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ORIGINAL ARTICLE

Pseudoxanthoma Elasticum-Like Phenotype with Cutis Laxa and Multiple Coagulation Factor Deficiency Represents a Separate Genetic Entity

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Data on six patients with a Pseudoxanthoma Elasticum (PXE)-like phenotype, characterized by excessive skin folding (resembling cutis laxa) and a deficiency of the vitamin K-dependent clotting factors (II, VII, IX, and X) are presented. A comparison is made between the clinical, ultrastructural, and molecular findings in these patients and those seen in classic PXE and cutis laxa, respectively. Clinical overlap with PXE is obvious from the skin manifestations of yellowish papules or leathery plaques with dot-like depressions at presentation, angioid streaks and/or ocular peau d'orange, and fragmentation and calcification of elastic fibers in the dermis. Important phenotypic differences with PXE include much more severe skin laxity with spreading toward the trunk and limbs with thick, leathery skin folds rather than confinement to flexural areas, and no decrease in visual acuity. Moreover, detailed electron microscopic analyses revealed that alterations of elastic fibers as well as their mineralization were slightly different from those in classic PXE. Molecular analysis revealed neither causal mutations in the *ABCC6* gene (ATP-binding cassette subfamily C member 6), which is responsible for PXE, nor in *VKORC1* (vitamin K 2,3 epoxide reductase), known to be involved in vitamin K-dependent factor deficiency. However, the *GGCX* gene (gamma-glutamyl carboxylase), encoding an enzyme important for γ-carboxylation of gla-proteins, harbored mutations in six out of seven patients analyzed. These findings all support the hypothesis that the disorder indeed represents a separate clinical and genetic entity, the molecular background of which remains to be unraveled.

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INTRODUCTION

Pseudoxanthoma Elasticum (PXE – OMIM no. 264800) is an autosomal-recessive multisystem disease, affecting mainly the eyes (peau d'orange (Pd'O), angioid streaks (AS), loss of central vision owing to neovascularization and subsequent exudation, and hemorrhage), the skin (yellowish papules or plaques of coalesced papules on the neck and in flexural areas), and the cardiovascular system (diffuse occlusive arterial disease). In some patients, the skin lesions can be progressive, evolving toward excessive skin folding in the flexural areas (McKusick, 1966; Neldner, 1988; Hu *et al.*, 2003; Chassaing *et al.*, 2005). Histologically, it is characterized by fragmentation and mineralization of the elastic fibers in the reticular dermis.

PXE is caused by mutations in the *ABCC6* gene (ATP-binding cassette subfamily C member 6 – chromosome 16p13.1) encoding an ATP-dependent transmembrane transporter whose substrate and pathophysiological role remain to be elucidated (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000; Struk *et al.*, 2000).

Cutis laxa (OMIM nos. 123700; 219100) is a heterogeneous group of acquired or inherited (autosomal-recessive or -dominant) disorders, characterized by loose, sagging, and redundant skin. The clinical findings can be limited to the skin, although extracutaneous manifestations (pulmonary emphysema, hernias, intestinal diverticuli, ocular anterior segment abnormalities) have also been described (Lewis *et al.*, 2004; Ringpfeil, 2005).

The histopathology of cutis laxa, using van Giesson stains, can reveal both loss and fragmentation of elastic fibers in the reticular dermis (Lewis *et al.*, 2004; Ringpfeil, 2005). Although the molecular background of cutis laxa is largely

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Abbreviations: ABCC6, ATP-binding cassette subfamily C member 6; AS, angioid streaks; GGCX, gamma-glutamyl carboxylase; Pd'O, peau d'orange; PXE, Pseudoxanthoma Elasticum; VKCFD1/2, congenital deficiency of the vitamin K-dependent factors; VKORC1, vitamin K 2,3 epoxide reductase

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unknown, causal mutations have been reported in the elastin gene (*ELN* on chromosome 7q11.2) and in the fibulin-5 gene (*FBLN5* on chromosome 14q32.1) (Tassabehji *et al.*, 1998; Loeys *et al.*, 2002; Markova *et al.*, 2003).

We describe six patients in whom a clinical diagnosis of PXE was initially made and subsequently supported by light microscopy on skin biopsy. However, these patients progressively developed excessive skin folds which, although initially confined to the flexural areas, spread both toward the abdomen and limbs. Moreover, all six patients were also found to have a deficiency of the vitamin K-dependent coagulation factors (factors II, VII, IX, X). Two patients had a history of or suggestive of cerebral aneurysms.

This phenotype has been previously described in a few case reports (MacMillan and Vickers, 1971; Rongioletti *et al.*, 1989; Le Corvaisier-Pieto *et al.*, 1996) but to our knowledge, no molecular data are available.

We have undertaken a detailed electron microscopy study of the ultrastructural characteristics of the dermis of these patients. Additionally, a molecular study of the *ABCC6* gene was performed as well as an initial candidate gene screening for the clotting disorder.

Based on the clinical, structural, and molecular findings on these six novel patients, we hypothesize that their condition represents a separate genetic entity.

RESULTS

Clinical description

Six Caucasian patients with initial signs of typical yellowish papules that coalesce and form plaques with regularly depressed dots, sometimes referred to as cutaneous ''Pd'O'' (Figure 1b), developed an increasing number of excessive, leathery skin folds, which gradually spread beyond the flexural areas of the body (Figure 1c–f). The papular and leathery skin lesions with dot-like depressions, however, remained stable. Fundoscopy revealed only limited AS and/or Pd'O without comets temporal to the macula in patients 1 through 4 and normal visual acuity in all patients (Figure 1a). During follow-up, both fundus appearance and visual acuity remained unchanged.

Cardiovascular investigations, including clinical examination, electrocardiography, and ultrasonography, showed subclinical atherosclerotic plaques in the lower limbs in two patients and vascular occlusion with intermittent claudication in one patient. In patient 1, two cerebral aneurysms were subsequently discovered at age 36 and 46 years and successfully treated.

Routine blood coagulation tests revealed a prolonged prothrombin time with decreased levels of the vitamin K-dependent clotting factors (Table 1). Clinical manifestations were present in only two patients. Patient 3 suffered three meningeal hemorrhages over a period of 20 years. Further bleeding events include a postpartum hemorrhage after delivery of her only child and one episode of unexplained hematemesis. Patient 5 had a history of epistaxis, spontaneous gingival bleeding, and severe vaginal hemorrhages.

Details on the individual phenotypes can be found in Table 2.

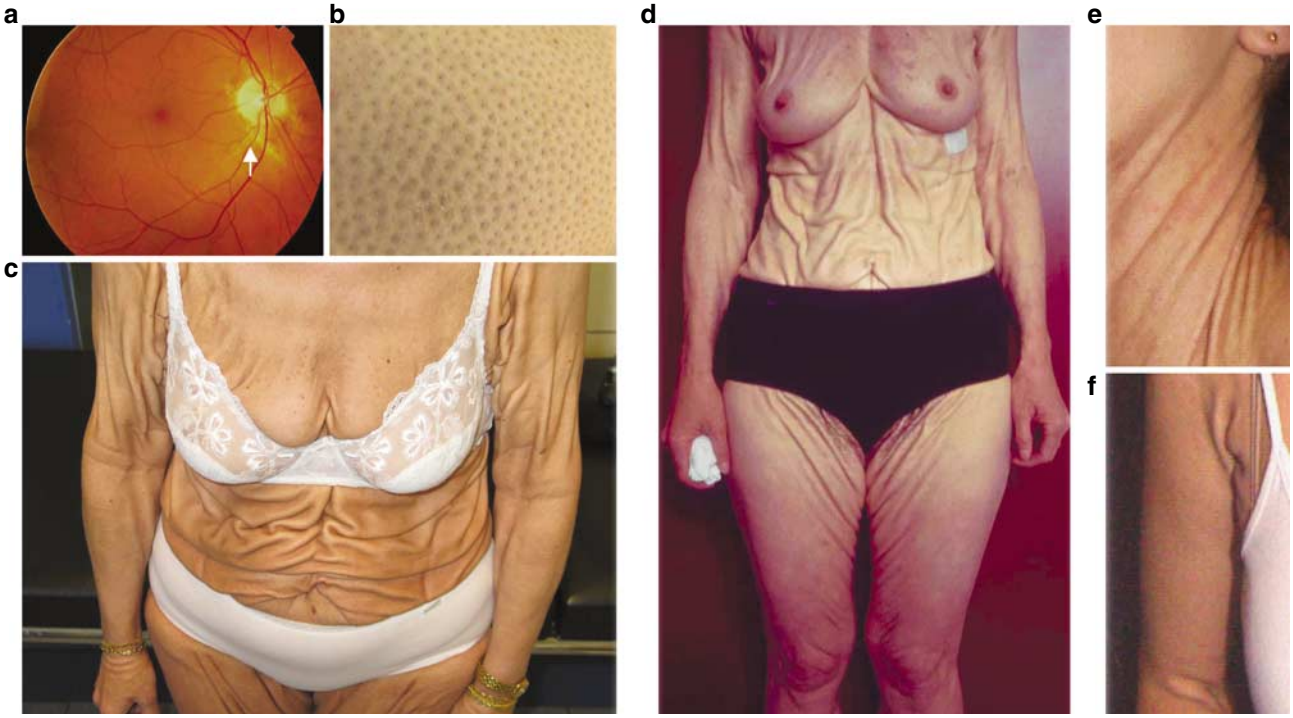

Figure 1. Clinical characteristics of the presented phenotype in several patients. Note (**a**) a mild retinopathy, (**b**) skin lesions featuring yellowish papules that coalesce and form plaques with regularly depressed dots, and (**c**–**f**) generalized excessive and leathery skin folds.

Table 1. Biochemical analysis results of the PT and vitamin K-dependent coagulation factor activity levels

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Normal
PT (INR)	1.81	1.84	1.97	2.19	1.7	1.9	0.8–1.2
Factor II (% nl)[1]	66	61	38	38	20	18	100 (90–150)
Factor VII (% nl)	26	39	50	62	74	88	100 (90–150)
Factor IX (% nl)	70	71	103	90	48	56	100 (90–150)
Factor X (% nl)	15	40	20	17	20	18	100 (90–150)

PT, prothrombin time; INR, International Normalized Ratio.
[1]Percentage of normal activity.

Table 2. Comparison of the phenotypical, histopathological, and molecular characteristics of the six presented patients (1–6) and the four previously reported cases (7–10)

	1	2	3	4	5	6	7	8	9	10
Sex	F	F	F	F	F	M	M	M	F	F
Age[1]	46	47	67	32	46	44	40	33	24	24
Age of onset skin symptoms[1]	18	13	3	18	NA	NA	16	Puberty	Puberty	Puberty
Positive familial history	–	–	–	–	NA	NA	–	–	+	+
Generalized skin folds/laxity	+	+	+	+	+	+	+	+	+	+
Yellowish papules	–	+	+	+	+	+	NA	NA	–	–
Dot-like depressions	+	+	+	+	+	+	NA	NA	–	–
Yellow mucosal pattern	–	+	+	–	NA	NA	NA	NA	NA	NA
Esthetic surgery performed	–	+	+	+	NA	NA	NA	NA	NA	NA
Positive calcium stain	+	+	+	+	+	+	+	+	+	+
AS	+	+	+	+	–	–	–	–	–	–
Ocular Pd'O	–	+	–	+	–	–	NA	–	–	–
Decreased visual acuity	–	–	–	–	–	–	–	–	–	–
Clotting deficiency	+	+	+	+	+	+	+	+	+	+
Abnormal bleeding tendency	–	–	+	–	+	–	+	–	+	+
Subclinical atherosclerosis	–	+	+	–	+	–	–	–	+	+
Weak peripheral pulsations	–	–	–	+	–	–	NA	NA	NA	NA
Cerebral aneurysms	+	–	?	–	NA	NA	–	–	–	–
ABCC6 mutations	–	–	–	–	NA	NA	–	NA	NA	NA
VKORC1 mutations	–	–	–	–	–	–	–	NA	NA	NA

AS, angioid streaks; F, female; M, male; NA, not available; Pd'O, peau d'orange.
[1]In years.
Case 7: Le Corvaisier-Pieto *et al.*, 1996; case 8: Rongioletti *et al.*, 1989; cases 9 and 10: MacMillan and Vickers, 1971.

Ultrastructural findings

By light microscopy, the overall appearance of the dermis was identical to that typical of PXE. The reticular dermis presented areas in which elastic fibers were polymorphous, fragmented, and mineralized, as shown by Von Kossa staining.

At the ultrastructural level, the alterations of the elastic fibers could be better analyzed and compared with those of PXE and revealed peculiar features. Although the two classical types of mineral precipitates, fine granular and bulky calcifications, were present, elastic fibers had a more fragmented and mottled appearance compared to those typical of PXE. In longitudinal sections, elastic fibers appeared to be made of loose, thin strands of polymorphous elastin material. Moreover, very often mineralization occupied only a limited area within huge elastic fibers and was organized as peculiar small electron-dense crystal-like precipitates. Several collagen fibrils were fused forming the so-called ''collagen flowers'' (Figure 2).

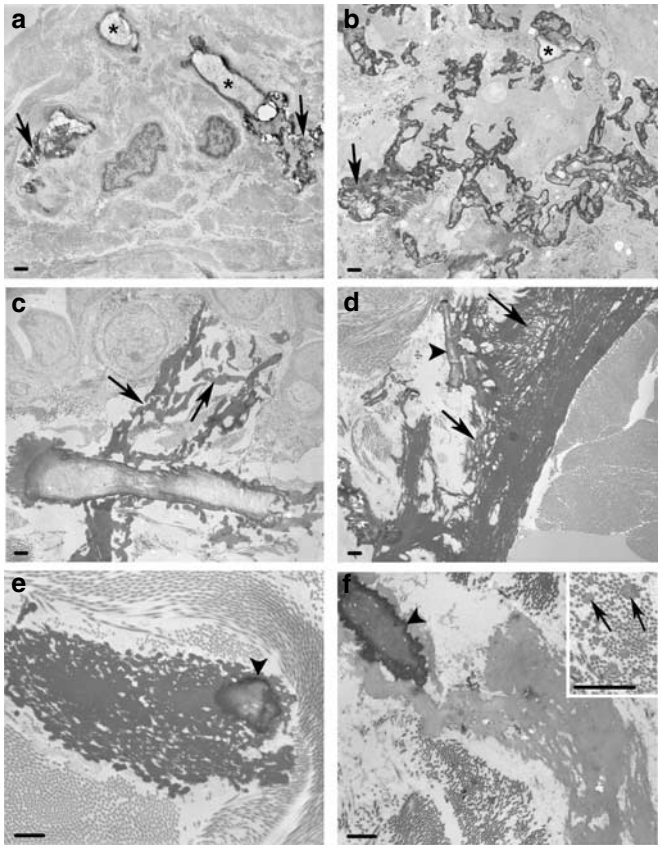

Figure 2. Ultrastructural characteristics of the presented phenotype. At low magnification electron microscopy, dermal alterations are mainly located in the reticular dermis and appear very similar to those in PXE. The great majority of elastic fibers are polymorphous, fragmented, and mineralized and are surrounded by rather thick and compact collagen bundles (**a** and **b**). Both the fine granular (**a** and **b**, asterisks) and the bulky types (**a** and **b**, arrows) of elastic fiber mineralization, always described in PXE, are present. However, different from classical PXE, the great majority of the apparently normal elastic fibers are organized as rather thin and polymorphous strands of elastin material connected into a loose network (**c** and **d**, arrows). Moreover, calcification affects often the marginal areas of elastic fibers (**d**–**f**, arrowheads), whereas in PXE mineralized fine deposits are always in the core of elastic fibers. Similar to PXE, collagen fibrils can be fused forming the so called ''collagen flowers'' (**f**, inset, arrows). Bar = 1 μm.

Table 3. Mutations found in the six presented cases (1–6) and one earlier reported case (7)

Patient	Allele 1		Exon	Allele 2		Exon
1	p.W493S	c.1506G>C	12	p.W493S	c.1506G>C	12
2	—	—	—	—	—	—
3	p.R476C	c.1454C>T	10	—	—	—
4	p.R476H	c.1455G>A	10	—	—	—
5	p.Q374X	c.1149C>T	8	p.G537Y	c.1339G>T	12
6	p.Q374X	c.1149C>T	8	p.G537Y	c.1339G>T	12
7	p.F299S	c.924T>C	8	p.G558R	c.1700G>A	12

Molecular results

Molecular analysis of the *ABCC6* gene, responsible for PXE, failed to identify mutations in all six patients, and in one patient reported earlier (Le Corvaisier-Pieto *et al.*, 1996).

Sequencing of the *VKORC1* gene (vitamin K 2,3 epoxide reductase), responsible for vitamin K recycling, did not reveal any mutation. Analysis of the second gene involved in the clotting disorder, *GGCX* (gamma-glutamyl carboxylase), revealed six novel missense mutations and one novel nonsense mutation in the seven patients, including the patient reported earlier (Table 3). Mutations occurred in homozygous or compound heterozygous form in four patients. In two other patients, only one mutation could be detected. These mutations affected amino acids which were fully or almost fully conserved in *Mus musculus*, *Rattus norvegicus*, *Fugu rubripes*, *Anopheles*, and *Drosophila*. None of these mutations were found in 200 control chromosomes of unrelated healthy Europeans.

DISCUSSION

The six patients described show clinical overlap with PXE, hence the initial diagnosis of a severe form of that disorder. All initially manifested skin manifestations considered typical of PXE, such as yellowish papules and/or plaques with dot-like depressions characterized by light microscopy as fragmentation and calcification of elastic fibers in the reticular dermis. Moreover, the observed retinopathy (AS originating from the optic disc, Pd'O lesions temporal to the macula) in these patients was anatomically similar if not identical to that seen in PXE.

However, clinical, ultrastructural, and molecular differences allow us to discriminate the phenotype of these patients from PXE and from cutis laxa (Table 4).

First, these patients are remarkable for the severity of their skin involvement. Increased skin laxity and excessive skin folding can be seen in PXE, but usually these features remain confined to the flexural areas of the body (McKusick, 1966; Neldner, 1988; Hu *et al.*, 2003; Chassaing *et al.*, 2005). In contrast, in these patients, the leathery skin folds extended toward the limbs and abdomen leaving only the face, hands, and feet unaffected. Interestingly, the speed of evolution of the skin laxity was quite different between patients. Whereas progression over several years in patients 1 and 4 led to the very severe skin phenotype in their early thirties, patient 3 already suffered from severe skin laxity during her childhood, and patient 2 manifested rapid progression during puberty.

Secondly, the fundus examination revealed only limited AS and/or mild Pd'O. Moreover, visual acuity remained stable in all four patients during follow-up. In contrast, in PXE, up to 60% of patients suffer from considerable loss of central vision. Although sample size is small, this suggests that in this PXE-like phenotype, the retinopathy is milder and has a more benign prognosis.

Third, electron microscopy revealed that dermis alterations, especially those of elastic fibers, were similar, but not identical, to those in PXE. Similarities with PXE were that (i) changes occurred in the same areas of the dermis; (ii) not

Table 4. Clinical, ultrastructural, and molecular characteristics of the PXE-like syndrome with a coagulation disorder *versus* PXE and cutis laxa

	PXE-like	PXE	Cutis laxa
Generalized skin folds/laxity	Always present	Not present	Often present
Positive Von Kossa stain of the dermis	Present	Present	Not present
Electron microscopy of the dermis	Mineralized elastic fibers	Mineralized elastic fibers	Scarce and mottled elastic fibers
Retinopathy (AS/Pd'O)	Present but mild	Present and often severe	Not present
Decreased visual acuity	Not present	60%	Infrequent
Clotting deficiency	Always present	Not described	Not described
Atherosclerosis	Subclinical in 50%	(Sub)clinical in 55%	Infrequent
Cerebral aneurysms	Present	Infrequent	Infrequent
Abnormal bleeding tendency	Present (50%)	Infrequent (10%)	Not described
ABCC6 mutations	Not present	Present (DR=96%)	Not present

AS/Pd'O, angioid streaks/peau d'orange; DR, detection ratio; PXE, Pseudoxanthoma Elasticum.

all elastic fibers were mineralized, but in those that were, the two types of mineralization already described in PXE were present; (iii) fibroblasts had huge cisternae of the endoplasmic reticulum; (iv) lateral fusion of collagen fibrils were observed in one patient. However, elastic fibers, either mineralized or not, were different from that typical of PXE. In patients 1, 2, and 3, they were often made of aggregates of distinct strands of elastin and mineralization was mostly confined to the periphery of the fibers (case 1) or was associated to the most compact areas of the fibers (cases 2 and 3). In contrast, in patient 4 elastic fibers were huge and compact and mineralization was very severe. Moreover, electron-dense crystal-like bodies, never observed in PXE, were present in all patients in the central core of finely mineralized elastic fibers.

Finally, molecular analysis of the *ABCC6* gene, responsible for PXE, failed to identify mutations in all of the patients, including the one reported earlier.

Classical cutis laxa syndromes could also be excluded because neither the retinopathy nor mineralization of elastic fibers is seen in those disorders (Krill and Archer, 1972; Lewis *et al.*, 2004).

A second feature of the phenotype is a deficiency of the clotting factors II, VII, IX, and X, which are synthesized in the liver and depend on vitamin K for their function. Such deficiency can either be acquired or congenital (Oldenburg *et al.*, 2000; Sadler, 2004; Zhang and Ginsburg, 2004). However, in our patients there was no evidence for an acquired form, as they did not exhibit any hepatic disease or malabsorption.

Congenital deficiency of the vitamin K-dependent factors (VKCFD) is a very rare autosomal-recessive disorder. It is caused either by mutations in the *GGCX* gene (VKCFD1 – OMIM no. 277450) on chromosome 2p12, or in the *VKORC1* gene (VKCFD2 – OMIM no. 607473) on chromosome 16p11.2. These genes encode a vitamin K-dependent carboxylase and vitamin K 2,3 epoxide reductase, respectively (Oldenburg *et al.*, 2000; Li *et al.*, 2004; Rost *et al.*, 2004a, b; Zhang and Ginsburg, 2004). Both enzymes are essential for post-translational γ-carboxylation of clotting factors, enabling them to attach to the phospholipid bilayer of membranes as an essential prerequisite for blood coagulation (Zhang and Ginsburg, 2004). The clinical features of VKCFD1 and -2 are highly variable and may include epistaxis, (neonatal) intracranial hemorrhage, hemarthrosis, etc., although several patients remain asymptomatic (Zhang and Ginsburg, 2004). Significant bleeding diathesis was observed in cases 3 and 5, although it remains uncertain whether this was due to the mild to moderate clotting factor deficiency. It is interesting though that two cerebral aneurysms were detected in case 1. Taken together with the potentially subarachnoid hemorrhages in case 3, this may indicate that patients with this disorder might have an increased risk for cerebral aneurysms. By contrast, in PXE, cerebrovascular complications are mostly ischemic (stroke).

In order to elucidate the molecular pathogenesis of this phenotype, we initially focused on the *VKORC1* gene, as it is also located on chromosome 16p, albeit 15 Mb away from *ABCC6*. However, sequencing of the whole coding region did not reveal any causal mutation. In contrast, analysis of the second gene involved in the clotting disorder, *GGCX*, has revealed seven different mutations in six patients. Of these, p.Q374X, p.W493S, p.R476C, and p.R476H are located in the propeptide binding site of the γ-carboxylase, important for binding of the substrates. This site has been mapped to amino acids 50–225, 349–500, and 425–513 (Yamada *et al.*, 1995; Wu *et al.*, 1997; Lin *et al.*, 2002). Thus, they could result in a shorter residence time of the substrate on the γ-carboxylase with formation of poorly carboxylated, less active proteins (Mutucumarana *et al.*, 2000).

Site-directed mutagenesis demonstrated that regions around residues 234, 406, and 503 partly define the propeptide binding sites (Sugiura *et al.*, 1996). The p.F299S, p.G537Y, and p.G558S mutations may therefore also influence the affinity of the γ–carboxylase for its substrates. Further bio- and immunohistochemical analysis of the

functional effects of these mutations is currently ongoing. It is worthwhile mentioning that the vitamin K-dependent enzymes responsible for carboxylation of clotting factors are also involved in modulating bone mineralization and in preventing calcium precipitation in soft tissues (Price and Williamson, 1985; Price *et al.*, 1998). Our molecular results are remarkable in that mutations in this phenotype seem to occur typically in exons 8, 10, and 12. Mutations in these exons have not been described so far in patients with the hereditary coagulation disorder without the cutaneous phenotype. It is our hypothesis that these exons are of critical importance in domains that are essential in the activating role of the GGCX enzyme in the coagulation cascade, but also for the activation of other gla-proteins such as, for example, matrix gla protein or osteocalcin. The latter are known inhibitors of calcification. Hence, a decreased or absent activation of such proteins can explain the calcification and subsequent fragmentation of the elastic fibers. The severity of the skin lesions might be due to the additive effect of decreased or absent activity of multiple calcification inhibitors.

A second theoretical possibility would be digenic inheritance, in which a second, as yet unknown gene, is responsible for the cutaneous phenotype. Linkage analysis was possible in only one family without known mutations; unfortunately, we were unable to obtain DNA from a sib of the proband, essential for the interpretation of the results.

Furthermore, apart from gene defects, the correct carboxylation of clotting factors and of modulators of mineralization in bone and soft tissues may depend on post-translational maturation of enzymes (Wajih *et al.*, 2004) as well as on cell metabolic alterations affecting, for instance, the structural organization of membranes of the endoplasmic reticulum where carboxylating enzymes are located (Wallin *et al.*, 1999). Therefore, the problem is rather complex and the involvement of vitamin K-dependent processes in mineralization of connective tissues and of elastic fibers in particular is under investigation.

At present 10 cases with this peculiar phenotype have been reported (Table 4). In the literature, the fundus examination has always been considered normal. However, owing to the very mild aspect of the retinopathy, it is possible that small AS were missed. The fact that visual acuity has always been reported as normal is yet another indicator of the rather mild nature of the ocular features.

Bleeding tendency was very variable, as can be expected from the hereditary form of VKCFD.

In conclusion, as (i) the fundus findings and the elastic fiber mineralization in the skin of patients exclude any known form of cutis laxa, and (ii) the distinct clinical manifestations and severity of cutaneous and retinal symptoms, as well as the slightly different ultrastructural features and the absence of *ABCC6* mutations in patients seem to exclude PXE, data support the hypothesis that these patients suffer from a new disorder. The molecular background of this peculiar phenotype, and more specifically the role of the *GGCX* gene, is currently under investigation.

MATERIALS AND METHODS

Patients

The patients were clinically examined (skin evaluation, fundoscopy, and cardiovascular work-up) at the Ghent University Hospital (Belgium) (case 1), l'Hôpital Porte Madeleine, Orléans (France) (cases 2 and 3), the University of Modena and Reggio Emilia, Modena (Italy) (case 4). Patients 5 and 6 who reside in the United States were not personally evaluated by the authors, but clinical data were obtained from their medical specialists. The first four patients had a full thickness skin biopsy taken in a skin lesion suggestive for PXE. Biopsies were evaluated with light microscopy using hematoxylin and eosin, van Giesson (elastin), and Von Kossa (calcium) stains.

For electron microscopy, skin biopsy fragments were immediately fixed in 3% glutaraldehyde in Tyrode's saline pH 7.2 for 2–4 hours at room temperature, washed in saline, post-fixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in ethanol and propylene oxide, and embedded in spurr resin. Semithin sections were stained with 1% toluidine blue and observed by light microscopy. Thin sections were stained with 1% uranyl acetate in 50% ethanol and lead citrate and observed in a Jeol EM1200 electron microscope. Skin biopsy fragments from patients 2 and 3 were first fixed in formalin and embedded in paraffin and then rescued and embedded in spurr resin.

Coagulation assays were performed on citrated blood samples. The activities of factors II, VII, IX, and X were measured in one-stage clotting assays using Diagnostica Stago (Asnières sur Seine, France) reagents. The prothrombin time was performed using the STA-Neoplastin CI plus kit (Diagnostica Stago, France).

Informed consent was obtained from all patients and the Declaration of Helsinki Principles was followed. This study was approved by the Ethical Committee of the Ghent University Hospital.

Molecular analysis

Molecular analysis of the *ABCC6* gene was performed in all six patients and one additional, already reported, patient (Le Corvaisier-Pieto *et al.*, 1996), using PCR primers described by Wang *et al.* (2001). In order to distinguish between *ABCC6* and its two pseudogenes a long-range PCR was performed of exons 1 through 10. The following primers were used: forward primer: 5′-ATA CTC AGT ATC AGC CAG GAT GTT-3′ and reverse primer: 5′-GGG ACT CCG TTC AAA TCC CG-3′. Subsequently, PCR reactions for the separate exons were performed on the long-range amplicon. For the detection of the deletion of exons 23–29, the primers described by le Saux *et al.* (2001) were used.

The *VKORC1* coding region was analyzed using following primers: exon 1: forward primer 5′-CTC CGT GGC TGG TTT TCT C-3′and reverse primer 5′-CCG ATC CCA GAC TCC AGA AT-3′; exon 2: forward primer 5′-ATG GGA GGT CGG GGA ACA-3′ and reverse primer 5′-TGA GCA GCT AGC TGG CTG-3′; exon 3: forward primer 5′-TCT GCC CTG GAG CCT CTT-3′ and reverse primer 5′-CAC ATC TAG GGC CTT CTA G-3′.

The *GGCX* coding region was analyzed using primers and PCR conditions described by Oldenburg *et al.* (2000).

The whole coding region and intron/exon boundaries of *ABCC6*, *VKORC1*, and *GGCX* were analyzed with direct sequencing using an Applied Biosystems 3100 Sequencer, with ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Lennik, Belgium).

Nucleotide numbers are derived from cDNA *GGCX* sequences (GenBank accession no. BC013979). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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