

Low serum vitamin K in PXE results in defective carboxylation of mineralization inhibitors similar to the GGCX mutations in the PXE-like syndrome

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Soft-tissue mineralization is a tightly regulated process relying on the activity of systemic and tissue-specific inhibitors and promoters of calcium precipitation. Many of these, such as matrix gla protein (MGP) and osteocalcin (OC), need to undergo carboxylation to become active. This post-translational modification is catalyzed by the gammaglutamyl carboxylase GGCX and requires vitamin K (VK) as an essential co-factor. Recently, we described a novel phenotype characterized by aberrant mineralization of the elastic fibers resulting from mutations in *GGCX*. Because of the resemblance with pseudoxanthoma elasticum (PXE), a prototype disorder of elastic fiber mineralization, it was coined the PXE-like syndrome. As mutations in *GGCX* negatively affect protein carboxylation, it is likely that inactive inhibitors of calcification contribute to ectopic mineralization in PXE-like syndrome. Because of the remarkable similarities with PXE, we performed a comparative study of various forms of VK-dependent proteins in serum, plasma (using ELISA), and dermal tissues (using immunohistochemistry) of PXE-like and PXE patients using innovative, conformation-specific antibodies. Furthermore, we measured VK serum concentrations (using HPLC) in PXE-like and PXE samples to evaluate the VK status. In PXE-like patients, we noted an accumulation of uncarboxylated Gla proteins, MGP, and OC in plasma, serum, and in the dermis. Serum levels of VK were normal in these patients. In PXE patients, we found similar, although not identical results for the Gla proteins in the circulation and dermal tissue. However, the VK serum concentration in PXE patients was significantly decreased compared with controls. Our findings allow us to conclude that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient protein carboxylation of VK-dependent inhibitors of calcification. Although in PXE-like patients this is due to mutations in the *GGCX* gene, a deficiency of the carboxylation co-factor VK is at the basis of the decreased activity of calcification inhibitors in PXE.

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Soft-tissue calcification is a highly regulated process relying on both systemic and tissue-specific balances of inhibitors and promoters of calcium precipitation. Fetuin-A (α 2-Schmid-Herremans glycoprotein), a member of the cystatin family of protease inhibitors produced predominantly by the liver, is considered an important circulating inhibitory factor of soft-tissue mineralization.¹ Among local regulators, matrix gla protein (MGP), osteopontin (OPN), and bone morpho-

genetic protein-2 (BMP-2) are diversely associated with tissue mineralization *in vivo*.^{2–4} Proteins primarily involved in the regulation of bone mineralization, such as osteocalcin (OC) or osteonectin (ON), have also been linked to local extra-osseous regulation of calcium homeostasis.⁵

Among all, MGP is considered the key physiological inhibitor of soft tissue calcification, acting as a direct inhibitor of calcium crystal formation, as MGP-deficient mice

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suffer from spontaneous and ultimately fatal calcification of arteries and cartilage.⁶ Together with OC and the clotting factors II, VII, IX, and X, MGP needs to undergo carboxylation of glutamate (Glu) into γ -carboxyglutamate (Gla) residues to become active. Carboxylation catalyzed by the endoplasmic gamma-glutamyl carboxylase (GGCX) to form Gla residues in all these proteins requires vitamin K (VK) as a co-factor, hence the terms 'VK dependent' or 'Gla' proteins.⁷ MGP, unique among the Gla proteins, also has the tandemly repeated Ser-X-Glu sequence at the N-terminus end, which is a recognition motif for serine phosphorylation. Phosphorylation is thought to be carried out by the Golgi casein kinase.^{8,9} It has been suggested earlier that—next to being carboxylated—MGP must be fully phosphorylated at each target serine residue to have its optimal inhibitory activity on soft tissue calcification.⁹

Besides the γ -carboxylase, the metabolic VK cycle also involves the reductase enzyme VKORC1. Mutations in the *GGCX* or *VKORC1* genes are associated with a hereditary deficiency of the VK-dependent clotting factors as well as a clinically relevant dosing dependency of anti-coagulants.^{10,11} Besides these enzymatic defects, a deficiency of VK has been described in association with coagulation, bone (osteoporosis, osteoarthritis), and vascular (arteriosclerosis) disorders resulting from insufficient carboxylation of Gla proteins.^{12,13}

We recently described an additional phenotype resulting from sequence changes in the *GGCX* gene (chrom. 2p12; OMIM#137167). This novel autosomal recessive disorder was characterized by a generalized deficiency of the VK-dependent clotting factors as well as mineralization and fragmentation of elastic fibers leading to thickened, inelastic skin, and limited retinopathy.¹⁴ This disease was coined the 'pseudo-xanthoma elasticum (PXE)-like syndrome' (OMIM#610842) because of its remarkable clinical and histological resemblance with PXE (OMIM#264800), a prototype disorder of elastic fiber mineralization.^{15,16} PXE is caused by mutations in the *ABCC6* gene (chrom. 16p13.1; OMIM#603234), coding for an ATP-dependent transmembrane transporter, which is most abundant in liver and kidney.^{17,18} Neither the substrate of *ABCC6* nor its relation to ectopic mineralization or elastic fiber changes observed in PXE is presently known.

As *GGCX* mutations in the PXE-like syndrome negatively affect protein carboxylation, we and others hypothesized that elastic fiber mineralization results from deficient VK-dependent inhibitors of calcification, such as MGP and OC. Recently reported findings already point toward a role of MGP in classic PXE, but neither the involvement of other Gla proteins nor the mechanism for such involvement have been studied yet.^{19–22} Because of the remarkable phenotypical similarities of the PXE-like syndrome and PXE and the absence in PXE of *GGCX* or *VKORC1* mutations, which both have the potential of disrupting the VK cycle, we wondered whether the VK cycle could also be disturbed by another mechanism in PXE. More precisely, we hypothesized that

disturbances in Gla proteins in PXE patients could be linked to VK, the co-factor in the VK cycle and an essential mediator of Gla-protein carboxylation.

We aimed to evaluate these hypotheses by studying the various forms of Gla proteins in serum and tissues of PXE-like syndrome and PXE patients by using innovative antibodies. The latter were designed to specifically measure and differentiate between carboxylated and uncarboxylated forms of the proteins in immunohistochemistry (IHC) as well as in ELISA assays. Furthermore, we evaluated the VK status in PXE-like and PXE patients by HPLC measurement of VK serum concentrations.

MATERIALS AND METHODS

Patients

PXE and PXE-like patients were clinically evaluated in the Department of Genetics of the Ghent University Hospital (Belgium), the Department of Dermatology of the Orleans Hospital (France), and the Department of Biomedical Sciences, University of Modena and Reggio Emilia (Italy). The diagnosis of PXE or the PXE-like syndrome was confirmed via molecular analysis of the *ABCC6* or *GGCX* gene, respectively, the methodology and results of which have been reported earlier.^{14–16} The demographic characteristics of the patient cohorts used in this study are summarized in Table 1. Informed consent was obtained from all patients and the Declaration of Helsinki protocols were followed. This study was approved by the Ethical Committee of the Ghent University Hospital.

Biochemical Measurements

Serum was prepared by incubating the samples for 20 min at RT and subsequent centrifugation (10 min, 3000 \times g); plasma was prepared in citrate tubes. All samples were frozen within 2 h after blood collection.

Mineralization inhibitor levels were quantified in serum and citrate plasma of 4 PXE-like and 16 PXE patients using the ELISA technique. For measurement of the total fraction of uncarboxylated MGP (ucMGP), a competitive ELISA assay was applied.^{23,24}

Two additional sandwich ELISAs were developed at VitaK BV, Maastricht, The Netherlands, to determine the respective plasma levels of desphospho carboxylated (dp-cMGP) or desphospho uncarboxylated (dp-ucMGP) MGP. In brief, monoclonal anti-dpMGP was coated to the microtiter plate. After blocking, either patient plasma or standards were incubated. The standard peptide was synthetic MGP, based on the non-phosphorylated 3–15 aa sequence and either the non-carboxylated 35–54 aa sequence or carboxylated 35–54 aa sequence, linked with a hydrophilic spacer (Pepscan, Lelystad, The Netherlands). After incubation and washing, the standard or sample was detected using a biotinylated monoclonal ucMGP antibody or biotinylated cMGP antibody.

Table 1 Demographic characteristics of the patient and control cohorts

Experiment	Patient cohort	F:M ratio	Mean age (years)	Mean duration of disease (years)
ELISA calcification inhibitors	16 PXE	13:3	56 (14; 28–75)	15 (11; 3–40)
	4 PXE-like	4:0	50 (13; 33–71)	15 (10; 6–32)
	55 Controls	29:26	58 (10; 30–75)	—
HPLC vitamin K	30 PXE	20:10	49 (15; 14–79)	16 (14; 2–41)
	4 PXE-like	4:0	50 (13; 33–71)	15 (10; 6–32)
	34 Controls	21:13	45 (14; 18–75)	—
Immunohistochemistry	9 PXE	5:4	52 (17; 31–79)	14 (10; 5–40)
	3 PXE-like	3:0	57 (9; 50–71)	15 (12; 6–32)
	7 Controls	5:2	55 (10; 33–76)	—

F, female; M, male.

Mean age (s.d.; range); mean duration of disease (s.d.; range).

For measurements of total OC, uncarboxylated (ucOC), and carboxylated (cOC) OC, we used conformation-specific sandwich ELISAs (Takara Shuzo, Shiga, Japan). Results of the MGP and OC measurements were compared with serum levels obtained in a for sex and age comparable control population of 55 individuals (Table 1).

Vitamin K₁ serum concentrations were assessed in 4 PXE-like and in 30 PXE patients using an HPLC technique with post-column reduction and fluorescence detection, as described earlier.^{25,26} None of the patients were taking VK supplements or coumarins at the time of measurement. Serum VK₁ levels in patients were compared with those of a sex and age comparable reference population of 34 healthy men and women (Table 1).

Immunohistochemistry

IHC was performed on skin biopsy specimens from three female patients with the PXE-like syndrome reported in an earlier study,¹⁴ and on skin samples from nine patients (one male and eight female patients) with a clinical, histological, and molecular diagnosis of PXE. Normal age- and sex-matched skin biopsy samples from seven individuals and lesional skin from three patients with solar elastosis or elastofibroma were used as controls (Table 1).

Light microscopy was performed on tissues embedded in paraffin. Antibodies against cMGP, ucMGP, fetuin-A, ON, BMP-2, gas-6, and OC were provided by VitaK (Maastricht, The Netherlands).²³ The mAb against OC and polyclonal antibodies against gas6, fetuin, OPN, ON, and BMP-2 stained total protein.²⁷ After deparaffinization, sections were heated in 0.2% citric acid at pH 6.0, washed with phosphate-buffered saline and incubated with the primary antibody. For the polyclonal antibodies, citric acid treatment was not added. All antibodies were diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Negative controls were

performed by omitting the primary antibody. Biotinylated sheep anti-mouse IgG (monoclonal antibodies—Amersham Biosciences, Little Chalfont, UK), swine anti-rabbit IgG (polyclonal antibodies—Dako, Golstrup, Denmark) or anti-goat IgG (BMP-2—Dako) were used as a second antibody (1 h at RT), followed by incubation with the avidin-linked peroxidase complex (Dako); staining was performed by the AEC revelation kit (brown stain; Dako). Sections were counterstained with hematoxylin and mounted with cover slips. All labelings were performed and evaluated independently by OMV and LM to assess reproducibility. The intensity of the staining was qualified in each section as 'absent' (0), 'light' (+), 'moderate' (++) or 'heavy' (+++).

Electron microscopy was performed on skin biopsies that were fixed in 2.5% glutaraldehyde in Tyrode's saline pH 7.2 (16–20 h at 4°C), post-fixed in 1% osmium tetroxide (Fluka AG Chem) in the same buffer for 2 h at RT, dehydrated in ethanol and propylene oxide and embedded in Spurr resin (Polysciences, Warrington, PA, USA). Some specimens were rescued from formalin-fixed and paraffin-embedded biopsies. Ultrathin sections were collected on nickel grids and processed for immunocytochemistry as described earlier.²⁸ Unspecific epitopes were neutralized by incubating sections on 0.5% bovine serum albumin in buffer. Monoclonal antibodies toward uncarboxylated and carboxylated species of MGP were used in parallel in all experiments where controls and patient samples had to be compared. Immunoreactions were revealed by secondary antibodies conjugated with 10 nm gold particles (E.Y. Laboratories, San Mateo, CA, USA). Controls were performed by omitting the primary antibody or by incubating sections with non-immune sera instead of the primary antibody. Sections were then stained with uranyl acetate and lead citrate and observed by transmission electron microscopy (Jeol, EM1200, Tokyo, Japan).

The intensity of immunostaining was evaluated by counting the number of gold particles per unit area. More than 20 different and randomly selected areas were analyzed and the mean values and s.d. were calculated. As the reactivity of the two antibodies is different, comparison was made only between samples processed in parallel with the same antibody.

Statistical Analysis

All data given are means of duplicate measurements. Normality distribution was checked by the Kolmogorov–Smirnov test. Differences between groups were compared using the non-parametric Mann–Whitney *U*-test and were considered significant at *P*<0.05. Ratios of uncarboxylated over carboxylated OC and MGP were calculated by dividing the measured level of the uncarboxylated form by the measured value of the carboxylated form. For VK measurements, also median values are given; differences were compared using the non-parametric median test and considered significant at *P*<0.05.

RESULTS

Biochemical Measurements

Serum and plasma concentrations of OC

The total amount of OC was significantly higher in the circulation of PXE-like patients compared with controls, being 17 and 9 ng/ml, respectively (Figures 1 and 2). Elevated levels of circulating ucOC were demonstrated (Figures 1A and 2a), whereas cOC levels were diminished (Figures 1C and 2b), resulting in significantly higher ucOC/cOC ratio compared with the reference population (Figures 1E and 2c). In PXE, the total amount of OC was slightly lower than in control sera, being 7.5 ng/ml in patients and 9.0 ng/ml in controls. No significant abnormalities were noted in levels of cOC or ucOC.

Serum and plasma concentrations of MGP

For MGP, both the sandwich and competitive ELISA assays were used (Figures 1 and 2). In PXE-like patients, an increase of the dp-ucMGP isoform (Figures 1G and 2d) with a very high uc/cMGP ratio compared with controls

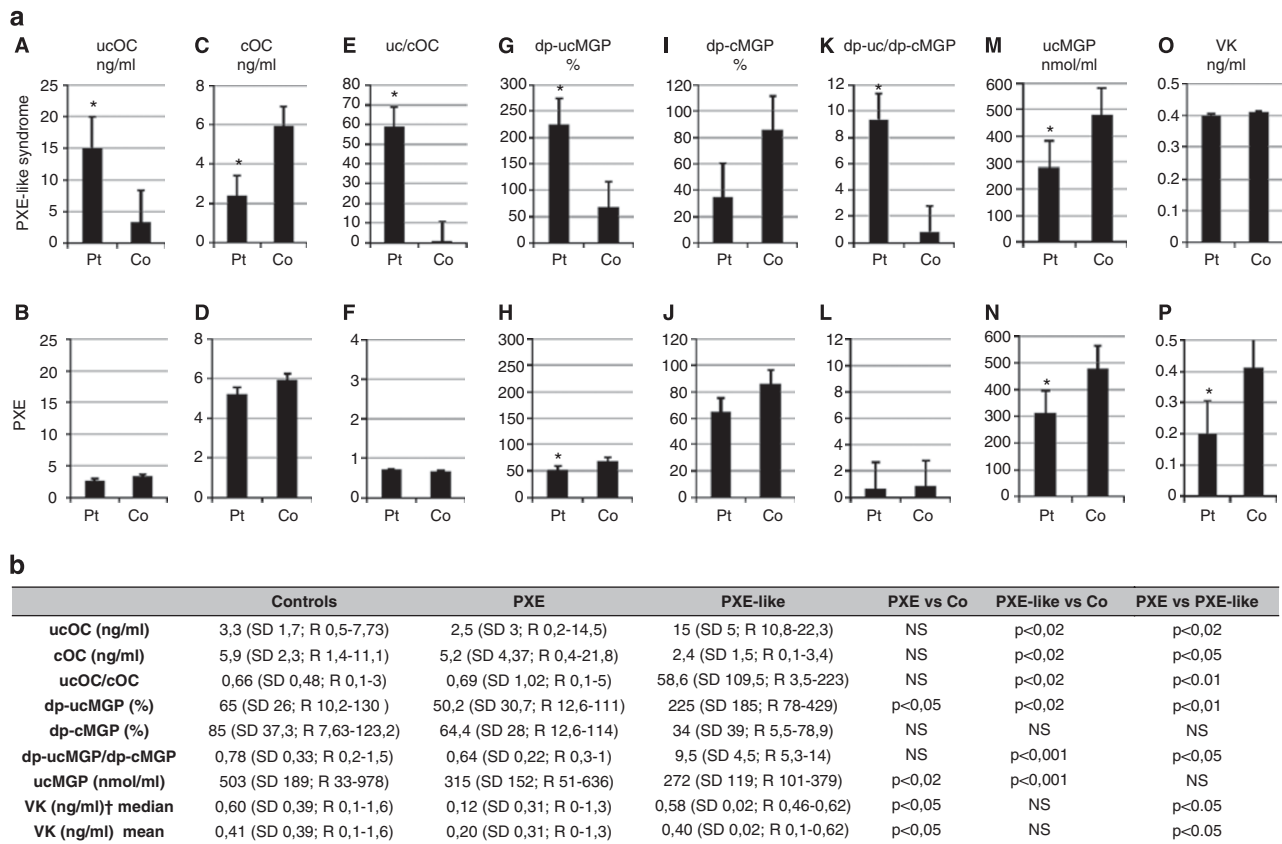


Figure 1 (a) Graphical representation of sandwich ELISA measurements for serum ucOC (A, B) and cOC (C, D), uc/cOC ratio (E, F), serum dp-ucMGP (G, H) and dp-cMGP (I, J), dp-uc/dp-cMGP ratio (K, L), serum VK (O, P) and competitive ELISA measurement results for ucMGP (M, N) in PXE-like and PXE patients, compared with controls. For PXE-like patients, significant differences are observed in all measurements except dp-cMGP and vitamin K serum concentrations. For PXE, significant differences in ucMGP and vitamin K can be seen. uc, uncarboxylated; c, carboxylated; dp, dephosphorylated; OC, osteocalcin; MGP, matrix gla protein; VK, vitamin K; †*P*<0.05. (b) Quantitative data on the sandwich and competitive ELISA measurements in PXE and PXE-like patients. Values are means, except w: median values. Co, controls; NS, not significant.

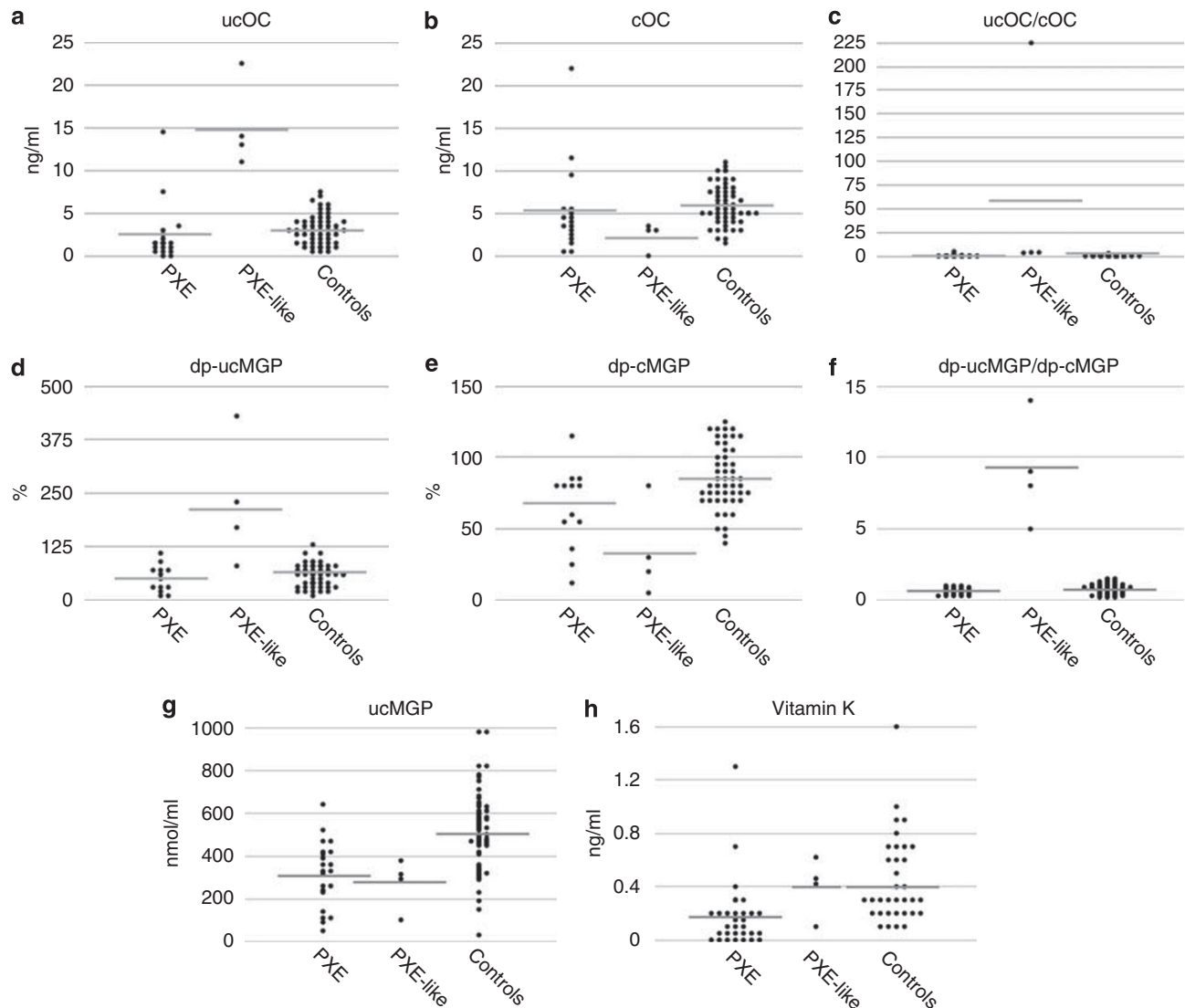


Figure 2 Scatter plot representation of sandwich ELISA serum measurements of ucOC (a), cOC (b), ucOC/cOC ratio (c), dp-ucMGP (d), dp-cMGP (e), dp-ucMGP/dp-cMGP ratio (f) and VK (h) and competitive ELISA measurement results for ucMGP (g) in PXE-like and PXE patients, compared with controls. Horizontal lines represent mean values. For PXE-like patients, significant differences are observed in all measurements except dp-cMGP and vitamin K serum concentrations. For PXE, significant differences can be seen. ucMGP and vitamin K can be seen. uc, uncarboxylated; c, carboxylated; dp, dephosphorylated; OC, osteocalcin; MGP, matrix gla protein.

(Figures 1K and 2f) was shown. Competitive ELISA measurement of total ucMGP revealed significantly decreased serum levels (Figures 1M and 2g). In PXE patients, no alterations were observed of dp-cMGP and dp-ucMGP, nor in their respective uc/c ratios using sandwich ELISA compared with control samples (Figures 1H, J, L, and 2d–f). The competitive assay for total ucMGP, however, showed decreased serum levels, similar to PXE-like patients (Figures 1N and 2g).

Serum concentration of VK

The serum VK concentration in PXE-like patients was normal compared with a control population (Figures 1 and 2) (0.58 vs 0.60 ng/ml in the reference population; $P > 0.1$) (Figures 1O

and 2h). In PXE patients, the median serum VK concentration was significantly decreased (0.12 ng/ml; s.d. = 0.31; $P < 0.05$) (Figures 1P and 2h). In some PXE patients ($n = 5$), the VK concentration was below the detection limit.

Pathology Findings

Light microscopy

In PXE-like patients (Figures 3 and 4; Table 2), labeling for cMGP and ucMGP was strongly positive in the mid-dermal elastorrhexis area (Figure 3e and f) and absent in elastorrhexis-free areas and in control samples (Figure 3a and b). In PXE patients, similar findings were observed for cMGP (Figure 3d). Although ucMGP stained somewhat less intense in the elastorrhectic fibers compared with those in PXE-like

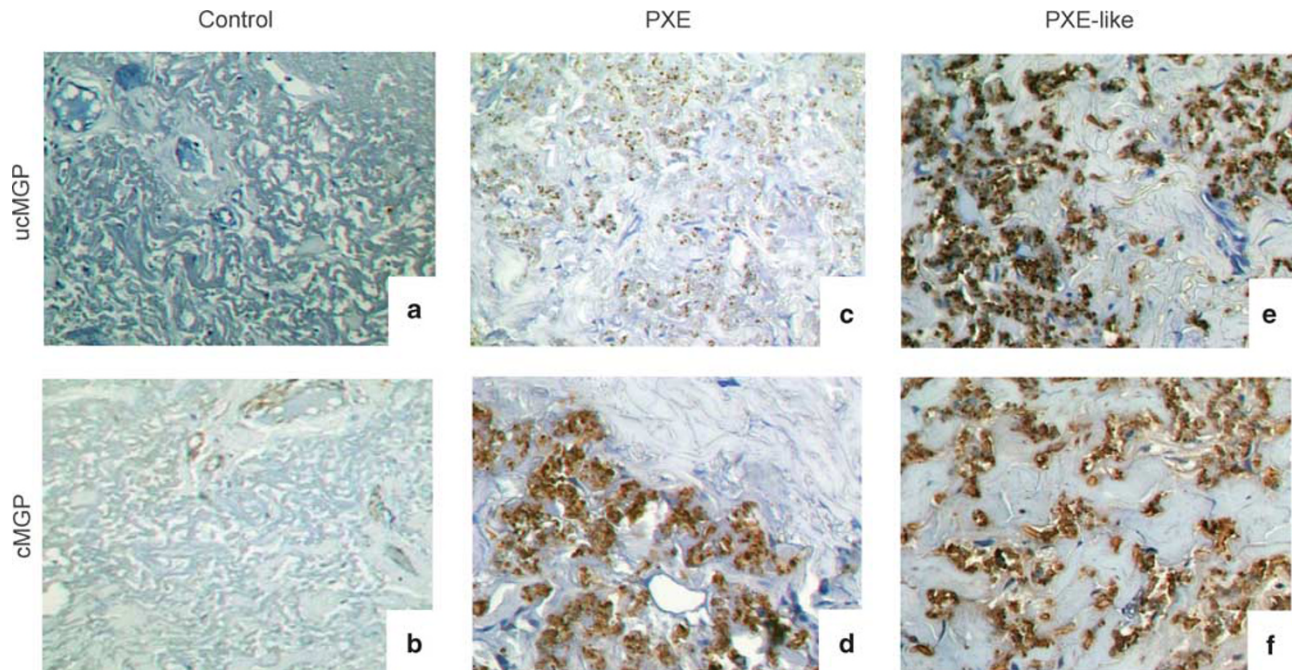


Figure 3 IHC staining results for carboxylated MGP (cMGP) and uncarboxylated MGP (ucMGP), respectively, in controls (**a, b**), PXE patients (**c, d**) and PXE-like patients (**e, f**). Significant staining of cMGP and ucMGP in the elastorrhexis areas is seen in PXE and PXE-like patients.

syndrome, it was still significantly more prominent compared with controls (Figure 3c).

Labeling for OC was weakly to moderately positive in the epidermis and elastorrhexis zone of PXE-like and PXE patients compared with controls (Figure 4c). OC was the only protein that also stained weakly positive in fibroblasts, compared with moderate staining in controls.

Fetuin-A stained heavily in the area of elastorrhexis as well as subepidermally—in the upper papillary dermis (Figure 4d). In PXE, significant staining was also seen in the elastorrhexis zone, whereas the subepidermal labeling was less prominent compared with PXE-like tissues.

Both OPN and BMP2 stained strongly in the elastorrhexis zone in PXE and PXE-like, compared with very weak or no staining in controls (data not shown).

Electron microscopy

In the dermis of PXE-like patients and controls, fibroblasts were negative for ucMGP (Figure 5; Tables 3 and 4). By contrast, fibroblasts were slightly positive for cMGP in controls and negative in PXE-like patients (Table 3). In the extracellular space, elastic fibers were the only matrix constituent positive for both forms of MGP in PXE-like patients and in controls (Table 3). The evaluation of the number of gold particles per $1 \mu\text{m}^2$ on the apparently normal elastic fibers revealed that ucMGP was more abundant within the fibers of PXE-like patients compared with controls, the number of gold particles being $46.7 \pm 26.5/\mu\text{m}^2$ in patients and $27 \pm 8.2/\mu\text{m}^2$ in controls ($P < 0.001$) (Figure 5a and e;

Table 4). The antibody recognizing cMGP was more abundant within the elastic fibers of controls than in the apparently normal ones of PXE-like patients, being $5.7 \pm 3.1/\mu\text{m}^2$ in controls and $2.1 \pm 1.8/\mu\text{m}^2$ in patients ($P < 0.001$; Table 4). The antibody recognizing ucMGP reacted with epitopes abundantly present in bulky calcium precipitates (data not shown) and in the core of calcified elastic fibers (Figure 5e). By contrast, the antibody recognizing cMGP primarily localized at the border of the finely mineralized areas within elastic fibers separating calcified from still normal elastin (Figure 5b and f).

In the dermis of PXE patients, fibroblasts were negative for ucMGP and barely positive for cMGP. In general, PXE fibroblasts were less positive for cMGP compared with controls (Table 3).²⁰ In the extracellular space, the distribution of positive immunostaining for cMGP and ucMGP gave results almost identical to those in patients with the PXE-like syndrome (Figure 5c and d). Irrespective of the calcification degree, elastic fibers were the only extracellular matrix component positive for both antibodies. The immunoreaction for ucMGP, the inactive form of MGP, was slightly more intense on PXE elastic fibers compared with controls (Tables 3 and 4). Moreover, as already reported,²⁰ it was confirmed that ucMGP antibodies were associated with either bulky and finely dispersed mineral precipitates inside elastic fibers (Figure 5c), whereas cMGP antibodies nicely and precisely localized at the border of the mineralized areas of calcified elastic fibers (Figure 5d).

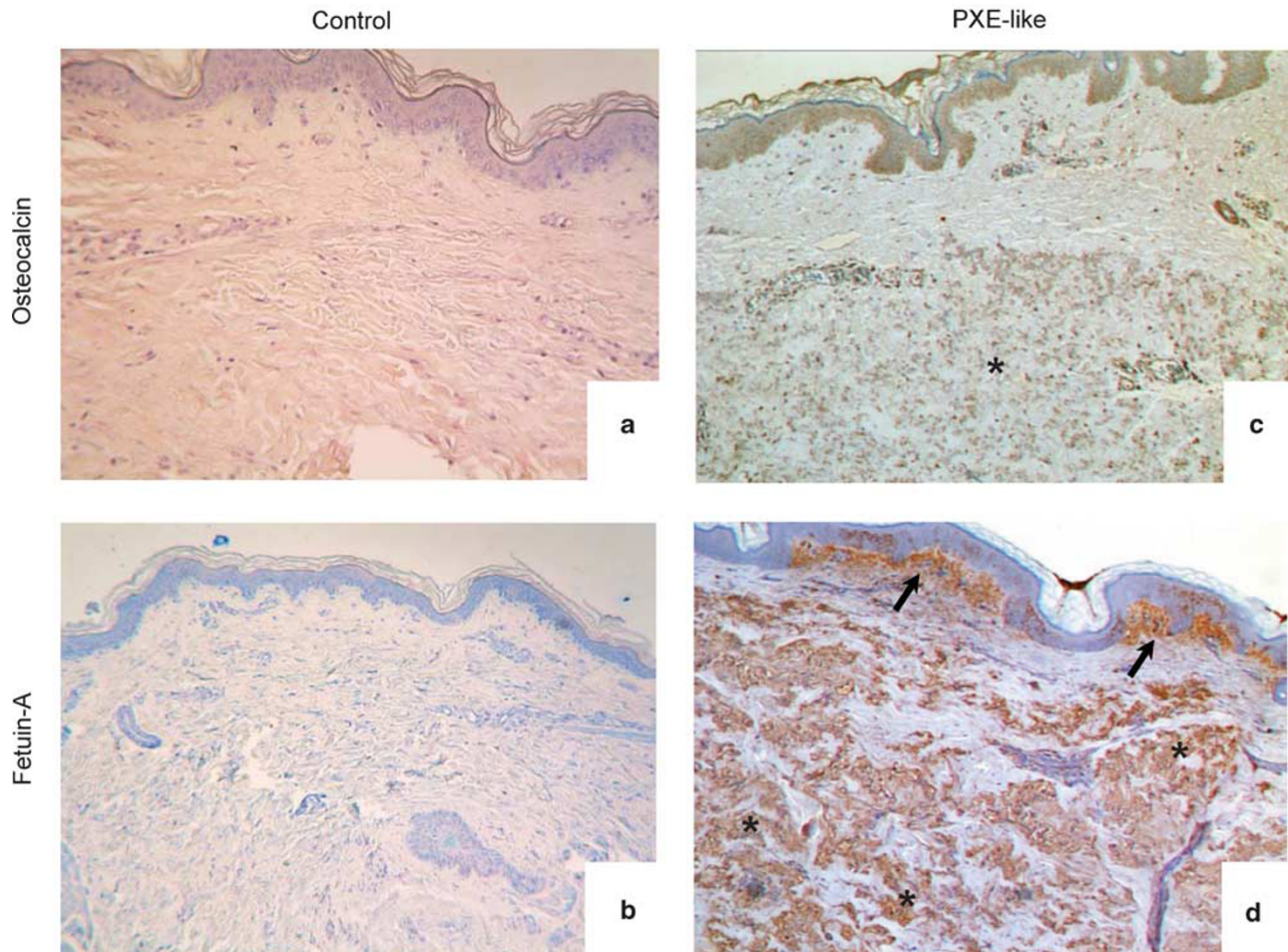


Figure 4 IHC staining for osteocalcin and fetuin-A in controls (**a, b**) and PXE-like patients (**c, d**). Weakly to moderately positive staining for OC and heavy staining for fetuin-A is seen in PXE-like patients. In PXE, similar labeling was observed as in PXE-like patients. Mid-dermal labeling is marked with asterisk; subepidermal labeling is arrowed.

Control Experiments

In both PXE-like and PXE patients, Gla proteins that are not involved in calcium homeostasis, such as gas-6, did not stain the elastorrhexic fibers (data not shown). MGP, OC, and fetuin-A labelings did not yield a positive stain in solar elastosis or elastofibroma samples used as controls of skin conditions with dystrophic but non-mineralized elastic fibers (data not shown).

DISCUSSION

This study confirms that inactive VK-dependent Gla proteins are present both in the circulation and locally in dermal tissue of PXE and PXE-like patients. Although in PXE-like patients this is likely due to the *GGCX* mutations, PXE patients tend to present decreased serum levels of VK, the essential co-factor of the VK cycle, necessary for γ -carboxylase activity.

VK-Dependent Proteins in PXE-Like Patients

In this study, we first evaluated the hypothesis that low levels of carboxylated species of MGP and OC results in elastic fiber mineralization in PXE-like patients affected by *GGCX* mutations. We noted a significant accumulation of uncarboxylated MGP in dermal elastorrhexis. Quantitative analysis at the ultrastructural level confirmed the abundance of uncarboxylated MGP and unusually low amounts of carboxylated protein compared with controls. Sandwich ELISA assays in serum and plasma of PXE-like patients revealed decreased levels of carboxylated forms of MGP and OC, whereas the levels of immature forms, respectively, uncarboxylated unphosphorylated MGP and the total fraction of uncarboxylated OC, were dramatically increased. As a result, carboxylated/uncarboxylated ratios of both MGP and OC were decreased. Indeed, all these findings are the direct consequences of *GGCX* deficiency and are factors likely to favor calcium precipitation in tissues such as the skin.

Table 2 Semiquantitative summary of immunohistochemical findings

	Papillary dermis	Normal elastic fibers	Elastorrhexis	Fibroblasts
<i>PXE ABCC6 (n = 9)</i>				
cMGP	0	0	+++	0
ucMGP	0	0	+ (punct.)	0
Fetuin-A	0	0	+++	0
Osteocalcin	0	0	+ (punct.)	+
<i>PXE-like GGCX (n = 3)</i>				
cMGP	0	0	+++	0
ucMGP	0	0	++ (> ABCC6)	0
Fetuin-A	+++	0	+++	0
Osteocalcin	0	0	+ (punct.)	+
<i>Controls (normal skin) (n = 7)</i>				
cMGP	0	0		0
ucMGP	0	0	NA	0
Fetuin-A	0	0		0
Osteocalcin	0	0		++
<i>Controls (dystrophic elastic fibers) (n = 3)</i>				
cMGP	0	0	Dystrophic	0
ucMGP	0	0	Fibers	0
Fetuin-A	0	0	0	0
Osteocalcin	0	0	(internal positive controls+; eg endothelial cells)	+

cMGP, carboxylated matrix gla protein; *n*, number; NA, not available; punct, punctuated; ucMGP, uncarboxylated matrix gla protein.

Two findings need further interpretation. First, competitive ELISA experiments, specifically measuring total uncarboxylated MGP (dp-ucMGP and p-ucMGP), showed unexpectedly decreased serum levels. This may be explained by the high affinity of the phosphorylated ucMGP fraction (the most abundant form) for calcium in contrast to the unphosphorylated ucMGP. Hence, the former may accumulate in the calcified tissues resulting in low serum levels. Second, labeling for cMGP and OC was clearly positive in all PXE-like skin samples. Interestingly, cMGP and ucMGP localized in different areas of mineralized elastic fibers in patients. Similarly to what was observed in the dermis of PXE patients,²⁰ cMGP was at the border of mineralization, whereas ucMGP was heavily associated with mineral precipitates (Figure 5). The presence of carboxylated species in lesional skin and plasma indicates that the VK metabolism

was not completely abolished. Yet, the amount of cMGP and OC accumulating at sites of calcium precipitation seem to be far below the critical threshold necessary to limit the calcification process. In a single pedigree displaying PXE-like features, Li *et al*²² recently reported that dermal elastorrhexis only weakly expressed carboxylated MGP. In the latter paper, both mutations detected in the *GGCX* gene were associated with different reduction in γ -carboxylase activity. We did not measure the γ -carboxylase activity in our own patients, but it seems likely that they keep a significant enzyme activity, as about 30% of carboxylated MGP and OC was still present in the circulation, although not sufficient to avoid connective-tissue calcification.

The intensity of OC labeling in mid-dermal elastorrhexis suggests an additional local role for this Gla protein, albeit more limited. Even though the lack of conformation-specific antibodies does not allow us to show that most OC in lesional skin is uncarboxylated, our finding of increased serum and plasma levels of uncarboxylated OC in PXE-like patients corroborates with this possibility.

Besides the local calcification inhibitors MGP and OC, we observed strong staining of the potent systemic inhibitor of calcification fetuin-A in the dermis of PXE-like patients. This serum protein carries calcium phosphate and has been shown to have a major function in preventing pathological calcification.²⁹⁻³¹ The extent of the protective mechanism by fetuin-A, however, seems to be less than that exerted by MGP, as fetuin-A-deficient mice only develop minor calcified lesions compared with the extensive calcifications in *Mgp*^{-/-} mice.³⁰ However, fetuin-A is closely related to the extracellular transport of MGP. Price *et al*⁸ have shown that cMGP, but not ucMGP, is carried by fetuin-A in plasma. This suggests that VK-dependent post-translational carboxylation of MGP is essential for fetuin-A binding and transport, and therefore could influence calcium deposition in the PXE-like syndrome resulting in a two-hit mechanism for the promotion of calcification via MGP.

Measurement of VK in serum of PXE-like patients revealed normal concentrations. This confirms the hypothesis that in PXE-like syndrome, ectopic mineralization is primarily the result of the *GGCX* mutations causing inactive Gla proteins.

VK-Dependent Proteins in PXE Patients

The identification of the PXE-like syndrome opened an interesting avenue of comparative research in PXE, because of the resemblance of both phenotypes. We therefore evaluated the involvement of MGP, OC, and fetuin-A in PXE skin samples and observed similar IHC results as in PXE-like tissues. Biochemical measurements also revealed a decrease of serum and plasma total ucMGP, but—in contrast to PXE-like patients—normal concentrations of dp-ucMGP were found.

Contrary to PXE-like patients, none of the PXE patients had mutations or functionally relevant polymorphisms in the *GGCX* gene, which could explain a possible dysregulation of the VK-cycle. We therefore also evaluated serum

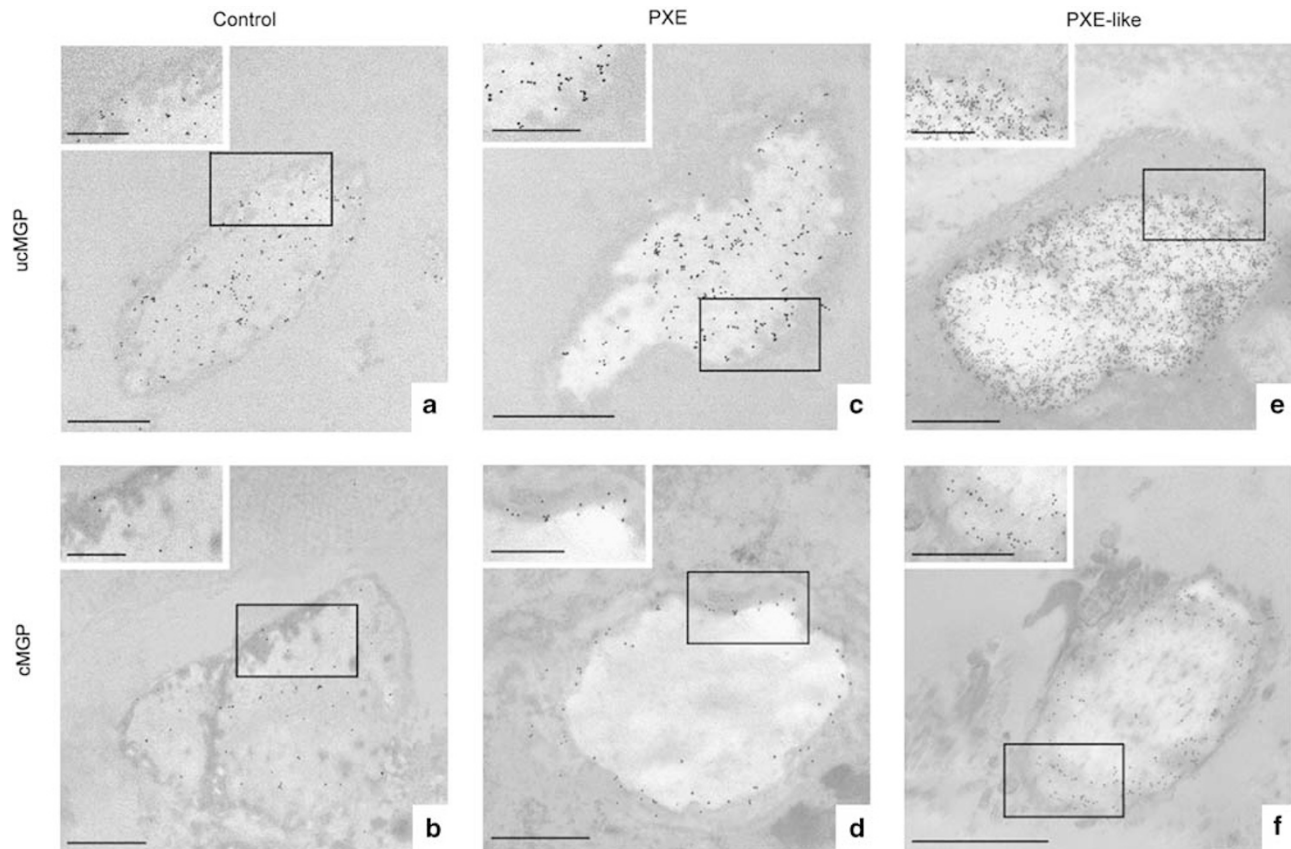


Figure 5 Transmission electron microscopy of elastic fibers in the skin of control subjects (a, b), PXE (c, d), and PXE-like patients (e, f). Gold particles represent epitopes positive to anti-ucMGP (a, c, e) and -cMGP (b, d, f) antibodies. In PXE-like patients, ucMGP was more abundant compared with controls, whereas the contrary was seen for cMGP. In PXE patients, similar results were obtained, although less pronounced, for staining of elastic fibers. In both diseases, ucMGP antibodies were located in the core of the elastic fibers, whereas cMGP antibodies were localized at the border of the mineralized areas. Bars: 1 and 0.5 μm (inserts).

Table 3 Relative positivity of the dermal components toward the antibodies recognizing cMGP and ucMGP assayed by immunoelectron microscopy

	Fibroblasts		Normal elastic fibers		Calcified elastin	
	cMGP	ucMGP	cMGP	ucMGP	cMGP	ucMGP
Controls	+	-	+++	+++	//	//
PXE-like	-	-	++	+++++	++	+++++
PXE	±	-	+	++++	++	+++++

cMGP, carboxylated matrix gla protein; ucMGP, uncarboxylated matrix gla protein.

Only reactions performed with the same antibody can be compared.

concentrations of VK, the essential co-factor of this metabolic cycle. We observed a poor vitamin K₁ status in PXE patients with median serum concentrations much lower than these measured in the reference population (0.12 vs 0.60 ng/ml, s.d. = 0.31, $P < 0.05$) or in other disorders associated with

Gla-protein-related abnormal mineralization such as Crohn's disease or osteoarthritis.^{13,32} In five PXE patients, the VK concentration was below the detection limit, as observed in disorders with malabsorption of fat-soluble vitamins such as cystic fibrosis.³³

These low VK serum concentrations would suggest that γ -carboxylation of OC is also negatively affected in PXE. Surprisingly, we did not detect abnormal OC levels in PXE serum samples, despite the IHC resemblance of labelings for OC with PXE-like syndrome, in which highly disturbed OC serum levels were noted. Osteoblasts, the cell type most expressing OC, have however a high level of low-density lipoprotein receptor-related protein 1 (LRP1) on their surface. LRP1 allows efficient uptake of the apoprotein E-containing chylomicron remnants that carry the bulk of the diet-derived VK. Therefore, it is plausible to assume osteoblasts to be more efficient in acquiring their share of the inadequate amounts of circulating VK in PXE patients than eg vascular smooth muscle cells in arteries or fibroblasts in skin.³⁴

The observation of such poor VK status in PXE patients supports the recently suggested hypothesis of VK as a

Table 4 Number of gold particles per μm^2 counted on the elastic fibers of control dermis and on the apparently normal elastic fibers of the dermis of patients

	Controls	PXE	PXE-like	PXE vs Co	PXE-like vs Co	PXE-like vs PXE
cMGP	5.7 ± 3.1	1.4 ± 1.3	2.1 ± 1.8	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.1
ucMGP	27.0 ± 8.2	30.7 ± 36.1	46.7 ± 26.5	<i>P</i> < 0.7	<i>P</i> < 0.001	<i>P</i> < 0.02

Co, controls; cMGP, carboxylated matrix gla protein; ucMGP, uncarboxylated matrix gla protein.

candidate substrate of the ABCC6 transporter, which could provide a link between mutations in the *ABCC6* gene, deficient protein carboxylation, and resulting mineralization of elastic fibers.³⁵

The increased expression of OPN, a mineral-binding phosphoprotein abundant in several mineralized tissues, in both PXE and PXE-like can be considered a rescue response to MGP dysfunction.³⁶ OPN upregulation in mineralized aorta of *Mgp*^{-/-} mice has previously been proposed to act as a secondary, inducible, calcification inhibitor, which limits further mineralization.³ In *Mgp*^{-/-} mice, OPN initiates removal of the mineral by macrophages.³⁷ Of note, macrophages have also been found to be abundant near calcified areas in PXE dermis.³⁸ MGP has been shown to negatively regulate BMP2, a known osteoinductive protein.³⁹ The observed upregulation of BMP2 may likewise be due to MGP dysfunction.

By immunoelectron microscopy it has been confirmed that ucMGP is specifically associated with calcification in both disorders and that the scarce cMGP present in the dermis of patients is precisely localized at the boundary separating calcified from normal elastin so appearing to inhibit/limit mineralization. Earlier IHC reports on ectopic mineralization, showing involvement of various glycoproteins such as vitronectin or bone sialoprotein were hampered by the low specificity of these findings.⁴⁰ In this study, samples of disorders with dystrophic elastic fibers but no mineralization, such as elastosis or elastofibroma, did not yield any MGP, OC, or fetuin labelings, suggesting that our observations are highly specific for PXE and the PXE-like syndrome. Similarly, labeling for Gla proteins not involved in calcium homeostasis, such as gas-6, were negative.

From our findings, we can conclude that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient carboxylation of VK-dependent inhibitors of calcification. In the PXE-like syndrome, this deficiency results directly from mutations in the γ -carboxylase gene. In PXE, deficiency of the carboxylation co-factor VK is at the basis of the decreased activity of calcification inhibitors. In both disorders MGP has a critical place. Whether VK is indeed the substrate of ABCC6 needs further study. However, our observations are promising toward therapeutic interventions in PXE, a disorder with no treatment to date. It has been shown in other disorders with poor VK status that exogenous VK

supplementation enhances the level of γ -glutamyl-carboxylation, increasing the levels of carboxylated Gla proteins, and as such (partially) inhibiting calcium precipitation. In animal models with deficient VK-dependent calcification inhibitors, ectopic calcification could be partially rescued with high doses of VK.²⁵ As it is possible that other inhibitory pathways of calcification, such as the Ank pathway in the *Abcc6*^{-/-} mice,⁴¹ or other genes are also involved in clinical manifestations of human PXE and PXE-like syndrome, the extent of the VK supplementation effect remains to be determined in a clinical setting.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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