup>*  
237 femora are most likely a result of compensatory bone remodeling to retain whole bone strength  
238 without increasing total bone mass. Interestingly, *Ma et al.* recently reported that local levels of  
239 extracellular citrate are also important for the osteogenic development of human mesenchymal  
240 stem cells<sup>17</sup>. It is therefore conceivable that ANKH-dependent citrate release into bone is not  
241 only important for the material properties of hydroxyapatite, but also contributes to osteogenic  
242 differentiation.

243 Relatively high extracellular citrate concentrations of approximately 400  $\mu$ M are found in the  
244 brain. Astrocytes actively release citrate, which is used by neurons as energy source under

245 hypoglycemic conditions <sup>14</sup>. Patients suffering from CMD due to inactivating mutations in *ANKH*  
246 suffer from mental retardation <sup>7</sup>, suggesting a function of ANKH in brain physiology.  
247 The highest extracellular citrate concentrations are found in prostatic fluid (up to 180 mM).  
248 ANKH is expressed at high levels in the epithelial cells of the prostate, known to release citrate  
249 into prostatic fluid. Although a specific splice variant of the mitochondrial citrate carrier SLC25A1  
250 has been implied in citrate efflux from the prostate <sup>24</sup>, ANKH likely contributes to this process.  
251 In summary, extracellular citrate is present in many tissues and body fluids and we anticipate  
252 that our discovery that ANKH/ANK is involved in extracellular citrate homeostasis will allow  
253 clarifying its function in other tissues for instance by using the *Ank<sup>ank/ank</sup>* mouse model.  
254  
255 A second important finding of the current study is that most, if not all, PPi found in the  
256 extracellular environment of ANKH/ANK containing cells, originates from released NTPs, which  
257 are extracellularly converted into their respective NMP and PPi by ENPP1. This contradicts  
258 earlier work, proposing direct ANKH/ANK-dependent cellular efflux of PPi <sup>1</sup>. Our current data  
259 strongly support the conclusion that ANKH/ANK mediates release of NTPs release, not PPi.  
260 First, *in vitro* experiments showed that the majority of PPi found in the culture medium of  
261 HEK293-*ANKH<sup>wt</sup>* cells was derived from NTP efflux. Two reports have appeared that suggest  
262 cells release ATP in an ANKH-dependent manner <sup>25-27</sup>. These studies did however not quantify  
263 the relative amounts of extracellular ATP, AMP and PPi and therefore did not allow assessment  
264 of the relative contribution of ANKH-mediated ATP release to extracellular PPi concentrations.  
265 Strong evidence arguing against direct PPi transport by ANKH/ANK also comes from our  
266 analysis of bones of mice lacking ENPP1. Moreover, 75% of the PPi present in bone depends  
267 on ANK activity (Fig. 4). If ANK would directly transport PPi, incorporation of this fraction into  
268 bone would not require ENPP1 activity. However, we found that PPi is virtually absent in bones  
269 of *Enpp1<sup>-/-</sup>* mice (*asj<sup>GrsrJ</sup>*) (Szeri et al, manuscript in preparation), which is only compatible with  
270 ANKH/ANK mediating NTP release with subsequent extracellular formation of PPi by ENPP1.

271 The function of PPI in bone tissue is not completely clear, but might be related to stabilization of  
272 hydroxyapatite and, consequently, bone mineral density. Such a function would fit data of  
273 previous studies showing that bones of *Enpp1*<sup>-/-</sup> mice, which virtually lack PPI (Szeri et al,  
274 manuscript in preparation), have a substantially greater reduction in mineral density<sup>28,29</sup> than  
275 bones of *Ank*<sup>ank/ank</sup> mice. These data also indicate that the residual 25% of PPI found in bones of  
276 *Ank*<sup>ank/ank</sup> mice suffices to a large extent to keep BMD close to the normal range. The proposed  
277 effects of PPI on mineral density are similar to the effects of bisphosphonates, pharmaceutical  
278 PPI analogues that are widely used in the treatment of osteoporosis<sup>30</sup>. Kim et al<sup>5</sup> have  
279 previously found a more dramatic effect of ANK on bone mineral density. A different genetic  
280 background of their *Ank*<sup>ank/ank</sup> mice might underlie this more dramatic effect. Food composition,  
281 specifically varying PPI content<sup>31</sup>, might also have contributed to the differences found in BMD  
282 between the two studies.

283

284 ANKH/ANK has previously been shown to inhibit ectopic mineralization in the microenvironment  
285 of the joint space<sup>1</sup>. Low plasma levels of PPI underlie several other genetic mineralization  
286 disorders<sup>18,32</sup>. Plasma citrate levels depend on ANK activity, demonstrating that ANK substrates  
287 end up in the blood circulation. This does not come as a surprise given the wide tissue distribution  
288 of ANKH/ANK<sup>1</sup>. Most likely, NTPs are also released into the blood circulation via ANK, allowing  
289 subsequent PPI formation in plasma. Under normal conditions, 60-70% of plasma PPI comes  
290 from ABCC6-mediated hepatic NTP secretion<sup>10,11</sup>. ANK can therefore be expected to be  
291 responsible for part of the remaining 30-40% of the PPI present in plasma that is independent of  
292 ABCC6 activity. The relatively small contribution of ANK together with the large variability in  
293 plasma PPI concentrations<sup>10,11,31</sup> prevents determination of the contribution of ANK to plasma PPI  
294 in *Ank*<sup>ank/ank</sup> mice. *Ank*<sup>ank/ank</sup>; *Abcc6*<sup>-/-</sup> compound mutant mice (*Ank*<sup>ank</sup>; *Abcc6*<sup>tm1Jfk</sup>) provide the  
295 optimal experimental model system to determine the contribution of ANKH/ANK to plasma PPI. If  
296 ANKH indeed contributes to plasma PPI homeostasis, it represents an attractive pharmacological

297 target in ectopic mineralization disorders caused by low plasma levels of PPI. For instance,  
298 stimulation of ANKH activity in patients suffering from pseudoxanthoma elasticum (PXE), a slowly  
299 progressive ectopic calcification disorder caused by inactivating mutations in the gene encoding  
300 the hepatic efflux transporter ABCC6<sup>32</sup>, might increase plasma PPI concentrations and halt  
301 disease progression. As citrate chelates calcium and has been shown to prevent kidney stone  
302 (uroliths) formation<sup>22</sup>, ANKH-mediated citrate release might also contribute to inhibition of ectopic  
303 mineralization in joints and other tissues. The previous observation of *Ho et al.*<sup>1</sup> that *Ank*<sup>ank/ank</sup>  
304 mice have an increased incidence of kidney calcification would fit a function of ANKH/ANK in  
305 prevention of ectopic mineralization in tissues different from those lining the joints.

306

307 In conclusion, we identified ANKH/ANK as an important player in cellular release of citrate and  
308 NTPs. Citrate might have a previously unanticipated role in the prevention of soft tissue  
309 mineralization, in addition to other major ectopic mineralization inhibitors like PPI, Mg<sup>2+</sup> and  
310 Fetuin-A<sup>33,34</sup>. Moreover, we found that ANKH/ANK is a crucial factor in normal bone physiology  
311 by determining the amount of citrate and PPI incorporated in bone tissue.

312

313

## 314 **Materials and methods**

315

### 316 **Cell culture**

317 HEK293 cells were passaged in HyClone DMEM (GE) supplemented with 5% FBS and 100 units  
318 pen/strep per ml (Gibco) at 37°C and 5% CO<sub>2</sub> under humidified conditions. Efflux experiments  
319 were performed in 6-well plates in 2.5 ml Pro293a medium (Lonza) supplemented with 2 mM L-  
320 glutamine and 100 units pen/strep per ml.

321

### 322 **Animals**

323 Mice heterozygous for the progressive ankylosis allele (*ank*) were obtained from The Jackson  
324 Laboratory (Bar Harbor, ME; C3FeB6 *A/A<sup>w-J</sup>-Ank<sup>ank/J</sup>*, stock number 000200). Heterozygote  
325 breeders were used to generate *Ank<sup>ank/ank</sup>*, heterozygous and wild type littermates. Protocols were  
326 approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University in  
327 accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.  
328 Animals analyzed were between 11-14 weeks old. Plasma samples were collected by cardiac  
329 puncture in heparinized syringes. Studies included similar numbers of male and female mice.

330

### 331 **Mutagenesis and overexpression of ANKH**

332 *ANKH<sup>wt</sup>* cDNA was obtained from Sino Biological and subcloned into pEntr223 by USER  
333 cloning. The *L244S* mutation was introduced by USER cloning with primers 5'-  
334 ACCAGAAGCUCAGCATCTTTCTTATTGTTGCATCTCCC-3' and  
335 AGCTTCTGGUGGCCTTCCGCTC TAATTCTGGCCACA. cDNAs were subsequently subcloned  
336 in a Gateway compatible pQCXIP expression vector<sup>10</sup>. HEK293 cells were transfected with  
337 pQCXIP-ANKH by calcium phosphate precipitation. *ANKH<sup>wt</sup>* and *ANKH<sup>L244S</sup>* in clones resistant  
338 to 2  $\mu$ M puromycin were determined by immunoblot analysis, with a polyclonal antibody directed  
339 against ANKH (OAAB06341, Aviva Systems Biology).

340

### 341 **Enzymatic quantification of PP<sub>i</sub>, AMP and citrate**

342 In medium samples, PP<sub>i</sub> and AMP were quantified as described<sup>11</sup> with modifications. PP<sub>i</sub>  
343 concentrations were determined using ATP sulfurylase from NEB, and adenosine  
344 5'phosphosulfate from Cayman Chemicals. AMP was quantified as follows: To 1  $\mu$ l of sample or  
345 standard, 100  $\mu$ l of a solution containing 0.14 U/ml pyruvate orthophosphate dikinase (PPDK, kind  
346 gift of Kikkoman Chemifa), 12.5  $\mu$ mol/L PP<sub>i</sub> (Sigma-Aldrich), 40  $\mu$ mol/L phosphoenol pyruvate  
347 (Cayman Chemicals), 50  $\mu$ mol/L dithiothreitol, 1 mmol/L EDTA, 7.5 mmol/L MgSO<sub>4</sub> and 30 mmol/L  
348 BES (pH 8.0) was added. Conversion of AMP into ATP was allowed to proceed for 20 min at 45

349 °C, after which PPK was inactivated by incubation at 80 °C for 10 min. To determine PPK and  
350 citrate amounts in bones, tibiae and femora of 13-week-old mice were collected and defleshed.  
351 Epiphyses were removed and bone marrow was spun out of the bones (30,000 RCF, 1 min).  
352 Bones were subsequently dissolved by incubation with continuous mixing in 10% formic acid (60  
353 °C, 750 RPM, 14 hrs). Samples were spun for 10 min at 30,000 RCF and the supernatant was  
354 analyzed for PPK and citrate content. For bone extracts a slightly modified, more sensitive, version  
355 of the PPK assay was used. A total reaction volume of 520 µl assay mix contained 100 µl of SL-  
356 ATP detection reagent (Biothema, Sweden), 0.1 µl ATP removal reagent (“apyrase”, BioThema,  
357 Sweden), 6 µM adenosine-5'-phosphosulphate (APS) (SantaCruz, TX), 0.15 U/ml ATP  
358 sulphurylase (ATPS) (New England Biolabs) and 400 µl of ATP-free Tris-EDTA buffer (BioThema,  
359 Sweden) was first incubated overnight at room temperature to convert PPK into ATP for  
360 subsequent degradation by apyrase. The overnight incubation removed background PPK from the  
361 assay mixture, resulting in a higher sensitivity of the assay. Next, the sample, diluted 500-fold in  
362 Tris-EDTA buffer, was added to 500 µl of the assay mixture, resulting in an increase in  
363 luminescence due to the conversion of PPK and APS into ATP, a reaction catalyzed by ATPS.  
364 Finally, a known amount of ATP was added as internal standard and the ratio between the  
365 increase in bioluminescent signal induced by the addition of PPK and by the increase induced by  
366 the addition of ATP was used to calculate the PPK concentration. The assay was performed in a  
367 Berthold FB12 luminometer in the linear range of the detector. Internal PPK standards were used  
368 to show robustness and sensitivity of the assay.

369 Citrate was quantified in medium samples using the Megazyme Citric Acid Kit (Megazyme,  
370 Ireland).

371

### 372 **Real-time ATP efflux assay**

373 Real-time ATP efflux assays were performed as described <sup>11</sup>, with modifications. To reduce ATP  
374 release by the initial buffer change, cells were incubated at 27°C, for 1 hr. Then an additional 50

375  $\mu$ l of ATP efflux buffer containing 10% of ATP-monitoring reagent (BactiterGlo, Promega),  
376 dissolved in ATP efflux buffer was added. Bioluminescence was followed in real-time for 1 hr at  
377 27 °C and 2 hrs at 37 °C in a Flex Station3 microplate reader (Molecular Devices).

378

### 379 **LC/MS-based global metabolite profiling**

380 Proteins were precipitated in 200  $\mu$ l of medium or 50  $\mu$ l plasma by adding 800  $\mu$ l and 200  $\mu$ l  
381 acetonitrile:methanol (1:1), respectively. Samples were shaken (10 minutes, 500 RPM, 21°C),  
382 centrifuged (15,000 g, 4°C, 10 min) and the supernatant dried in a Speed-Vac. Pellets were  
383 stored at -20°C until analysis. For analysis pellets were suspended in 45  $\mu$ l mobile phase A of  
384 which 10  $\mu$ l was analyzed by ion-pairing LC/MS as described <sup>12</sup>.

385 Analytes were identified based on accurate mass and retention time, which matched reference  
386 standards. Peak areas were determined using Masshunter Qualitative Analysis software version  
387 7.0SP2 (Agilent Technologies).

388

### 389 **LC/MS-based quantification of citrate**

390 Plasma proteins were removed as described above and resuspended in 50  $\mu$ l mobile phase A,  
391 while urine and bone samples were diluted in mobile phase A (5 and 20-fold, respectively). A  
392 volume of 5  $\mu$ l of each sample was analyzed as described under LC/MS global metabolite  
393 profiling, along with calibration curves consisting of mobile phase A spiked with citrate  
394 concentrations ranging from 1 to 1000  $\mu$ M. Quantification was performed using Masshunter  
395 Profinder Quantitative Analysis software version B.08.00, service pack 3 (Agilent Technologies).

396

### 397 **NMR spectroscopy**

398 One-dimensional <sup>1</sup>H-NMR spectroscopy of urine samples was performed as described <sup>35</sup>. Briefly,  
399 urine samples were centrifuged for 10 min at 3,000 g and trimethylsilyl-2,2,3,3-  
400 tetradeuteriopropionic acid (TSP; sodium salt; Sigma) in D<sub>2</sub>O was added before analysis to serve

401 both, as an internal quantity reference and a chemical shift reference. The pH of each sample  
402 was adjusted to  $2.50 \pm 0.05$  with concentrated HCl.  $^1\text{H-NMR}$  spectra were obtained using a Bruker  
403 500-MHz spectrometer (pulse angle:  $90^\circ$ ; delay time: 4 s; no. of scans: 256; relaxation delay: 2s).  
404 Assignment of peak positions for compound identification was performed by comparing the peak  
405 positions in the spectra of the metabolites with the reference spectral database of model  
406 compounds at pH 2.5 using Amix version 3.9.14 (Bruker BioSpin).

407

#### 408 **Calculation of the contribution of NTP release to ANKH-dependent accumulation of PPi in** 409 **the culture medium**

410 To estimate the contribution of ANKH<sup>wt</sup>-mediated NTP release to 24-hour extracellular PPi  
411 concentrations, PPi concentrations in medium of HEK293 parental cells were subtracted from the  
412 PPi concentrations detected in medium of HEK293-ANKH<sup>wt</sup> cells, yielding an ANKH-specific PPi  
413 accumulation in the 24-hr culture medium samples of 2.4  $\mu\text{M}$ . The same calculation demonstrated  
414 an ANKH-specific accumulation of 1.4  $\mu\text{M}$  AMP in the culture medium. This demonstrated that  
415 ATP release underlies at least 60% of the ANKH-dependent PPi accumulation detected in the  
416 culture medium ( $1.4/2.4 \times 100 = 58$ ). GMP, UMP and CMP were also found to increase in culture  
417 medium in an ANKH-dependent manner. Based on the relative LC/MS signals of the NMPs, we  
418 estimated that AMP was responsible for 80% of the total NMP concentration in the culture  
419 medium, whereas GMP, UMP and CMP together were responsible for the remaining 20%.  
420 Together these data demonstrate that nucleoside monophosphate (NMP) concentrations could  
421 explain 70% of the ANKH-dependent PPi that had accumulated in the culture medium after 24  
422 hrs. The calculated 70% is most likely an underestimation, as generated NMPs will be further  
423 metabolized by the HEK293 cells, as we have observed before <sup>10</sup>.

424

#### 425 **MicroCT**



426 Each bone was scanned using a Bruker Skyscan 1275 microCT system equipped with a 1 mm  
427 aluminum filter. One femur from each mouse was scanned at 55 kV and 181  $\mu$ A with a 74 ms  
428 exposure time. Transverse scan slices were obtained by placing the long axis of the bone  
429 parallel to the z axis of the scanner using a custom 3D printed sample holder. An isometric  
430 voxel size of 13  $\mu$ m was used. Images were reconstructed using nRecon (Bruker) and analyzed  
431 using CTan (Bruker).

432

### 433 **Three-point Bending assay**

434 Three-point bending was performed on bones that had been stored at -20 °C in PBS-soaked  
435 gauze after harvest. Femora were scanned with microCT before performing three-point bending.  
436 Briefly, each femur was oriented on a standard fixture with femoral condyles facing down and a  
437 bending span of 8.7 mm. Next, a monotonic displacement ramp of 0.1 mm/s was applied until  
438 failure, with force and displacement acquired digitally. The force-displacement curves were  
439 converted to stress-strain using microCT-based geometry and analyzed using a custom GNU  
440 Octave script.

441

### 442 **Statistical analyses.**

443 P-values of group comparisons were calculated using one-way Anova using Prism 7.0d version  
444 (GraphPad Software Inc.). Significance is indicated in the figures, with \* < 0.05, \*\* < 0.01, \*\*\* <  
445 0.001 and \*\*\*\* < 0.0001.

446

447

### 448 **Author contributions**

449 FS            Wrote manuscript, performed experiments

450 SL            Performed experiments

451 SD            Performed experiments

452 UE Performed NMR analysis of urine samples  
453 RE Provided access to essential equipment  
454 KR Provided access to critical equipment  
455 CW Provided essential reagents  
456 JPS Provided essential reagents  
457 RW Provided patient sample and analyzed data  
458 RET performed experiments, analyzed data  
459 RJ Performed metabolomics analyses, analyzed data  
460 KvdW Wrote manuscript, analyzed data, conceptualized project, supervised project

461

462 All Authors have seen and reviewed the manuscript

463

#### 464 **Acknowledgements**

465 We thank our colleagues Piet Borst (The Netherlands Cancer Institute), Susan Cole (Queens's  
466 University) and Jouni Uitto (Thomas Jefferson University) for critically reviewing our manuscript  
467 and valuable discussions. FSz received financial support from the Fulbright Visiting Scholar  
468 Program sponsored by the U.S. Department of State and a mobility grant from the Hungarian  
469 Academy of Sciences. Further funding for this work was provided by PXE International and the  
470 National Institutes of Health Grant R01AR072695 (KvdW).

471

#### 472 **Competing Interests**

473 The authors declare they have no financial or non-financial competing interests.

474

475 **References**

- 476 1. Ho, A. M., Johnson, M. D. & Kingsley, D. M. Role of the mouse ank gene in control of  
477 tissue calcification and arthritis. *Science* **289**, 265–270 (2000).
- 478 2. Kawasaki, K., Buchanan, A. V. & Weiss, K. M. Biomineralization in humans: making the  
479 hard choices in life. *Annu. Rev. Genet.* **43**, 119–142 (2009).
- 480 3. Gurley, K. A. *et al.* Mineral formation in joints caused by complete or joint-specific loss of  
481 ANK function. *J. Bone Miner. Res.* **21**, 1238–1247 (2006).
- 482 4. Abhishek, A. & Doherty, M. Pathophysiology of articular chondrocalcinosis--role of ANKH.  
483 *Nat. Rev. Rheumatol.* **7**, 96–104 (2011).
- 484 5. Kim, H. J., Minashima, T., McCarthy, E. F., Winkles, J. A. & Kirsch, T. Progressive  
485 ankylosis protein (ANK) in osteoblasts and osteoclasts controls bone formation and bone  
486 remodeling. *J. Bone Miner. Res.* **25**, 1771–1783 (2010).
- 487 6. Wu, C. *et al.* BioGPS: an extensible and customizable portal for querying and organizing  
488 gene annotation resources. *Genome Biol.* **10**, R130–8 (2009).
- 489 7. Morava, E. *et al.* Autosomal recessive mental retardation, deafness, ankylosis, and mild  
490 hypophosphatemia associated with a novel ANKH mutation in a consanguineous family.  
491 *J. Clin. Endocrinol. Metab.* **96**, E189–98 (2011).
- 492 8. Orriss, I. R., Arnett, T. R. & Russell, R. G. G. Pyrophosphate: a key inhibitor of  
493 mineralisation. *Curr. Opin. Pharmacol.* **28**, 57–68 (2016).
- 494 9. Nitschke, Y. & Rutsch, F. Inherited Arterial Calcification Syndromes: Etiologies and  
495 Treatment Concepts. *Curr. Osteoporos. Rep.* **15**, 1–16 (2017).
- 496 10. Jansen, R. S. *et al.* ABCC6 prevents ectopic mineralization seen in pseudoxanthoma  
497 elasticum by inducing cellular nucleotide release. *Proc. Natl. Acad. Sci. U.S.A.* **110**,  
498 20206–20211 (2013).
- 499 11. Jansen, R. S. *et al.* ABCC6-mediated ATP secretion by the liver is the main source of the  
500 mineralization inhibitor inorganic pyrophosphate in the systemic circulation-brief report.  
501 *Arterioscler. Thromb. Vasc. Biol.* **34**, 1985–1989 (2014).
- 502 12. Goncalves, M. D. *et al.* High-fructose corn syrup enhances intestinal tumor growth in  
503 mice. *Science* **363**, 1345–1349 (2019).
- 504 13. Alfrey, A. C. & Solomons, C. C. Bone pyrophosphate in uremia and its association with  
505 extraosseous calcification. *J. Clin. Invest.* **57**, 700–705 (1976).
- 506 14. Mycielska, M. E., Milenkovic, V. M., Wetzels, C. H., Rümmele, P. & Geissler, E. K.  
507 Extracellular Citrate in Health and Disease. *Curr. Mol. Med.* **15**, 884–891 (2015).
- 508 15. Petrarulo, M., Facchini, P., Cerelli, E., Marangella, M. & Linari, F. Citrate in urine  
509 determined with a new citrate lyase method. *Clin. Chem.* **41**, 1518–1521 (1995).
- 510 16. Hu, Y.-Y., Rawal, A. & Schmidt-Rohr, K. Strongly bound citrate stabilizes the apatite  
511 nanocrystals in bone. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 22425–22429 (2010).
- 512 17. Ma, C. *et al.* Citrate-based materials fuel human stem cells by metabonegenic regulation.  
513 *Proc. Natl. Acad. Sci. U.S.A.* **115**, E11741–E11750 (2018).
- 514 18. Uitto, J., Li, Q., van de Wetering, K., Váradi, A. & Terry, S. F. Insights into  
515 Pathomechanisms and Treatment Development in Heritable Ectopic Mineralization  
516 Disorders: Summary of the PXE International Biennial Research Symposium-2016. *J.*  
517 *Invest. Dermatol.* **137**, 790–795 (2017).
- 518 19. Shin, S.-Y. *et al.* An atlas of genetic influences on human blood metabolites. *Nat. Genet.*  
519 **46**, 543–550 (2014).
- 520 20. Sanchez, M.-P. *et al.* Sequence-based GWAS, network and pathway analyses reveal  
521 genes co-associated with milk cheese-making properties and milk composition in  
522 Montbéliarde cows. *Genet. Sel. Evol.* **51**, 34–19 (2019).
- 523 21. Rudman, D. *et al.* Hypocitraturia in patients with gastrointestinal malabsorption. *N. Engl.*  
524 *J. Med.* **303**, 657–661 (1980).
- 525 22. Raffin, E. P. *et al.* The Effect of Thiazide and Potassium Citrate Use on the Health

- 526 Related Quality of Life of Patients with Urolithiasis. *J. Urol.* **200**, 1290–1294 (2018).  
527 23. Harvey, J. A., Zobitz, M. M. & Pak, C. Y. Bioavailability of citrate from two different  
528 preparations of potassium citrate. *J. Clin. Pharmacol.* **29**, 338–341 (1989).  
529 24. Mazurek, M. P. *et al.* Molecular origin of plasma membrane citrate transporter in human  
530 prostate epithelial cells. *EMBO reports* **11**, 431–437 (2010).  
531 25. Rosenthal, A. K. *et al.* The progressive ankylosis gene product ANK regulates  
532 extracellular ATP levels in primary articular chondrocytes. *Arthritis Res. Ther.* **15**, R154  
533 (2013).  
534 26. Costello, J. C. *et al.* Parallel regulation of extracellular ATP and inorganic pyrophosphate:  
535 roles of growth factors, transduction modulators, and ANK. *Connect. Tissue Res.* **52**,  
536 139–146 (2011).  
537 27. Mitton-Fitzgerald, E., Gohr, C. M., Bettendorf, B. & Rosenthal, A. K. The Role of ANK in  
538 Calcium Pyrophosphate Deposition Disease. *Curr. Rheumatol. Rep.* **18**, 25 (2016).  
539 28. Albright, R. A. *et al.* ENPP1-Fc prevents mortality and vascular calcifications in rodent  
540 model of generalized arterial calcification of infancy. *Nature Commun.* **6**, 10006 (2015).  
541 29. Li, Q. *et al.* Mutant Enpp1<sup>asj</sup> mice as a model for generalized arterial calcification of  
542 infancy. *Dis. Models Mech.* **6**, 1227–1235 (2013).  
543 30. Russell, R. G. G. Bisphosphonates: From Bench to Bedside. *Ann. N. Y. Acad. Sci.* **1068**,  
544 367–401 (2006).  
545 31. Dedinszki, D. *et al.* Oral administration of pyrophosphate inhibits connective tissue  
546 calcification. *EMBO Mol. Med.* **9**, 1463–1470 (2017).  
547 32. Borst, P., Váradi, A. & van de Wetering, K. PXE, a Mysterious Inborn Error Clarified.  
548 *Trends Biochem. Sci.* **44**, 125–140 (2019).  
549 33. Herrmann, M., Kinkeldey, A. & Jahnen-Dechent, W. Fetuin-A Function in Systemic  
550 Mineral Metabolism. *TCM* **22**, 197–201 (2012).  
551 34. Rüfenacht, H. S. & Fleisch, H. Measurement of inhibitors of calcium phosphate  
552 precipitation in plasma ultrafiltrate. *Am. J. Physiol.* **246**, F648–55 (1984).  
553 35. Engelke, U. F. H. *et al.* Guanidinoacetate methyltransferase (GAMT) deficiency  
554 diagnosed by proton NMR spectroscopy of body fluids. *NMR Biomed.* **22**, 538–544  
555 (2009).  
556 36. Kirejczyk, J. K. *et al.* Urinary citrate excretion in healthy children depends on age and  
557 gender. *Pediatr. Nephrol.* **29**, 1575–1582 (2014).  
558

559

560 **Fig 1. HEK293-ANKH<sup>wt</sup> cells release ATP, which is rapidly converted into pyrophosphate**  
561 **(PPi) and AMP.** Detection of ANKH in HEK293 parental, HEK293-ANKH<sup>wt</sup> and HEK293-  
562 ANKH<sup>L244S</sup> cells by immunoblot analysis (A). Concentrations of pyrophosphate (PPi) (B) and AMP  
563 (C) were quantified enzymatically and followed in medium samples of HEK293 parental, HEK293-  
564 ANKH<sup>wt</sup> and HEK293-ANKH<sup>L244S</sup> cells over the course of 24 hours. ATP release by HEK293  
565 parental, HEK293-ANKH<sup>wt</sup> and HEK293-ANKH<sup>L244S</sup> cells was followed in real time using a  
566 luciferase-based assay (D). Results of representative experiments performed in triplicate are  
567 shown. In panels B and C data are expressed as mean +/- SD. Panel D shows mean +/- SEM.

568

569 **Fig 2. Medium of HEK293-ANKH<sup>wt</sup> cells contains large amounts of nucleoside**  
570 **monophosphates.** LC/MS-based global metabolite profiling was applied to 24-hour medium  
571 samples of HEK293 parental, HEK293-ANKH<sup>wt</sup> and HEK293-ANKH<sup>L244S</sup> cells. The relative  
572 abundance of masses corresponding to AMP (A), CMP (B), GMP (C) and UMP (D) were  
573 determined. Authentic standards were used to confirm the identity of NMPs. Data are expressed  
574 as mean +/- SD of an experiment performed in triplicate. \* p < 0.05, \*\* p < 0.01.

575

576 **Fig 3. Medium of HEK293-ANKH<sup>wt</sup> cells contains large amounts of citrate, succinate and**  
577 **malate.** LC/MS-based global metabolite profiling was applied to 24-hour medium samples of  
578 HEK293 parental, HEK293-ANKH<sup>wt</sup> and HEK293-ANKH<sup>L244S</sup> cells. The relative abundance of  
579 masses corresponding to citrate (A), malate (B) and succinate (C) were determined. Authentic  
580 standards were used to confirm the identity of the Krebs-cycle intermediates. Using an  
581 enzymatic assay, citrate concentrations were followed for 24 hours in (D). Data are expressed  
582 as mean +/- SD of an experiment performed in triplicate. \*\*\*\* p < 0.0001.

583

584 **Fig 4. PPI content of bone tissue depends on ANK activity.** Pyrophosphate content of tibiae  
585 (A) and femora (B) of wild type (n=10), heterozygous (HET, n=10) and *Ank<sup>ank/ank</sup>* (*ank/ank*, n=8)  
586 mice. Data are expressed as mean +/- SD. \*\* p < 0.01, \*\*\*\* p < 0.0001.

587

588 **Fig 5. Extracellular Citrate depends on ANK/ANKH activity.** (A) Citrate plasma concentrations  
589 in wild type (n=8), heterozygous (HET, n=8) and *Ank<sup>ank/ank</sup>* (n=8) mice. Citrate concentrations in  
590 urine of wild type (n=6), heterozygous (HET, n=10) and *Ank<sup>ank/ank</sup>* (n=9) mice. (C) Urine of a patient  
591 suffering from craniometaphyseal dysplasia (CMD) due to biallelic inactivating mutations in *ANKH*  
592 is virtually devoid of citrate. NMR spectra of urine of a patient with biallelic  
593 pathogenic *ANKH<sup>L244S</sup>* mutations (C, upper panel). A representative sex- and age-matched control  
594 urine sample contained 370  $\mu\text{mol}/\text{mmol}$  creatinine (C, lower panel). Spectra are scaled on  
595 creatinine. Citrate resonates as a typical AB-system (2.98 ppm; four peaks between 2.80 and  
596 3.05 ppm). Reference values for urinary citrate for this age group are 208-468  $\mu\text{mol}/\text{mmol}$   
597 creatinine (n=20 healthy controls)<sup>36</sup>. Succinate resonates as a singlet resonance at 2.66 ppm.  
598 For unknown reasons, urinary lactate was somewhat increased in urine of the CMD patient  
599 (120  $\mu\text{mol}/\text{mmol}$  creatinine; reference <75  $\mu\text{mol}/\text{mmol}$  creatinine). Citrate content of tibiae (D) and  
600 femora (E) of wild type (n=10), heterozygous (HET, n=10) and *Ank<sup>ank/ank</sup>* (n=10) mice. Data are  
601 expressed as mean +/- SD. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

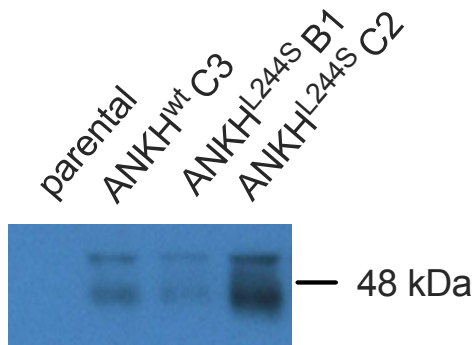
602

603 **Fig 6. Bone geometry and mechanical performance is altered in the absence of ANK**  
604 **activity.** microCT was used to determine (A) bone area, (B) cortical thickness, (C) tissue mineral  
605 density (TMD), (D) bone area fraction (B.Ar/T.Ar), (E) bone perimeter, and (F) eccentricity in  
606 femora of wild type (n=9), heterozygous (HET, n=10) and *Ank<sup>ank/ank</sup>* (n=8) mice. (G) To compare  
607 whole bone bending strength, a linear regression between section modulus and ultimate bending  
608 moment was analyzed for each genotype ( $r^2 = 0.84$  wild type, 0.73 HET, 0.67 *Ank<sup>ank/ank</sup>*). The  
609 slope was not different between genotypes, but the intercept was significantly different in femora

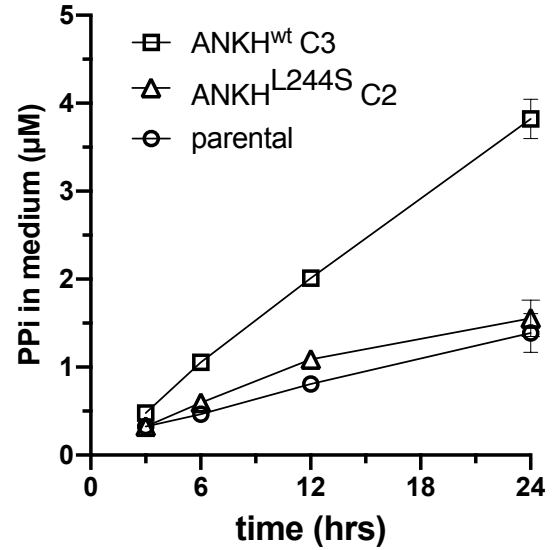
610 from *Ank*<sup>ank/ank</sup> mice, which utilized an increased section modulus to achieve the corresponding  
611 ultimate moment. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .  
612

# Figure 1

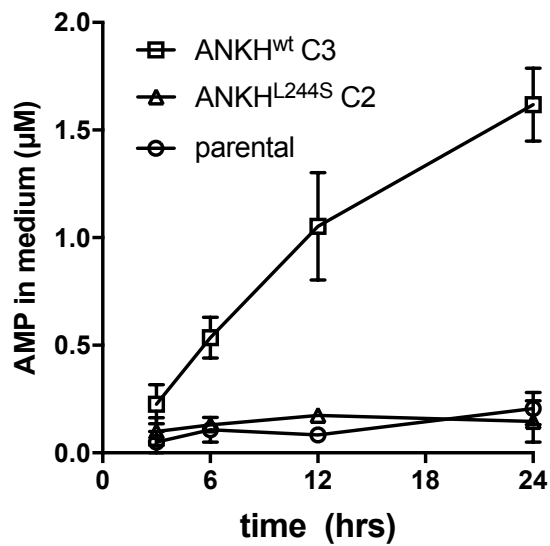
**A**



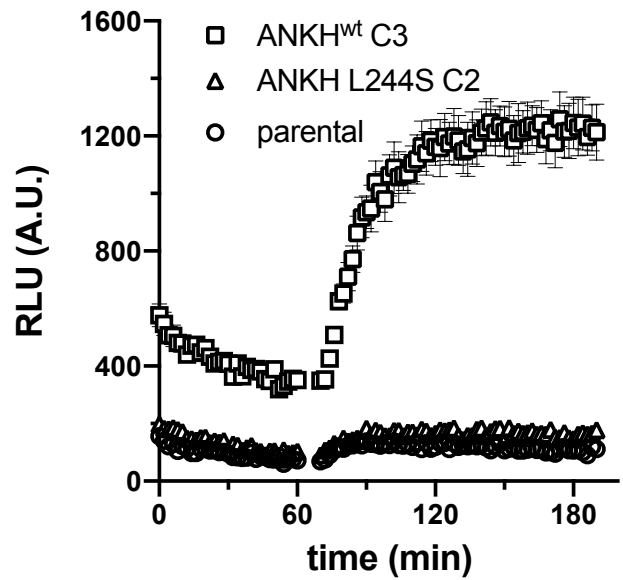
**B**



**C**



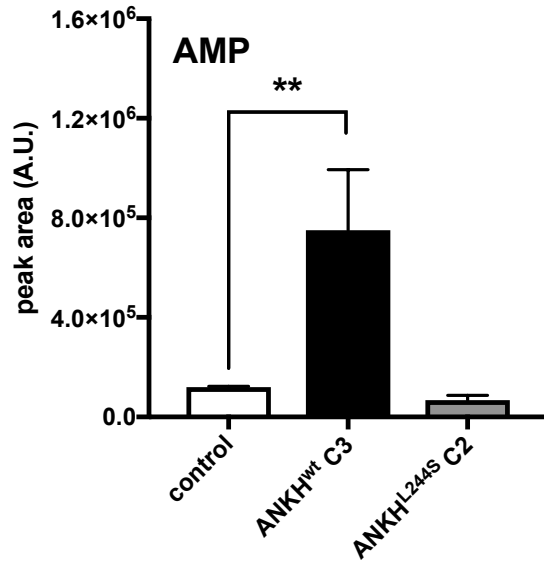
**D**



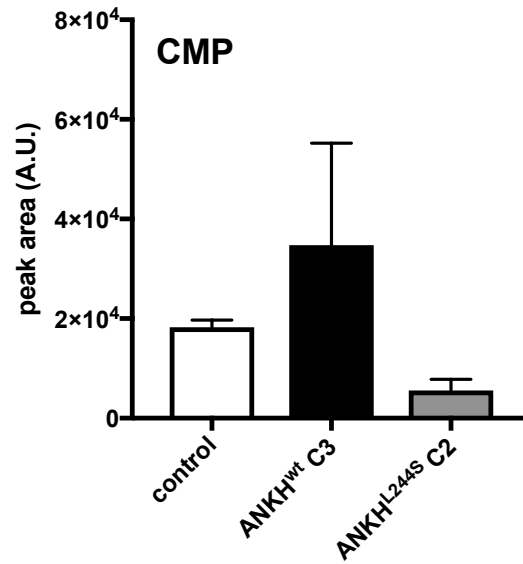


## Figure 2

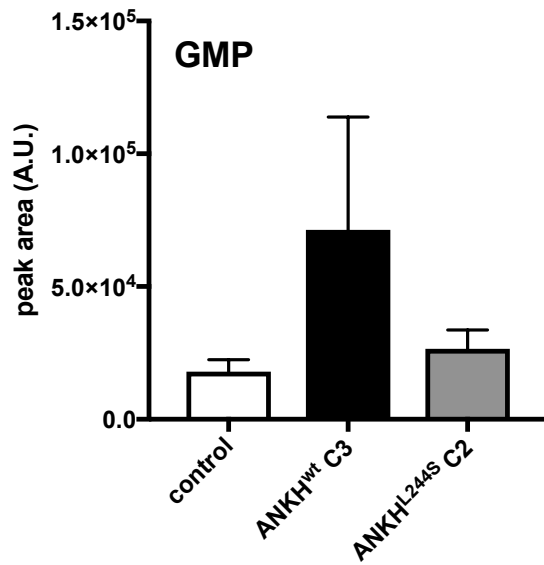
**A**



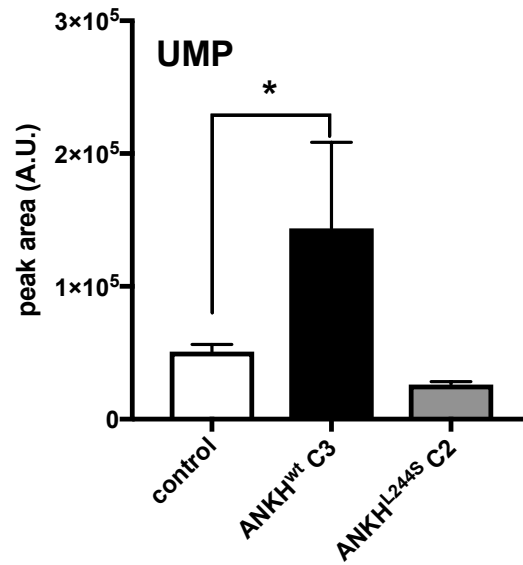
**B**



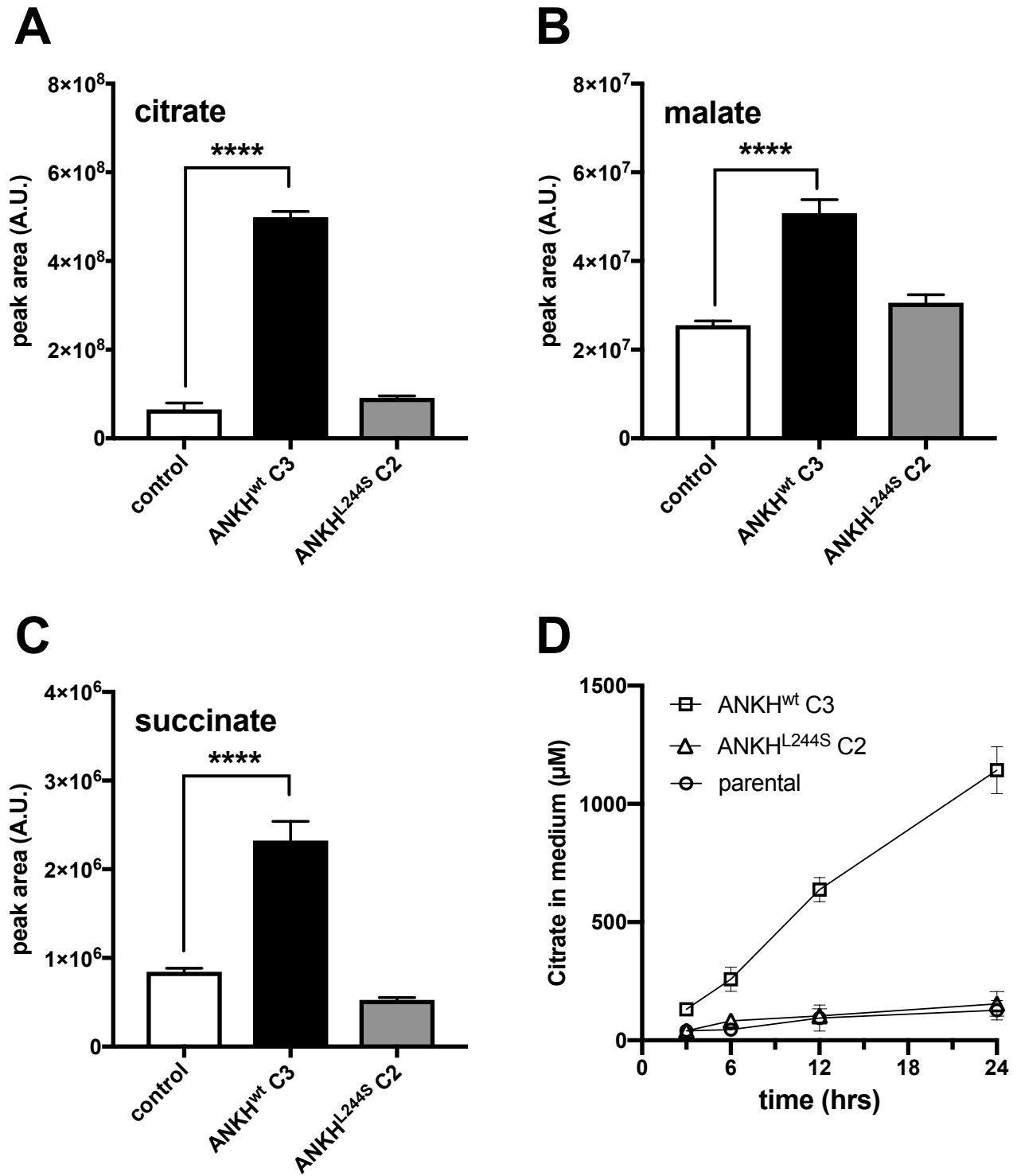
**C**



**D**

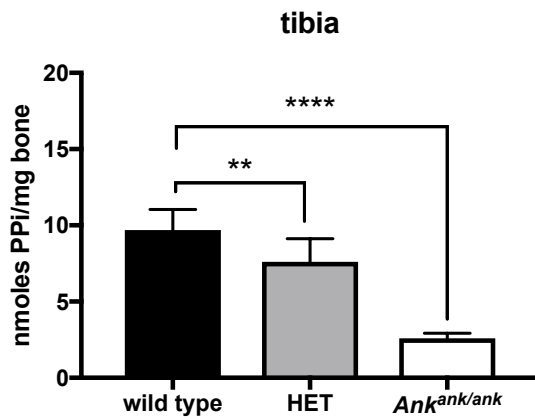


## Figure 3

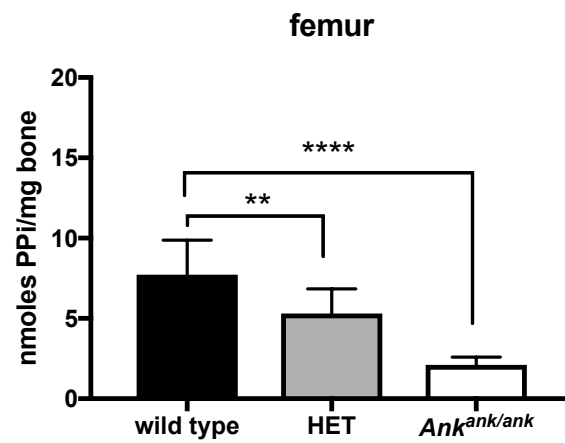


## Figure 4

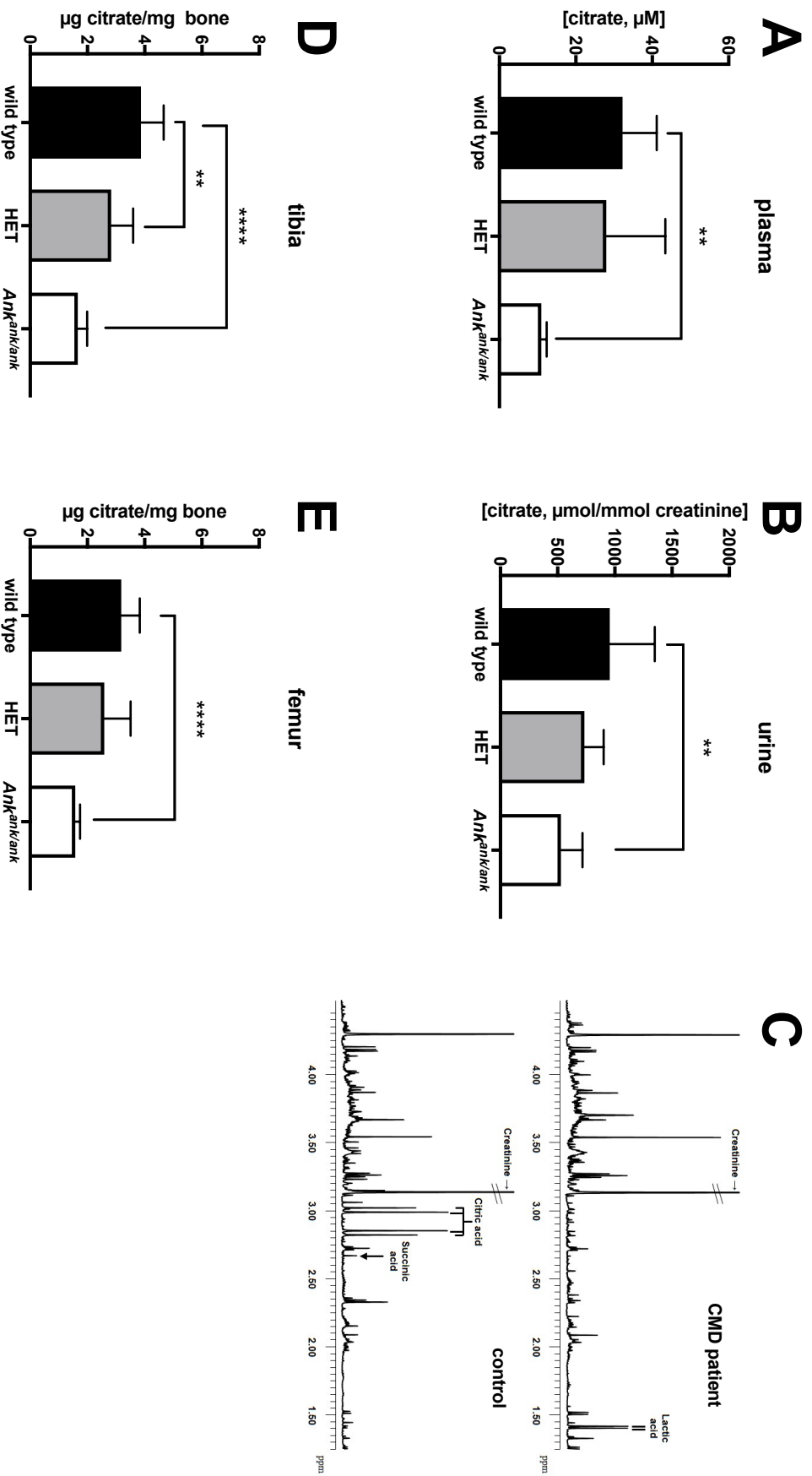
**A**



**B**



# Figure 5



# Figure 6

