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Restricting dietary magnesium accelerates ectopic connective tissue mineralization in a mouse model of pseudoxanthoma elasticum (*Abcc6*^{-/-})

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Abstract

Ectopic mineralization, linked to a number of diseases, is a major cause of morbidity and mortality in humans. Pseudoxanthoma elasticum (PXE) is a heritable multisystem disorder characterized by calcium phosphate deposition in various tissues. The mineral content of diet has been suggested to modify the disease severity in PXE. The aim of this study is to explore the role of diet with reduced magnesium in modifying tissue mineralization in a mouse model of PXE. *Abcc6*^{-/-} mice were placed on either standard rodent diet (control) or an experimental diet low in magnesium at weaning (4 wks) and examined for mineralization in the skin and internal organs at the ages of 1.5, 2 or 6 months by computerized morphometric analysis of histopathologic sections and by chemical assay of calcium and phosphate. Experimental *Abcc6*^{-/-} mice demonstrated an accelerated, early-onset mineralization of connective tissues, as compared to control mice. Wild-type or heterozygous mice on experimental diet did not show evidence of mineralization up to 6 months of age. All mice on experimental diet showed decreased urinary calcium, increased urinary phosphate and elevated parathyroid serum levels. However, no difference in bone density at 6 months of age was noted. Our findings indicate that the mineral content, particularly magnesium, can modify the extent and the onset of mineralization in *Abcc6*^{-/-} mice, and suggest that dietary magnesium levels may contribute to the phenotypic variability of PXE. The control of mineralization by dietary magnesium may have broader implications in general population in the context of vascular mineralization.

Keywords

Connective tissue; mineralization; modifier; diet-genetics

Introduction

The pathological processes of ectopic mineralization have been linked to a number of diverse conditions, such as arteriosclerosis, cancer and autoimmune diseases, major causes of morbidity and mortality in humans (1–3). A recent study examined the risk of death

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Conflict of Interest

The authors have no conflict of interest.

Author Contributions

QJ designed and performed the research and analyzed the data; JU conceived the data, wrote the manuscript, and provided financial support.

associated with coronary artery calcification (CAC) in a cohort of 25,253 patients and found that CAC was an independent risk factor to death (3). The relative risk of death associated with CAC was up to 12.5-fold, depending on the CAC score, thus establishing ectopic calcification as a major cause of mortality in the general population.

Two major types of acquired ectopic calcification processes involving peripheral connective tissues, particularly in the skin and the arterial blood vessels, have been recognized. Metastatic calcification refers to calcium deposition in association with elevated circulating levels of phosphate and/or calcium, as in chronic renal failure, while dystrophic calcification is usually secondary to a form of insult to the tissues, as seen in autoimmune diseases and cancer. Metastatic calcinosis in the skin can be characterized clinically by large nodular subcutaneous calcifications that often occur in periarticular distribution and are reversible upon correction of the calcium and/or phosphate abnormalities (4). It can be seen in association with vascular calcification, as in calciphylaxis which has very high mortality rate (5). It commonly occurs in patients with chronic renal failure in whom poor renal clearance of phosphate results in hyperphosphatemia (6). Dystrophic calcification occurs frequently in previously damaged or diseased tissue. Localized involvement occurs in many cutaneous inflammatory lesions, such as acne, stasis ulcers and granulomas, as well as in benign and malignant neoplasms (1). While calcinosis can be present as small cutaneous deposits primarily on the fingers or elbows, as in patients with scleroderma, particularly the CREST syndrome (limited scleroderma), it has also been reported in patients with systemic lupus erythematosus (1). Dystrophic calcification occurs without calcium/phosphate abnormalities in the plasma levels.

A number of Mendelian genetic disorders share phenotypic similarities with the acquired forms of metastatic and dystrophic calcification (4). One of them, the heritable counterpart of acquired dystrophic calcinosis, is pseudoxanthoma elasticum (PXE), an autosomal recessive multisystem disorder (7). Specifically, these patients develop ectopic mineralization of calcium phosphate deposits in the skin and a number of internal organs, with major clinical consequences in the eye and the cardiovascular system. The diagnosis is frequently not made until in early teens and occasionally delayed to the twenties and thirties. Once the mineralization process ensues, it progressively continues throughout the life of the affected individual. PXE is caused by mutations in the *ABCC6* gene which encodes a transmembrane efflux transporter protein, ABCC6, primarily expressed in the liver and the kidneys (8–10). The pathomechanistic details how the *ABCC6* gene mutations result in mineralization of the peripheral connective tissues are currently unknown, and specifically, the nature of the substrate(s) transported by ABCC6 remain to be identified (11). It has been suggested, however, that PXE is a metabolic disorder, and in the absence of functional ABCC6 transporter activity, the plasma and peripheral tissues become deficient in an anti-mineralization factor(s) that are required to prevent aberrant mineralization under physiologic calcium and phosphate homeostasis (7,12). In this context, it has been demonstrated that the calcium and phosphate blood levels, as well as the parathyroid hormone and vitamin D concentrations are within the normal limits in PXE (13).

Understanding of the pathologic nature of PXE has been advanced by development of *Abcc6* knockout (KO) mice which recapitulate the genetic, histopathologic, and ultrastructural features of PXE (14,15). Specifically, these mice develop mineral deposits in the same organs as in patients with PXE, *i.e.*, the skin, the eyes, and the arterial blood vessels (14). The mineralization process in the traditional *Abcc6* KO mouse, initially developed on J129Sv/C57BL/6J background and fed standard laboratory mouse diet, ensues at 5–6 weeks of age (14). It is of interest that the first site of mineralization is the connective tissue sheath of vibrissae, and quantitation of the mineral deposits either by computerized morphometric analysis of histopathological sections or by direct chemical assay of calcium

and phosphate serves as a biomarker reflecting the progress of the overall mineralization in these mice (16). Recent studies have indicated that changing the genetic background of these mice can significantly alter the age of onset and the severity of the mineralization, suggesting the presence of modifier genes (17).

Several lines of evidence have suggested a role for the mineral content of diet in modifying the severity of PXE (18). Early on, retrospective surveys suggested that individuals with a history of high intake of dairy products, rich in calcium and phosphate, during adolescence developed a more severe disease later in life (13,19). More recently, genetically controlled studies utilizing the *Abcc6*^{-/-} mice as the preclinical platform have suggested that specifically magnesium, but not calcium or phosphate, content of the diet can influence the extent of ectopic mineralization in peripheral connective tissues (20–22). Specifically, supplementation of the mouse diet with magnesium in amounts that increase the magnesium content by 5-fold over the standard diet, abolished the mineralization noted in the *Abcc6*^{-/-} mice. In this study we have specifically asked the question whether, conversely, an experimental diet low in magnesium might accelerate the mineralization process using the *Abcc6*^{-/-} mice as a model system.

Methods

Mice

Genetically modified *Abcc6*^{-/-}, *Abcc6*^{+/-} and *Abcc6*^{+/+} mice were generated by cross-breeding the traditional *Abcc6*^{-/-} mice (*Abcc6*^{tm1Jfk}, ref. 14) with an immunodeficient *Rag1*^{-/-} mouse (B6.129S7-*Rag1*^{tm1mom/J}) in C57/BL6 background (strain: 002216F; Jackson Labs, Bar Harbor, ME, USA). The mice were housed in a temperature- and humidity-controlled room with a 12 hour light/dark cycle. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University where the study was conducted.

Experimental design and diet

The experiments were initiated at weaning when the mice were approximately 4 weeks of age. The mice with the same genotype were divided into two groups, either fed with regular mouse diet provided by the animal facility (Laboratory Autoclavable Rodent Diet 5010*; LabDiet; Brentwood, MO, USA) or with experimental diet (TD.00442; Harlan Teklad Laboratories, Madison, WI, USA), for either 2, 4 or 20 weeks. The experimental diet is a chemically defined diet which contains 0.4% Ca, 0.85% P, 0.04% Mg and 100 IU/g vitamin D3; this diet is referred to as “acceleration diet”. In comparison, the composition of the control diet is 1% Ca, 0.43% P, 0.22% Mg and 4.4 IU/g vitamin D3, respectively.

Sample collection

All samples were collected when the mice were sacrificed, except the urine samples which were collected once a week initiating at 2 weeks after beginning the diet. The urine samples pooled from the different time points were used for chemical quantitation of calcium and phosphate. Muzzle skin biopsies, 6-μm in diameter, were obtained from both sides. One biopsy was used for chemical quantitation of calcium and phosphate, and another one was used for computerized morphometric quantitation of tissue mineralization after staining for histopathology (12,23). Similarly, the eyes, heart, blood vessels and the kidneys were processed and stained using histopathology. Both femurs from each mouse were excised, cleaned of soft tissue, and placed either in 0.15 N HCl for dissolution and chemical quantitation of calcium and phosphate or in 10% phosphate-buffered formalin for non-destructive 3-dimensional evaluation of bone density by micro-computed tomography.

(micro-CT). The whole blood was collected by cardiac puncture, and serum was obtained by centrifugation for parathyroid hormone (PTH) and serum calcium and phosphate assays.

Histology

For histopathological evaluation of mineralization of soft tissues including muzzle skin and the internal organs, the tissue specimens were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned (5 μ m), and stained with hematoxylin-eosin (H&E), Alizarin-Red and von Kossa utilizing standard procedures.

Computerized morphometric quantitation of connective tissue mineralization

Examination of H&E-stained sections of muzzle skin was performed using computerized morphometric analysis. The Nikon model TE2000 microscope equipped with an Auto Quant Imaging system was employed to examine the sections (Watervliet, New York, NY, USA). The total number of vibrissae, including those with perceptible mineralization of the dermal sheath and those with absence of mineralization, was established in all sections, and the degree of mineralization was expressed as both the percentage of the mineralized vibrissae of the total number of vibrissae examined, as well as the percentage of area of mineralization per total area of vibrissae.

Chemical quantitation of calcium or phosphate deposition

Specimens of muzzle skin, which embodies the vibrissae, were harvested and decalcified with 0.15 N HCl for 48 h. The *o*-cresolphthalein complexone method was utilized to measure the calcium content in a colorimetric fashion [Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX, USA]. The content of phosphate was determined with the Malachite Green Phosphate Assay kit (Bioassay Systems, Hayward, CA, USA). The values for calcium and phosphate were standardized to tissue weight. The femurs were decalcified with 0.15 N HCl for two weeks and the calcium content in the solute was measured using the chemical assays as above. The urine and serum calcium levels were measured using Calcium (CPC) Liquicolor kit.

Serum parathyroid hormone assay

Mouse serum PTH concentrations were measured using Mouse Intact PTH Elisa Kit (Immutopics Inc., San Clemente, CA, USA).

Micro-computed tomography

The femurs were scanned for bone density by Computer Axial Tomography (CAT scan) in a MicroCAT II scanner (ImTek, Inc), and the data were analyzed using the Inveon Research Workplace software (Siemens Corporation).

Statistical analysis

The disease probability overall was analyzed by Fisher exact test. The statistical differences between the means in distinct groups of mice receiving various diets were first calculated by the Kruskal-Wallis test, followed by Student's two-tailed *t*-test if the data were normally distributed or by Wilcoxon test. Differences were considered significant when $p < 0.05$. All data are reported as mean $\pm$ SE.

Results

Experimental design

This study utilized genetically modified *Abcc6*^{-/-} mice with targeted ablation of the *Abcc6* gene either on C57BL/6 or *Rag1*^{-/-} background. This double knockout mouse has demonstrated progressive mineralization of connective tissues in the same pattern as observed in traditional single *Abcc6* knockout mouse (*Abcc6*^{tm1JfK}) (14,23). Thus, it serves as an expedient model system to explore various treatment modalities and modifying factors for PXE. The *Abcc6*^{-/-} mice, males and females separately, were placed at weaning, *i.e.*, at 4 weeks of age, on either standard laboratory diet (control diet) or experimental “acceleration” diet (experimental) with mineral modifications as detailed in Materials and Methods section. The mice were then followed either for 2, 4 or 20 weeks on the respective diets, and sacrificed at the age of 1.5, 2 or 6 months, respectively.

Mineralization of the connective tissue sheath of vibrissae

The first site of mineralization in the *Abcc6*^{-/-} mice is the dermal sheath of vibrissae, consisting of connective tissues components, collagen and elastin (14). Mineralization of this sheath ensues in traditional *Abcc6*^{tm1JfK} mice kept on control diet at ~5–6 weeks of age, and subsequently progresses with the advent of the overall mineralization process in these mice.

Histopathologic examination of the muzzle skin containing the vibrissae either by routine H&E stain or with special stains, Alizarin Red and von Kossa, which recognize calcium phosphate complexes, suggested increased mineralization both at the age of 2 and 6 months in mice on experimental diet, as compared to the corresponding age matched KO mice on the control diet (Fig. 1). It should be noted that wild-type control mice or *Rag1*^{-/-} mice fed the experimental diet for 5 months (at 6 months of age) did not show any evidence of mineralization (Fig. 1A–C). The mineralization was quantitated by computerized morphometric analysis of the corresponding H&E stained sections. The results indicated a significant, up to 40-fold, enhancement of the mineralization in the mice on experimental acceleration diet, both in female and male mice, at 2 months of age (Table 1). Increased mineralization was reflected both by the increased number of vibrissae that were mineralized as well as the total area occupied by mineralization in the vibrissae examined.

The quantitation of the mineralization by computerized morphometric analysis was complemented by direct calcium and phosphate assays from the muzzle skin containing the mineralized vibrissae. At 2 months of age the calcium phosphate content in the muzzle skin was significantly increased in knockout mice on experimental diet, as compared to the corresponding mice on control diet, both in males and females separately (Fig. 2A,B). An increase in the mineralization of KO mice on experimental diet was noted in males already after two weeks of feeding (Fig. 2A; KO exp. diet, 1.5 mon). It should be noted that wild type mice (WT) or heterozygous *Abcc6*^{+/-} mice (HET) on experimental diet for one month did not show any evidence of mineralization of the vibrissae.

Computerized morphometric quantitation of mineralization of the vibrissae in KO mice at 6 months of age on experimental diet showed 1.7–1.9-fold increase over the KO mice fed control diet; however, due to considerable variability, this difference was not statistically significant (Table 2). Chemical quantitation of calcium and phosphate in 6 month old KO mice kept on either control or experimental diet for 5 months also demonstrated a marked increase in mineralization, as compared to the corresponding WT controls, and the difference between the KO mice either on control or experimental diet was statistically significant (*p*=0.024) (Fig. 2C). Thus, quantitation of the mineralization of the dermal sheath of vibrissae in mice on experimental diet demonstrates a rapid onset of mineralization, noted

as early as at two weeks on this diet, but the difference between the KO mice either on experimental or control diet was less pronounced, yet statistically significant, at 6 months of age.

Since the *Rag1*^{-/-} background could conceivably influence the degree of mineralization in the double KO mice (24), the acceleration diet was also tested in *Abcc6*^{-/-} mice on C57BL/6 background. The results revealed that the acceleration diet enhanced mineralization of various connective tissues including muzzle skin in a time-dependent pattern, which is consistent to the findings in the *Abcc6*^{-/-} mice on *Rag1*^{-/-} background (Fig. S1). Moreover, such mineralization was not observed in *Abcc6*^{+/-} or *Abcc6*^{+/+} mice on *Rag1*^{-/-} background when placed on the acceleration diet even up to 5 months.

Mineralization of internal organs

Previous studies by us have indicated that mineralization of the vibrissae in *Abcc6*^{-/-} mice is accompanied by development of calcium phosphate deposits in a number of internal organs (14,23). To examine whether the experimental diet also accelerates the mineralization of the internal organs, KO mice on either control or experimental diet for 1 month were necropsied at 2 months of age. Histopathologic examination revealed extensive mineralization in various tissues of KO mice on experimental diet, including the cartilage, kidney, and the heart as well as in arterial blood vessels (Fig. S2), but it was not seen in age-matched KO mice on control diet. Quantitation of the internal organ involvement, in comparison of the mineralization of the vibrissae, was also examined at 6 months old KO mice which had been kept either on control or experimental diet for 5 months, in comparison to the corresponding WT controls. The overall calcification probability in KO mice on experimental or control diet was statistically higher in comparison to WT mice ($p < 0.05$, Fisher exact test Table S1). The results indicated that KO mice, 100% of which demonstrated mineralization of vibrissae, also had involvement in the kidneys and the eyes, and to a lesser extent in the heart, both in mice kept on control and experimental diet (Table S1). Again, no evidence of mineralization in the internal organs was noted in WT mice kept either on control or experimental diet for 5 months.

Serum and urinary calcium and phosphate levels, and serum parathyroid hormone levels

To examine the metabolic consequences of the experimental diet, serum and urinary calcium and phosphate levels were determined by chemical assays. Serum calcium levels in two month old (Fig. S3A,B) or 6 month old (Fig. S3C) mice were not different in mice on experimental diet compared to the control diet, and the values were not different from those noted in WT or HET mice on either one of the diets. Similarly, measurements of serum phosphate levels at two months did not reveal any differences (results not shown).

Urinary calcium expression was also measured in the mice on experimental diet in comparison to those on control diet. The urinary samples represent weekly collections pooled from each individual mouse, and the calcium concentration was determined by a chemical assay. The results indicated that all mice, males or females, both KO and WT, demonstrated marked reduction in urinary calcium when the mice were placed on the experimental diet. Similar findings were noted both at the age of 2 month (Fig. S4A,B) and 6 months (Fig. S4C) of age. Assay of urinary phosphate in the mice demonstrated marked, statistically significant increase both in KO and WT mice placed on the experimental diet (Fig. S4D).

To explore the mechanisms leading to reduced calcium and elevated phosphate urinary excretion, PTH levels were also determined at 2 and 6 months of age of KO and WT mice either on experimental or control diet (Fig. S5). PTH serum levels in KO mice, both male

and female, were significantly increased at 2 months of age (Fig. S4A). A statistically significant increase in PTH levels was also noted in KO mice kept on experimental diet for 5 months, and similar increase was also recorded in WT mice (Fig. S5B).

Bone density and calcium content analysis

Considering the changes noted in urinary calcium and serum PTH levels in mice fed experimental diet, we asked the question whether this diet would have consequences on the density of bones in these mice. The density of bones was measured by two independent approaches. First, the calcium content of the bones in 6 month old KO mice, which had been on experimental or control diet for 5 months, was determined. No statistical difference in the bone mineral content of these two groups of mice was noted (Fig. S6). Similarly, the experimental diet did not change the calcium content of bones in WT mice as compared to WT mice kept on control diet. The apparent increase in the mineral content of the KO mice in comparison to WT mice was statistically not significant. Secondly, the bone density was quantitated by micro-CT scan of femurs in 6 month old KO mice fed experimental diet or control diet for 5 months. No difference in the bone density was noted (Table S2).

Discussion

This study clearly demonstrates that an experimental diet with mineral modifications, in particularly low in magnesium, accelerates the connective tissue mineralization in the genetically altered mouse with targeted ablation of the *Abcc6* gene. This study complements previous demonstrations that diet rich in magnesium can prevent mineralization in this mouse model (20,21). It should be noted that the experimental acceleration diet differed from the control diet also in the content of other minerals, including Ca and P. However, previous studies by us have demonstrated that variation in the calcium or phosphate content of diet does not affect the mineralization in this mouse model (20,25). Collectively, these observations suggest that mineral content of the diet can modify the ectopic mineralization in mice, a finding potentially relevant to soft tissue mineralization in humans as well. While the mechanism by which magnesium modulates the mineralization process is currently unknown, previous studies have shown that there is a marked increase in the urinary output of calcium and concomitant reduction in phosphate in the mouse treated with high magnesium-containing diet (20,21). These findings were highlighted by our studies reported here indicating reduced calcium and increased phosphate excretion in the urine of mice kept on the experimental diet low in magnesium. It is possible, therefore, that increased magnesium replaces calcium in the calcium/phosphate complexes, and since magnesium phosphate is more soluble than calcium phosphate in the corresponding concentrations, this may result in reduced or absent mineralization in peripheral tissues. Conversely, diet low in magnesium may allow excessive deposition of calcium phosphate complexes as a result of reduced magnesium/calcium ratio. These findings support the notion that changes in the diet, specifically an increase in dietary magnesium, might be helpful for patients with PXE. It should be noted the serum levels of PTH were increased in mice on low-magnesium diet. Since there is a clear relationship between PTH levels and calcium metabolism, one concern would relate to bone density as a result of changes in dietary magnesium. No change in bone density was observed, possibly due to the fact that changes in PTH levels were relatively modest.

The dietary allowance of magnesium recommended by the Food and Drug Administration is up to 420 mg per day for adults (26). It should be noted that various vegetables (spinach, tomato and pumpkin seeds) and grains (buckwheat flour, oat bran, barley and wheat flour) are particularly rich in magnesium (18,25). Based on the preclinical mouse studies cited above, a clinical trial exploring the effects of supplementary magnesium on the progression of cutaneous and ocular signs of PXE is currently being developed (Dr. Mark Lebwohl, Mt.

Sinai School of Medicine, personal communication). One difficulty of such clinical trials in patients with PXE is that the clinical course is slow and quite variable, and there are no reliable biomarkers to predict the improvement in the clinical progression short term (11). One should also consider potential side effects of long-term magnesium supplementation (27). Specifically, there is the theoretical possibility that removal of calcium from the bones would result in osteoporosis with clinical consequences. It should be noted, however, that the calcium content of the bones measured by chemical assays, and bone density determined by micro-CT scan did not reveal any long-term adverse effects on the bones as the result of extended increase of magnesium intake in the *Abcc6*^{-/-} mice up to nine months of age (21). This observation is supported by demonstration that elevated magnesium diet prevents the deposition, but does not reverse the existing calcium phosphate deposits in the *Abcc6*^{-/-} mice (20,21). These observations suggest that any putative magnesium treatment of PXE patients should be initiated as soon as the diagnosis is made, and such treatment may not have a major influence at the late stages of the disease process. Nevertheless, long-term supplementation of diet in humans not only in patients with PXE, but also in general population might be helpful in reducing the aberrant mineralization, such as in arterial blood vessels, a major cause of morbidity and mortality in general population (28,29). However, additional long-term preclinical studies utilizing the available animal models are required to test the potential consequences of dietary magnesium on bone metabolism and to monitor toxicity. Finally, it is appropriate to point out that the mineral metabolism in the mice may differ from that in humans, and the clinical efficacy and potential side-effects in humans remain to be seen in carefully controlled clinical trials.

It should be noted that control of mineralization by dietary magnesium may have broader implications beyond PXE in situations such as arteriosclerosis. It is of interest that a study by investigators at the Centers for Disease Control and Prevention (CDC) in the United States, based on a National Health and Nutrition Examination Survey 1999–2000, concluded that substantial numbers of US adults failed to consume adequate amounts of magnesium in their diets (30). Collectively, these observations suggest that careful assessment of dietary magnesium, not only in patients with PXE but in populations at large as well, is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histopathology demonstrating mineralization of dermal sheath of vibrissae in *Abcc6*^{-/-} mice at the age of 2 or 6 months, fed either control diet (Contr.) or experimental acceleration diet (Exp.); Hematoxylin & Eosin (left panel), Alizarin Red (middle panel) and von Kossa (right panel) stains. The *Abcc6*^{-/-} mice on experimental diet showed more mineral deposits than their age-matched KO mice on control diet (G–I vs. D–F; M–O vs. J–L). No evidence of mineralization was noted in the 6 month-old WT mice on the experimental diet for 5 months (A–C). The magnification in all figures is the same; scale bar = 100 µm.

Figure 2.

Calcium phosphate content in the muzzle skin containing the vibrissae of male (A) and female (B) *Abcc6*^{-/-} (KO), *Abcc6*^{+/-} (HET), and *Abcc6*^{+/+} (WT) mice at the age of 1.5, 2 (A,B) or 6 (C) months, after having been 0.5, 1 or 5 months on the experimental acceleration diet (Exp.), in comparison to *Abcc6*^{-/-} mice on control diet (Contr.). The data is presented as mean ± S.E. (*p<0.05).

Table 1

Quantitation of Mineralization of Vibrissae of 2 Month Old *Abcc6*^{-/-} Mice Fed the Control or Experimental Diet^{a)}

Experimental Group	Total vib. per mouse ^{b)} (n)	Mineralized vib. per mouse ^{b)} (n)	Percent mineralized (%)	Area of mineralization per total area ^{c)} (%)	Fold ^{d)}	p-value
KO Female Contr. Diet for 1 month (n=4)	13.8	1.8	12.7	0.08 ± 0.071 (0.016; 0-0.292)	1.0	
KO Female Exp. Diet for 1 month (n=4)	13.8	4.8	34.5	3.13 ± 1.689 (2.305; 0-7.927)	39.1	< 0.05 ^{e)}
KO Male Contr. Diet for 1 month (n=3)	15.0	1.0	6.7	0.12 ± 0.119 (0.002; 0-0.359)	1.5	
KO Male Exp. Diet for 1 month (n=5)	10.0	4.0	40.0	1.95 ± 1.016 (1.382; 0-6.821)	24.4	< 0.05 ^{f)}

^{a)} Skin sections from KO mice at the age of 2 months *i.e.* four weeks on the diets, were stained with H&E and examined for mineralization by light microscopy followed by quantitative computerized morphometric analysis.

^{b)} n, numbers of vibrissae examined.

^{c)} Values presented as mean ± S.E., as well as median and range in parenthesis.

^{d)} Calculated using KO Female on the control diet group as 1.0.

^{e)} p-value for comparison between KO Female Contr. Diet and KO Females Exp. Diet groups (Wilcoxon test).

^{f)} p-value for comparison between KO Males Contr. Diet and KO Males Exp. Diet groups (Wilcoxon test).

Table 2

Quantitation of Mineralization of Vibrissae of 6 Month Old *Abcc6*^{-/-} (KO) or *Abcc6*^{+/+} (WT) Mice Fed a Control or Experimental Diet ^{a)}

Group ^{b)}	Total vibrissae per mouse ^{c)} (n)	Mineralized vibrissae per mouse ^{c)} (n)	Percent Vibrissae mineralized (%)	Area of mineralization per total area ^{d)} (%)	Fold ^{e)}	p-value ^{f)}
KO Males Contr. Diet (n=5)	11.4	7.2	63.2	2.99 ± 0.49 (2.6; 2.26–4.91)	1.0	>0.05 ^{g)}
KO Females Exp. Diet (n=4)	15	8.5	56.7	5.60 ± 1.37 (4.48; 3.74–9.68)	1.87	>0.05 ^{h)}
KO Males Exp. Diet (n=4)	11	6.8	61.3	5.03 ± 0.60 (5.47; 3.28–5.90)	1.68	>0.05 ⁱ⁾
WT Males Contr. Diet (n=3)	10.0	0	0	0	N/A	N/A
WT Males Exp. Diet (n=5)	13.4	0	0	0	N/A	N/A

- ^{a)} Skin sections from mice at the age of 6 months, fed either a control diet or the experimental diet, were stained with H&E and examined for mineralization by light microscopy, followed by quantitative computerized morphometric analysis.
- ^{b)} *Abcc6*^{-/-}/*Rag*^{-/-} knockout (KO) mice fed a control diet (Contr.) or experimental diet (Exp.).
- ^{c)} n, numbers of vibrissae examined.
- ^{d)} Values presented as mean ± S.E., as well as median and range in parenthesis.
- ^{e)} Calculated using KO Males Contr. Diet group as 1.0.
- ^{f)} The total exact p-value for the Kruskal-Wallis test of the entire experimental group is p = 0.06
- ^{g)} p-value for comparison between KO Males Contr. Diet and KO Males Exp. Diet groups (Wilcoxon Test).
- ^{h)} p-value for comparison between KO Males Contr. Diet and KO Females Exp. Diet groups (Wilcoxon Test).
- ⁱ⁾ p-value for comparison between KO Males Exp. Diet and KO Females Exp. Diet groups (Wilcoxon Test).