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Atherogenic Diet Accelerates Ectopic Mineralization in a Mouse Model of Pseudoxanthoma Elasticum

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Abstract

Objective: Pseudoxanthoma elasticum (PXE) is a multisystem heritable disorder caused by mutations in the *Abcc6* gene. The disease is characterized by ectopic mineralization of the skin, eyes, and arterial blood vessels. Previous studies have suggested that cardiovascular complications in patients with PXE are caused in part by premature atherosclerosis. The aim of this study is to determine the effect of an atherogenic diet on ectopic mineralization.

Methods: We used *Abcc6*^{tm1JfK} mice (*Abcc6*$^{-/-}$ mice) as an established preclinical model of PXE. The offspring at age of 4 weeks were divided into two groups and fed the standard control laboratory diet (control group) and the atherogenic diet. Serum lipid profiles and bile acids were measured, and steatosis and tissue mineralization were evaluated by histopathologic analysis and chemical calcium quantification assay, respectively.

Results: After 50–58 weeks of feeding an atherogenic diet, the concentrations of total cholesterol, low-density lipoprotein/very-low-density lipoprotein cholesterol, and bile acids were significantly higher in the *Abcc6*$^{-/-}$ mice on the atherogenic diet (180.9 ± 14.8 g/L, 145.9 ± 12.9 g/L, and 9.7 ± 1.4 μmol/L, respectively) than in *Abcc6*$^{-/-}$ mice on a control diet (85.2 ± 4.8 g/L, 25.1 ± 5.5 g/L, and 3.3 ± 0.5 μmol/L, respectively) ($P < 0.001$). Hypercholesterolemia was accompanied by extensive lipid accumulation in the liver and aorta, a characteristic feature of steatosis. The direct calcium assay demonstrated significantly increased mineralization of the muzzle skin containing the dermal sheath of vibrissae (57.2 ± 4.4 μmol Ca/gram tissue on the atherogenic diet and 43.9 ± 2.2 μmol Ca/gram tissue on control diet; $P < 0.01$), a reproducible biomarker of the ectopic mineralization process in these mice. An increased frequency of mineralization was also observed in the kidneys and eyes of mice on the atherogenic diet ($P < 0.01$).

Conclusion: These observations suggest that the atherogenic diet caused hypercholesterolemia and accelerated ectopic mineralization in the *Abcc6*$^{-/-}$ mice. Our findings have clinical implications for patients with PXE, a currently intractable disorder with considerable morbidity and occasional mortality.

Keywords: pseudoxanthoma elasticum, ectopic mineralization, atherogenesis, mouse model

Introduction

Pathological calcification of connective tissues, also termed ectopic mineralization, is a complicated process leading to deposition of calcium phosphate complexes in the extracellular matrix. This process particularly affects the arterial blood vessels and is common in patients with age-associated disorders.[1] In the clinical setting, ectopic mineralization has been encountered in both acquired diseases and heritable Mendelian single-gene disorders with phenotypic similarities. Diseases that cause ectopic mineralization, among which pseudoxanthoma elasticum (PXE) is the paradigm heritable disorder, are prime targets of the efforts to elucidate the precise pathomechanistic pathways.

PXE (OMIM# 264800) is an autosomal recessive connective tissue disorder in which fragmentation and mineralization of elastic fibers result in cutaneous, ocular, and cardiovascular manifestations.[2-3] The first organ system affected is often the skin, which develops small yellowish papules primarily in flexural areas of the body.

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These lesions tend to progressively coalesce into larger plaques, and the skin eventually loses its elasticity and becomes leathery and redundant with loss of recoil. Histopathologic examination of the skin lesions reveals accumulation of pleomorphic elastotic material that progressively becomes mineralized. The characteristic ophthalmologic finding in patients with PXE is the presence of angioid streaks; these lesions reflect the breakage of the mineralized Bruch's membrane, an elastin-rich sheath between the pigmented retina and choroid of the eye. Angioid streaks are associated with subretinal neovascularization and hemorrhage, which cause progressive loss of central vision and can lead to legal blindness if left untreated. Cardiovascular complications are also prominent in patients with PXE, often manifesting as nephrogenic hypertension, intermittent claudication, occasional bleeding from the intestinal arteries, and sometimes premature myocardial infarction and stroke. PXE is caused by loss-of-function mutations in the *ABCC6* gene; this gene encodes an adenosine triphosphate-dependent transporter protein, ABCC6, which is primarily expressed in the liver and kidneys.[2] Our understanding of the disease pathology has been advanced by the study of *Abcc6*-knockout mice, which recapitulate the features of PXE, including progressive mineralization of the skin, eyes, and arterial blood vessels.[4-5] Emerging evidence suggests that PXE is a metabolic disease caused by the absence of ABCC6-mediated adenosine triphosphate release from liver into the circulation, resulting in reduced plasma levels of inorganic pyrophosphate, a potent inhibitor of ectopic mineralization.[6–8] However, the pathomechanistic details and the precise nature of the molecules transported by ABCC6 are currently unknown.

ABCC6 has been suggested as a new player in cellular cholesterol and lipoprotein metabolism, as described for other ABC transporters.[9] An *ABCC6* gene polymorphism (p.R1268Q, rs2238472) may be associated with variations in the plasma levels of lipoproteins.[10] In addition, intronic variants of the *ABCC6* gene (rs150468 and rs212077) have been shown to influence the risk of coronary artery disease through their effects on high-density lipoprotein (HDL) cholesterol.[11] In addition, some studies have suggested that *ABCC6* carriers, particularly those with p.R1141X, the most frequent mutation in Caucasian populations, have an increased risk of premature coronary artery disease and peripheral artery disease.[12-13] These findings indicate that carriers of *ABCC6* loss-of-function mutations might benefit from therapy aimed at preventing coronary artery disease. In contrast, however, another study failed to demonstrate the association between heterozygosity of p.R1141X and the risk of ischemic vascular disease.[14] Atorvastatin, a cholesterol-lowering drug commonly used in the clinical setting, was recently shown to counteract tissue mineralization in the *Abcc6*-knockout mouse; this suggests that patients with PXE might benefit from statin treatment.[15]

In this study, we used the *Abcc6*-knockout mouse as a model of PXE to examine the consequences of an atherogenic diet on serum lipid profiles and the degree of ectopic mineralization. The results demonstrated that the atherogenic diet induced hypercholesterolemia and steatosis accompanied by increased mineralization in the soft connective tissues. These findings have clinical implications for patients with PXE.

Materials and methods

Mice had free access to water and were maintained in the climate-controlled Animal Facility of Thomas Jefferson University. Euthanization was performed by carbon dioxide asphyxiation and opening of the chest as approved by the American Veterinary Medical Association. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Proper handling and care protocols were followed according to the animal welfare policies of the U. S. Public Health Service.

Experimental design and diets

The mice were placed on a standard control laboratory diet (Laboratory Autoclavable Meal Rodent Diet 5010; PMI Nutrition, Brentwood, MO) during maintenance and breeding. Offspring were genotyped for the *Abcc6* status by previously described polymerase chain reaction protocols and primers.[4] At the age of 4 weeks, the *Abcc6*$^{-/-}$ offspring were divided into two groups and fed specific diets for another 50–58 weeks. Six male and nine female *Abcc6*$^{-/-}$ mice in the first group continued the standard control laboratory diet. Seven male and three female *Abcc6*$^{-/-}$ mice in the second group were placed on the atherogenic diet at 4 weeks of age (Teklad Diet TD.02028; Envigo, Madison, WI). This atherogenic diet has higher fat, sucrose, cholesterol, and cholic acid content than the standard control rodent diet. The specific contents of the control diet are provided at http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028443.pdf, and those of the atherogenic diet are provided at http://www.envigo.com/resources/data-sheets/02028.pdf. These two diets are compared in Table 1. All mice were fasted overnight prior to euthanasia for blood and tissue analysis.

Measurement of serum lipids and bile acids

The cholesterol and triglyceride levels in fasted serum samples were determined by colorimetric assays. The

Table 1

Composition of the experimental diets.

	Content of (%)			
Diet	**Cholesterol**	**Sucrose**	**Fat**	**Cholic acid**
Control diet	0.0283	1.16	6.40	0.00
Atherogenic diet	1.25	32.50	21.20	0.50

concentrations of total cholesterol, HDL cholesterol, and low-density lipoprotein/very-low-density lipoprotein (LDL/VLDL) cholesterol were measured with an EnzyChrom™ HDL and LDL/VLDL Assay Kit (BioAssay Systems, Hayward, CA, USA). The triglyceride content was measured with an EnzyChrom™ Triglyceride Assay Kit (BioAssay Systems). The absorbance values of the samples were obtained with an Epoch Model microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The serum level of total bile acids was determined by an assay kit from Cell Biolabs, Inc. (San Diego, CA, USA). All measurements were performed in duplicate.

Quantification of serum phosphorus and magnesium

The serum phosphorus and magnesium contents were determined with a Malachite Green Phosphate Assay Kit and a QuantiChrom™ Magnesium Assay Kit (BioAssay Systems, USA), respectively.

Histopathological analysis

Necropsy tissue samples from muzzle skin and internal organs were fixed in 10% phosphate-buffered formalin for 2 days, transferred to 30% sucrose in phosphate-buffered saline overnight, and then embedded in Shandon Cryomatrix (Thermo Fisher Scientific, Waltham, MA, USA). The tissues were sectioned (6 μm) and stained with hematoxylin and eosin using standard procedures. The slides were examined for ectopic mineralization under a light microscope. Oil Red O staining was performed on adjacent sections to detect lipid accumulation (Hitobiotec Corp., Kingsport, TN, USA).

Chemical quantification of calcium

To quantify mineral deposition, muzzle skin specimens were harvested and the calcium was solubilized with 0.15 N hydrochloric acid for 48 hours at room temperature, followed by an assay of the solubilized calcium in the supernatant. Colorimetric analysis by the *o*-cresolphthalein complexone method [Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX, USA] was performed to measure the calcium content. The values of calcium in muzzle skin were normalized to tissue weight. The calcium in the serum samples was analyzed using the same quantitative assay.

Statistical analysis

The data were presented as mean ± standard error (SE). The results in different groups of mice receiving different diets were analyzed using non-parametric Mann-Whitney U test using Prism 8 (GraphPad, San Diego, CA, USA). Fisher's exact test was used to determine the differences between the proportions of mineralization in the organs of mice fed with different diets. Statistical significance was reached at $P < 0.05$.

Results

Serum lipid profiles and bile acids in *Abcc6*$^{-/-}$ mice fed control or atherogenic diet

The mice fed the atherogenic diet showed significantly higher serum levels of total cholesterol (52.9% increase) and LDL/VLDL cholesterol (82.8% increase) than the mice fed the control diet; however, the serum HDL cholesterol and triglyceride levels were not significantly different between the two groups (Fig. 1). In addition, a > 1.9-fold increase in the serum bile acid level was noted in mice on the atherogenic diet (Table 2). No sex-related differences in the serum lipid panels or total bile acid levels were observed between the two groups of mice.

To examine the metabolic consequences of the atherogenic diet, the serum concentrations of calcium, phosphorus, and magnesium were determined in all mice at the end of the experimental diet. No significant differences were noted in the serum concentrations of these components (Table 2).

Steatosis in *Abcc6*$^{-/-}$ mice fed the atherogenic diet

Oil Red O staining of the liver and aorta showed significantly higher amounts of lipids in the *Abcc6*$^{-/-}$ mice on the atherogenic diet than in *Abcc6*$^{-/-}$ mice on the control diet (Fig. 2A). Thus, the elevations in the serum concentrations of total cholesterol and LDL/VLDL cholesterol were accompanied by steatosis. Although ectopic mineralization affects several tissues in patients with PXE, the clinical manifestations are primarily evident in the skin, eyes, and cardiovascular system. Therefore, the muzzle skin, eyes, and arterial blood vessels (characteristic sites of ectopic mineralization in PXE) were examined for mineralization by hematoxylin and eosin staining, and the lipid distribution was examined by Oil Red O staining

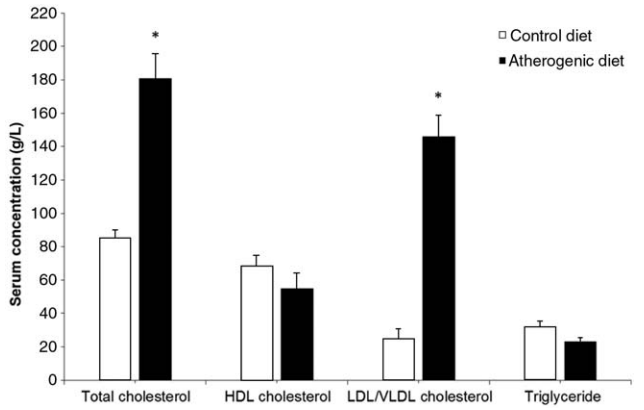

Figure 1. Serum cholesterol and triglyceride concentrations in *Abcc6*$^{-/-}$ mice maintained on either the control diet or atherogenic diet. The mice were placed on specific diets at 4 weeks of age, and blood samples were obtained at 54–62 weeks of age for analysis of lipid concentrations. The values are expressed as mean ± SE. Control diet, $n = 15$; atherogenic diet, $n = 10$. *$P < 0.001$ compared with mice on the control diet.

Table 2

Calcium, phosphorus, magnesium, and bile acid concentrations in the serum of *Abcc6*$^{-/-}$ mice placed on different diets (mean ± SE)

Diet	Concentration in group			
	Calcium (g/L)	Phosphorus (g/L)	Magnesium (g/L)	Bile acid (μmol/L)
Control diet	8.03 ± 0.21	9.29 ± 0.30	2.19 ± 0.09	3.33 ± 0.47
Atherogenic diet	8.40 ± 0.22	9.50 ± 0.50	2.11 ± 0.14	9.73 ± 1.43*

* $P < 0.001$.

(Fig. 2B). Mineralization was noted in the dermal sheath of vibrissae in the muzzle skin, the retinas, and the arterial blood vessels in the kidneys (Fig. 2B). However, mineralization in these tissues was not associated with deposition of lipids (Fig. 2B).

Effects of atherogenic diet on tissue mineralization in Abcc6$^{-/-}$ *mice*

The degree of mineralization in *Abcc6*$^{-/-}$ mice on the atherogenic diet, as determined by the content of calcium in the muzzle skin, was significantly higher (30.4% increase) than that in mice on the control diet (Fig. 3). In addition to mineralization of the dermal sheath of vibrissae in the muzzle skin, mineralization was evaluated by histopathological analysis in the kidneys and the eyes by counting the number of mice with mineralization as a percent of the total mice examined. The results demonstrated that 70.0% of mice (7 of 10) on the atherogenic diet had mineralization in the kidneys, a significantly higher proportion than the 13.3% of mice (2 of 15) on the control diet. The incidence of eye mineralization was also higher in mice on the atherogenic diet (26.7%, 4 of 15 mice) than in mice on the control diet (6.7%, 1 of 15 mice).

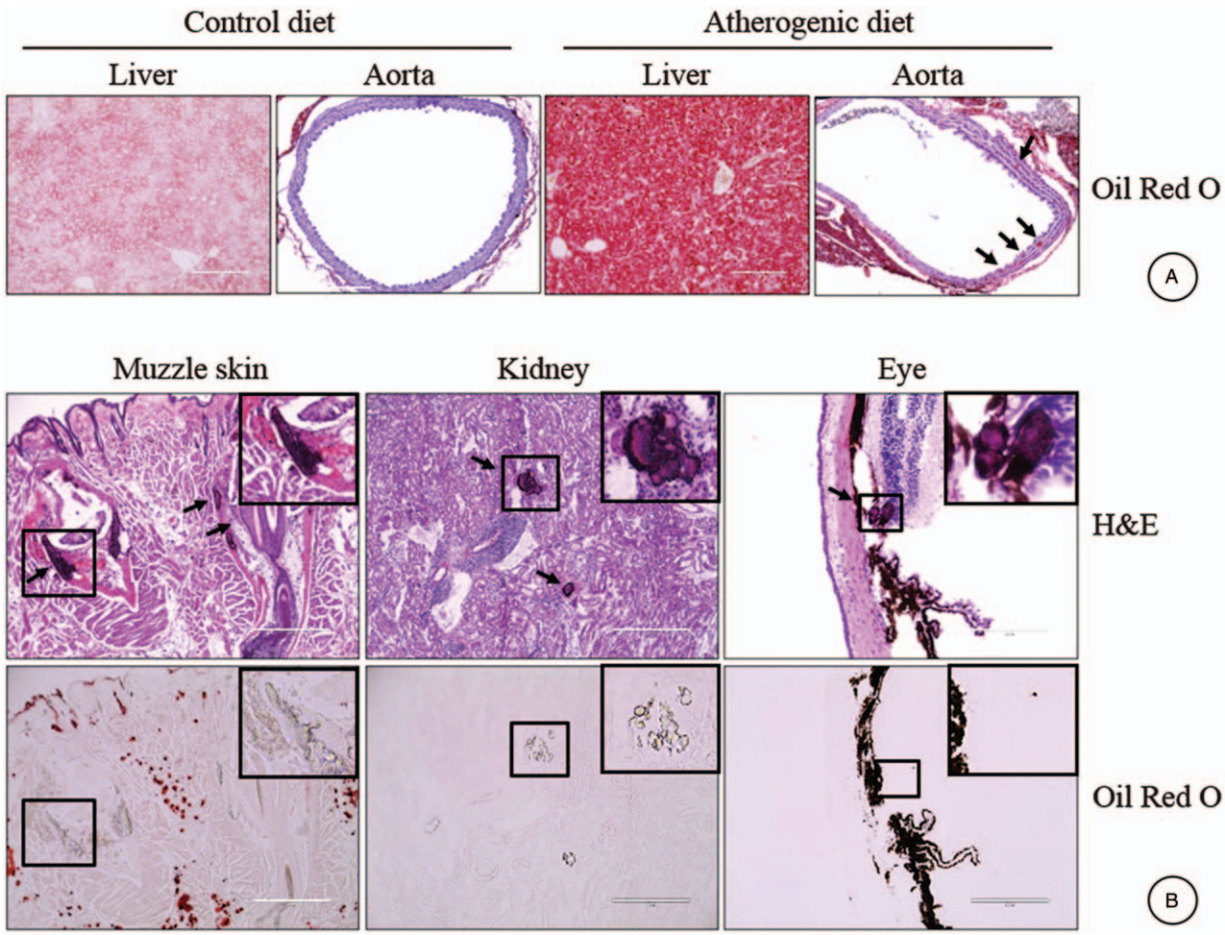

Figure 2. Demonstration of the presence of steatosis in the liver and absence of lipid accumulation at sites of ectopic mineralization in *Abcc6*$^{-/-}$ mice fed the atherogenic diet. (A) Comparison of lipid accumulation in the liver and aorta of *Abcc6*$^{-/-}$ mice on the atherogenic versus control diet, as revealed by Oil Red O staining. Scale bar = 200 μm. (B) The *Abcc6*$^{-/-}$ mice on the atherogenic diet developed ectopic mineralization (the arrows) in muzzle skin containing the dermal sheath of vibrissae, kidney, and eye as demonstrated by hematoxylin and eosin staining. However, Oil Red O staining did not show lipid accumulation in these tissues in the areas of ectopic mineralization. Scale bar = 400 μm.

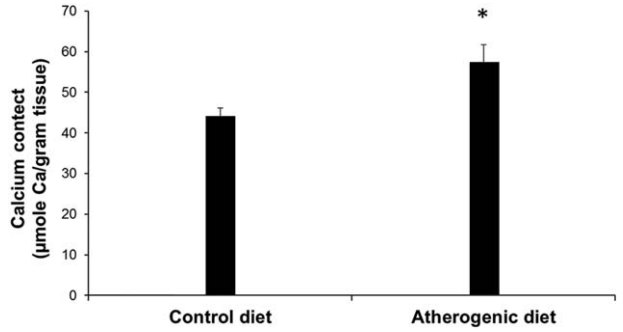

Figure 3. Quantitation of ectopic mineralization in muzzle skin by direct chemical assay of calcium. The *Abcc6*$^{-/-}$ mice were maintained on a control diet and atherogenic diet, respectively, and the degree of mineralization in muzzle skin was determined. The values are expressed as mean ± SE. Control diet, $n = 15$; atherogenic diet, $n = 10$. *$P = 0.0019$ compared with mice on the control diet.

Discussion

Cardiovascular disease is the leading cause of death in the Western world. Hypercholesterolemia (ie, elevated total cholesterol and LDL cholesterol) is a well-known risk factor for atherosclerotic vascular disease and stroke in humans.[16] "Western-type diets" or "atherogenic diets" high in saturated fat and cholesterol have been linked to elevated circulating cholesterol levels, particularly LDL cholesterol,[17] prompting the recommendation that humans limit their intake of these dietary constituents.[16]

Atherogenic diets are often used to induce hypercholesterolemia and accelerate the rate of atherosclerotic lesion development in certain models of atherosclerosis, such as mice, hamsters, guinea pigs, and rabbits. Therefore, the use of such diets to promote atherosclerosis in these models has been a valuable tool for both understanding the pathophysiology of the disease and developing therapies that can potentially prevent or reverse it. The study of murine atherosclerosis was greatly stimulated by the generation and characterization of *ApoE*- and *Ldlr*-deficient mice, which develop accelerated atherosclerotic lesions when challenged by atherogenic diets.[18-19]

The atherosclerotic lesions in animals fed an atherogenic diet are usually more lipid-rich than those in animals fed a control diet.[20] Researchers have used several different diets that vary in their cholesterol content, amount and type of fatty acids, and absence or presence of bile salts, such as cholic acid, which help further in the development of atherosclerosis because they aid cholesterol and fat absorption and reduce cholesterol disposal via conversion to bile acid. These components have been shown to influence lipoprotein levels and/or atherosclerosis, with dietary cholesterol being the major proatherogenic component. A common feature of each of these models is the increase in the serum levels of total cholesterol and VLDL and/or LDL cholesterol.[20] The atherogenic rodent diet TD.02028 is one such diet and has been studied in animal models of lipid metabolism, hepatic steatosis, and atherosclerosis.[21–24]

The first site of mineralization, noted as early as approximately 5–6 weeks of age in *Abcc6*$^{-/-}$ mice kept on the control diet, was the dermal sheath of vibrissae in the muzzle skin.[4] Mineralization of the vibrissae serves as an early biomarker of the overall mineralization process, and its quantitation by a direct calcium assay of the muzzle skin allows determination of the overall extent of mineralization in these mice.[4,15] In the present study, when the *Abcc6*$^{-/-}$ mice were challenged with an atherogenic diet (TD.02028) containing higher fat, sucrose, cholesterol, and cholic acid content than the standard control rodent diet, their serum levels were significantly higher with concurrent increase of mineral deposition in the soft connective tissues of the muzzle skin. Serum concentrations of calcium, phosphorus, and magnesium were not different from those in mice on the control diet, suggesting that mineral homeostasis was not altered by the atherogenic diet. The atherogenic diet also exacerbated ectopic mineralization in the kidneys and eyes despite normal serum mineral homeostasis. Although the detailed pathomechanisms of the apparent increase in ectopic mineralization as a result of atherogenesis is not clear, recent studies have suggested that inflammatory atherosclerosis precedes and drives ectopic mineralization.[25] In the present study, the increased tissue mineralization in mice fed the atherogenic diet was associated with elevated serum levels of total cholesterol and LDL/VLDL cholesterol, risk factors for cardiovascular disease. In contrast, cholesterol-lowering drugs such as atorvastatin have been shown to ameliorate the extent of ectopic mineralization in the same mouse model.[15] These findings have clinical relevance for the management of PXE in humans. In this context, the prevalence of PXE (approximately 1:50,000) suggests that more than 150,000 individuals are affected by PXE worldwide. Sequence variants in the *ABCC6* gene are associated with plasma levels of lipoprotein.[10-11] Early detection of hypercholesterolemia and atherosclerotic disease is of paramount importance to institute possible prevention strategies and monitor treatment. Limiting the intake of dietary saturated fat and cholesterol is expected to provide significant benefits to a large number of patients in terms of preventing cardiovascular complications and worsened tissue mineralization.

One limitation of the study is that the degree of vascular mineralization as a result of hypercholesterolemia was not analyzed in depth. This is due to the fact that PXE is a late onset, yet progressive disease. While multisystem mineralization is reproduced in the *Abcc6*$^{-/-}$ mice, mineralization of the vasculature occurs later in life.[4] Further studies should examine the *Abcc6*$^{-/-}$ mice to preceded death or a moribund state when vascular mineralization becomes fully penetrant.

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