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Intraperitoneal pyrophosphate treatment reduces renal calcifications in Npt2a null mice

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Abstract

Mutations in the proximal tubular sodium-dependent phosphate co-transporters NPT2a and NPT2c have been reported in patients with renal stone disease and nephrocalcinosis, however the relative contribution of genotype, dietary calcium and phosphate, and modifiers of mineralization such as pyrophosphate (PPI) to the formation of renal mineral deposits is unclear. In the present study, we used Npt2a−/− mice to model the renal calcifications observed in these disorders. We observed elevated urinary excretion of PPI in Npt2a−/− mice when compared to WT mice. Presence of two hypomorphic Extracellular nucleotide pyrophosphatase phosphodiesterase 1 (Enpp1asj/asj) alleles decreased urine PPI and worsened renal calcifications in Npt2a−/− mice. These studies suggest that PPI is a thus far unrecognized factor protecting Npt2a−/− mice from the development of renal mineral deposits. Consistent with this conclusion, we next showed that renal calcifications in these mice can be reduced by intraperitoneal administration of sodium pyrophosphate. If confirmed in humans, urine PPI could therefore be of interest for developing new strategies to prevent the nephrocalcinosis and nephrolithiasis seen in phosphaturic disorders.

Introduction

Mutations in the sodium phosphate co-transporters NPT2a [1–3] and NPT2c [4, 5] have been associated with intraluminal stones (nephrolithiasis) and mineral deposits in the renal parenchyma (nephrocalcinosis) in patients with familial forms of hypophosphatemia. In genome-wide association studies, NPT2a has also been associated with nephrolithiasis [6] and altered renal function [7, 8]. With both genetic abnormalities affected individuals show renal phosphate-wasting, high circulating levels of 1,25(OH)2D, and absorptive hypercalciuria as a result of increased intestinal uptake of calcium [4, 5, 9, 10], and oral phosphate supplements are currently thought to reduce the risk for renal mineralization by lowering circulating levels of 1,25(OH)2D and absorptive hypercalciuria [11]. However, the relative contribution of genotype,
Pyrophosphate reduces calcification in kidneys of Npt2a<sup>-/-</sup> mice

dietary calcium and phosphate, and modifiers of mineralization to the formation of renal mineral deposits is unclear. Our recent work suggests that reduced levels of osteopontin (Opn), an extracellular matrix factor affecting binding of phosphate to hydroxyapatite crystals, contribute to the development of nephrocalcinosis in Npt2a<sup>-/-</sup> mice [12]. This may be due to the fact that Npt2a<sup>-/-</sup> mice respond differently to dietary phosphate when compared to WT mice [13]. Further evaluation in the Npt2a<sup>-/-</sup> cohort on different diets suggests that urinary calcium excretion, plasma phosphate, and FGF23 levels appear to be positively correlated to renal mineral deposit formation, while urine phosphate levels and the urine anion gap, an indirect measure of ammonia excretion, appear to be inversely correlated [13]. In addition, local tissue levels of Pi generated by tissue nonspecific alkaline phosphatase (Tnsalp) and ectonucleoside triphosphate diphosphohydrolase 5 (Enpd5) may be important as suggested by decreased skeletal mineralization in the absence of these enzymes [14, 15].

In the present report, we hypothesize that genes involved in the synthesis of pyrophosphate (PPI) in the interstitial matrix may be associated with renal mineralization in these mice [16, 17].

PPI is present in plasma at a concentration of 1–6 μM [18] and in urine levels are around 10 μM [19]. Calcium phosphate stone formers appear to have reduced urinary PPI excretion when compared with control subjects [20–23]. Intravenous [32]PPI is rapidly hydrolyzed in plasma by tissue nonspecific alkaline phosphatase (Tnsalp) that is expressed in the proximal tubules of the kidneys [24] and less than 5% of intravenous [32]PPI appears in urine. These data indicate that urine PPI is generated locally in the kidneys [25, 26].

**Extracellular nucleotide pyrophosphatase phosphodiesterase 1 (Enpp1)** hydrolyzes extracellular ATP into AMP and PPI and may be an important source of extracellular PPI in the body [27, 28]. Enpp1 is the founding member of the ENPP or NPP family of enzymes [29]. It has phosphodiesterase activity [27] and is a type II extracellular membrane bound glycoprotein located on the mineral-depositing matrix vesicles of osteoblasts and chondrocytes [30] and the vascular surface of cerebral capillaries [28]. Enpp1 is also expressed in the kidney collecting duct and possibly other segments [25]. The second source of PPI generation in the kidney is the mevalonate pathway inside mitochondria [26]. Intracellular PPI is released into the interstitium and the urine by the transporter progressive ankylosis gene product (Ank) [31]. Ank is located at the apical membrane of collecting ducts suggesting that it may function to inhibit mineralization within the tubule lumen. Additionally, ecto-5-prime nucleotidase (NT5E/CD73), which inhibits Tnsalp by further hydrolyzing AMP to adenosine, and adenosine triphosphate-binding cassette, member 6 (Abcc 6), recently shown to secrete ATP from hepatocytes [32], may both be involved in PPI generation.

In the present study, we used Npt2a<sup>-/-</sup> mice to model these disorders. Renal mineral deposits in Npt2a<sup>-/-</sup> mice are found at intraluminal and interstitial sites, they contain calcium, phosphorus and osteopontin, and it has been suggested that they ultrastructurally resemble the composition of Randall’s plaques [33, 34]. The extent of renal mineralization is highest between newborn and weaning age Npt2a<sup>-/-</sup> mice [35]. Mineralization resolves subsequently on 0.3–0.6% dietary phosphate, but persists beyond weaning age when diets are supplemented with 1.65% phosphate [35] or 1.2% phosphate [12, 36]. Ablation of 25(OH)-vitamin D-1-alpha hydroxylase (Cyp27a1) prevents renal mineralization, as shown in Cyp27a1<sup>-/-</sup>/Npt2a<sup>-/-</sup> double-knockout mice [35].

We here report that urine PPI levels are increased in Npt2a<sup>-/-</sup> mice when compared to WT mice, possibly to protect from renal mineralization in the setting of hyperphosphaturia. Presence of two hypomorphic Enpp1<sup>1as1as</sup> alleles decreases urine PPI and worsens renal calcium phosphate deposit formation in Npt2a<sup>-/-</sup> mice. Conversely, development of mineral deposits in these mice can be reduced by intraperitoneal administration of sodium pyrophosphate. These...
studies suggest that PPI may be a thus far unrecognized factor modulating the development of renal calcifications in Npt2a−/− mice which may be, if confirmed in humans, of diagnostic and therapeutic relevance for phosphaturic disorders.

Materials and methods

Animals

Male and female C57BL/6 mice were obtained from Charles River Laboratory, MA. Male and female Npt2a−/− mice (B6.129S2-Slc34a1tm1Hten/J, Stock No: 004802), and Enpp1asj/asj mice (C57BL/6J-Enpp1asj/GrsrJ, Stock No: 012810) were purchased from The Jackson Laboratory, ME. The Enpp1asj allele is partially active and shows approximately 15% level of Enpp1 activity compared to wild-type controls [37]. Mice were genotyped by PCR amplification of genomic DNA extracted from tail clippings as described [29, 38–40]. Mice were weaned at 3 weeks of age and allowed free access to water and regular chow (1.0% calcium, 0.7% phosphorus, of which 0.3% phosphorus is readily available for absorption, Harlan Teklad TD.2018S). Mice received daily intraperitoneal (i.p.) injection of Hanks Buffered Saline (Gibco, Life Sciences) or sodium pyrophosphate in HBSS for two weeks until age four weeks as previously described (160 micromole/Kg/day) according to [41]. To determine whether renal mineral deposits persist beyond weaning age mice were followed for an additional 10 weeks of age after weaning on regular chow. The background of all mouse lines is C57Bl6, use of littermates for controls further reduced bias based on genetic background. No difference in renal mineral deposits was observed between sexes as previously reported by us [12, 36] and thus genders were combined here.

Mice were euthanized following orbital exsanguination in deep anesthesia with isoflurane and vital organs were removed as described [12, 36]. The research under IACUC protocol 2014–11635 was first approved Oct. 22 2014 by the Yale Institutional Animal Care and Use Committee (IACUC), was renewed Sept. 7 2016, and is valid through Sept. 30 2017. Yale University has an approved Animal Welfare Assurance (#A3230-01) on file with the NIH Office of Laboratory Animal Welfare. The Assurance was approved May 5, 2015.

Blood and urine parameters

Biochemical analyses were done on blood samples (taken by orbital exsanguination) and spot urines collected following an overnight fast at the same time of day between 10 AM and 2 PM. Following deproteinization of heparinized plasma by filtration (NanoSep 300 K, Pall Corp., Ann Arbor, MI), plasma and urinary total pyrophosphate (PPI) concentrations were determined using a fluorometric probe (AB112155, ABCAM, Cambridge, MA). Urine PPI was corrected for urine creatinine, which was measured by LC-MS/MS or by ELISA using appropriate controls to adjust for inter-assay variability.

Kidney histology

Left kidneys were fixed in 4% formalin/PBS at 4˚C for 12 h and then dehydrated with increasing concentration of ethanol and xylene, followed by paraffin embedding. Mineral deposits were determined on 10 um von Kossa stained sections counterstained with 1% methyl green. Hematoxyline/eosin was used as counterstain for morphological evaluation. Histomorphometric evaluation of sagittal kidney sections that includes cortex, medulla and pelvis was performed blinded by two independent observers using an Osteomeasure System (Osteometrics, Atlanta, GA). Percent calcified area was determined using the formula: % calc. area = 100°calcified area/total area (including cortex, medulla and pelvic lumen), and is dependent
on number of observed areas per section. Mineralization size was determined using the formula: calc. size = calcified area/number of observed calcified areas per section.

For transmission electron microscopy, a 1 mm³ block of the left kidney was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffered saline for 2 hrs., followed by post-fixation in 1% osmium liquid for 2 hours. Dehydration was carried out using a series of ethanol concentrations (50% to 100%). Renal tissue was embedded in epoxy resin, and polymerization was carried out overnight at 60˚C. After preparing a thin section (50 nm), the tissues were double stained with uranium and lead and observed using a Tecnai Biotwin (LaB6, 80 kV) (FEI, Thermo Fisher, Hillsboro, OR) at the Yale Center for Cellular and Molecular Imaging (YCCMI).

Renal gene expression analysis

Right kidneys were used for preparation of total RNA using Trizol (Thermo Fisher Sci, Inc., Waltham, MA). qRT-PCR (Omniscript, QuantiTect, Qiagen, Valencia, CA) was performed in an ABI-Step One Plus Cycler (Fisher, Life Technologies, Waltham, MA) using the mouse beta actin forward primer: GGCTGTATTCCCTCCATCG, and reverse primer: CCAGTTGGTAACAA TGCCATGT, the mouse Enpp1 forward primer: CTGTTTTTGCAGTATGGTGC and reverse primer: CTCACCACCTCTGAAATTTGTT, the mouse Entpd5 forward primer: CCAAGAGCTCGA TCCCCAGAA and reverse primer: TGTTAGAAAGTCTACGGTAACC, the mouse Ank forward primer: TACGGGCTGGCGTATTCTTTG and reverse primer: CACTGTAGGCTATCAGGGTGT, and the mouse Tnsalp forward primer CCAACTCTTTTGTGCCAGAGA and reverse primer: GGCTACATTGGTGTTGAGCTTT.

Statistical analysis

Data are expressed as means±SEM and analyzed in Microsoft Excel 2010 or Graphpad Prism 6.0. Differences were considered significant if p-values, calculated using the unpaired, two-tailed Student’s t-test, linear regression analysis, or one-way ANOVA using Tukey’s adjustment for multiple comparisons, were smaller than 0.05.

Results

Renal PPI excretion is increased Npt2a⁻/⁻ mice

Humans with loss-of-function of NPT2a [1–3] and NPT2c [4, 5] develop renal mineralization, which may manifest during early childhood prior to specific therapy or when inappropriately receiving active vitamin D analogs, but can also occur throughout life [9]. To model these kidney abnormalities, we used 2 months old Npt2a⁻/⁻ mice [39, 40] placed on a diet containing 0.6% calcium and 0.7% phosphorus (Harlan Teklad TD.2018S).

Interestingly, the urine PPI concentration was increased in Npt2a⁻/⁻ mice (1257 ± 272 micromole/l, n = 19 vs. WT 157 ± 13 micromole/l, n = 7, p = 0.042) (Fig 1A).

Similarly, urine PPI excretion corrected for urine creatinine was increased in Npt2a⁻/⁻ mice (3.0 ± 0.53 micromole/mg, n = 19 vs. WT 1.3 ± 0.42 micromole/mg, n = 9, p = 0.038) (Panel A in S1 Fig). Evaluation of whole kidney gene expression was unchanged for the PPI-generating enzyme Enpp1 (0.004 ± 0.001, n = 9 vs. WT 0.005 ± 0.001, n = 7, p = ns) and decreased for the PPI transporter Ank (0.00015 ± 2.8e-5, n = 9 vs. WT 0.001 ± 0.00014, n = 10, p = 0.007) (Fig 1B and 1C). Expression of the Pi-generating enzyme Entpd5 was decreased (0.06 ± 0.01, n = 9 vs. WT 0.66 ± 0.15, n = 10, p = 0.0073) and expression of Tnsalp, which hydrolyses PPI to Pi, was increased (0.07 ± 0.02, n = 9 vs. WT 0.02 ± 0.004, n = 10, p = 0.0043) (Fig 1D and 1E). Thus, the source of urine PPI in Npt2a⁻/⁻ mice remains unclear and may be extrarenal, localized to a
specific tubular segment inside the kidneys, or regulation may occur on the post-translational level.

To further evaluate the role of PPI in renal mineral deposit formation in the setting of renal phosphate wasting we next reduced endogenous PPI production using the hypomorphic murine Enpp1<sup>asj</sup> allele [37] or administered sodium pyrophosphate by intraperitoneal injection as previously described [41] to increase PPI.

Presence of the hypomorphic Enpp1<sup>asj</sup> allele blunts urine PPI excretion and worsens renal mineralization in Npt2a<sup>-/-</sup> mice

Enpp1<sup>asj/asj</sup> mice develop renal mineralization on a “stone-forming” high phosphorus, low magnesium diet, while they develop no renal mineralization on regular chow [17, 42].
Presence of two hypomorphic \textit{asj} alleles of \textit{Enpp1} blunted the increase of the urine PPI concentration of double-mutant mice when compared to \textit{Npt2a}\textsuperscript{-/-} mice on regular chow, albeit non-significantly (67 ± 21, n = 4 vs. \textit{Npt2a}\textsuperscript{-/-} 1257 ± 272 micromole/l, n = 19, p = 0.084, Fig 1A). Similarly, urine PPI excretion corrected for urine creatinine was decreased in double mutant mice (0.43 ± 0.084 micromole/mg, n = 4 vs. \textit{Npt2a}\textsuperscript{-/-} 3.0 ± 0.53 micromole/mg, n = 19, p = 0.044, panel A in S1 Fig). One or two hypomorphic \textit{asj} alleles of \textit{Enpp1} furthermore increased the calcified area of double-mutant mice when compared to \textit{Npt2a}\textsuperscript{-/-} mice on regular chow in a gene dose-dependent fashion (0.3 ± 0.07, n = 8 in \textit{Enpp1}\textsuperscript{\textit{asj}/+}/\textit{Npt2a}\textsuperscript{-/-}, p = ns vs. 0.26 ± 0.04% in \textit{Npt2a}\textsuperscript{-/-} and 0.69 ± 0.15% in \textit{Enpp1}\textsuperscript{\textit{asj}/\textit{asj}}/\textit{Npt2a}\textsuperscript{-/-}, p < 0.0001 vs. \textit{Npt2a}\textsuperscript{-/-}) while no mineral deposits were found in \textit{Enpp1}\textsuperscript{\textit{asj}/\textit{asj}} mice on regular chow (Fig 2A). Since increased calcified area in double mutants was due to an increase in number of calcifications, no difference was observed for mineralization size between \textit{Npt2a}\textsuperscript{-/-}, \textit{Enpp1}\textsuperscript{\textit{asj}/+}/\textit{Npt2a}\textsuperscript{-/-}, and \textit{Enpp1}\textsuperscript{\textit{asj}/\textit{asj}}/\textit{Npt2a}\textsuperscript{-/-} mice (Fig 2B). Renal calcified area inversely correlated with spot urine PPI concentration (slope = -5.226e-005 ± 2.391e-005, R\textsuperscript{2} = 0.126, p = 0.036) (Fig 3A). No significant correlation was found for calcification area (Fig 3B) or when area and size were correlated with urine PPI corrected for urine creatinine (Panels C and D in S1 Fig).

**Intraperitoneal sodium PPI injection decreases renal mineral deposits in \textit{Npt2a}\textsuperscript{-/-} mice**

Intraperitoneal injection of sodium pyrophosphate was previously shown to reduce arterial calcification in an uremic mouse model [41]. We used the dose of 160 micromole/Kg/day published by these authors and two weeks old \textit{Npt2a}\textsuperscript{-/-} pups for this experiment, because renal calcification is more pronounced when compared to older mice (Fig 4A and 4C). Size and body weight (BW) of mice in the treatment group were indistinguishable from vehicle and the animals appeared to be thriving well. Following sacrifice at four weeks of age we observed a reduction of renal mineral deposits by 33% in the treatment group (0.4 ± 0.04, n = 9 vs. vehicle 0.7 ± 0.06%, n = 12, p = 0.01) (Fig 4C and 4D) while mineralization size again was unaffected (Fig 4E). Plasma PPI levels at sacrifice were increased, albeit non-significantly (3.9 ± 0.8, n = 9 vs. vehicle 2.0 ± 0.4 micromole/l, n = 5, p = ns) (Fig 4F). Likewise, the U-PPi concentration was increased (244.9 ± 33.2, n = 14 vs. vehicle 149.4 ± 28.8 micromole/l, n = 14, p = 0.039) (Fig 4G and panel B in S1 Fig).

Histological evaluation showed large interstitial mineral deposits that displaced the surrounding renal tubules. In addition, we observed small intraluminal mineral deposits in cortical and medullary tubular segments of the kidneys of \textit{Npt2a}\textsuperscript{-/-} and double-mutant mice (Fig 4A). Transmission electron images showed concentric spheres of similar morphology in \textit{Npt2a}\textsuperscript{-/-} and double-knockout mice (Fig 4B) as previously described for \textit{Npt2a}\textsuperscript{-/-} mice by us [13, 43] and others [33, 34]. No mineralization was observed in renal vasculature or in the renal pelvis of our mice.

**Discussion**

Oral phosphate supplements are currently thought to be the primary intervention to reduce risk for renal mineralization in human carriers of \textit{NPT2a} and \textit{NPT2c} mutations. However, there is concern that oral phosphate therapy might contribute to the formation of renal mineralization despite reduced 1,25(OH)\textsubscript{2}D levels and reduced urinary calcium excretion under certain conditions, for example in patients with X-linked hypophosphatemia (XLH) treated with oral phosphate supplements given multiple times throughout the day [44, 45] and in otherwise healthy individuals following treatment with phosphate enema [46].
Fig 2. The hypomorphic Enpp1sj allele worsens renal mineralization area seen in Npt2a-/- mice on regular chow. Histomorphometric analysis of renal mineralization (%calcified area = 100 × mineralization area/tissue area, A; calcification size = mineralization area/number of calcifications, um², B) in 10 um sections of kidneys from mice fed regular chow for 10 weeks. The data represent individual animals (closed circles) with the means ± SEM, p-values shown above the lines of comparisons were calculated by one-way ANOVA.
We recently reported that reduced urine levels of osteopontin (Opn), an extracellular matrix factor affecting binding of phosphate to hydroxyapatite crystals, contribute to the development of nephrocalcinosis in Npt2a−/− mice [12]. The present report describes that the urine PPi concentration may be an additional modifier of renal calcifications in this mouse model.

Reduced Enpp1 activity increased the % calcified area in double mutant mice when compared to Npt2a−/− mice (Fig 4A), while the size of the calcium phosphate deposits was not affected. Similarly, intraperitoneal sodium PPi treatment reduced % calcified area, while calcification size was unchanged. Although further studies are required to define cause and effect, these data suggest that PPI inhibits nucleation (Figs 2A and 4A), which is different from the effect of Opn reported by us [12], that predominantly decreases mineralization size, consistent with the known role of Opn in calcium phosphate crystal growth. Interventions that increase both PPI and Opn would therefore be predicted to be additive.

Enpp1 expression is positively regulated by phosphate in osteoblast cultures [47], and therefore we expected that expression is likewise increased in Npt2a−/− mice to explain the increased urine PPI levels. Instead, we found that Enpp1 expression is unchanged, possibly as a result of reduced Pi sensing in the absence of Npt2a. Furthermore, Ank expression was decreased and Tnsalp was increased, all predicted to reduce local PPI production. These findings suggest that PPI may be generated outside of the kidneys contrary to previous reports [25, 26], and elevate urine PPI despite unchanged or decreased local gene expression for Enpp1 and Ank, respectively. Consistent with this hypothesis is our finding that global reduction of Enpp1 activity in Enpp1asj/asj mutant mice decreased urine PPI levels and that intraperitoneal injection of sodium pyrophosphate increased urine PPI levels (Fig 4G). Alternatively, PPI production may be regulated locally by increased renal activities of Enpp1 and Ank on a post-transcriptional level.

Interestingly, urine PPI in 10 weeks old Npt2a−/− mice is higher than in 4 weeks old weanlings (1257±272 micromole/l vs. 149.4 ± 28.8 micromole/l). This may be a developmental change of urine PPI over the first 10 weeks of life and could be a contributing factor explaining the initial observation in Npt2a−/− mice reported by the Tenenhouse lab [33], that renal calcifications peak with weaning age and subsequently decrease during adult life in these mice.

Tissue specific ablation of Enpp1 (and possibly Ank) could help determine in future studies whether PPI is produced renally or extrarenally. Injection of recombinant Enpp1 may be able to reduce the renal calcifications in Npt2a−/− mice [26, 29] and provide further evidence of the causal relationship of this extracellular enzyme, urine PPI, and renal mineralization.

Also, separate evaluation of interstitial and luminal mineralization and PPI levels and/or activity of PPI generating enzymes may be of interest in future studies. Finally, determining how urinary pH, anion gap, citrate, oxalate, magnesium, and the expression of uromodulin (Tamm-Horsfall protein, THP) or Opn [48] modify PPI action may help better understanding the pathogenesis of renal mineralization in Npt2a−/− mice.

In summary, we show here that urine PPI is increased in Npt2a−/− mice. Presence of one or two hypomorphic Enpp1asj alleles decreases urine PPI and increases renal mineral deposits in Npt2a−/− mice. Furthermore, the development of nephrocalcinosis and nephrolithiasis in these mice can be reduced by intraperitoneal administration of sodium pyrophosphate. These studies suggest that PPI may be a thus far unrecognized factor modulating the development of renal calcifications in Npt2a−/− mice which may be, if confirmed in humans, of diagnostic and therapeutic relevance for phosphaturic disorders.
Fig 3. Urinary pyrophosphate concentration is inversely associated with renal mineralization size in a combined bivariate linear regression analysis of all mice. All experimental WT and mutant mice from Fig 2 (n = 28) for which urine was available were evaluated using linear regression analysis to determine the association of renal mineralization with the urine pyrophosphate concentration (U-PPi) (% calcified area = 100 × calcified area/total area A and calcification size = calcified area/number of mineralization B). Data points represent values of individual animals. Results of the linear regression analysis are shown as solid line with 95% confidence interval (stippled lines), $R^2$ and p-values.

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**Supporting information**

**S1 Fig. U-PPi corrected by U-creatinine.** Urine pyrophosphate excretion of mice fed regular chow for 10 weeks (U-PPi/U-crea, A) and urine pyrophosphate excretion (U-PPi/U-crea) of two weeks old Npt2a⁻/⁻ pups treated with i.p. injections of vehicle or sodium pyrophosphate (160 micromole/Kg/day) for two weeks (B), measured after overnight fast and 18–24 hrs. following the last treatment. Linear regression analysis to determine the association of renal pyrophosphate levels (F) and urine pyrophosphate (U-PPi) (G) of two weeks old Npt2a⁻/⁻ pups treated with i.p. injections of vehicle or sodium pyrophosphate (160 micromole/Kg/day) for two weeks, measured after overnight fast and 18–24 hrs. following the last treatment. The data represent individual animals (closed circles) with the means±SEM, p-values shown above the lines of comparisons were calculated by Student’s t-test.

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mineralization with the ratio of urine pyrophosphate/urine creatinine (U-PPi/U-crea) (% calcified area = 100\*calcified area/total area C and calcification size = calcified area/number of mineralization D). The data represent individual animals (closed circles) or means±SEM, p-values shown above the lines of comparisons were calculated by one-way ANOVA using Tukey’s adjustment for multiple comparisons (A) and Student’s t-test (B-D).

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