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Effects of etidronate on the Enpp1\(^{-/-}\) mouse model of generalized arterial calcification of infancy

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Abstract. Generalized arterial calcification of infancy (GACI) is an autosomal recessive disorder of spontaneous infantile arterial and periarticular calcification which is attributed to mutations in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) gene. Whilst the bisphosphonate, etidronate, is currently used off-label for the treatment for GACI, recent studies have highlighted its detrimental effects on bone mineralisation. In the present study, we used the Enpp1\(^{-/-}\) mouse model of GACI to examine the effects of etidronate treatment (100 µg/kg), on vascular and skeletal calcification. Micro-computed tomography (µCT) analysis revealed a significant decrease in trabecular bone mass, as reflected by the decrease in trabecular bone volume/tissue volume (BV/TV; %), trabecular thickness, trabecular separation, trabecular number and pattern factor (P<0.05) in the Enpp1\(^{-/-}\) mice in comparison to the wild-type (WT) mice. Mechanical testing revealed that in the WT mice, treatment with etidronate significantly improved work to fracture and increased work post-failure (P<0.05, in comparison to the vehicle-treated WT mice). This significant increase, however, was not observed in the Enpp1\(^{-/-}\) mice. Treatment with etidronate had no effect on bone parameters in the WT mice; however, the Enpp1\(^{-/-}\) mice displayed an increased structural model index (SMI; P<0.05). We used a recently developed 3D µCT protocol to reconstruct and quantify the extensive aortic calcification in Enpp1\(^{-/-}\) mice in comparison to the WT mice. However, treatment with etidronate did not prevent de novo calcification, and did not arrest the progression of established calcification of the aorta.

Introduction

Generalized arterial calcification of infancy (GACI) is an autosomal recessive disorder of spontaneous infantile arterial and periarticular calcification (1-3). This life-threatening disease is caused by loss-of-function mutations in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene, a key regulator of biomineralisation and vascular calcification (4-8).

ENPP1 is a cell-surface glycoprotein enzyme that functions in synergy with the anklyosis protein (ANK) to respectively form and intracellularly channel inorganic pyrophosphate (PPi), an inhibitor of hydroxyapatite formation, from nucleoside triphosphates (9-12). The extracellular concentration of PPi is further influenced by tissue non-specific alkaline phosphatase (TNSALP), another cell-surface enzyme located on the cell membrane of osteoblasts and chondrocytes, as well as on the membranes of their matrix vesicles (MV) (13). TNSALP exerts its effects by hydrolysing PPi, reducing the concentration of this mineralisation inhibitor and establishing a phosphate (Pi)/PPi ratio permissive for the formation of hydroxyapatite crystals (14-17). Phosphatase, orphan 1 (PHOSPHO1) is another essential phosphatase, located within osteoblast- and chondrocyte-derived MVs with high phosphohydrolase activity toward phosphaethanolamine and phosphocholine (18-22), which contributes Pi for the initiation of skeletal mineralisation. Together, ENPP1, ANK, TNSALP and PHOSPHO1 control the Pi/PPi ratio conducive to physiological skeletal mineralisation. Thus, ENPP1 in GACI reduces extracellular Pi levels and predisposes to ectopic calcification. This was further exemplified in a previous study of ours, in which we determined that vascular smooth muscle cells from mice deficient in Enpp1 have increased TNSALP levels (23). In naturally occurring mouse models, the link between defective Enpp1 expression and altered mineralisation was initially demonstrated in ‘tip toe walking’ (ttw/ttw) mice (24-28). These animals are homozygous for a G→T substitution resulting in the introduction of a stop codon in the NPP1 coding sequence. The subsequent truncated protein leads to the loss of a vital calcium binding domain and two putative glycosylation sites (25). The phenotype of this mouse includes the postnatal development of progressive ankylosing intervertebral and peripheral joint hyperostosis, as well as spontaneous arterial and articular cartilage calcification and increased vertebral cortical bone formation (24-28). Transgenic mice that are homozygous for a...
disruption in exon 9 of the Enpp1 gene (Enpp1\(-/-\) mice) exhibit abnormalities that are almost identical to those present in ttw/ttw mice (29). These include decreased levels of extracellular PP\(_i\), with phenotypic characteristics, including significant alterations in bone mineralisation in long bones and calvariae, and pathological, severe peri-spinal soft tissue and arterial calcification (30-32).

Effective treatment for infants and young children with GACI is critical as without it, 85% of patients succumb to the disease within 6 months of age. First used off-label in the treatment of Fibrodisplasia ossificans progressiva, the ‘first generation’ bisphosphonate, etidronate (EHDP; ethane-1-hydroxy-1,1-diphosphonic acid, also known as 1-hydroxyethylidene-bisphosphonate) is an analogue of PP, and has also been used in the treatment of GACI. Bisphosphonates are potent inhibitors of osteoclast activity, and are widely used in clinical practice to prevent the bone loss associated with conditions, such as Paget's disease, metastatic bone disease and osteoporosis (33). The inhibitory effects of bisphosphonates on osteoblast function have also been demonstrated (34-37).

In 2008, a retrospective observational analysis of 55 patients with GACI revealed survival beyond infancy with etidronate therapy (38) corroborated by a recent study highlighting that 15 out of 22 GACI survivors received etidronate (39). However, studies on uremic rats have suggested that the administration of etidronate may not be able to prevent arterial calcification without inhibiting bone formation (40). Furthermore, a recent case report has highlighted the profound inhibition of skeletal mineralisation with paradoxical joint calcifications following protracted etidronate therapy in a 7-year-old boy with GACI (41). Taken together, these findings have led us to herein assess the effects of etidronate on bone architecture and arterial calcification in the Enpp1\(-/-\) mouse model of GACI.

Materials and methods

Animals. Enpp1\(-/-\) and wild-type (WT) mice were generated and maintained as previously described (1,5,29,42). Male mice were administered etidronate at 100 µg/kg, intraperitoneally twice a week from 11 to 22 weeks of age. The dosage of etidronate used in this study was based on the dose reported in a previously study (43). Animals were administered saline as a placebo (vehicle treatment). All animals were weighed once a week. The animals were sacrificed at 22 weeks of age and the tissues were dissected for further analysis. All animal experiments were approved by The Roslin Institute’s Animal Users Committee and the animals were maintained in accordance with the UK Home Office guidelines for the care and use of laboratory animals (PIL number DD 60/3828).

Preparation of tissue. The aortae and tibiae were dissected as previously described (5). The aortae were fixed in 10% neutral buffered formalin (NBF) for 48 h before being transferred to 70% ethanol. The tibiae were immediately frozen in distilled water pending analysis.

Micro-computed tomography (\(\mu\)CT) of the aortae. Prior to scanning, the aortae were immersed for a minimum period of time (10 min) in a macro-molecular iopamidol-based contrast agent (Niopam 300; Brako UK Ltd., High Wycombe, Buckinghamshire, UK) diluted 1:4 in water as previously described (44). To allow tissue differentiation, aortic luminae were filled with corn oil and the aortae were submersed in oil for the duration of the scan. Tissues were imaged using a Skyscan 1172 X-Ray Microtomograph (Bruker Daltonics, Brussels, Belgium). Sequential high-resolution scans were acquired using a rotation step of 0.3\(^\circ\) with the averaging of 3 frames at each step, applying a 0.5-mm aluminium filter, with an X-ray source set at 60 kV and 167 µA, and with an isotropic voxel size of 7 µm. The scans were reconstructed using NRecon (Bruker Daltonics). Noise in the reconstructed images was reduced by applying a median filter (radius = 1). The region of interest was selected to be the aortic arch, 200 lices (1.4 mm) under the subclavian artery. Soft and calcified tissue was identified by thresholding using CTAn software (Bruker Daltonics).

\(\mu\)CT of the tibiae. High-resolution scans with an isotropic voxel size of 5 µm were acquired with a \(\mu\)CT system (60 kV, 0.5 mm aluminium filter, 0.6\(^\circ\) rotation, Skyscan 1172; Bruker Daltonics). Scans were reconstructed using NRecon software (Bruker Daltonics). A 1,000-\(\mu\)m section of the metaphysis 250 \(\mu\)m off the reference plate was taken for analysis of the trabecular bone. The base of the growth plate was used as a standard reference point. A 250-\(\mu\)m metaphysis section of the mid-diaphysis, 1,500 \(\mu\)m below the reference plate, was scanned for the analysis of cortical structure. Data were analysed with CTAn software (Bruker Daltonics). The following parameters were analysed using CTAn software (Bruker Daltonics): percentage bone volume/trabecular bone volume (%BV/TV), trabecular number (Tb.N; /mm), trabecular patten factor (Tb.Pf), bone mineral density (BMD; g/cm\(^2\)), trabecular thickness (Tb.Th; mm), trabecular separation (Tb.Sp) and the structure model index (SMI) were evaluated. In the cortical bone, %BV/TV, BMD (g/cm\(^2\)), cortical thickness, cross-sectional area (mm\(^2\)), the percentage of closed pores and polar moment of inertia (mm\(^4\)) were evaluated.

Mechanical testing. Mechanical testing of the cortical bone was carried out using a Zwick materials testing machine (Zwick Armaturen GmbH, Ennepetal, Germany) and data were analysed as previously described (45). The span was fixed at 6.0 mm. The cross-head was lowered at 1 mm/min and data were recorded after every 0.1 mm change in deflection. Each bone was tested to fracture. Failure and fracture points were identified from the load-extension curve as the point of maximum load and where the load rapidly decreased to zero, respectively. The maximum stiffness was defined as the maximum gradient of the rising portion of this curve, and the yield point, the point at which the gradient reduced to 95% of this value. Both values were calculated from a polynomial curve fitted to the rising region of the load-extension curve.

Serum marker analysis. To determine differences in bone formation and resorption, plasma serum was collected from the mice at 22 weeks of age. A sandwich ELISA PINP (IDS Ltd., Boldons, UK) and a C-terminal telopeptide of type I collagen (CTx) ELISA kit ( RatLaps\(^{TM}\); IDS) were used respectively, and analyses were performed according to the manufacturer's instructions.
Statistical analysis. General linear model analysis, the Student's t-test, the Mann-Whitney non-parametric test and Pearson's correlation analysis were used to assess the data where appropriate. All data are expressed as the means ± SEM. Statistical analysis was performed using SPSS (IBM Software, New York, NY, USA). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Enpp1−/− mouse growth phenotype. In initial experiments, we examined whether the treatment of Enpp1−/− and WT mice with 100 µg/kg etidronate affects their growth. In accordance with our previous study, the Enpp1−/− mice exhibited a reduced growth in comparison to the WT mice (18.4% smaller than the age-matched WT controls; P<0.05) (5). Intraperitoneal injections of etidronate had no effect on the total body weight of the WT mice, nor the Enpp1−/− mice in comparison to the respective vehicle-treated mice (Fig. 1). Notably, the Enpp1−/− mice appeared to lose weight from approximately 12 weeks of age, which may be a consequence of their limited movement due to excessive joint calcification (Fig. 1) (1,5).

Aortic calcification. We have previously demonstrated that Enpp1−/− mice exhibit arterial calcification from 11 weeks of age (31). In this study, we employed our recently developed three-dimensional (3D) µCT protocol (44) for the quantification of aortic calcification to examine the effects of treatment with etidronate on mice lacking Enpp1. As expected, the Enpp1−/− mice exhibited extensive aortic calcification in comparison to the WT mice at 22 weeks of age (Fig. 2B). However, treatment with etidronate did not prevent de novo calcification, and did not arrest the progression of established calcification of the aorta in these mice (Fig. 2).

µCT analysis of bone microarchitecture. Enpp1−/− mice have previously been reported to display reduced mineral content in bone, with a reduction in bone volume fraction and trabecular thickness (5). The present study extended these observations by fully examining the effects of the administration of etidronate on the bone phenotype of Enpp1−/− mice. µCT analysis of the tibiae from Enpp1−/− mice in comparison to those from WT mice (both vehicle-treated) at 22 weeks of age revealed a significant decrease in trabecular bone mass, as reflected by a decrease in %BV/TV, trabecular thickness and trabecular number (P<0.05; Table I). Moreover, we observed a significant decrease in cortical parameters in the tibiae of the 22-week-old Enpp1−/−
mice in comparison to the age-matched WT mice, except for cortical porosity (P<0.05; Table II). Treatment with etidronate had no significant effect on cortical or trabecular bone parameters in the WT mice (Tables I and II). In the Enpp1−/− mice, treatment with etidronate resulted in an increase in trabecular number and %BV/TV, as reflected by the significant decrease in trabecular separation (P<0.05, in comparison to the vehicle-treated Enpp1−/− mice) (Table I). The Enpp1−/− mice treated with etidronate did show a significant decrease in SMI [quantification of the plate- or rod-like geometry of trabecular structures, as previously described (46)] compared to the vehicle-treated Enpp1−/− mice (P<0.05; Table I).

**Mechanical testing.** The changes in bone geometry observed as a result of treatment with etidronate in the Enpp1−/− mice are likely to alter the biomechanical properties of long bones. In order to examine this hypothesis, we carried out 3-point bending analysis of the tibiae. Mechanical testing revealed a significant decrease in all mechanical parameters examined (stiffness, load at failure, work to fracture, load at fracture, work to fracture, and yield) except work post-failure, in the Enpp1−/− mice compared to the WT mice at 22 weeks of age (Table III; P<0.05), reflecting reduced bone strength and stiffness as we have previously reported (5). In the WT mice, treatment with 100 µg/kg etidronate significantly improved work to fracture and increased work post-failure (Table III; P<0.05, in comparison to the vehicle-treated WT mice); this suggests that more energy is required to fracture these etidronate-treated bones in comparison to the vehicle-treated bones. This significant increase, however, was not observed in the Enpp1−/− mice treated with etidronate (Table III).

**Plasma biochemical markers.** The level of osteoblast and osteoclast activity was assessed by ELISA of serum taken from the etidronate-treated and vehicle-treated 22-week-old male Enpp1−/− and WT mice. The plasma concentrations of P1NP, a marker of bone formation, were unaltered in the Enpp1−/− and WT mice at 22 weeks of age (Fig. 3A). Moreover, no significant differences in bone formation were observed upon the administration of etidronate in the WT or Enpp1−/− mice (Fig. 3A). The plasma concentrations of CTX, a marker of bone resorption, were increased in the Enpp1−/− mice in comparison to the WT mice in

**Table I. µCT analysis of trabecular bone in male placebo (vehicle)- and etidronate-treated Enpp1−/− and WT mice.**

|          | WT          | Enpp1−/−     |
|----------|-------------|--------------|
|          | Placebo     | Enpp1−/−     |
|          | Etidronate  |              |
| BV/TV (%)|             |              |
|          | 8.79±1.59   | 8.75±1.79    |
| BMD (g/cm³) | 0.139±0.021 | 0.13±0.024   |
| Tb.Th (µm) | 60.62±4.31  | 57.34±8.34   |
| Tb.Sp (µm) | 298.24±12.68 | 290.47±26.56 |
| Tb.N      | 0.00146±0.00023 | 0.0015±0.00029 |
| Tb.Pf     | 0.027±0.0026 | 0.026±0.0033 |
| SMI       | 2.48±0.11   | 2.33±0.23    |
| DA        | 2.30±0.19   | 2.08±0.15    |
|          |             |              |

Results are expressed as the means ± SEM. *Significant difference compared to wild-type (WT) mice; †significant difference compared to placebo-treated mice. P<0.05; P<0.01; P<0.001. µCT, micro-computed tomography; BV/TV, bone volume/trabecular bone volume; BMD, bone mineral density; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; Tb.Pf, trabecular pattern factor; SMI, structure model index; DA, degree of anisotropy.

**Table II. µCT analysis of cortical bone in male placebo (vehicle)- and etidronate-treated Enpp1−/− and WT mice.**

|          | WT          | Enpp1−/−     |
|----------|-------------|--------------|
|          | Placebo     | Enpp1−/−     |
|          | Etidronate  |              |
| Co.BMD (g/cm³) | 1.18±0.018  | 1.18±0.020   |
| Co.Po (%)  | 53.27±7.26  | 54.79±5.64   |
| Co.Th (µm) | 197.90±1.91 | 195.64±3.84  |
| Co.Area (µm²) | 5.14E+06±5.57E+05 | 5.09E+06±3.72E+05 |

Results are expressed as the means ± SEM. *Significant difference compared to wild-type (WT) mice; †significant difference compared to placebo-treated mice. P<0.05; P<0.01; P<0.001. µCT, micro-computed tomography; BV/TV, bone volume/trabecular bone volume; BMD, bone mineral density; Co.BMD, cortical bone mineral density; Co.Po; cortical porosity; Co.Th, cortical thickness; Co.Area, cortical area.
resorption. Results are expressed as the means ± SEM. (A) P1NP, a marker of bone formation; (B) CTx (RatLaps™), a marker of bone

Figure 3. Serum marker analysis in demonstrating that tibiae from etidronate on the present study was undertaken to determine the effects of inhibition (4,47). In particular, the Enpp1-/- mouse is of particular importance in advancing our understanding of GACI. Thus, the present study was undertaken to determine the effects of etidronate on Enpp1-/- mice.

We data confirm and extend those of our previous study (5), with improved survival in patients with GACI, an autosomal recessive disorder of spontaneous infantile arterial and periarticular calcification which is attributed to mutations in the ENPP1 gene (38). Animal models have proven to be key to the understanding of pathological ectopic mineralisation (4,47). In particular, the Enpp1-/- mouse is of particular importance in advancing our understanding of GACI. Thus, the present study was undertaken to determine the effects of etidronate on Enpp1-/- mice.

|                      | Placebo          | Etidronate       |
|----------------------|------------------|------------------|
| Stiffness (N/mm)     | 44.56±7.08       | 48.82±23.43      |
| Load at failure (N)  | 17.22±2.90       | 15.34±4.13       |
| Work to failure (J)  | 0.066±0.00066    | 0.0064±0.0019    |
| Load at fracture (N) | 1.72±0.29        | 1.53±0.41        |
| Work to fracture (J) | 0.0069±0.00087   | 0.0086±0.0019    |
| Work post-failure (J)| 0.00037±0.00041  | 0.0023±0.0014   |
| Yield (N)            | 14.41±3.95       | 12.97±4.42       |

Table III. Measurements of tibia mechanical properties in male placebo (vehicle)- and etidronate-treated Enpp1-/- and WT mice.

Results are expressed as the means ± SEM. *Significant difference compared to wild-type (WT) mice; **Significant difference compared to placebo-treated mice; *P<0.05; **P<0.01; ***P<0.001.

both the etidronate- and vehicle-treated mice (P<0.05; Fig. 3B). This is in concordance with our previous observation of this marker in Enpp1-/- mice (5). However, there were no significant differences observed between the etidronate- and vehicle-treated mice in either parameter (Fig. 3).

Discussion

Studies have associated treatment with bisphosphonates, chiefly etidronate, with improved survival in patients with GACI, an autosomal recessive disorder of spontaneous infantile arterial and periarticular calcification which is attributed to mutations in the ENPP1 gene (38). Animal models have proven to be key to the understanding of pathological ectopic mineralisation (4,47). In particular, the Enpp1-/- mouse is of particular importance in advancing our understanding of GACI. Thus, the present study was undertaken to determine the effects of etidronate on Enpp1-/- mice.

Our data confirm and extend those of our previous study (5), demonstrating that tibiae from Enpp1-/- mice have a reduced trabecular bone mass and cortical thickness in comparison to WT mice, which explains the altered bone mechanical properties noted in the present study. This, therefore, is consistent with the depletion of NPP1 activity reducing extracellular PP, to abnormally low levels, resulting in insufficient PP, a substrate for TNAP to generate P, for normal mineral formation. In the present study, we also used our novel 3D µCT protocol to provide evidence of the severe hypermineralisation of the arteries in Enpp1-/- mice, consistent with reduced extracellular PP, levels predisposing the vascular system to ectopic calcification.

Bisphosphonates are typically prescribed for the treatment of osteoporosis and to reduce fracture risk, preventing bone loss primarily by the inhibition of osteoclast function (33). However, there is evidence to suggest that bisphosphonates impair the anabolic response of bone to parathyroid hormone (48), inhibiting osteoblast function and suppressing bone formation (34-37). Furthermore, the hydrolysis-resistant P-C-P motif of bisphosphonates resembles the TNSALP susceptible core of PP, (49) permitting bisphosphonates to impair calcium phosphate crystallisation (50). First used off-label in the treatment of Fibrodysplasia ossificans progressiva, the ‘first generation’ bisphosphonate, etidronate, is an analogue of PP, and whilst it is currently being investigated as a treatment for GACI, a number of studies have highlighted a number of detrimental effects with this approach, including the development of rickets or osteomalacia during the protracted administration of etidronate (40,41,51-56).

In this study, to clarify the effects of bisphosphonates on GACI, we treated WT and Enpp1-/- mice with 100 µg/kg etidronate. In the WT mice, treatment with etidronate had no effect on cortical or trabecular parameters as determined by µCT analysis. Despite this, treatment with etidronate significantly improved work to fracture and increased work post-failure, thus suggesting that more energy is required to fracture these bones in comparison to the vehicle-treated bones. The assessment of bone architecture in Enpp1-/- mice treated with etidronate revealed a significant decrease in SMI, a method for the determination of the plate- or rod-like geometry of trabecular structures. This change in bone geometry did not, however,
affect the bone mechanical properties. Consistent with this, the assessment of plasma markers of bone formation and resorption revealed that this dosage of etidronate did not significantly affect the bone remodelling process in the Enpp1−/− mice, nor in WT mice. These findings add further support to those of a recent in vivo study using mice, which also reported no effects of the administration of etidronate on bone resorption (57). Furthermore, the anti-resorptive effects of alendronate, risedronate and minodronate were revealed to be 1,000-, 3,300- and 10,000-fold greater than those of etidronate, respectively (57). However, this initiation of treatment may have been too late to resolve underlying, pre-existing calcifications and additional investigation into this would allow for further conclusions to be drawn.

In conclusion, despite the changes in bone microarchitectures, we did not observe an inhibition of aortic calcification or bone formation in the Enpp1−/− mice treated with etidronate. Additional studies are, therefore, required to fully determine whether etidronate is the most appropriate therapy for the treatment of GACI.

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