Dietary magnesium, not calcium, prevents vascular calcification in a mouse model for pseudoxanthoma elasticum

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Abstract  Pseudoxanthoma elasticum (PXE) is a heritable disorder characterized by ectopic calcification of connective tissue in skin, Bruch’s membrane of the eye, and walls of blood vessels. PXE is caused by mutations in the ABCC6 gene, but the exact etiology is still unknown. While observations on patients suggest that high calcium intake worsens the clinical symptoms, the patient organization PXE International has published the dietary advice to increase calcium intake in combination with increased magnesium intake. To obtain more data on this controversial issue, we examined the effect of dietary calcium and magnesium in the Abcc6−/− mouse, a PXE mouse model which mimics the clinical features of PXE. Abcc6−/− mice were placed on specific diets for 3, 7, and 12 months. Disease severity was measured by quantifying calcification of blood vessels in the kidney. Raising the calcium content in the diet from 0.5% to 2% did not change disease severity. In contrast, simultaneous increase of both calcium (from 0.5% to 2.0%) and magnesium (from 0.05% to 0.2%) slowed down the calcification significantly. Our present findings that increase in dietary magnesium reduces vascular calcification in a mouse model for PXE should stimulate further studies to establish a dietary intervention for PXE.

Keywords  PXE · ABCC6 · Calcium · Magnesium · Vascular calcification · ABC transporter · Histopathology · Calcium metabolism · Cardiovascular · Connective tissue · Diet

Introduction  Pseudoxanthoma elasticum (PXE) is a connective tissue disease characterized by progressive mineralization and fragmentation of elastic fibers [1–3]. Clinical manifestations generally appear in the second or third decade of life and occur mainly in skin, eye, and blood vessels. In the skin, PXE is characterized by papules and plaques typically present in the neck and in flexural areas. The eye of PXE patients can develop angioid streaks, which represent breaks in Bruch’s membrane. Through these breaks, neovascularization occurs with subsequent hemorrhages, which lead to acute, irreversible loss of vision. Cardiovascular symptoms of PXE include calcification of blood vessels and bleedings in internal organs. The prevalence of PXE is estimated to be between 1:25,000 and 1:100,000 [4]. PXE inherits in an autosomal recessive fashion [5]. In 2000, the causative gene was identified as ABCC6, a member of the large family of ATP-binding cassette transporters.
tion with increased magnesium intake [18]. While this may have the negative side effect of osteoporosis. Indeed, the organs affected in PXE show little or no expression of the protein, which suggests that PXE is a systemic disease caused by changes in blood composition when ABCC6 transport function is deficient [12].

To investigate the biological function of ABCC6, we and others have disrupted the homologous gene in the mouse [13, 14]. Abcc6−/− mice readily develop ectopic calcification in the walls of blood vessels. At advanced age, also Bruch’s membrane of the eye and the skin become mineralized. Thus, Abcc6 deficiency in the mouse essentially re-creates the clinical features of PXE. This confirms that mutations in ABCC6 cause PXE. It also indicates that the Abcc6−/− mouse is a suitable animal model for studies on etiology and treatment of PXE.

While serum ion balance is not altered in PXE, several studies on patients suggest that mineral intake influences disease severity in PXE. First, Renie et al. found, after examination of 32 unrelated PXE cases, that clinical severity correlated positively with calcium ingestion [15]. Second, Martinez-Hernandez and colleagues observed regression of the calcification in the dermis in a PXE patient upon reduction of calcium intake [16]. Finally, a case report [17] described that normalization of the serum calcium–phosphate product resulted in regression of the skin lesions in a patient with chronic renal failure. These studies suggested that restriction of dietary calcium intake may be beneficial for PXE patients. However, this has not led to a general advice to PXE patients to restrict dietary calcium intake. Reducing serum calcium levels is difficult to achieve, as the supply of the calcium from the bones is practically unlimited. Moreover, reducing calcium intake may have the negative side effect of osteoporosis. Indeed, the patient organization PXE International has given PXE patients the advice to increase calcium intake in combination with increased magnesium intake [18]. While this dietary advice allegedly has been of value in treatment of kidney stones [18], there is currently no evidence that this diet is beneficial for PXE patients.

In the present study, we used the Abcc6−/− mouse model to obtain experimental data on the putative effects of dietary calcium and magnesium on PXE pathology. The results presented here show that dietary magnesium influences the development of pathology in this animal model for PXE, whereas calcium does not.

Materials and methods

Mice and diets

Generation and PCR-mediated genotyping of the Abcc6−/− mice has been described [13]. Abcc6−/− mice were generated in a hybrid background of C57Bl/6-129/ola and then backcrossed to C57Bl/6. The genetic background of the Abcc6−/− and wild-type mice used in the present experiment was backcross number 5. After weaning, female Abcc6−/− and wild-type mice were fed one of three custom designed diets, which differed in calcium and magnesium concentration. We first designed a “baseline” diet, which contained the Ca and Mg nutrient requirements for health and maximal growth, which are 0.5% (w/w) Ca and 0.05% (w/w) Mg [19]. In addition, this Ca and Mg content corresponds to the formulation of the standard purified rodent diet AIN-93. Next, in view of the reports which suggested that high calcium intake aggravates PXE, we designed a “4×Ca” diet, with four times increased calcium content: 2% Ca. Finally, following the advice of PXE International [18], the third diet was supplemented with both Ca and Mg. This “4×Ca, 4×Mg” diet contained a fourfold increased calcium and magnesium (2% Ca and 0.2% Mg). All other ingredients were kept the same for all three diets (Table 1). P for example was kept at 0.5%. Diets were purchased from Harlan Teklad (Madison, Wi, USA).

In this study, we employed 58 Abcc6−/− mice and 58 wild-type mice, five to nine mice per genotype, diet, and diet duration. Mice were kept in a controlled environment with food and water ad libitum and a 12-h light/dark cycle with light intensity of 70–90 lx. They were checked regularly for general parameters of well-being, and their weight was measured. All animal studies were approved by an independent animal ethical committee (Dutch equivalent of the IACUC).

The diets were given for 3, 7, or 12 months. At the end of the diet period, the mice were killed. At 9:00 A.M., the food was removed, and after fasting for 6–8 h, mice were killed by CO2/O2 inhalation and decapitated, and blood was collected in tubes containing heparin. Plasma was obtained after centrifugation and stored at −80°C. Plasma values were determined by the clinical laboratory of the Academic Medical Center, University Hospital of Amsterdam, following standard procedures and assays (Roche Diagnostics). The following plasma parameters were measured: calcium, magnesium, phosphate, iron (all by colorimetric assays), HDL-cholesterol, creatinine, urea (by enzymatic assays), and total protein (biuret assay).

For histopathology, heart and kidney were collected and fixed in 2% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin. Sections were stained with hematoxylin–eosin and von Kossa using standard procedures.
We quantified the calcification of blood vessels in the cortex of the kidney with the use of histology. Sagittal sections of 6 μm were cut from the kidney and stained for calcification according to von Kossa. For quantification, we examined five sections per mouse, each separated by 180 μm. We counted the number of von Kossa positive blood vessel profiles in the kidney cortex and calculated the average number per section. Von Kossa positive structures in the pelvis, tubules, or in adjacent adipose tissue were excluded.

Micrographs were taken with a Zeiss Axioplan 2 (Carl Zeiss AG, Germany) equipped with an Evolution MP camera (MediaCybernetics, Bethesda, USA) and Image-Pro Plus acquisition software (version 6, MediaCybernetics, Bethesda, USA). A ×5 and a ×20 Plan apo achromat objective lens with 0.16, respectively, 0.60 numerical aperture were used. Image processing was done with Adobe Photoshop (Adobe Systems Inc., USA).

Statistics

A one-way ANOVA was used to determine the independent effects of genotype, diet, and diet duration on the various parameters measured. Significance was accepted at p<0.05. In case of statistical significance, the ANOVA was followed by a post hoc test of Student–Newman–Keuls. Since the number of calcifications in blood vessels of the kidney cortex did not show a normal distribution, we used the non-parametric Kruskal–Wallis test (followed by Mann–Whitney U test) to analyze the effect of diet on the number of calcifications in blood vessels of the kidney at 3, 7, and 12 months of diet. In addition, for determining correlation between parameters, Spearman’s rho was calculated.
Results

Diet schedule

To examine the effect of dietary calcium and magnesium concentrations on PXE pathology, we put Abcc6−/− and wild-type mice on one of three custom diets: the baseline diet (0.5% Ca and 0.05% Mg), 4×Ca diet (2% Ca and 0.05% Mg), and the 4×Ca, 4×Mg diet (2% Ca and 0.2% Mg). The diets were started immediately after weaning, at 4 weeks after birth, and lasted for 3, 7, or 12 months. None of the diets had overt adverse effects on behavior or well-being of the mice. In addition, no significant weight differences were found between the genotypes.

Diet mineral content influences blood vessel calcification in the kidney

Since calcification of blood vessels is a prominent clinical feature both of PXE patients [1] and Abcc6−/− mice [13], we measured this parameter in the mouse as function of the diets. Histology was used to quantify calcification of small arteries in the cortex of the kidney (Fig. 1).

Wild-type mice hardly developed calcifications in the kidney. In only four of the 58 wild-type mice examined, we detected calcified blood vessels. Two of these mice had received the baseline diet for 12 months; the other two mice had received the 4×Ca diet for 12 months. The low number of calcifications in wild-type mice precluded statistical analysis of possible differential effects of the diets.

In contrast with wild-type mice, Abcc6−/− mice readily developed calcifications. When fed the baseline diet, calcifications were already found after 3 months (about one per section), and this increased to 18 per section after 12 months (Fig. 2). Surprisingly, supplementation of the diet with calcium did not increase the calcification. In contrast, simultaneous supplementation with both Ca and Mg slowed down the calcification significantly (compared to baseline diet or 4×Ca diet after 3, 7, and 12 months; Kruskal–Wallis test, $p<0.05$). After 12 months of the 4×Ca, 4×Mg diet, the number of calcifications was less than one third of that in the other diet groups.

MicroCT scans of the heart confirm the effect of dietary magnesium

To confirm and extend our finding that dietary Mg modulates ectopic calcification, we employed μCT to measure calcification in the hearts of the mice. Since the calcifications were most abundant after 12 months of diet, we performed this analysis at this time point. The μCT scans revealed multiple calcifications localized mostly in the ventricles (Fig. 3b, c). These calcifications had an

![Fig. 1 Light micrographs of sections of the kidney cortex of a wild-type (a) and an Abcc6−/− mouse (b, with detail in c), stained for calcification with von Kossa. The mice were killed after 12 months of a diet supplemented with Ca (4×Ca diet). Extensive calcification (black deposits) is present in arteries in the kidney cortex of the Abcc6−/− mouse (b). Please notice that the calcification is located within the wall of the artery (arrows in b and c, which shows a detail of b). Bar represents 100 μm](image)
average size of $10^5 \mu m^3$. Histology localized them in walls of blood vessels (Fig. 4). In agreement with the findings in the kidney, the 4×Ca, 4×Mg diet reduced calcification as compared to the 4×Ca diet (on average 17 vs. 51 per heart; Fig. 5). This underscores that dietary magnesium modulates calcification in this PXE mouse model.

Analysis of plasma parameters

In order to assess possible systemic consequences of the diets, we collected fasting blood of the mice at sacrifice and measured several plasma parameters. Using ANOVA, we analyzed the influence of genotype, diet, and diet duration (eq. age) on these parameters. In addition, we determined whether there was a correlation of these values with disease severity as reflected in the number of calcifications in kidney blood vessels.

The plasma levels of the minerals examined (Ca, PO₄, Mg, and Fe) did not significantly depend on genotype. In contrast, the choice of diet caused significant changes in plasma Ca and Mg levels. Mice fed with extra calcium had higher plasma levels of calcium. Extra magnesium in the food led to higher plasma Mg levels. However, these changes were small and were not statistically significant at all time points (see Table 2 for Abcc6⁻/⁻ mice after 7 months of diet). In addition, no correlation was observed between the levels of these minerals and the extent of calcification in the kidney. For example, Spearman’s correlation coefficient between plasma Mg levels and kidney calcification in Abcc6⁻/⁻ mice was −0.189 (P value 0.501) after 7 months of diet and 0.228 (P value 0.320) after 12 months of diet. PO₄ and Fe levels did not change significantly by diet.

Since calcifications in kidney blood vessels may affect kidney function, we measured plasma indicators of kidney function. Chronic renal dysfunction is among others associated with elevated plasma levels of urea, creatinine, and total protein. We found no significant effect of genotype on the levels of these parameters. Apparently, in none of the diet groups, the kidney calcifications caused a measurable decrease in kidney function. In addition, no correlation was found between these plasma values and kidney blood vessel calcifications.

In a previous study, we found that HDL-cholesterol levels were decreased in Abcc6⁻/⁻ mice. In the present study, Abcc6⁻/⁻ mice again had reduced plasma HDL-cholesterol levels (for all Abcc6⁻/⁻ mice vs. wild-type mice: 8.55 vs. 11.57 mmol/L, p=0.04). However, the HDL-cholesterol levels did not depend on the diet, and no correlation existed between decreased HDL-cholesterol levels and increased kidney calcification.
Discussion

The clinical expression of PXE is remarkably variable even between patients that share exactly the same ABCC6 mutation [8]. This implies that other factors contribute to PXE pathology, including perhaps dietary factors [20]. The aim of the present study was to investigate in our mouse model for PXE whether we could influence age of onset, progression, and severity of the pathology by mineral intake. The clinical relevance of this research question is obvious as presymptomatic DNA diagnosis for PXE is possible, and mineral intake can be easily modified by PXE patients themselves.

The use of the animal model has the advantage that all experimental factors can be controlled. The Abcc6−/− mouse develops calcification in the skin, eye, and cardiovascular system and thus essentially recapitulates all clinical features of PXE [13, 14]. While in PXE patients the involvement of the various organs (skin, eye, and cardiovascular system) is highly variable [8], in the Abcc6−/− mouse, the vascular calcification is the most prominent feature. The skin and eye pathology develop in the Abcc6−/− mouse only at advanced age. In the present study, we therefore focused on the cardiovascular aspect of PXE, which we quantified in the kidney by histology and in the heart by μCT scan.

In addition to the Abcc6−/− mouse, another mouse model recently became relevant for PXE as well. A number of inbred mouse strains are very susceptible to dystrophic cardiac calcinosis (DCC), which refers to a spontaneous peri/myocardial calcification. This calcification occurs in the presence of normal calcium and phosphate levels [21]. In 2007, Meng and coworkers demonstrated that Abcc6 is the major causative gene for DCC as they rescued the calcification phenotype in DBA mice by transgenic complementation with a BAC uniquely containing Abcc6. DCC susceptible strains reportedly have an alternative splice variant, which leads to ABCC6 protein deficiency [22]. Interestingly, a number of studies have already been done on the influence of diet on DCC.

Ca intake does not influence vascular calcification in the Abcc6−/− mouse

The first factor which we investigated was the calcium concentration in the food. Previous observations on PXE patients suggested that high calcium intake increased the severity of disease [15–17]. We examined the effect of a fourfold increase in dietary calcium over a period of up to 1 year. In this period, we noticed small increases in plasma calcium concentration. Most importantly, however, severity and pace of soft tissue calcification did not change. This
indicates that, within the range of the calcium concentrations used, the vascular calcification in the Abcc6−/− mouse does not depend on Ca intake. These results certainly do not provide a justification to diminish PXE patient Ca intake.

Mg intake does influence vascular calcification in the Abcc6−/− mouse

The next dietary mineral we tested was magnesium, since there are indications that this mineral influences soft tissue calcification [23]. Moreover, a dietary advice has been given to PXE patients to increase magnesium intake along with increased calcium intake [18]. In agreement with this advice, we found that a fourfold increase in both Ca and Mg concentration reduced blood vessel calcification in our PXE mouse model. This effect was clearly caused by the magnesium concentration since the fourfold increase of calcium alone did not produce this effect.

Recently, LaRusso and coworkers [24] evaluated the effect of dietary magnesium in a different, but highly comparable Abcc6 knockout mouse [14]. Their experimental setup consisted of a diet period of 8 weeks, and calcification was measured in the capsule surrounding the vibrissae. Three Mg concentrations were used: 0.04%, 0.22%, and 1.10%. The lowest two Mg concentrations resembled the concentrations of our baseline diet (0.05%) and of our 4×Ca, 4×Mg diet (0.2%). In contrast to our study, they did not find a significant difference in calcification between these two concentrations. This may be due to the relative short duration of their diet period, compared to our study. An important finding was that their highest Mg concentration (1.10%) completely prevented calcification during the 8-week diet period. Since our highest concentration was 0.2%, this extends our findings. It underscores the importance of further studies to establish the optimal Mg concentration and to determine the dependence of the effect on other factors, such as calcium and phosphate levels, as well as diet duration. In addition to these findings on Abcc6 knockout mice, also diet studied on DCC susceptible mice demonstrated an influence of dietary Mg on ectopic calcification [25].

The mechanism by which dietary magnesium exerts its influence on ectopic calcification is currently unclear. Magnesium ions function as co-factor in over 300 enzymatic reactions [23]. For a number of obvious mechanisms, we found no support in our data: For example, magnesium can bind phosphate, but we did not observe changes in plasma phosphate levels as result of higher magnesium intake. Alternatively, magnesium is a competitor of calcium and may substitute for Ca2+ in the crystal lattice, causing crystal poisoning [26]. For such a direct effect, the local magnesium concentrations clearly are of importance. We found indeed that the high Ca high Mg diet increased the plasma magnesium level, but no correlation between plasma magnesium levels and calcifications in the kidney was apparent. Finally, magnesium may also influence calcification by suppressing parathyroid hormone (PTH) secretion. As PTH is an important regulator of calcium homeostasis, LaRusso et al. [24] measured plasma PTH concentrations in Abcc6−/− mice in their dietary intervention study. However, high magnesium intake apparently did not change PTH levels.

While the present study shows that dietary magnesium modulates PXE severity in Abcc6−/− mice, it should be pointed out that we have no indications that a disturbed magnesium level is cause or part of the etiology of PXE. The present study as well as a previous study revealed no difference in plasma Mg levels between Abcc6−/− and wild-type mice [13]. Therefore, we have no concrete indications that the ABCC6 protein is directly involved in (co-)transport of magnesium.

Other dietary factors and PXE

The effect of dietary phosphorus has been studied in Abcc6−/− mice as well as in DCC susceptible mice. In Abcc6−/− mice, no significant effect was demonstrated, nor did the use of phosphate binders produce a clear effect [24, 27]. However, a high phosphorus diet exacerbated the DCC phenotype [25]. Probably, the combination of minerals in the food plays an important role as the most severe calcifications were found in DCC mice that received the combination of low magnesium and high phosphorus [25]. Interestingly, a study on PXE

| Table 2 | Plasma minerals (mmol/L ± SD) in Abcc6−/− and wild-type mice after 7 months of diet |
|-------|----------------------------------|----------------|-----------------|-----------------|----------------|
|       | Calcium                         | Phosphate       | Ca×PO4          | Ca/PO4          | Magnesium      |
| Baseline diet | 2.62±0.10a                      | 3.49±0.47       | 9.12±1.30       | 0.76±0.10       | 1.23±0.12b     |
| 4×Ca, 4×Mg diet | 2.85±0.12a                      | 3.97±0.52       | 11.35±1.74      | 0.73±0.08       | 1.46±0.20b     |
| 4×Ca diet     | 2.96±0.33a                      | 3.75±0.56       | 11.16±2.77      | 0.79±0.07       | 1.31±0.18b     |

Data of wild-type and Abcc6−/− mice were pooled since there was no difference between the genotypes

a Significant (P<0.05) differences between the baseline diet and the other two diets

b Significant differences (P<0.05) between 4×Ca, 4×Mg diet and other two diets
patients suggested that the use of aluminum hydroxide as oral phosphate binder may halt or reverse calcification in some patients [28].

Other dietary factors that have been studied in relation to DCC are dietary fluoride and dietary fat concentrations. Excessive fluoride consumption reduced pathological calcifications in the DCC mice [29]. The involvement of dietary fat in the DCC pathology is controversial. In one study, a high fat diet apparently did not affect calcification [30], but other studies suggest that a high fat content in the diet aggravates this phenotype [31, 32]. The possible effect of dietary fat is particularly interesting since we noticed that HDL-cholesterol level was decreased in the Abcc6−/− mice. We have found this before [13], but failed to reproduce this observation in plasma of PXE patients (unpublished results), so the relation with PXE pathology is at present unclear. The mouse model may differ in this aspect from the human situation.

In conclusion, while serum mineral balance in PXE is not altered, the findings on the Abcc6−/− mouse and on the DCC susceptible mouse suggest that cardiovascular features of PXE can be effectively modulated by dietary minerals. The task ahead of us now is to elaborate the formula of the optimal diet: a diet which maximizes the reduction of ectopic calcification while minimizing adverse effects of toxicity and osteoporosis. The Abcc6−/− mouse can be of great value for testing and design of such a diet. In addition, by examining old mice and extending the diet duration to more than 1 year, this animal model may also give an indication of the effect of dietary minerals on the calcification that occurs in the skin and eye of PXE patients.

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