Mutant Enpp1\textsuperscript{asj} mouse as a model for generalized arterial calcification of infancy

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SUMMARY

Generalized arterial calcification of infancy (GACI), an autosomal recessive disorder, is characterized by early mineralization of blood vessels, often diagnosed by prenatal ultrasound and usually resulting in demise during the first year of life. It is caused in most cases by mutations in the ENPP1 gene, encoding an enzyme that hydrolyzes ATP to AMP and inorganic pyrophosphate, the latter being a powerful anti-mineralization factor. Recently, a novel mouse phenotype was recognized as a result of ENU mutagenesis – those mice developed stiffening of the joints, hence the mutations was named “ages with stiffened joints” (asj). These mice harbor a missense mutation, p.V246D, in the Enpp1 gene. Here we demonstrate that the mutant ENPP1 protein is largely absent in the liver of asj mice, and the lack of enzymatic activity results in reduced inorganic pyrophosphate (PPi) levels in the plasma, accompanied by extensive mineralization of a number of tissues including arterial blood vessels. The progress of mineralization is highly dependent on the mineral composition of the diet, with significant shortening of the lifespan on a diet enriched in phosphorus and low in magnesium. Collectively, this mouse serves as an animal model for GACI.
INTRODUCTION

Several Mendelian genetic disorders have recently been shown to result in vascular mineralization, with profound phenotypic manifestations (Li and Uitto, 2013; Nitschke and Rutsch, 2012b). The prototype of such conditions is generalized arterial calcification of infancy (GACI), an autosomal recessive disorder characterized by early mineralization of blood vessels, often diagnosed prenatally through ultrasound (Rutsch et al., 2011). The newborns manifest with severe hypertension, cardiomyopathy and heart failure, resulting in demise of the affected individuals in most cases during the first year of life. GACI is caused by loss-of-function mutations in the ENPP1 gene, which codes for ectonucleotide pyrophosphatase/phosphodiesterase1 (ENPP1), an enzyme that hydrolyzes ATP to AMP and inorganic pyrophosphate (PPi) (Ruf et al., 2005). Since PPi is a powerful local inhibitor of ectopic mineralization, in the absence of the ENPP1 activity, progressive vascular mineralization ensues.

A number of mouse models have been identified to recapitulate the features of human diseases with vascular mineralization (Li and Uitto, 2013; Mackenzie et al., 2012). A mutant mouse with a missense mutation (p.V246D) in the Enpp1 gene was recently identified by the neuromutagenesis program at The Jackson Laboratory as a result of ENU treatment (Harris et al., 2012). These mice demonstrated stiff posture, abnormalities in the front legs, and stiffening of the joints. The standard pathological screen done at 7 months of age revealed very stiff and unbendable joints with severe osteoarthritis, hence this mutation was named “ages with stiffened joints” (asj). An interesting histopathological observation in these mice was mineralization of the dermal sheath of vibrissae, an observation that we had previously made in Abcc6tm1Jfk knockout mice, a model for pseudoxanthoma elasticum (PXE), which develop late-onset mineralization of
the dermis, arterial blood vessels and Bruch’s membrane in the eye (Klement et al., 2005).

Considering the apparent overlap of aberrant mineralization between GACI and PXE, we have now carefully characterized the *Ennp1<sup>aj</sup>* mouse as a potential model for GACI.
RESULTS

Phenotypic manifestations of Enpp1<sup>asj</sup> mice

The Enpp1<sup>asj</sup> mice were obtained from The Jackson Laboratory, and as previously described, by approximately 2 months of age they were noted to have stiffening of the joints, particularly the forepaws, which resulted in a slow, hobbling gait, which worsens as they age (Fig. 1A). This process was clearly accelerated when the mice were placed on an “acceleration diet”, rich in phosphorus and low in magnesium (Jiang and Uitto, 2012).

In spite of the limited locomotion, the asj mice kept on normal laboratory diet had a normal lifespan. However, if the mothers were placed on the acceleration diet during pregnancy and the pups were placed on the same diet at weaning at 4 weeks of age, the lifespan of the mice was drastically reduced (Fig. 2). Specifically, more than 50% of the asj mice died spontaneously before the age of 6 weeks, and the average age of demise was 6.4±0.6 weeks (mean ± SE; n=15). There was no difference in the age of death between males and females (P=0.47; Student’s t-test). Only 4 out of 28 asj mice survived to 12 weeks of age, which were sacrificed for analysis.

We also looked for evidence of embryonic lethality in the asj mice by genotyping a total of 136 newborn pups representing 35 litters from heterozygous mating pairs. The distribution between wild-type, heterozygous and homozygous asj mice was 37:71:28. This distribution did not differ from the expected Mendelian distribution of 34:68:34 (χ² = 1.456; P=0.483). Thus, the asj mutant mice have a significantly shortened lifespan when placed on a special diet, but there is no evidence of embryonic lethality.

Evidence of aberrant mineralization
Histopathologic examination of mice whose mothers were on the acceleration diet during pregnancy demonstrated extensive mineralization in the dermal sheath of vibrissae as well as in a number of internal organs when examined by hematoxylin-eosin or Alizarin red stains (Fig. 1). Specifically, the dermal sheath of vibrissae was noted to be mineralized as early as at 4 weeks of age, and the mineralization progressed up to 12 weeks of age, the latest time point available for examination due to early demise of the affected mice (Fig. 1B). The extent of mineralization was quantitated by chemical assay for calcium in the biopsies of muzzle skin containing the vibrissae in mice in the range of 4-12 weeks old, which showed marked, up to 17.7-fold, increase in mineral content in asj mice compared to wild-type littermates (Fig. 3A). The heterozygotes were phenotypically and histopathologically normal, and the calcium content of the muzzle skin was low, the same as in normal wild-type mice (Fig. 3A).

The mineralization of the dermal sheath of vibrissae was also monitored noninvasively by computed tomography. At 7 weeks of age, the asj mice on the acceleration diet showed evidence of severe mineralization in the muzzle area, a finding that was not present in the wild-type mice (Fig. 4). The composition of mineral was further analyzed by energy dispersive X-ray (EDAX) of the deposits in the asj mouse vibrissae from histopathological sections (Kavukcuoglu et al., 2012). The analysis revealed calcium and phosphorus as the principal ions in ~2:1 ratio, similar to that in enchondral bone (Fig. 5A). Topographic “radar” mapping co-localized calcium and phosphate, suggesting the presence of hydroxyapatite (Fig. 5B).

In addition to mineralization of the dermal sheath of vibrissae, extensive mineralization was noted in the aorta, as well as in the coronary and carotid arteries, and in the retina of the eye (Fig. 1B; Table 1). Also, blood vessels in the liver were mineralized, but no mineralization was noted in the liver parenchyma of the asj mice. No mineralization was noted in the dermal sheath
of vibrissae, aorta or eyes in the heterozygote mice, but 2 of 13 heterozygotes showed mineralization in the heart. Only one wild-type mouse (1/13) showed evidence of mineralization in the eyes (Table 1). An interesting observation was that there was extensive mineralization in the kidneys of the asj mice kept on the acceleration diet (Fig. 1C). The mineralization affected primarily the medullary tubules as well as arcuate and renal arteries. Similar mineralization was noted in Enpp1\textsuperscript{+/asj} heterozygous mice as visualized by histopathology (Fig. 1C) and quantitated by direct chemical assay of calcium (Fig. 3B). Evidence of mineralization of the kidney of wild-type mice was also noted when kept on acceleration diet, but to a much lower extent than in homozygous and heterozygous asj mice (Figs. 1C and 3B).

The asj mice on normal laboratory diet had a normal lifespan, and these asj mice had much less mineralization in the dermal sheath of vibrissae, as determined by histopathology, as compared to mice on the acceleration diet. In addition, the vascular mineralization phenotype is delayed until at ~5 months of age, as compared with early onset of 4 weeks when the mice were kept on the acceleration diet. Thus, the asj mice manifest with extensive mineralization of a number of connective tissues, and the extent of mineralization is clearly modulated by the diet.

**Genetic and molecular characterization**

Sequencing of the Enpp1\textsuperscript{asj} mice confirmed that they were homozygous for a p.V246D substitution as a result of a T-to-A transversion mutation in position 771 within exon 7 of the Enpp1 gene (Fig. 6A). This nucleotide substitution allows distinction of the corresponding wild-type and mutant asj alleles by restriction enzyme digestion with Taq\textsuperscript{α}I, forming the basis of genotyping of these mice (Fig. 6B).
To examine the consequences of the missense mutation in *Enpp1* at the mRNA and protein levels, quantitative PCR (qPCR) and Western analyses were performed on 4 *asj* and 4 wild-type mice sacrificed at 4-12 weeks of age. The mRNA levels were not different in the liver of *asj* and wild-type mice by qPCR (Fig. 6A). However, isolation of the protein from liver with subsequent Western analysis with an ENPP1 specific antibody clearly revealed the presence of a band of the appropriate size, 110 kD, in wild-type mice, but the level of protein was below the detection limit of the Western analysis in *asj* mice (Fig. 7B). It should be noted that while the antibody used is clearly specific for wild-type *Enpp1*, its precise epitope is not known, and consequently, our results do not exclude the possibility that it does not recognize the mutant protein. This possibility, however, is unlikely since the antibody used is polyclonal. The enzyme kinetics revealed that the ENPP1 isolated from the liver of *asj* mice was markedly reduced in activity (Fig. 7C). The $K_m$ for the wild-type *Enpp1* mouse, as determined by Hanes-Woolf plot, was $213.6 \pm 14.0 \mu M$ (mean ± SE), as compared to $K_m$ of $192.0 \pm 6.7 \mu M$ for *asj* mice (Fig. 7C) ($P > 0.05$). However, the $V_{max}$ for the wild-type enzyme was $33.4 \pm 2.1$ nmol p-nitrophenol released/min/mg protein vs. $8.1 \pm 0.3$ nmol in *asj* mice ($P < 0.01$).

Since the *asj* mice demonstrated residual enzyme activity, yet Western analysis showed the presence of little, if any, ENPP1 protein, two critical experiments were performed. First, the ENPP1 enzymatic activity was measured in heterozygous *asj* mice in comparison to wild-type and homozygous mutant mice. The results showed that the $K_m$ in heterozygous mice was $203.8 \pm 13.1 \mu M$. The $V_{max}$ was $20.8 \pm 0.8$ nmol, a 38% reduction compared to wild-type enzyme measured at linear range of reaction ($P < 0.05$). Secondly, liver from an *Enpp1tm1Gdg* knock-out mice, developed by targeted ablation of the gene, was used for the corresponding enzyme assay (Sali et al., 1999). These mice showed very low $V_{max}$, $2.9 \pm 0.4$ nmol, even less than in *asj* mice.
(P < 0.05), suggesting that the latter mice may have some residual activity, thus not being completely null (Fig. 7C).

To examine the consequences of reduced ENPP1 activity in asj mice, the concentrations of PPi in plasma of wild-type, heterozygous and homozygous mice were determined in a three-step enzymatic reaction. As expected, the asj mice showed markedly reduced PPi levels, ~20% from the wild-type controls with concomitant reduction in PPi/Pi ratio, and the heterozygous mice showed intermediate levels (Table 2). The serum calcium and phosphorus levels and the corresponding Ca/P ratio were not statistically different in these three groups of mice (Table 2).
DISCUSSION

In this study, we have extensively characterized a mutant Enpp1<sup>asj</sup> mouse as a model for generalized arterial calcification of infancy. This autosomal recessive disorder manifests with profound arterial mineralization often suspected from the results of prenatal ultrasound and commonly diagnosed in early postnatal period (Nitschke and Rutsch, 2012b; Rutsch et al., 2011). In most cases, the affected children die within the first 6 months of life as a consequence of vascular insufficiency causing end organ damage. This disease in its classic form is caused by mutations in the ENPP1 gene, which encodes ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1), which is also known as plasma cell membrane glycoprotein 1 (PC-1) (Ruf et al., 2005). This enzyme converts ATP to AMP and PP<sub>i</sub>, this pathway being the main source of inorganic pyrophosphate. The extracellular PP<sub>i</sub> plays a critical role as an inhibitor of hydroxyapatite formation, while P<sub>i</sub> promotes formation of hydroxyapatite crystals. Thus, the mineralization is controlled by the PP<sub>i</sub>/P<sub>i</sub> ratio as a result of activities of a number of enzymes, including ENPP1, TNAP, as well as transporter proteins mediating the extracellular transport of P<sub>i</sub> and PP<sub>i</sub>, including ankylosis protein (ANK) and type III sodium-dependent P<sub>i</sub>co-transporter 1 (P<sub>i</sub>T1) (Mackenzie et al., 2012). Thus, reduced PP<sub>i</sub>/P<sub>i</sub> ratio in patients with GACI as a result of reduced ENPP1 activity, mechanistically leads to aberrant mineralization of extracellular connective tissues.

The asj mouse examined in this study has several features that recapitulate GACI in patients. The hallmark of the disease, extensive arterial calcification can be demonstrated in the asj mice as early as 4 weeks of age when on the acceleration diet, and the homozygous mice often die most likely as a consequence of aberrant mineralization of the aorta as well as of blood vessels in other tissues, including heart and carotid arteries. Notably, these mice also
demonstrated mineral deposits in the retina of the eye as well as in the dermal sheath of vibrissae in the muzzle skin. Similar to patients with GACI, the plasma concentration of PPi was markedly reduced (<20% of the control mice) and the heterozygotes demonstrated intermediate levels. It should be noted that the heterozygous littermates did not demonstrate vascular mineralization, with the exception of the kidneys, and were indistinguishable from the wild-type controls, attesting to the autosomal recessive mode of the asj mutant allele. In this context, it should be noted that there currently is no evidence for modulation of the Enpp1<sup>asj</sup> mutation by other genes, such as Abcc6 in PXE mice (Klement et al., 2005) or in some inbred strains of mice (KK/HIJ, DBA/2J and C3H/HeJ) (Berndt et al., 2013) associated with vascular mineralization.

The asj mouse was developed at The Jackson Laboratory as part of the ENU mutagenesis program (Harris et al., 2012). The phenotypic features of these mice include stiffening of the joints (hence “ages with stiffened joints”, asj), and these mice were shown to harbor a missense mutation p.V246D, in the Enpp1 gene. Initial necropsies of these mice at 7 months of age revealed periarticular mineralization of the ligaments and mineralization of the dermal sheath of vibrissae. Previously, mutations affecting the Enpp1 gene have been described in a mutant mouse, “tip toe walking” (ttw/ttw) shown to harbor a stop codon mutation in the Enpp1 coding sequence (Okawa et al., 1998). Similarly, Enpp1<sup>tm1Gdg</sup> knock-out mice exhibit abnormalities similar to those in ttw/ttw mice (Sali et al., 1999). Furthermore, another Enpp1 mutant mouse with p.C397S missense mutation has been characterized by low bone mineral density, crystal-related arthropathy and vascular calcification (Babij et al., 2009). Characterization of these mice has largely focused on alterations in bone mineralization in long bones and calvariae and periarticular as well as perispinal soft tissue mineralization. While arterial calcification was documented in some of these previously described mice, the changes were frequently not noted.
to be present until at 16-22 weeks of age (Babij et al., 2009; Mackenzie et al., 2012). In asj mice, the mineralization was noted to occur as early as 4 weeks of age, with an early demise at ~6 weeks of birth. Consequently, this mouse model accurately recapitulates features of GACI, a disease usually lethal within the first 6 months of life. This difference at the age of onset of mineralization can be attributable, at least in part, to the special diet that was used in our study to accelerate the mineralization process. This “acceleration diet” consists of increased phosphate and reduced magnesium in comparison to the standard mouse laboratory diet (Jiang and Uitto, 2012; Li and Uitto, 2010). Specifically, the phosphate concentration was increased 2-fold (8.5 mg/g of food), and the magnesium content was reduced to 20% of the control (0.4 mg/g of food). All the mice were placed on this experimental diet at weaning at 4 weeks of age, but in addition, the mothers during the pregnancy and lactation were on this special diet. We previously showed that this diet also accelerates the aberrant mineralization noted in Abcc6tm1Jfk null mice, a model of another aberrant mineralization disorder, pseudoxanthoma elasticum (Jiang and Uitto, 2012). It should be noted, however, that this diet does not cause any mineralization in the arterial vessels, the eyes or in the vibrissae in wild-type control mice or in heterozygotes of mutations in either Enpp1 or Abcc6 genes.

An interesting observation was extensive mineralization in the kidneys of homozygous and heterozygous asj mice, and wild-type control mice also showed some evidence of aberrant mineralization, when the mice were placed on the experimental acceleration diet. The observed mineralization of kidneys in the heterozygous mice differs from that in humans in that heterozygous carriers of ENPP1 mutations do not show any evidence of mineralization. Similar findings of nephrocalcinosis have previously been noted in mice with high phosphorus-containing diet, similar to patients with hyperphosphatemia (Markowitz and Perazella, 2009).
The increased mineralization in the kidney suggests that this may be a process determined by a more complex genetic background in which ENPP1 plays a role but is not the sole contributor.

In their classic forms, GACI and PXE are two clinically distinct conditions, GACI manifesting with extensive arterial calcification at birth leading to early demise of the affected patients, while the clinical manifestations and tissue mineralization in PXE is of late onset and slowly progressive (Nitschke and Rutsch, 2012b; Uitto et al., 2010). However, recently patients with features of early GACI with development of PXE with characteristic skin findings have been noted (Le Boulanger et al., 2010; Li et al., 2012). In addition, a subset of patients with GACI has been recently shown to harbor mutations in the ABCC6 gene, instead of ENPP1 (Nitschke and Rutsch, 2012b). These observations suggest the presence of common pathomechanistic pathways leading to aberrant tissue mineralization (Nitschke and Rutsch, 2012a). While the pathomechanistic details of tissue mineralization particularly in the case of PXE are currently unknown, it should be noted that the PPi levels in plasma of Abcc6+/− mice are not altered (Li et al., unpublished). Finally, there are a number of additional heritable aberrant mineralization disorders resulting in calcium deposits in the skin, including normophosphatemic and hyperphosphatemic familial tumoral calcinosis, and arterial calcification with CD73 deficiency, each due to mutations in different genes (Li and Uitto, 2013). The presence of hydroxyapatite crystal deposition in these, phenotypically diverse conditions, attest to the complex mineralization/anti-mineralization network required for normal homeostasis. Dissection of the pathomechanistic details leading to aberrant mineral deposition in these single gene disorders will assist us in development and testing of efficient treatment modalities. In this context, the asj mice could well serve as a model system to study potential treatment modalities for GACI under genetically and environmentally controlled conditions. In this regard, a few
studies have reported improvement in some patients with GACI with treatment with bisphosphonates, but controlled studies attesting to the efficacy of this treatment strategy are lacking (Edouard et al., 2011; Ramjan et al., 2009). Furthermore, this mouse model could be used to test other compounds, such as pyrophosphate and sodium thiosulfate, which have been suggested to counteract vascular mineralization (Hayden and Goldsmith, 2010; Ning et al., 2013; O'Neill et al., 2011). Collectively, the ability to carefully dissect and analyze asj mice may provide enhanced clinical understanding of GACI towards improved treatment.
METHODS

Animals and diet

$Enpp1^{asj}$ mice on a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME) (Harris et al., 2012). $Enpp1^{+/+}$ and $Enpp1^{asj}$ mice were generated from heterozygous matings. Mice were maintained either on standard laboratory diet (Laboratory Autoclavable Rodent Diet 5010; PMI Nutritional International, Brentwood, MO) or fed an “acceleration diet” (Harlan Teklad, Rodent diet TD.00442, Madison, WI) which we have previously shown to accelerate the ectopic mineralization in $Abcc6^{-/-}$ mice; this diet is enriched in phosphorus and has reduced magnesium content (Jiang and Uitto, 2012). The mice were maintained under standard conditions at the Animal Facility of Thomas Jefferson University, and all protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Proper handling and care were followed according to the Animal Welfare Policies of the Public Health Service.

Genotyping and gene sequencing

A primer pair with sequences 5’-TGATCTGCATCCTGGGATAA-3’ and 5’-TAAGGAAAGACCAATTGCAGA-3’ was used to amplify exon 7 of the $Enpp1$ gene. To identify the $Enpp1^{asj}$ wild-type and mutant alleles, PCR products were digested with TaqαI (New England BioLabs, Ipswich, MA), producing a band of either 300 bp or 150 bp corresponding to the wild-type and $asj$ alleles, respectively.

Gene sequencing was performed at the Kimmel Cancer Center Nucleic Acid Facility at Thomas Jefferson University using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Products were analyzed on the 3730 DNA Analyzer (Applied
Biosystems), and the results were visualized with Chromas software (Technelysium, South Brisbane, Australia).

**Quantitative PCR**

Quantitative PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems) with Power SYBR Green PCR Master Mix, as described previously (Li et al., 2007). *Enpp1* primers had sequences 5’-GCCAAAGACCCCCAACCTACAAA-3’ and 5’-ACAGGTCTCCTGGAAATCCAGACA-3’. The amount of *Enpp1* mRNA per sample was quantified and normalized to *Gapdh* mRNA, and relative expression levels were calculated by the \( \Delta \Delta Ct \) method. The dissociation curve was generated with Dissociation Curve software, version 1.0 (Applied Biosystems), to determine reaction specificity.

**EDAX analysis and topographic mapping**

For energy dispersive X-ray analysis (EDAX), paraffin sections of muzzle skin containing mineral deposits were mounted onto carbon carriers and analyzed for elemental composition with a JEOL-T330A scanning electron microscope (JEOL Ltd., Tokyo, Japan) fitted with an EDAX microanalysis analyzer. X-ray topographic maps of calcium and phosphorus were collected with Thermo Scientific NSS software, version 2.3 (Swedesboro, NJ).

**Western blot**

Liver lysates were prepared by homogenizing tissues in lysis buffer containing PMSF (Sigma, St. Louis, MO), phosphatase inhibitor cocktail (Sigma), protease inhibitor cocktail (Thermo, Rockford, IL), and 8M urea (Fisher, Pittsburgh, PA) in RIPA buffer (Sigma). A BCA kit (Thermo) was used to determine the protein concentration of all lysates.
Protein, ~90 μg, was loaded per lane in an 8% gel (Thermo) for SDS-PAGE, and the proteins were then transferred to a PVDF membrane. The membrane was blocked in 5% milk/TBS + 0.1% Tween-20 at room temperature for one hour and then incubated with anti-ENPP1 primary antibody (Cell Signaling, Danvers, MA), 1:500 in dilution buffer (2% milk/TBS + 0.1% Tween-20) at 4°C overnight. To visualize the signal, the membrane was incubated in anti-rabbit secondary antibody (LI-COR, Lincoln, NE) in dilution buffer for one hour at room temperature and then scanned with an Odyssey Infrared Imager (LI-COR). The membrane was then stripped and reprobed with anti-β-actin antibody (Bioorbyt, San Francisco, CA), 1:750 in dilution buffer.

**Enzyme activity assay**

ENPP1 activity in wild-type, heterozygous and *asj* mouse liver, as well as in *Enpp1*<sup>tm1Gdg</sup> knock-out mice (kindly provided by Dr. Robert Terkeltaub, University of California, San Diego), was determined using the substrate thymidine 5′-monophosphate p-nitrophenyl ester (p-Nph-5′-TMP, Sigma). Total proteins were extracted from whole liver with lysis buffer containing 50 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl, 0.5% NP-40, pH 7.5, PMSF, and complete protease inhibitor (Roche) (Babij et al., 2009). A BCA kit (Pierce) was used to determine the protein concentration of lysates. Protein lysates were diluted with assay buffer (50 mM Tris-HCl, pH 9.5, and 250 mM NaCl in water) to 100 ng/μl. In 96-well plates, 50 μL of p-Nph-5′-TMP (diluted with assay buffer to 9 different concentrations) was added to 50 μL of protein lysate. All samples were tested in duplicate. The samples were then incubated at 37°C, and absorbance (400 nm) was measured with a microplate reader (Bio-Rad 800) every 5 minutes for up to 30 minutes to ensure linearity of the reaction. A molar extinction coefficient of the
reaction product, p-nitrophenol, of 18.4 x 10^3 M\(^{-1}\) cm\(^{-1}\) was used in determination of enzyme kinetics. Enzyme activity was expressed as nmol p-nitrophenol released per minute per mg of protein.

**Quantification of calcium and phosphate**

To quantify the calcium deposition in the dermal sheath of mouse vibrissae, and the kidneys, muzzle skin and kidney were harvested and decalcified with 0.15 N HCl for 48 hours (skin) or with 0.6 N HCl for one week (kidney) at room temperature. The calcium content in these samples as well as in serum was determined colorimetrically by the o-cresolphthalein complexone method (Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX). The phosphate content of serum was determined with Malachite Green Phosphate Assay kit (BioAssay Systems, Hayward, CA). The values for calcium and phosphate were normalized to tissue weight.

**Inorganic pyrophosphate assay**

PP\(_i\) was measured by an enzymatic assay using uridine-diphosphoglucose (UDPG) pyrophosphorylase as previously described (Lomashvili et al., 2005; O’Neill et al., 2010), with modifications. Heparinized plasma samples (20 µl; 1:4 dilution) were heated at 65°C for 10 min, followed by three different assays performed on each sample: (1) no addition of PP\(_i\) standard, (2) pre-incubation with 0.35 U pyrophosphatase at 37°C for 1 hour, and (3) addition of 3 µM PP\(_i\). Samples were then added to 100 µl of reaction buffer that contained 5.2 mM Mg Acetate, 57 mM Tris Acetate (pH 7.8), 4 µM NADP, 7.5 µM UDPG, 18.6 µM glucose-1, 6-diphosphate, 0.14 U UDPG pyrophosphorylase, 2.5 U phosphoglucomutase, 0.4 U glucose-6-phosphate.
dehydrogenase, and 0.02 µCi [3H]UDPG. After a 30 min incubation at 37°C, 200 µl of 4% activated charcoal was added to each sample with occasional stirring to bind residual UDPG. After centrifugation, the radioactivity in 100 µl of supernatant was counted. The plasma [PPi] was determined as (CPM1-CPM2)/(CPM3-CPM1) x 3 µM.

Histopathological analysis

Muzzle skin and internal organs from euthanized mice were fixed in 10% phosphate-buffered formalin, routinely processed, and embedded in paraffin. Tissues were sectioned (6 µm) and stained with hematoxylin eosin (H&E) and Alizarin Red using standard procedures. Slides were examined under light microscopy for mineralization and other lesions by an experienced veterinary pathologist (JPS).

Small animal computed tomography (CT scan)

Enpp1 wild-type and asj mice were examined for mineralization at 7 weeks of age by CT scan, as described (Le Corre et al., 2012). Briefly, mice were anesthetized with a Xylazine-Ketamine-Acetopromazine cocktail (160 µL per 25 g body weight of 10 mg/kg Xylazine, 200 mg/kg Ketamine, 2 mg/kg Acetopromazine) and then scanned with a MicroCAT II (ImTek Inc., Oak Ridge, TN). A 3-dimensional facial rendering was created for each mouse using Amira software, version 3.1 (Visualization Sciences Group, Burlington, MA).

Statistical analysis

The comparisons in different groups of mice were completed using two-sided Kruskal–Wallis nonparametric tests. The Kruskal–Wallis test is comparable to one-way analysis of variance, but
without the parametric assumptions. Fisher’s exact test was used to determine the difference between proportions in mineralization phenotypes in mice. All statistical computations were completed using SPSS version 15.0 software (SPSS Inc., Chicago, IL).

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COMPETING INTEREST

The authors declare that they do not have any competing interest.

AUTHOR CONTRIBUTION

QL, HG and DWC performed the experiments; AB and JPS performed histopathologic analyses; JU developed the concept, interpreted the data and prepared the manuscript.

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FIGURE LEGENDS

Figure 1. Phenotypic presentation and aberrant mineralization in asj mice at 12 weeks of age. (A) The Enpp1<sup>asj</sup> mice develop progressive stiffening of the joints leading to contractures as shown on the front paws (lower panel) in comparison to a corresponding wild-type mouse (upper panel). (B) Histopathology reveals extensive mineralization in the heart, aorta, carotid artery, retina of the eye, and dermal sheath of vibrissae, but not in the liver parenchyma of asj mice. (C) Extensive aberrant mineralization in the kidneys of heterozygous (middle panel) and homozygous (lower panel) asj mice. Focal areas of mineralization are also noted in the kidneys of wild-type mice (upper panel). (Alizarin red stain; original magnifications: heart, carotid artery, eye, vibrissae, liver, x150; aorta, kidney; x100).

Figure 2. Kaplan-Meier survival curves of asj mice on the acceleration diet. Note that >50% of asj mice died spontaneously prior to age 6 weeks, while the heterozygote mice (Enpp1<sup>+/asj</sup>) had a survival similar to wild-type controls.

Figure 3. Quantitation of mineralization by chemical assay of calcium in the dermal sheath of vibrissae and in the kidneys of mice in the range of 4-12 weeks of age in mice kept on acceleration diet. (A) Marked increase in mineralization is noted in the vibrissae of homozygous asj mice in comparison to wild-type or heterozygous animals (n=8). (B) Markedly increased mineralization in the kidneys of heterozygous and homozygous asj mice is noted (n=7-9; Statistical significance: *P < 0.05 vs. wild-type; **P < 0.001 vs. wild-type).
Figure 4. Computed tomography demonstrating extensive mineralization of dermal sheath of vibrissae in a 7-week old asj mouse (B) in comparison to a wild-type littermate (A). Single slices demonstrate evidence of mineralization (arrowheads in B), and computerized reconstruction reveals the mineral deposits in association with dermal sheath of vibrissae (upper left panel in B, arrowheads).

Figure 5. Energy dispersive X-ray analysis of the mineral deposits in the dermal sheath of vibrissae in asj mice. (A) Elemental composition analysis reveals the presence of calcium and phosphorus in ~2:1 ratio. (B) X-ray topography of the distribution of P and Ca reveals colocalization as demonstrated by merging of images.

Figure 6. Genotyping and mutation analysis of asj mice. (A) TaqI restriction enzyme digestion of PCR products corresponding to exon 7 of the Enpp1 gene reveals a 300 bp fragment representing the wild-type allele and a 150 bp fragment corresponding to the mutant allele. (B) Sequence analysis reveals in asj mice a homozygous T-to-A nucleotide substitution (arrows) which results in substitution of valine 246 by an aspartic acid (p.V246D).

Figure 7. Enpp1 mRNA, protein, and enzymatic analysis in asj mice. (A) Quantitative PCR reveals similar relative mRNA levels in the wild-type (Enpp1+/+) and in mutant (Enpp1asj) mice (mean ± SE; n=7). (B) Western analysis of the liver protein with an ENPP1-specific antibody demonstrates the presence of a band of 110 kDa in wild-type mice (lanes 1-4), while the protein is below the level of detection in asj mice (lanes 5-8). (C) Assay of ENPP1 enzymatic activity in the liver of wild-type, heterozygous and homozygous asj mice (n=4), as well as of an Enpp1+/−.
knock-out mouse (n=3). Note the markedly reduced activity in *asj* mice and intermediate activity in the heterozygotes in comparison to the wild-type mice.

REFERENCES


TRANSLATIONAL IMPACT

Clinical issue

A number of heritable disorders manifest with aberrant mineralization of the skin and vascular connective tissues, with a broad spectrum of phenotypic variability and often with considerable morbidity and mortality. One such condition is generalized arterial calcification of infancy (GACI), diagnosed with prenatal or perinatal calcification of arterial blood vessels, and the children usually die within the first year of life. Most patients with GACI harbor mutations in the ENPP1 gene encoding an enzyme, ectonucleotide pyrophosphatase/phosphodiesterase1 that hydrolyzes ATP to AMP and inorganic pyrophosphate (PPi), the latter one being a powerful local inhibitor of ectopic mineralization. Thus, in the absence of ENPP1 activity, progressive vascular mineralization ensues.

Results

A novel mouse model for GACI was recently identified as a result of ENU mutagenesis with a characteristic feature of stiffening of the joints which worsened as the mice age; thus this mouse phenotype was designed as “ages with stiffened joints”, asj. These mice develop progressive mineralization of the skin, the aorta, coronary arteries and arterial blood vessels in a number of tissues. The development of the mineralization phenotype could be noted soon after birth when the mice were placed on an “acceleration diet” rich in phosphorus and low in magnesium. The underlying molecular defect was demonstrated to be a homozygous T-to-A transversion mutation in position 771 of the Enpp1 gene resulting in homozygous p.V246D substitution. The mutant allele resulted in normal levels of the corresponding mRNA transcript, but the level of ENPP1 protein was below the detection limit by Western analysis, and the ENPP1 enzymatic activity was reduced to <20% of the corresponding wild-type mouse. As a result of reduced Enpp1
enzymatic activity the PPi/Pi ratio was markedly reduced. The heterozygote \textit{Enpp1}^{+/asj} mice did not demonstrate the mineralization phenotype in their arterial blood vessels, and their enzymatic activity was between that of the wild-type and the \textit{asj} mutant mouse.

\textbf{Implications and future directions}

This novel \textit{Enpp1}^{asj} mutant mouse recapitulates the genetic, molecular, and phenotypic features of human patients with GACI, including autosomal recessive inheritance, inactivating mutations in the \textit{ENPP1} gene, and profound mineralization of arterial blood vessels noted shortly after birth and resulting in early demise. Thus, this \textit{asj} mouse serves as a model to study the pathomechanistic features of GACI, and provides a means to test pharmacologic approaches, such as bisphosphonates, towards treatment of this, currently intractable, disease.
Cumulative Survival (%) vs. Age (weeks)

- **Enpp1^{+/+}**
- **Enpp1^{+/asj}**
- **Enpp1^{asj}**
A  Calcium in Vibrissae

B  Calcium in Kidney

Enpp1+/+  Enpp1+/asj  Enpp1asj

Enpp1+/+  Enpp1+/asj  Enpp1asj

µmol Ca/gram tissue

**  

*  

0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150  160

µmol Ca/gram tissue
A

Enpp1<sup>+/+</sup>

Enpp1<sup>+/asj</sup>

Enpp1<sup>asj</sup>

B

bp

Enpp1<sup>+/+</sup>  Enpp1<sup>+/asj</sup>  Enpp1<sup>asj</sup>
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Number of mice examined</th>
<th>Soft tissue mineralization (%)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>vibrissae</td>
<td>liver</td>
<td>kidneys</td>
<td>heart</td>
<td>aorta</td>
<td>eyes</td>
<td>carotid artery</td>
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<td>Enpp1+/+</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>85</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Enpp1asj</td>
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<td>100**</td>
<td>56+</td>
<td>100</td>
<td>67**</td>
<td>56+</td>
<td>44</td>
<td>78++</td>
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</table>

* Mice were placed on acceleration diet at 4 weeks of age and tissues were collected for histopathology at 3 months or earlier at the time of demise of the Enpp1asj mice. The values represent the percent of tissues affected by mineralization as examined by Hematoxylin-Eosin stain on one section.
* Statistical analyses were performed with Fisher’s Exact test; * P < 0.01; ** P < 0.001.
Table 2. Calcium, phosphorus and pyrophosphate concentrations in serum/plasma of mice on acceleration diet*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (mean ± S.E.)</th>
<th>Enpp1+/+ (n=9)</th>
<th>Enpp1+/asj (n=9)</th>
<th>Enpp1asj (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.81 ± 0.17</td>
<td>10.27 ± 0.21</td>
<td>10.87 ± 0.27</td>
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<tr>
<td>Phosphorus (mg/dL)</td>
<td>6.82 ± 0.31</td>
<td>7.10 ± 0.58</td>
<td>9.83 ± 2.00</td>
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<tr>
<td>Ca/P ratio</td>
<td>1.61 ± 0.06</td>
<td>1.52 ± 0.13</td>
<td>1.29 ± 0.20</td>
<td></td>
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<tr>
<td>PPi (mg/dL)</td>
<td>47.61 ± 8.23</td>
<td>21.69 ± 1.64*</td>
<td>8.62 ± 1.49*</td>
<td></td>
</tr>
<tr>
<td>PP/Pi ratio</td>
<td>6.30 ± 1.22</td>
<td>3.03 ± 0.30i</td>
<td>1.17 ± 0.24*</td>
<td></td>
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</table>

* The mice were placed on acceleration diet (low in magnesium and high in phosphorus) at 4 weeks of age. Blood samples were collected by cardiac puncture, Ca and P concentrations were determined in serum and PPi levels were measured in heparinized plasma. Statistical significance in comparison to Enpp1+/+ mice: * P < 0.01; ‡ P < 0.05