Vitamin K does not prevent soft tissue mineralization in a mouse model of pseudoxanthoma elasticum

Christopher Brampton,1 Yukiko Yamaguchi,1 Olivier Vanakker,2 Lut Van Laer,2 Li-Hsieh Chen,1 Manoj Thakore,1 Anne De Paepe,2 Viola Pomozi,3 Pál T. Szabó,4 Ludovic Martin,5 András Váradi3 and Olivier Le Saux1,*

1Department of Cell and Molecular Biology; John A. Burns School of Medicine; University of Hawaii; Honolulu, HI USA 2Center for Medical Genetics; Ghent University Hospital; Ghent, Belgium; 3Institute of Enzymology; 4Chemical Research Center; Hungarian Academy of Sciences; Budapest, Hungary; 5Integrated Neurovascular and Mitochondrial Biology; University of Angers; Angers School of Medicine; Angers, France

Introduction

Pseudoxanthoma elasticum (PXE, OMIM 264800) in humans is defined by dystrophic and progressive mineralization of elastic fibers in cutaneous, ocular and vascular tissues. PXE is caused by mutations in ABCC6, which encodes a protein of the ATP-driven organic anion transporter family. The inability of this transporter to secrete its unknown substrate towards the circulation is hypothesized to be the cause of the PXE disease progression and describes PXE as a metabolic disorder.8 Control of bone mineralization and calcification of soft tissues is a highly regulated process, requiring a fine balance between inhibitors and promoters at both the circulatory and local level.9,10 Among the primary local regulators of mineralization, Matrix Gla Protein (MGP) and osteocalcin (OC) are reliant on carboxylation for their activation and function.10,11 Gheduzzi et al. described PXE patients with a lower serum concentration of MGP and a higher ratio of under carboxylated MGP than in controls. In addition, this under carboxylated MGP co-localized with calcified elastic fibers.10 Uitto et al. also found the connective tissue capsule surrounding the vibrissae of the Abcc6-/- mouse whisker was calcified and contained exclusively under

Key words: pseudoxanthoma elasticum, vitamin K, mineralization, Abcc6, mouse

Abbreviations: PXE, pseudoxanthoma elasticum; MGP, matrix gla protein; OC, osteocalcin; MK4, menaquinone-4; MRP6, multidrug resistance protein 6; ABCC, ATP-binding cassette subfamily C; VKD, vitamin K-dependent proteins; GGCX, gamma-glutamyl carboxylase; VKORC1, vitamin K 2,3 epoxide reductase; WT, wild-type
carboxylated MGP. Vitamin K is an essential component in the post-translational carboxylation of glutamate residues (Glu) in these inhibitor proteins. The reduced form of vitamin K acts as a co-factor in the conversion of the Glu (inactive) form to the Gla form (γ-carboxyglutamate). With this in mind, it was recently hypothesized that a vitamin K precursor or a conjugated form was the potential substrate(s) for ABCC6.13

It is also known that PXE patients have low serum vitamin K levels and it was proposed that this low level might result in insufficient carboxylation of the circulating inhibitors of mineralization thus leading to mineral deposition in peripheral tissues. A subgroup of mutations in the gamma-glutamyl carboxylase—gene (GGCX) that encodes an enzyme essential in the carboxylation of Glu proteins results in a PXE-like phenotype and some coagulation factor deficiency. Mutations in the vitamin K 2,3 epoxide reductase (VKORC1) however, only result in deficiencies in the vitamin K dependent clotting factors (II, VII, IX, X).14 The link between vitamin K, vitamin K-dependent (VKD) proteins and PXE or PXE-like phenotypes has been previously discussed in references 15 and 16.

Vitamin K is a group of lipophilic, hydrophobic compounds. Of the two forms of naturally occurring vitamin K, vitamin K1- phylloquinone (phytomenadione), is the primary source for humans. Obtained from the leafy green plants and vegetables, it is poorly absorbed but has a high hepatic concentration. Vitamin K2 or menaquinones, are more unsaturated and have a variable number of isoprenyl groups (four in the case of MK4). There is an increased concentration of the menaquinones in extra-hepatic tissue compared to vitamin K1. MK4 is synthesized by mammalian tissues and additionally converted from vitamin K1.17,18 Some functions of the menaquinones and vitamin K1 appear interchangeable, notably in the activation of clotting factors but others such as bone resorption are more specific to the particular chemical structure of the type of vitamin K. However, one should note that depending on the type of vitamin K being tested, either phylloquinone or menaquinone, the efficacy of these compounds seems to depend on the specific circumstances being investigated.19,20 Of particular relevance to this investigation are studies using a rat model of arterial calcification. Here vitamin K1 and vitamin K2 (MK4) were shown to be equally effective in reversing the warfarin-induced calcification, thus implying interchangeable roles for these two compounds within this particular model.21

The phenotype of the Abcc6-/- mice we used has previously been described in reference 23. Two Abcc6 knockout mice essentially recapitulate the PXE disease progression with mineralization of the skin, the Bruch’s membrane of the eye and small size arteries.23,24 One notable feature of these mice, and of particular interest in this study, is the calcification of the vibrissae’s capsule in the whiskers, the connective tissue capsule surrounding the hair follicle.23,24 The calcification of this tissue (not present in humans) occurs at approximately 5 weeks of age and is reported to be progressive23 and quantifiable and thus serves as a marker of disease progression.25

To address the hypothesis that a vitamin-K dependent calcification inhibition process plays a role in the pathology of PXE15,16 we investigate whether a high dietary intake of vitamin K could be beneficial to PXE patients. For this purpose, we used an Abcc6-/- mouse model of the disease. We aimed to discover whether the provision of a diet enriched with two active forms of vitamin K (K1 and MK4) could compensate any negative effects of the Abcc6 knockout on the development of mineralization. Secondly, we investigated whether the lack of Abcc6 had any effect on the absorption, metabolism or distribution of vitamin K compared to WT controls under normal diet conditions.

### Results

#### Progression of the PXE phenotype.

In order to determine the optimum time range to conduct our experiments to stop or minimize the progression of calcification, we precisely quantified the mineralization of the whisker over the life span of Abcc6-/- mice (approximately 28–32 months). We established that within the period from 3 weeks to approximately 6 months of age there is a near-exponential rate of deposition of calcium in the vibrissae. This rate of calcification slows after 6 months of age and reaches a gently sloping plateau thereafter up to the natural demise of the animal (Fig. 1).

**Effects of vitamin K supplementation.** The diet schedule of 5 mg (low dose) or 100 mg/kg (high dose) vitamin K1 or vitamin MK4 corresponded to a 5-fold and 100-fold increase respectively in vitamin K1 above normal diet. For the MK4 supplementation, the actual increase could only estimated to be in the similar range of 5 to 100-fold because this form of vitamin K2 can be readily converted in vivo from vitamin K1, which is naturally present as traces in the animal chow and can also be synthesized by extra-hepatic tissues.26 However, given the large increase in serum concentration of MK4 in mice fed the MK4 enriched diet, the diet supplementation in vitamin MK4 produced the expected boost in the circulation.

Interestingly, mice fed with both concentrations of the enriched vitamin K1 diet showed no overt adverse effects on physiology or behavior, whereas Abcc6-/- mice fed with a high dose of MK4 had marked liver discoloration, with small distinct lesions on the liver surface. These mice also showed an apparent increased propensity to blood clotting observed upon blood withdrawal. Approximately 50% of the WT mice fed the enriched high dose MK4 diet also had areas of hair loss and skin lesions consistent with increased scratching. However, no change in the gross appearance of their liver was observed.

**Vitamin K1 or MK4 enriched diets do not affect tissue mineralization in Abcc6-/- mice.** Neither of the vitamin K enriched diets or treatment times had any effect on the total calcium content of the vibrissae tissue for WT mice, which contained very low levels of calcium (Figs. 2 and 3). In contrast to WT mice, Abcc6-/- mice fed with the normal diet up to 5 months of age had significantly elevated total calcium content levels in the vibrissae area. Importantly, we measured no significant change in the total calcium content of the vibrissae between Abcc6-/- mice fed on a normal diet or the vitamin K1 enriched diet for any of the three treatment groups (in utero, from pre-onset or from post-onset of phenotype). In each case, we measured significant increases
circulating levels of vitamin K1 and MK4 in the serum of mice fed with either a normal diet, or vitamin K1 or vitamin MK4 low and high dose enriched diets. Baseline vitamin K1 serum content was defined from WT mice fed a normal diet. In both the control WT pre-onset and 3 months feeding groups fed with a high dose of vitamin K1, we detected a significant increase in vitamin K1 levels above baseline. However, we were unable to detect significant increases in vitamin K1 from WT mice fed on the low dose diets, thus indicating that 5 mg/kg was ineffective at significantly raising the concentration of circulating vitamin K1 in these animals (Fig. 5). Although we saw some increase in the levels of MK4 circulating in the serum of Abcc6-/− mice fed with the high dose, 100 mg/kg, vitamin K1 at pre-onset and the in utero group, it was not a statistically significant increase (Fig. 5). Moreover, in comparison to paired WT controls, the Abcc6−/− mice in the pre-onset group fed the high dose diet had significantly less vitamin K1 circulating thus indicating that Abcc6 could play some role in vitamin K1 absorption or circulation (Fig. 5).

Our studies also showed interesting differences in the levels of circulating MK4 in mice fed exclusively with normal or vitamin K1 enriched diets. In mice fed a normal diet, we measured significantly more circulating MK4 in WT mice than in Abcc6−/−.
detected very low levels of circulating MK4 in the Abcc6-/- in utero group fed with the high, 100 mg/kg, vitamin K2 diet and at present we are unable to explain this result, though one may suggest an adaptation of the mice to a greater influx of the vitamin from the diet over a longer period of time. The carboxylation status of MGP and OC in the capsule of the vibrissae. We found no change in the carboxylation status of MGP in the capsule surrounding the vibrissae. The ucMGP was found mainly associated with the calcified area. MGP detected with an antibody that targets both under-carboxylated and carboxylated MGP was found also throughout the calcified regions of the capsule. We found no major association between OC and the calcification in the vibrissae of Abcc6-/- mice. Furthermore, the diet, whether normal or enriched with vitamin K, produced no visible change in OC levels in the vibrissae’s capsule (data not shown).

Blood chemistry. We found no significant difference in the blood chemistry between mice fed either a normal diet or the vitamin K1 5 or 100 mg/kg diets for all parameters. Ion levels, blood glucose, pH etc., were all within normal physiological

Similarly in mice fed the high dose vitamin K1 diet, WT mice had significantly more circulating MK4 than Abcc6-/- mice (Fig. 6). In general, in Abcc6-/- mice fed the enriched vitamin K1 diet, we were able to measure an increase in circulating MK4 compared to Abcc6-/- mice fed on a normal diet. However, this increase was not statistically significant (Fig. 6).

A vitamin MK4-enriched diet boost MK4 circulating levels. Serum analysis from WT mice fed on a diet enriched with 5 mg/kg of vitamin MK4 showed significantly increased levels of this compound indicating that this dosage was quite effective in raising the serum levels of MK4. In Abcc6-/- mice however, the increase in circulating MK4 from mice fed on the low dose MK4 diet was not significant. In contrast, the Abcc6-/- mice fed with the high dose of MK4 from mice fed on the low dose MK4 diet was not significant. In contrast, the Abcc6-/- mice fed with the high dose of MK4 in both the pre- and post-onset groups had significantly increased levels of circulating MK4 compared to WT and Abcc6-/- mice fed a normal diet. It is important to note that this data indicates that in Abcc6-/- mice, MK4 was available to the periphery in quantities greater than seen in WT or Abcc6-/- animals on a normal diet (Fig. 7). Also, we detected very low levels of circulating MK4 in the Abcc6-/- in utero group fed with the high, 100 mg/kg, vitamin K2 diet and at present we are unable to explain this result, though one may suggest an adaptation of the mice to a greater influx of the vitamin from the diet over a longer period of time.

The carboxylation status of MGP and OC in the capsule of the vibrissae. We found no change in the carboxylation status of MGP in the capsule surrounding the vibrissae. The ucMGP was found mainly associated with the calcified area. MGP detected with an antibody that targets both under-carboxylated and carboxylated MGP was found also throughout the calcified regions of the capsule. We found no major association between OC and the calcification in the vibrissae of Abcc6-/- mice. Furthermore, the diet, whether normal or enriched with vitamin K, produced no visible change in OC levels in the vibrissae’s capsule (data not shown).
levels as seen with the control normal diet fed mice (data not shown).

**Discussion**

Mutations in \( ABCC6 \) alone cannot explain the high phenotypic variation in PXE patients.\(^{27,28} \) More emphasis is now being placed on environmental factors such as: diet, smoking and hormone balance to explain the complexity of the intra/inter familial phenotypic variation.\(^{29,30} \) Although PXE patients have no deficiency in the vitamin K-dependent clotting factors synthesized within the liver, which incidentally suggest an adequate supply of vitamin K in this tissue, they have very low concentrations of this vitamin in the periphery.\(^{16} \) Additional work by Vanakker et al. established a strong link between low serum concentration of vitamin K and decreased carboxylation of inhibitors of the mineralization process in the plasma and serum such as MGP and OC.\(^{16} \) Studies in rats have also shown that supplementation with vitamin K can reverse arterial calcification induced by warfarin, a known inhibitor of the vitamin K cycle. This is probably due to an increase in carboxylated MGP levels at the site of the calcium deposit.\(^{22} \) This and the fact that PXE is a metabolic disease, and that a PXE-like disease with a similar phenotype is related to vitamin K, has led to the hypothesis of \( ABCC6 \) as a transporter of vitamin K.\(^{13} \) The aim of this study was therefore to investigate whether a large increase in vitamin K from the diet could raise the peripheral availability of vitamin K and thus have an effect on the calcification process in \( Abcc6^{\text{--/--}} \) mice, an animal model for PXE.

The link between vitamin K and mineralization is now well established,\(^{31} \) but due to the nature of vitamin K being a family of chemically related compounds with differing chemical structures, their respective activities are not necessarily fully understood. The type and dosage of vitamin K can also have vastly different effects on the model being studied. For example the Rotterdam study, a prospective, population-based cohort study, has shown that menaquinone but not phylloquinone is important in preventing vascular calcification and coronary heart disease.\(^{32} \) However, studies in rats using plasma prothrombin levels as a marker for vitamin K status have shown that low dietary doses of phylloquinone is more effective at maintaining vitamin K status than menaquinone.
introduced by choice and depth of sections to analyze. This method is very sensitive as we were able to reliably detect a significant increase in amount of calcification between groups of Abcc6-/- mice aged 4 weeks apart. Using total calcium quantification, we confirmed our findings initially obtained with Alizarin Red S staining and found no effect of any of the feeding regimes of either vitamin K1 or MK4 on total calcium content of the muzzle tissue in Abcc6-/- mice (Figs. 2 and 3). In addition, we compared kidney tissue sections from vitamin K treated Abcc6-/- mice and found typical levels of calcification (data not shown).23,33 Because of the lack of effect of vitamin K supplementation on the progression and severity of the mineralization phenotype of Abcc6-/- mice, we verified how much of the dietary increase in vitamin K1 and MK4 was actually absorbed, (MK9), but at higher doses the activity is the same.21 For this reason we designed our experiments using two types of commercially available vitamin K (K1 and MK4) to increase the probability of measuring an effect of this vitamin on the disease progression.

One advantage of using the Abcc6-/- mouse model is the consistency of its biomarkers of disease progression. The level of calcification in the bulb of the vibrissae between the Abcc6-/- mice is very consistent and also easily measurable. When compared with staining typically observed in Abcc6-/- mice fed with a control diet, we found no alteration in the staining patterns around the bulb of the vibrissae in any of the vitamin K treatment groups (Fig. 4). Analyzing the total calcium content of the muzzle tissue negates the need for time-consuming morphometric analysis of stained tissue sections and additionally removes any bias introduced by choice and depth of sections to analyze. This method is very sensitive as we were able to reliably detect a significant increase in amount of calcification between groups of Abcc6-/- mice aged 4 weeks apart. Using total calcium quantification, we confirmed our findings initially obtained with Alizarin Red S staining and found no effect of any of the feeding regimes of either vitamin K1 or MK4 on total calcium content of the muzzle tissue in Abcc6-/- mice (Figs. 2 and 3). In addition, we compared kidney tissue sections from vitamin K treated Abcc6-/- mice and found typical levels of calcification (data not shown).23,33

Because of the lack of effect of vitamin K supplementation on the progression and severity of the mineralization phenotype of Abcc6-/- mice, we verified how much of the dietary increase in vitamin K1 and MK4 was actually absorbed,
This discrepancy in circulating serum K1 levels between the two strains indicates that in Abcc6−/− mice the vitamin K1 is absorbed from the chow and re-circulated into the bloodstream but with less efficiency than in WT. In our mice treated with the high dose diet of MK4, we measured no difference between circulating MK4 in WT or Abcc6−/− mice indicating a good absorbance of this form of vitamin K from the diet. Additionally, these data show that elevated levels of vitamin K1 and MK4 were effectively absorbed from the diet and are able to pass into the circulation in WT and Abcc6−/− animals, and thus potentially available to peripheral tissues for the activation of MGP by carboxylation. We detected an increase in total MGP in the vibrissae of Abcc6−/− mice in association with areas of mineralization as expected. However, large proportion of this MGP remained un-carboxylated despite the increase in the availability of vitamin K. Nevertheless, this increase appears ineffectual in overcoming the calcium deposition because the vast majority of this overproduced MGP is not carboxylated.

**Figure 5.** HPLC-MS analysis of serum levels of vitamin K1 from mice fed diets enriched with 5 or 100 mg/kg of vitamin K1. We measured a general, non-significant increase in circulating vitamin K1 levels in the serum of both WT and Abcc6−/− mice fed on a diet enriched with 5 mg/kg of vitamin K1. When fed with a diet enriched with 100 mg/kg vitamin K1, WT mice had significantly more circulating vitamin K1 than the Abcc6−/− mice, with no significant increase in circulating vitamin K1 measured in Abcc6−/− mice fed with this diet.

Pass into the circulation and was available to peripheral tissues. The reduction in circulating vitamin K1 levels measured in human PXE patients was not reproduced in our mouse model. We measured no significant difference (Fig. 5) in the serum baseline concentration of circulating vitamin K1 in the Abcc6−/− and WT animals fed with a normal diet, with both strains of mice having a low concentration of this vitamin K form and therefore providing the first indication that a reduced peripheral vitamin K1 concentration is not a factor in the mouse mineralization phenotype. However, we did measure a significantly lower baseline circulating level of MK4 in the Abcc6−/− mice compared to WT. This not only indicates less availability of this form of vitamin K for carboxylase function at the periphery, but also suggests a lower rate of conversion from vitamin K1 in the knockout animals. Both WT and Abcc6−/− mice fed with high dose vitamin K1 diet had increased levels of both vitamin K1 and MK4 (Figs. 5 and 6), indicating that the conversion of MK4 from vitamin K1 followed the increase in the availability of phyloquinone. However, although the high vitamin K1 diet induced a significant increase in vitamin K1 serum concentration in Abcc6−/− mice, the increase was significantly less than the equivalent serum K1 levels in control WT mice (Fig. 5).
Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

Based on the discrepancy in MK4 serum levels between WT and Abcc6−/− mice fed on a normal diet and the lack of increase of MK4 serum levels in the vitamin K1 fed Abcc6−/− mice, which was most likely due to less K1 being available for conversion to MK4 (Fig. 6), we suggest that Abcc6 might be involved in vitamin K transport, absorption or metabolism in the body, but only to a limited degree. This would be in agreement with Vanaker et al. (2010).6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

Based on the discrepancy in MK4 serum levels between WT and Abcc6−/− mice fed on a normal diet and the lack of increase of MK4 serum levels in the vitamin K1 fed Abcc6−/− mice, which was most likely due to less K1 being available for conversion to MK4 (Fig. 6), we suggest that Abcc6 might be involved in vitamin K transport, absorption or metabolism in the body, but only to a limited degree. This would be in agreement with Vanaker et al. (2010).6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.

In summary, the results of the present study and those from Vanakker et al. (2010)6 Along with the fact that the livers of the Abcc6−/− mice on the highest vitamin MK4 dose (but not the livers of the WT animals) were discolored and had an altered physical appearance leads us to infer that a reduced amount of vitamin K reaches the periphery, with a potential hepatic bottle neck. It is known that Abcc6 is present in the gut. If the transport from the gut is adversely affected by the loss of Abcc6 and therefore isn’t fully absorbed, and/or additional transport from the liver into the peripheral tissue would also be reduced leading to the observed changes in liver appearance. This hypothesis could be relatively easily explored using other routes of delivery to raise the vitamin K levels in the periphery, such as intravenous administration, but due to our results showing that inducing an increase in MK4 is possible in the Abcc6−/− mice (however not effective in treating the disease progression), it is not easily rationalized.
maximal mineralization and to cover several treatment options for different developmental ages: (1) Vitamin K-enriched feeding from in utero to 4.5 months aimed to prevent calcification with the maximum time of increased vitamin K exposure. These mice were exposed to vitamin K in utero, breast-fed from vitamin K-fed mothers and then weaned on enriched vitamin K diets. Next, (2) by treating mice from weaning age at 3 weeks, which is before the onset of the phenotype (pre-onset), to 4.5 months old we sought to test whether the mineralization phenotype could be averted by exposing young mice to the diets. Finally, (3) mice were fed from 3 months of age, which is after the onset of the phenotype (post-onset) for 12 weeks. The latter experiment was designed to test whether treatment with the vitamin K diets could stop or possibly reverse calcification progression. This particular treatment regime is likely the most applicable as a potential treatment to the PXE condition since diagnosis is usually made after the phenotype has occurred.

K-dependent inhibition of calcification may not be as preponderant in the development of this disease as previously thought.

**Materials and Methods**

**Animals.** C57BL/6J mice designated here as wild-type (WT) controls were purchased from Jackson laboratories. *Abcc6*−/− mice were obtained from the laboratory of Dr. J. Uitto at the Thomas Jefferson University via a standard UBMTA. Mice used in these study have been housed and cared for in an approved Animal Care facility in the BioScience Building of the University of Hawaii School of Medicine. The mice were kept under routine laboratory conditions with 12 hours light-dark cycle with access ad libitum to water and standard chow. The Institutional Animal Care and Use Committee of the University of Hawaii approved this study. Experiments have been conducted according to national guidelines.

**Diet.** *Abcc6*−/− and WT mice were placed on a diet enriched with either 5 (low dose) or 100 mg/kg (high dose) of vitamin K1 (phyloquinone) or vitamin K2, specifically MK4. The specifically formulated diets were purchased from Bio-Serv, Inc., (AIN-93M). We designed our vitamin K-enriched diet experiment to take place within the first 5 months of life during the phase of maximal mineralization and to cover several treatment options for different developmental ages: (1) Vitamin K-enriched feeding from in utero to 4.5 months aimed to prevent calcification with the maximum time of increased vitamin K exposure. These mice were exposed to vitamin K in utero, breast-fed from vitamin K-fed mothers and then weaned on enriched vitamin K diets. Next, (2) by treating mice from weaning age at 3 weeks, which is before the onset of the phenotype (pre-onset), to 4.5 months old we sought to test whether the mineralization phenotype could be averted by exposing young mice to the diets. Finally, (3) mice were fed from 3 months of age, which is after the onset of the phenotype (post-onset) for 12 weeks. The latter experiment was designed to test whether treatment with the vitamin K diets could stop or possibly reverse calcification progression. This particular treatment regime is likely the most applicable as a potential treatment to the PXE condition since diagnosis is usually made after the phenotype has occurred.

**Histochemistry and immunohistochemistry.** Direct histological visualization of calcium deposition following Alizarin red S staining on paraffin-embedded sections was carried out on the left muzzle skin of each mouse. Briefly, 5 μm slides were deparaffinized and incubated in Alizarin red S solution (Sigma) for 5 minutes, excess dye was removed with a 50% mixture of acetone.
eral deposition in the different groups of mice we carried out a
CS3 (Adobe, San Jose, CA).

Individual images were collected and processed with Photoshop
an Axioscope 2 fluorescent microscope (Zeiss, Thornwood, NY).

in the capsule of the vibrissae was determined by imaging using
(Invitrogen, Carlsbad, CA). The distribution of MGP and OC
Temecula, CA). The secondary antibody was Alexafluor 488
was visualized with a rabbit polyclonal antibody (Millipore,
tify total MGP (carboxylated and uncarboxylated) while OC
detect the uncarboxylated MGP. A rabbit polyclonal anti-tMGP
ucMGP antibody (VitaK BV, Maastricht) was used to specifically
using 5 μ

staining of whisker samples from high dose diets was performed
(OCT) compound and stored at -80°C. Immunofluorescent
quickly harvested, placed in Optimum Cutting Temperature
Scientific). Selected sections from each treatment group were
examined for calcium deposition and images were collected using
an Axioscope 2 fluorescent microscope.

For immunofluorescent staining, muzzle skin samples were
quickly harvested, placed in Optimum Cutting Temperature
(OCT) compound and stored at -80°C. Immunofluorescent
staining of whisker samples from high dose diets was performed
using 5 μm-thick frozen sections. A mouse monoclonal anti-
ucMGP antibody (VitaK BV, Maastricht) was used to specifically
detect the uncarboxylated MGP. A rabbit polyclonal anti-tMGP
antibody (Santa Cruz biotech, Santa Cruz, CA) was used iden-
tify total MGP (carboxylated and uncarboxylated) while OC
was visualized with a rabbit polyclonal antibody (Millipore,
Temecula, CA). The secondary antibody was Alexafluor 488
(Invitrogen, Carlsbad, CA). The distribution of MGP and OC
in the capsule of the vibrissae was determined by imaging using
an Axioscope 2 fluorescent microscope (Zeiss, Thornwood, NY).
Individual images were collected and processed with Photoshop
CS3 (Adobe, San Jose, CA).

Colorimetric measurement. To quantify the levels of mineral
deposition in the different groups of mice we carried out a
colorimetric assay35 that measures directly the amount of cal-
cium within excised muzzle tissue, which is then corrected for
tissue weight. Briefly, the entire skin from one side of the muzzle
was harvested, minced and incubated at room temperature for
48 hours in 0.15 N HCl. The total calcium content of the HCl
supernatant was assessed by measuring the absorbance at 550 nm
using the Calcium (CPC) Liquicolor kit (Stanbio). Calcium con-
tent was normalized to total tissue dry weight prior to mincing.
The obtained absorbance values were quantified against a known
standard to provide calcium concentration in mg per deciliter
and per gram of tissue.

Blood analysis. Blood samples were harvested from each
mouse and the serum was immediately separated by low-speed
centrifugation and stored at -80°C until HPLC-MS analysis
of total serum vitamin K1 and vitamin MK4 content could be
performed. Vitamin K1 and K2 were extracted as described in
Spronk et al. (2003),36 with some modifications. Briefly: 25 μl
of plasma sample was diluted 1:1 with PBS. For deproteination, 200 μl
ethanol was added and vigorously vortexed. Vitamin Ks were
extracted with 600 μl n-hexane. After one minute vortexing the
samples were centrifuged (5 min at 1,000 rpm) and the hexane-
phase was separated and dried under a stream of nitrogen. The
extracted material was dissolved in 50 μl isopropanol, and kept at
-20°C until HPLC-MS analysis in sealed glass vials. For calibra-
tion purposes 0, 1, 5, 25 and 125 nM vitamin K1 and vitamin K2
was added to WT mouse serum and the extraction was carried out
in the same way.

Mass spectrometric measurements were run on an AB Sciex
3200 QTrap tandem mass spectrometer. The components were
ionized in negative ion mode using an atmospheric pressure
chemical ionization (APCI) source. The instrument was scanned
in multiple reaction-monitoring (MRM) mode. The MRM
transition was 444/185 for MK4 and 450/185 for vitamin K1.
The samples were separated prior to mass spectrometric analysis
using a Perkin Elmer HPLC system. Mobile phases were: water
and acetonitrile with a flow rate of 0.4 ml/min. A Phenomenex
LUNA Phenyl-hexyl column (C18, 55 x 2 mm, 3 μm particle
size) was used for the separation. The oven temperature was kept
at 40°C.

Additionally, analysis of blood chemistry of treated and con-
trol mice was performed using the i-STAT EC8+ system (Abbott).

Statistical analysis. Data were analyzed with the statistical
software PRISM® (GraphPad). Statistical analyses were per-
formed using two-tailed unpaired Student’s t-tests to determine
statistical difference between WT and Abcc6-/- groups. Results
were expressed as mean ± SEM and considered significant for p <
0.05, *p < 0.05, **p < 0.01 ***p < 0.001.

Acknowledgments
The authors would like to acknowledge Dr. Jouni Uitto’s labo-
atory for providing the Abcc6-/- mice used for this study. This
work is supported by funding from NIH HL087289, AHA
11GRNT5840005 and a research grant from PXE France (O.
le Saux); Dr. A. Varadi was funded by NIH R01AR055225, by
PXE Int. and Hungarian research grants OTKA CK 80135 and
OTKA 81204.
References