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# The Multifaceted Complexity of Genetic Diseases: A Lesson from Pseudoxanthoma Elasticum

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## 1. Introduction

Pseudoxanthoma elasticum (PXE), also known as Grönblad-Strandberg syndrome, is an autosomal recessive disorder mainly affecting skin, eyes and the cardiovascular system due to progressive mineralization of elastic fibres (Gheduzzi et al., 2003) in the presence of normal levels of calcium and phosphorus in blood and urine.

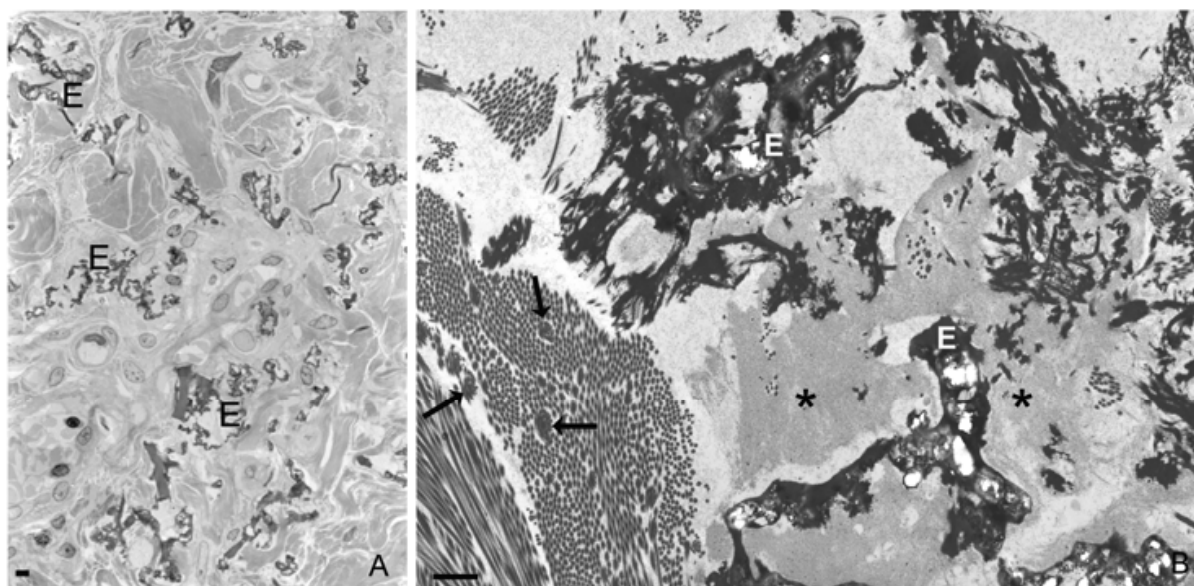


Fig. 1. Dermal biopsy from a patient affected by pseudoxanthoma elasticum (PXE).  
A) Semi-thin section stained with toluidine blue and observed by light microscopy.  
B) Ultrathin section stained with uranyl acetate and lead citrate visualized by transmission electron microscopy. Deformed, fragmented and mineralized elastic fibres (E) are clearly visible in the reticular dermis of the patient both at low and high magnifications. Collagen flowers (arrows) and electron-dense amorphous aggregates (\*) can be recognized at the ultrastructural level. Bar= 1  $\mu$ m

Although the elastic component is dramatically modified in terms of structural characteristics and functional properties, many other components of the extracellular matrix,

although not calcified, appear altered. Collagen fibrils, for instance, can be laterally fused giving rise to collagen flowers, whereas glycoproteins, abnormally secreted within connective tissues, are deposited in form of large amorphous aggregates (Gheduzzi et al., 2003; Pasquali-Ronchetti et al. 1981) (Figure 1).

The disease is due to mutations in the *ABCC6* gene, encoding for a transmembrane protein (MRP6) highly expressed in liver, kidney and at a lesser extent in several other tissues, although clinically affected. The physiological substrate of MRP6 is still elusive, even though functional studies reported that the protein may be involved in the transport of complex molecules as glutathione S-conjugate leukotriene C4 and of the synthetic cyclopentapeptide BQ123 (an endothelin 1 receptor antagonist) (Belinski et al., 2002; Ilias et al., 2002). Therefore, despite the exponentially increased number of studies performed in the last decade, the pathogenesis of ectopic calcifications in PXE is a still unresolved puzzle (Uitto et al. 2010).

PXE is present in all world's populations, with an estimated prevalence of 1 in 25.000–50.000 and a 2:1 female to male ratio (Neldner & Struk, 2002). Carriers of only one mutated allele do not develop evident clinical manifestations, however they cannot be considered completely healthy carriers, since they may be, for instance, at higher risk for cardiovascular complications (Vanakker et al., 2008).

## 2. Clinical manifestations

The clinical expression of PXE is heterogeneous, with considerable variation in age of onset, progression and severity of the disease, even within the same family and in the presence of identical mutations (Gheduzzi et al., 2004; Hu et al., 2003a).

### 2.1 Skin

Patients usually develop skin lesions, mainly at puberty, starting at the posterior side of the neck and in flexural areas such as armpits, antecubital and popliteal fossae, which may later expand to the inguinal region and the periumbilical area. Alterations are usually in form of round yellowish papules, 1–3 mm in diameter, that may coalesce with time into larger protruding plaques. In a relevant number of cases, the skin becomes wrinkled and redundant hanging in folds (Neldner & Struk, 2002) (Figure 2).

In the most severely affected patients, lesions on the mucosal membranes, especially on the inner side of the lower lip, can be observed. Occasionally, calcium deposits may extrude from the skin in advanced state of the disease, a condition described as “perforating PXE” (Lund & Gilbert, 1976). Other unusual clinical presentations of PXE include acneiform lesions (Heid et al., 1980), chronic granulomatous nodules (Heyl, 1967) and brown macules in a reticulate pattern (T.H. Li et al., 1996).

### 2.2 Cardiovascular system

Cardiovascular manifestations, although not frequent, can be observed already before the third or fourth decade of life and are mainly related to calcium deposition and degeneration of the elastic laminae of medium sized arteries (Mendelsohn et al., 1978). The most common cardiovascular complications, in approximately 20–25% of PXE patients, are: diminished or absent peripheral vascular pulsations, early onset of reno-vascular hypertension, echographic opacities due to calcification of arteries (especially in kidneys, spleen and pancreas), arterial hypertension, angina pectoris, intermittent claudication (often regarded

as the first sign of accelerated atherosclerosis), gastrointestinal haemorrhages, arteriosclerosis and increased risk of myocardial and cerebral infarction (Neldner & Struk, 2002). Marked calcification of valves and of atrial and ventricular myocardium, as well as calcified thrombi, which can result in mitral valve prolapse or stenosis and restrictive cardiomyopathy, can be clearly revealed by echocardiography (Rosenzweig et al., 1993).



Fig. 2. Typical dermal alterations in PXE.

Papules (B) as well as wrinkled and redundant skin (A) are classical dermal lesions observed in PXE patients

About 10% of PXE patients experience bleeding complications, especially gastrointestinal haemorrhages, due to fragility of calcified submucosal vessels (Golliet-Mercier et al., 2005). Bleeding may infrequently affect other organs such as urinary tract, uterus, joints and the cerebrovascular system (Bock & Schwegler, 2008; Heaton & Wilson, 1986).

### 2.3 Eyes

PXE is also characterized by severe ocular alterations due to calcification of the Bruch's membrane, that is a thin layer of connective tissue bridging the pigmented retinal epithelium to the choriocapillaries and that consists of a network of interwoven elastic and collagen fibres (Booij et al., 2010). Eye abnormalities are firstly represented by peau d'orange (diffuse mottling of the fundus) that, on an average to 1 to 8 years, precedes angioid streaks (greyish irregular lines radiating outward from the optic papilla corresponding to breaks of the calcified Bruch's membrane) (Figure 3). Within 20 years from diagnosis, almost all PXE patients develop angioid streaks, that, in the course of the disease, may become pale and



give way to a generalized atrophy of the adjacent tissue. In later stages, fibrovascular tissue as well as secondary choroidal neovascularization may develop. These new vessels have brittle walls, and this may cause recurrent, spontaneous, or trauma-induced retinal haemorrhages resulting in disciform scarring of the macula, which is responsible for decreased central visual acuity up to legal blindness (Georgalas et al., 2011).

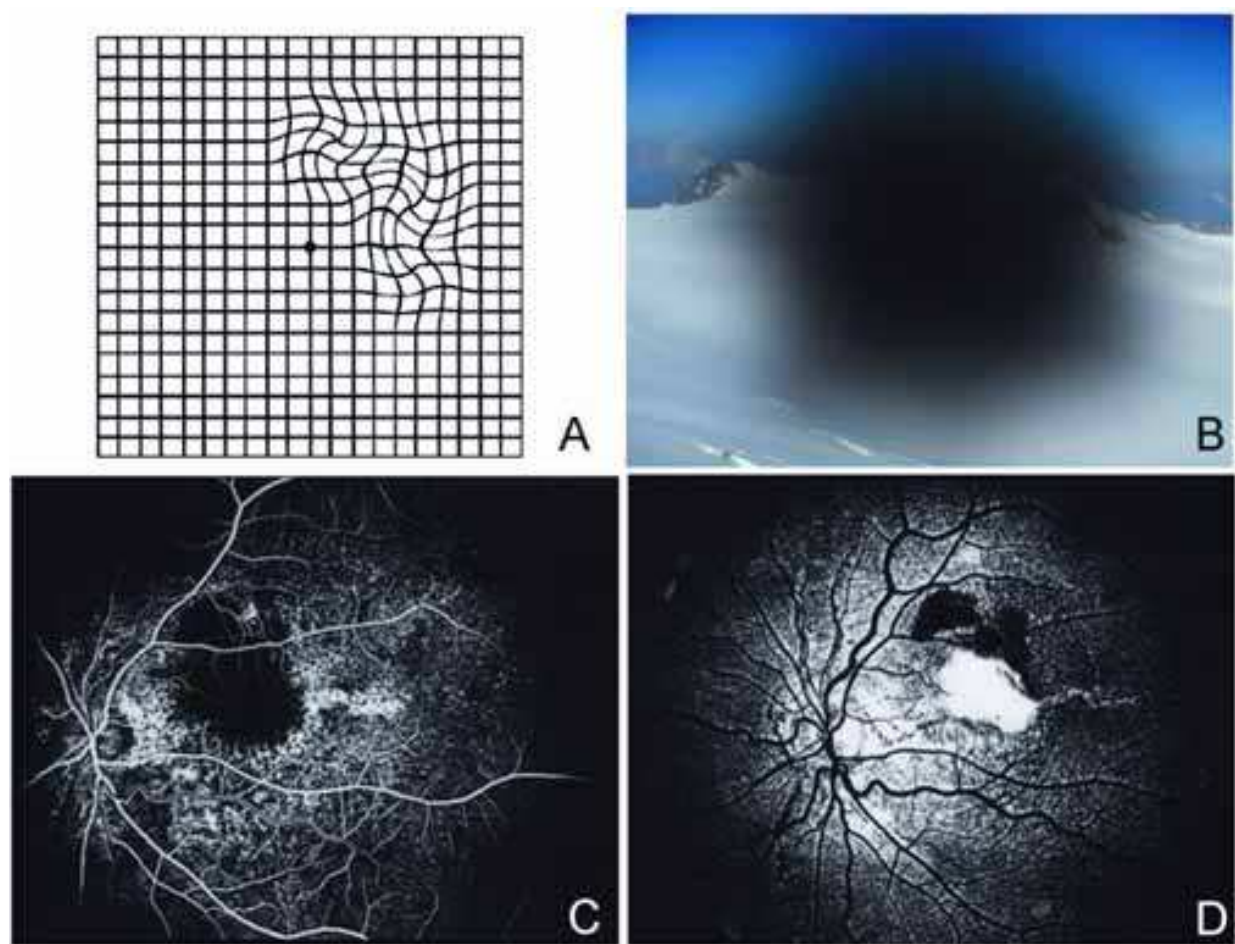


Fig. 3. Typical ocular alterations in PXE. Elastic fibre mineralization within the Bruch's membrane, haemorrhage, neovascularization and fibrosis (C-D) are the major causes of visual abnormalities. Distortion of the Amsler grid (A) is generally the first clinical sign of ocular involvement. Progression of the disease will end up with central vision loss (B) up to legal blindness

## 2.4 Other organs

Interestingly, microcalcifications can be detected in several organs, as testis and breast (Bercovitch et al. 2003; Vanakker et al., 2006), as well as in liver, kidneys and spleen (59% of patients and 23.5% of healthy carriers). On renal and abdominal ultrasonography, for instance, a characteristic hyperechogenicity with dotted pattern, possibly reflecting the calcified elastic layers of arteries, has been frequently reported (Suarez et al., 1991), as well as bilateral nephrocalcinosis (Chraïbi et al., 2007). To be noted, however, that parameters of kidney and liver functions are always normal in PXE patients, suggesting that calcification does not affect the activity of these organs (Vanakker et al., 2006).

During pregnancy, the placenta is abnormal, being hypoplastic and atrophic with focal calcifications; moreover, striking anomalies of the elastic lamellae are found in the maternal vessels (Gheduzzi et al., 2001). These alterations do not negatively affect pregnancy, however early delivery can be recommended if foetus stops growing.

### 3. Genetics

Pseudoxanthoma elasticum is inherited in an autosomal recessive manner. As a general rule, each parent of an individual with an autosomal recessive condition carries one copy of the mutated gene, without showing or showing very mild signs and symptoms of the disorder. In a few cases, however, an affected individual may have one parent without signs and the other parent with some sign of the disease. Also these cases have to be considered autosomal recessive because the normal-appearing parent, in fact, carries an *ABCC6* gene mutation, and the affected offspring inherits two altered genes, one from each parent (Ringpfeil et al., 2006). This situation is called pseudodominance, because it resembles autosomal dominant inheritance, in which one copy of an altered gene is sufficient to cause a disorder.

Because PXE is characterized by calcification of elastic fibres, genes involved in the synthesis and assembly of the elastic fibre network were initially considered as primary candidates for mutations. These included elastin (ELN) on chromosome 7, elastin-associated microfibrillar proteins, such as fibrillin 1 and fibrillin 2 (FBN1 and FBN2) on chromosomes 15 and 5, and lysyl oxidase (LOX) also on chromosome 15. However, genetic linkage analyses excluded all these chromosomal regions (Christiano et al., 1992; Raybould et al., 1994). Subsequent studies, employing positional cloning approaches, provided strong evidence for linkage to the short arm of chromosome 16, limiting a region of approximately 500 kb (Le Saux et al., 1999).

Examination of the existing genome database revealed that this candidate region contained four genes, none of which had actually an obvious connection to elastic fibres or more generally to the extracellular matrix, but after a long systematic sequencing approach, it appeared that the *ABCC6* gene (16p.13.1) is the main site of mutations in PXE (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000) (Figure 4).

This gene, spanning ~73 kb genomic DNA, is composed of 31 exons, belongs to the subfamily C of the ABC genes (ATP-binding cassette) and encodes for MRP6 (a transmembrane protein of 1503 aminoacids) composed of three hydrophobic membrane segments comprising five, six, and six transmembrane spanning domains, respectively, and two evolutionary conserved intracellular nucleotide binding folds (NBFs). The NBFs contain conserved Walker A and B domains, and a C motif critical for ATP binding and transmembrane transporter functions (Chassaing et al., 2005; Hu et al., 2003b) (Figure 5).

So far, approximately 300 different mutations have been reported in *ABCC6* (Costrop et al., 2010; Gheduzzi et al., 2004; Miksch et al., 2005; Plomp et al., 2008) and more than 80 have been detected in Italian PXE patients. The most frequent sequence changes are missense (55%) and nonsense (15%) mutations, as well as small deletions (15%), whereas less frequent alterations are represented by splicing errors, large deletions and insertions. Although the consequences of splicing mutations have not been investigated, at least 30% of all mutations cause a frameshift and the introduction of a stop-codon, which leads to premature chain termination. At protein level, the vast majority of mutations involve the cytoplasmatic domains and the carboxy-terminal end of MRP6. Mutations especially target the NBF1 and

NBF2 domains, and the 8<sup>th</sup> intracellular loop, consistent with the critical role of NBFs in ATP-driven transport. Functional studies have already shown that MRP6 transport is abolished by missense mutations located in NBF2 (Ilias et al., 2002).

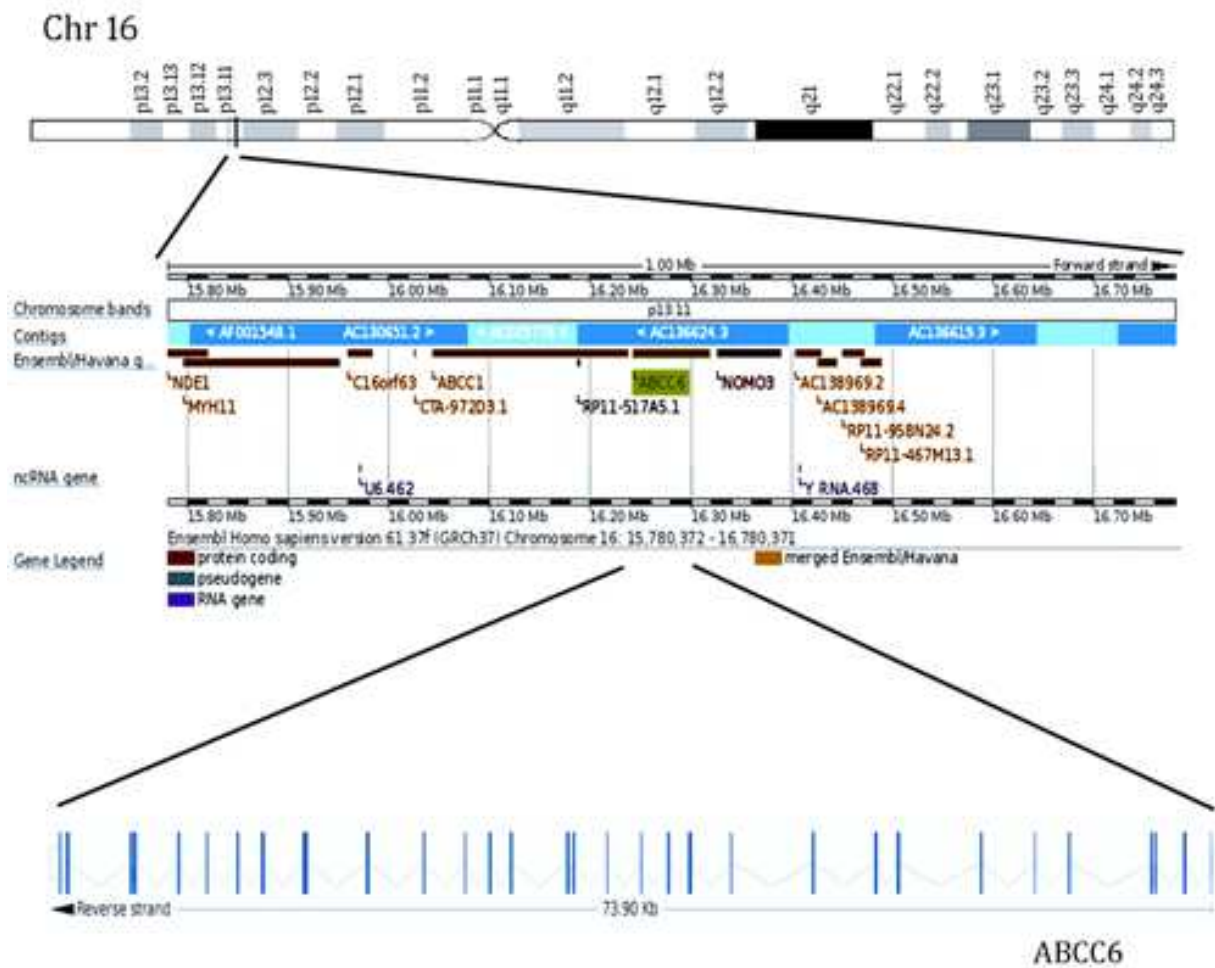


Fig. 4. Localization and structure of the *ABCC6* gene on chromosome 16

Two *ABCC6* mutations, R1141X and del (ex23\_29), occur very frequently, probably due to founder effects and genetic drift. R1141X may produce an instable mRNA which is rapidly degraded by nonsense mediated RNA decay (Hu et al., 2003a; Le Saux et al., 2000). The frequency of these two recurrent mutations differs according to the population studied: of the detected mutations, ex23\_29del is observed with a frequency of 28% in USA and 4% in Europe, whereas R1141X has a frequency of 4% in USA and 28% in Europe (Le Saux et al., 2001), with additional differences among European Countries, being 30% in Dutch patients (Bergen et al., 2004; Hu et al., 2003b) and about 26% and 13% in Italian and French patients, where a common founder effect was identified (Chassaing et al., 2004; Gheduzzi et al., 2004).

By contrast, in Japanese patients, neither R1141X nor ex23\_29del mutations were identified, whereas mutations 2542delG and Q378X account for 53% and 25%, respectively (Noji et al., 2004). In South African families of Afrikaansers, mutation R1339C represents more than half of the detected mutations, with a common haplotype indicating, also in this case, a founder effect (Le Saux et al., 2002).

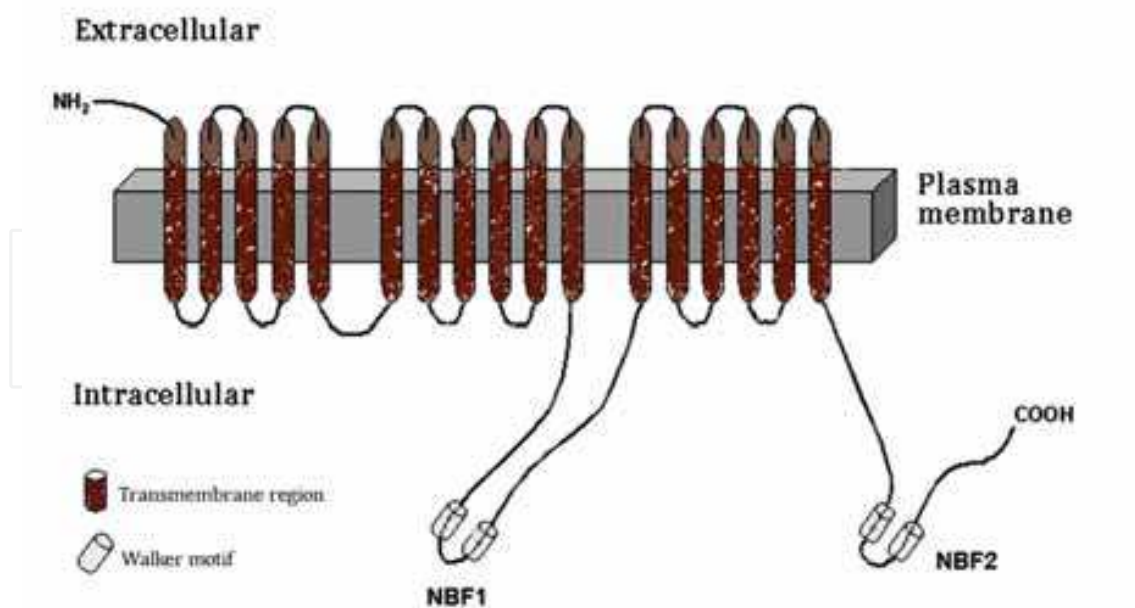


Fig. 5. Schematic drawing showing the typical transmembrane organization of the MRP6 protein

The *ABCC6* mutation detection rate is around 80-90%, since there are cases in which mutations cannot be identified in the coding region on one of the alleles, although the other allele harbours a recessive mutation (Chassaing et al., 2004; Gheduzzi et al., 2004); moreover, lack of mutation detection in some patients could reflect the occurrence of deletions (for example, deletion of exon 15 or deletion of the whole gene).

To date, no correlations have been established between the clinical phenotype and the nature or the position of the mutations (Pfender et al., 2007).

#### 4. Pathogenesis

The physiological function and the natural substrate(s) of MRP6 are currently unknown, however, because of its homology to MRP1, it has been classified as a multidrug resistance associated protein, thus belonging to the large family of membrane proteins that transport organic anions and/or other molecules against a concentration gradient at the cost of ATP hydrolysis (Borst & Elferink, 2002; Haimeur et al., 2004). The role of MRP6 in drug resistance is actually limited to low-level resistance of a small number of chemicals, like etoposide, teniposide, doxorubicin and daunorubicin (Belinsky et al., 2002; Kool et al., 1999). Consistently with its assumed functional role, MRP6 is highly expressed in liver and kidneys being localized to the basolateral side of hepatocytes and of proximal kidney tubules, suggesting that it may transport biomolecules from cells into the blood (Bergen et al., 2000; Kool et al., 1999; Scheffer et al., 2002).

However, in spite of the high level of *ABCC6* expression, liver and kidney do not suffer from mutations in this gene. By contrast, tissues as skin, retina and vessels, which are deeply altered in PXE, express very low levels of MRP6 (Bergen et al., 2000; Kool et al., 1999). These findings raised a still unsolved dilemma concerning the pathogenesis of PXE: how do mutations in a gene expressed primarily in the liver result in the mineralization of peripheral connective tissues?



To explain the putative mechanisms leading to ectopic calcifications from *ABCC6* mutations under normal calcium and phosphorus homeostatic conditions, two theories have been reported in the literature (Uitto et al., 2010): “the liver metabolic hypothesis” and “the peripheral cell hypothesis”. The metabolic hypothesis considers liver dismetabolism the only responsible for ectopic calcifications, whereas the peripheral cell hypothesis points to the role of mesenchymal cell metabolism on the homeostatic control of connective tissue calcifications in PXE.

The liver metabolic hypothesis postulates that the absence of functional MRP6 activity in hepatocytes results in deficiency of circulating factor(s) physiologically required to prevent aberrant mineralization (Jiang & Uitto, 2006; Uitto et al., 2010). In support of this hypothesis are clinical and experimental observations in PXE patients, as well as in the *Abcc6*<sup>-/-</sup> mouse (Klement et al., 2005), that serves as a model for human PXE. Firstly, clinical findings in PXE patients are rarely present at early childhood and the onset of clinical manifestations as well as the slow progression of the disease, due to continued accumulation of minerals in soft connective tissues of affected organs, can be regarded as the typical consequence of metabolic impairments that worsen with time. Secondly, serum from PXE patients, as well as from *Abcc6*<sup>-/-</sup> mice, lacks the capacity to prevent calcium/phosphate precipitation in an *in vitro* assay with smooth muscle cell cultures (Jiang et al., 2007). Furthermore, serum from PXE patients, when added to the culture medium, has been shown to modify the organization of elastic fibres without altering gene expression, thus suggesting the involvement of specific circulating factors directly acting on the assembly of elastic fibres (Le Saux et al., 2006), even if these changes occur in the absence of any *in vitro* calcification. Finally, recent skin grafting studies in wild-type and *Abcc6*<sup>-/-</sup> mice have further focused on the importance of circulating factor(s), hypothesizing that the mineralization process can be countered or even reversed by modifications of the homeostatic milieu (Jiang et al., 2009). In particular, it has been shown that the *Abcc6*<sup>-/-</sup> mouse skin graft does not develop mineralization, when placed onto the *Abcc6*<sup>+/+</sup> mouse, but calcification occurs in the skin of wild-type mouse after grafting onto the *Abcc6*<sup>-/-</sup> mouse, indicating that circulating factors in the recipient's blood could play a critical role in determining the degree of mineralization, irrespective of the graft genotype. However, in these skin graft experiments the possible modulation of fibroblast metabolism upon effect of circulating factors cannot be ruled out.

Actually, several studies have reported alterations in circulating factors in PXE patients, such as proteoglycans (Götting et al., 2005; Passi et al., 1996), plasma lipoproteins (Wang et al., 2001) and mineralization inhibitors, such as fetuin-A and Matrix Gla Protein (MGP) (Hendig et al., 2006, 2008). Moreover, a number of circulating molecules have been shown to be modified in the plasma of PXE patients by effect of a systemic altered redox balance (Garcia-Fernandez et al., 2008).

However, a number of questions remain to be elucidated. First of all, in PXE patients, despite of the absence and/or of the presence of one or more circulating factor(s), mineralization affects only a certain number of elastic fibres and only in peculiar areas of the body. Calcification seems, in fact, a rather specific phenomenon, since in PXE patients extracellular matrix components other than elastin (i.e. collagens or matrix glycoproteins) never undergo mineralization, furthermore, not all elastic fibres are calcified and not all areas of affected tissues are clinically involved (Gheduzzi et al., 2003; Pasquali-Ronchetti et al., 1981). In addition, the observation that patient's serum interferes with elastin assembly is in agreement with the above mentioned plasma modifications in PXE patients and especially with the abnormalities in glycosaminoglycan's content and species (Maccari et al., 2003, 2008;

Passi et al., 1996; Tiozzo Costa et al 1998), since it is well known that these matrix constituents are capable to greatly influence tropoelastin assembly (Gheduzzi et al., 2005; Tu & Weiss, 2008). Finally, if changes in the circulating environment can effectively modify the extent of ectopic calcifications, it is not clear why PXE mesenchymal cells, as dermal fibroblasts, maintain their abnormal phenotype even when they are cultured *in vitro* in optimal nutritional supplements and conditions far from their original environment (Boraldi et al., 2009).

Actually, in support to the “peripheral cell hypothesis”, it has been demonstrated that *in vitro* skin fibroblasts isolated from PXE patients exhibit a modified biosynthetic expression profile, altered cell-cell and cell-matrix interactions associated with changes in proliferative capacity (Boraldi et al., 2009; Quaglini et al., 2000), abnormal synthesis of elastin and of glycosaminoglycan/proteoglycan complexes (Passi et al., 1996) and enhanced degradation potential due to elevated matrix metalloproteinase-2 activity (Quaglini et al., 2005). Consistently, histopathological and ultrastructural observations showed that, in PXE, mineralization occurs only on elastic fibres (Gheduzzi et al., 2003; Pasquali-Ronchetti et al., 1981), suggesting a peculiar composition and/or organization of elastic fibre components (Lebwohl et al., 1993; Sakuraoka et al., 1994). By immuno-electron microscopy, it has been demonstrated that aberrant matrix proteins known for their high affinity for calcium and normally involved in mineralization processes (such as alkaline phosphatase, vitronectin, fibronectin, bone sialoprotein, osteonectin and proteoglycans) are accumulated within PXE elastic fibres (Contri et al., 1996; Kornet et al., 2004; Passi et al., 1996).

All these data undoubtedly highlight the importance of mesenchymal cells in the pathogenesis of ectopic calcifications, never the less it is still unclear whether these changes depend or not upon the expression of the *ABCC6* gene in mesenchymal cells (Matsuzaki et al., 2005). It has to be noted, in fact, that even normal fibroblasts, possibly due to aberrant splicings, do not seem to express the full MRP6 protein (Matsuzaki et al., 2005) and that immunologically positive epitopes have been recognized only on membranes of the endoplasmic reticulum of isolated dermal fibroblasts (Boraldi et al., 2009). What could be the significance and importance of the presence of at least part of MRP6 in the endoplasmic reticulum of mesenchymal cells have not been investigated.

However, in the light of these observations, changes in membrane transport properties described in PXE cultured fibroblasts (Boraldi et al., 2003) would seem likely the result of the high level of reactive oxygen species (ROS) on the structural organization of cell membranes (Boraldi et al., 2009) and consequently on cell permeability. It has been in fact demonstrated *in vitro* (Pasquali-Ronchetti et al., 2006) and *in vivo* (Garcia-Fernandez et al., 2008) that PXE is characterized by an altered redox balance. At cellular level, the chronic oxidative stress condition is due, at least in part, to the loss of mitochondrial membrane potential ( $\Delta\Psi$  (m)) with overproduction of ROS. Consistently, cultured fibroblasts produce more malondialdehyde, a product of lipid peroxidation, and accumulate higher amounts of carbonylated proteins compared to controls (Boraldi et al., 2009; Pasquali-Ronchetti et al., 2006). Likewise, in the circulation of patients, the redox unbalance leads to significantly high amount of oxidised proteins and lipids, which might have relevant effects on peripheral mesenchymal cells (Garcia-Fenandez et al., 2008).

Interestingly, among the molecular pathways which are sensitive to the redox potential is the vitamin K-cycle that, within connective tissues, is essential for the  $\gamma$ -glutamyl carboxylation of MGP (Matrix Gla Protein), a potent inhibitor of calcification in soft connective tissues (Schurgers et al., 2008). Consistently, in PXE fibroblasts (Gheduzzi et al., 2007) and in the *Abcc6*<sup>-/-</sup> mice (Li et al., 2007) there is a reduced carboxylation of MGP.

A recent characterization of the PXE fibroblast's protein profile revealed that numerous endoplasmic reticulum-associated proteins are differentially expressed in pathological cells. Among these proteins, calumenin and disulfide isomerase are involved in the recycling of vitamin K, leaving open the question if insufficient carboxylation of MGP in PXE cells could be due to reduced availability or to diminished recycling of vitamin K (Boraldi et al., 2009) (Figure 6).

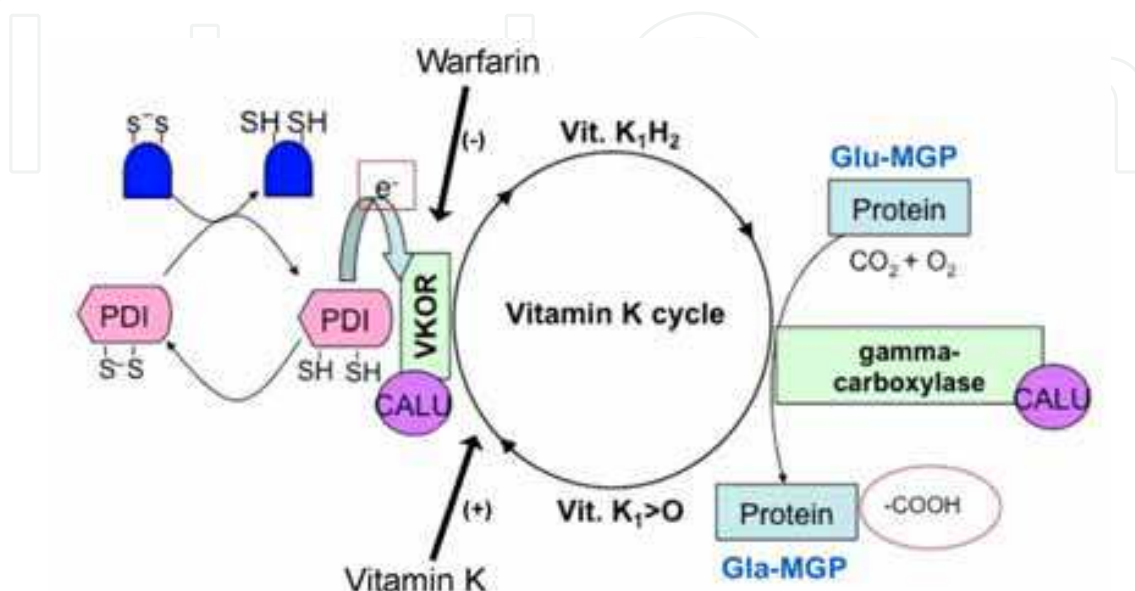


Fig. 6. Drawing illustrating the numerous factors involved in vitamin K cycle.

Vitamin K represents an important cofactor of protein carboxylation. Within the endoplasmic reticulum of mesenchymal cells, MGP (Matrix Gla protein) is activated by gamma-carboxylase from the inactive form (Glu-MGP) to the active form (Gla-MGP). Protein disulfide isomerase (PDI) and calumenin (CALU) are important modulators of these reactions. Warfarin, by inhibiting the action of vitamin K epoxide reductase, reduces the efficiency of the carboxylation process and favours the development of vascular calcifications. Modified from Wajih (Wajih et al., 2007)

To further confirm the importance of efficient MGP carboxylation in controlling the mineralization process, there are experimental evidences showing that antibodies specific for carboxylated (Gla-MGP) and non-carboxylated MGP (Glu-MGP) are differently localized within human dermal elastic fibres. In particular, both forms of MGP are rather heterogeneously distributed within elastin of control subjects, whereas in PXE patients Glu-MGP is markedly present in calcified areas and Gla-MGP is exclusively localized at the mineralization front (Gheduzzi et al., 2007). Although it has been suggested that MRP6 could function as a vitamin K transporter from the liver to the periphery, and that, in PXE, the mutated protein may prevent connective tissue from an adequate supply of the vitamin necessary for efficient carboxylation processes (Borst et al., 2008; Vanakker et al., 2010), *in vivo* and *in vitro* treatments with different forms of vitamin K do not appear to interfere and/or to inhibit the mineralization process (Jiang et al., 2011; Annovi et al., 2011).

Therefore, it could be suggested that mutated MRP6 in liver and kidney is responsible for the altered release in the circulation of factors that modify plasma components, among which proteins, lipids and, eventually, other constituents, thus contributing to produce an abnormal environment at the periphery, and to influence mesenchymal cell behaviour and



metabolism. Among peripheral alterations it would appear that there is an imbalance between production and degradation of oxidant species, abnormal protein and glycosaminoglycan synthesis, changes in post-translational protein modifications and abnormal DNA-methylation (Boraldi et al., 2009). Moreover, the alterations in the *in vitro* behaviour of PXE fibroblasts may suggest that permanent epigenetic changes have occurred, thus causing the inability of these cells to produce mature inhibitors and/or modulators of calcification (Figure 7).

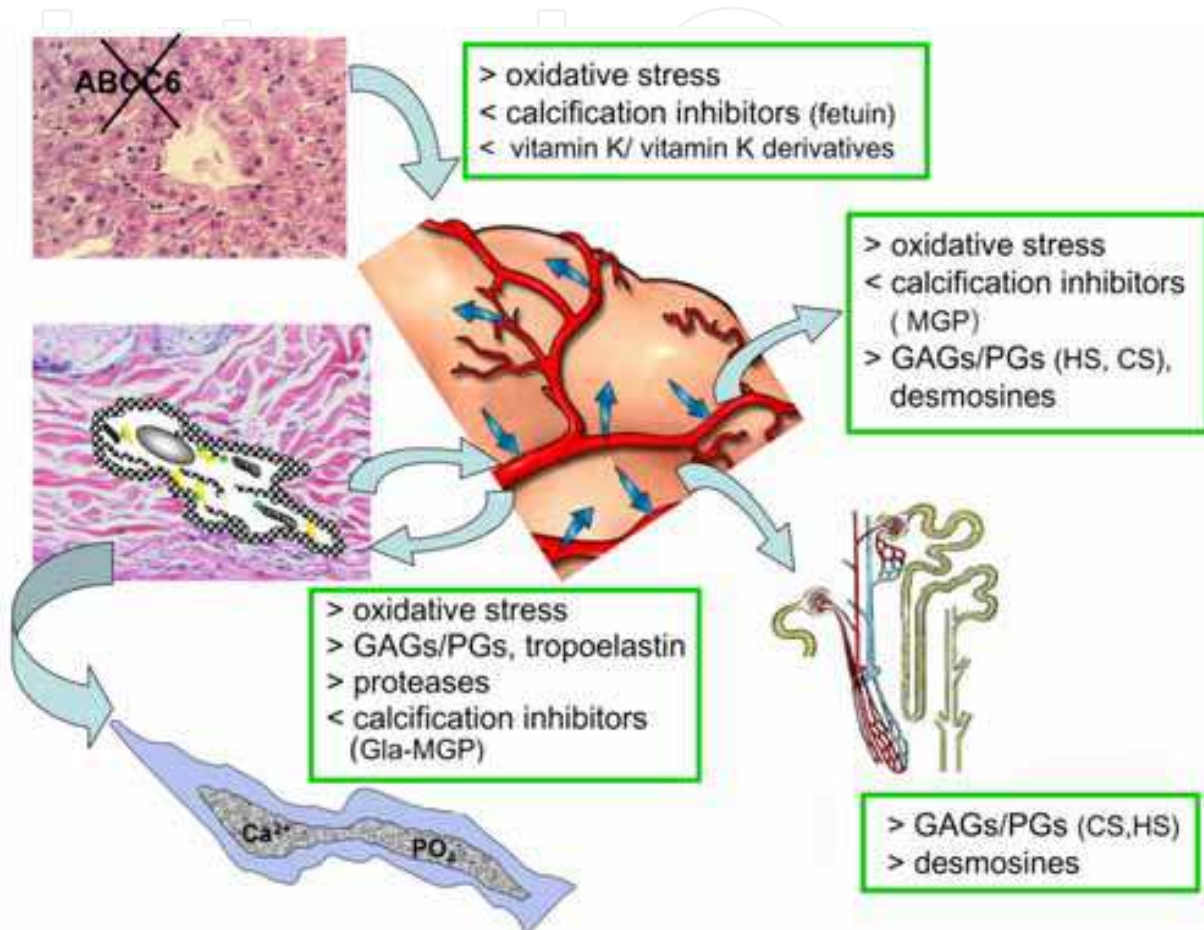


Fig. 7. Drawing that summarizes the major metabolic abnormalities observed in PXE and the relationships between organs and tissues possibly involved in the pathogenesis of elastic fibre calcification.

Absent or altered expression of the *ABCC6* gene in hepatocytes may be responsible for abnormal extrusion in the circulation of still unknown factor/s which is/are responsible for or contributing to oxidative stress, to ineffective inhibition of ectopic calcification, to reduced levels of vitamin K and/or vitamin K derivatives. Therefore, through the circulation, abnormal signals could reach mesenchymal cells epigenetically modifying their phenotype, i.e. chronic oxidative stress, altered synthesis of tropoelastin, production of glycosaminoglycans (GAGs) or proteoglycans (PGs) with peculiar physical-chemical properties, augmented proteolytic potential, lower expression of carboxylated Matrix Gla Protein (Gla-MGP), thus causing accumulation of calcium (Ca<sup>2+</sup>) and phosphate (PO<sub>4</sub><sup>-</sup>) mineral precipitates on elastic fibres. Abnormal products from mesenchymal cells can be found in the circulation or in the urine, i.e. parameters of redox balance, desmosines as indicators of elastin degradation, heparan sulfate (HS) and chondroitin-sulfate (CS) GAGs and inhibitors of calcification as MGP



The various levels of mineralization, even within the same tissue, could be explained by the heterogeneity of different mesenchymal cell subtypes and their peculiar functional imprinting related to structural and functional requirements of organs and tissues (Jelaska et al., 1999; Sorrell & Caplan, 2004).

On the basis of all these considerations, both “the liver metabolic” and “the peripheral cell” hypotheses, together, can actually help to understand the pathogenesis of clinical manifestations in PXE. On one side, there is the involvement of the liver that, expressing MRP6, has an important role in controlling metabolic processes and plasma composition, on the other side it cannot be underestimated the crucial role of peripheral mesenchymal cells, as fibroblasts, in regulating connective tissue homeostasis.

## 5. The role of modifier genes

Understanding PXE pathogenesis is further complicated by the fact that the age of disease onset and the expression of clinical symptoms are highly variable (Gonzales et al., 2009) and marked phenotypic variations have been observed in affected siblings bearing the same *ABCC6* mutation (Gheduzzi et al. 2004).

Although, there is no evidence for the involvement of other genes in the pathogenesis of PXE (Li et al., 2009), however, a number of modifying factors, both genetic and environmental, have been suggested to play a role in the phenotypic expression of the disease (Hovnanian, 2010).

One recently identified genetic factor involves polymorphisms in the promoter region of the *SPP1* gene (secreted phosphoprotein 1, also known as osteopontin) (Hendig et al., 2007). Osteopontin is a secreted, highly acidic phosphoprotein that is involved in immune cell activation, wound healing, bone morphogenesis (Denhardt et al., 2001), thus playing a major role in regulating the mineralization process in various tissues, including skin and aorta, where osteopontin is localized to elastic fibres (Baccarani-Contri et al., 1994). Higher expression of this protein has been observed in skin biopsies from PXE patients compared to samples from unaffected regions or from healthy individuals (Contri et al., 1996) and also in mice suffering from dystrophic cardiac calcification, suggesting that its expression is influenced by the *Dyscalc1* locus on chromosome 7 (Aherrahrou et al., 2004). Although several polymorphisms in the *SPP1* gene have been described and associated with various disorders such as systemic lupus erythematosus and arteriosclerosis (Giacopelli et al., 2004), the role of osteopontin in regulating the calcification process, strongly suggested that sequence variations in the *SPP1* promoter region might account for the higher expression observed in PXE patients, thus affecting the disease outcome. Consistently, mutational screening revealed nine different sequence variations, and three *SPP1* promoter polymorphisms (c.-1748A>G, c.-155\_156insG and c.244\_255insTG), in particular, were significantly associated with PXE. Until now, no functional studies have been carried out with the *SPP1* promoter polymorphisms c.-1748>G, whereas the polymorphism variant c.244\_245ins TG does not have a major regulatory effect. By contrast, the discovery that polymorphism c.155\_156insG generates a Runx2-binding site opened a new field of investigations, since Runx2-binding sites are in fact very important for regulating *SSP1* expression in bone tissue (Giacopelli et al., 2004). A constitutive expression of Runx2, combined with a glucocorticoids' supplementation, results in a strong upregulation of *SPP1* expression and finally in a biological matrix mineralization by primary dermal fibroblasts (Phillips et al., 2006). Therefore, polymorphisms in the *SPP1* promoter may represent a genetic risk factor contributing to PXE susceptibility.

Other studies have correlated the incidence of cardiovascular complications in PXE with polymorphisms of genes encoding for xylosyltransferase 1 (*XT-1*) and xylosyltransferase 2 (*XT-2*), a set of key enzymes involved in proteoglycan biosynthesis and considered biochemical markers of fibrosis (Schön et al., 2006). The altered proteoglycan metabolism, already observed *in vitro* (Passi et al., 1996) and *in vivo* (Maccari et al., 2003), suggests that enzymes from these pathways may function as genetic co-factors in the severity of PXE. Furthermore, PXE patients have elevated serum XT-I activity. On the basis of these observations Authors suggested a connection between the severity of the disease and genetic variations in the *XYLT* genes (Schön et al., 2006).

More recent studies have shown that polymorphisms in genes associated with redox balance as catalase (*CAT*), superoxide dismutase 2 (*SOD2*) and glutathione peroxidase 1 (*GPX1*) are associated with early onset of clinical manifestations (Zarbock et al., 2007), whereas polymorphisms of the *VEGF* gene (vascular endothelial growth factor) are involved in the pathogenesis of ocular manifestations (Zarbock et al., 2009). The distribution of 10 single nucleotide polymorphisms (SNPs) in the promoter and coding region of the *VEGFA* gene has been evaluated in DNA samples from 163 German patients affected by PXE and in 163 healthy subjects. Five SNPs showed significant association with severe retinopathy. The most significant association was with polymorphism c.-460C>T. In the light of these results *VEGF* gene polymorphisms might be considered useful prognostic markers for the development of PXE-associated retinopathy, thus allowing earlier therapeutic intervention in order to prevent loss of central vision, one of the most devastating consequences of PXE (Zarbock et al., 2009).

By contrast, very few data are available on the role of *ABCC6* polymorphisms on the occurrence and/or severity of clinical manifestations in PXE patients. The *ABCC6* pR1268Q polymorphism has been associated with lower plasma triglycerides and higher plasma HDL-cholesterol, suggesting that *ABCC6* may contribute to modulate plasma lipoproteins and possibly cardiovascular complications (Wang et al., 2001). In a larger study conducted on a German cohort of PXE patients, in addition to the complete screening of the *ABCC6* gene, the *ABCC6* promoter region was also analyzed and the following polymorphisms were found: c.-127C>T, c.-132C>T and C.-219A>C. Interestingly, the difference in the c.-219A>C frequencies between PXE patients and controls was statistically significant and this polymorphism appeared located in a transcriptional activator sequence of the *ABCC6* promoter, functioning as a binding site for a transcriptional repressor predominantly found in genes involved in lipid metabolism (Schulz et al., 2006), further sustaining a possible correlation between *ABCC6* and lipid metabolism.

Surprisingly, the observation that the c.3421C>T loss-of-function mutation on one allele of *ABCC6* (R1141X) is significantly associated to coronary artery disease (CAD), in the apparently normal population (Köblös et al., 2010), was not confirmed in a cohort of Italian PXE patients (Quaglino, unpublished data), further sustaining the difficulty to perform a genotype-phenotype correlation, although not excluding the possibility that carriers of *ABCC6* loss-of-function mutations may benefit from cardiovascular prevention programs (Vanakker et al., 2008).

## 6. Diagnosis and treatments

In spite of the impressive progress in understanding the genetic/molecular basis of inherited diseases, also in PXE, similarly to other genetic disorders, there have been limited improvements in terms of treatment and cure. Major advances concern diagnosis, due to

ability to recognize a continuously increased number of mutations (mutation detection rate varies from 80-90%). Attempts to establish genotype/phenotype correlations have yielded little clinically useful information other than the fact that, as PXE patients age, symptoms get worse, probably because of progressive accumulation of mineralized elastic fibres, associated to other age-related degenerative features (Garcia-Fernandez et al., 2008).

PXE is an important cause of blindness and of early death from cardiovascular manifestations (Neldner, 1988), therefore an early diagnosis is important in order to minimise the risk of systemic complications. One of the major problems encountered by patients affected by rare diseases, as PXE, is the difficulty to find physicians who are aware of the disorder and of the possible related complications. Therefore, strenuous efforts are necessary to spread the knowledge on these disorders not only in the scientific community, but also among practitioners, who represent the first medical reference point for patients. An additional help may derive from the definition of commonly accepted criteria for clinical diagnosis, which, in the case of PXE, include the presence of retinal angioid streaks (a fluorescein angiogram may be necessary) in combination with characteristic skin lesions (calcification of fragmented elastic fibres confirmed by von Kossa stain in a biopsy of lesional skin) (Figure 8) with or without a positive family history of PXE (two or more family members clinically diagnosed). It is however important to note that mild forms of the disorder can be easily overlooked and a negative family history does not exclude the diagnosis.

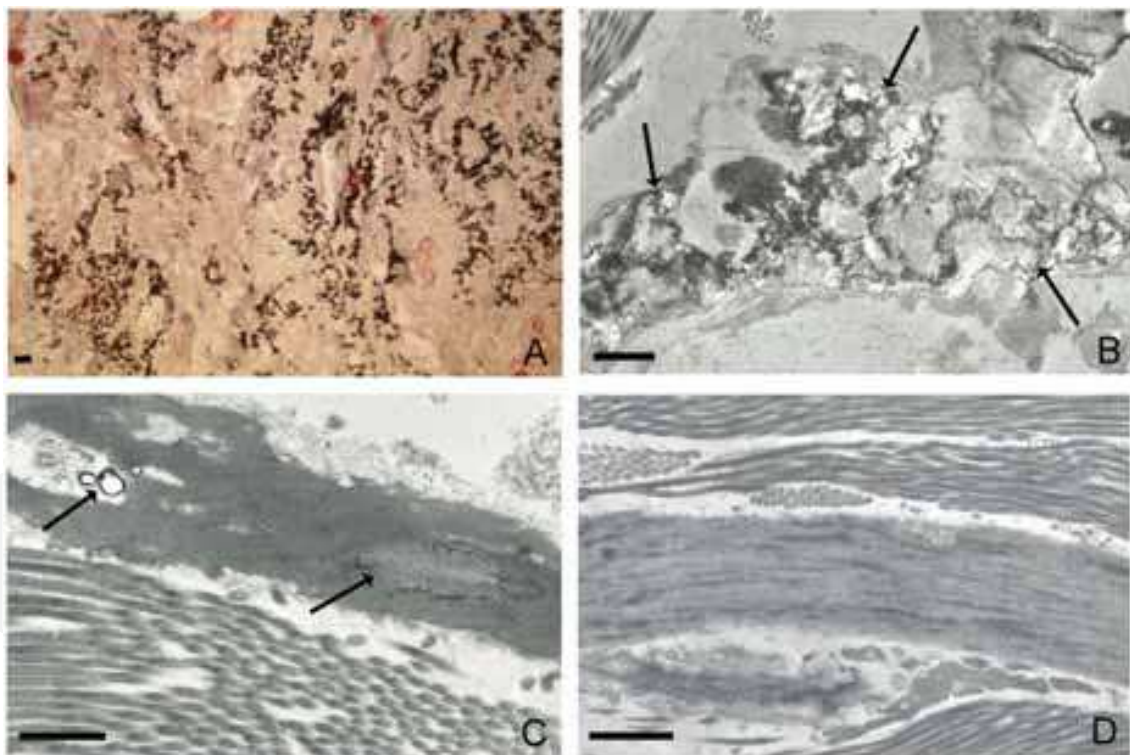


Fig. 8. Demonstration of mineralized elastic fibres is the gold standard diagnostic criteria in PXE.

A) Light microscopy of a dermal biopsy from a PXE patient stained with Von Kossa for the visualization of brownish calcified elastin. B-D) Transmission electron microscopy showing dramatically deformed and mineralized elastic fibres (arrows) in the dermis of a PXE patient (B) and small clinically irrelevant alterations (arrows) in the skin of PXE carriers (C), compared to the typical amorphous structure of normal elastic fibres (D). Bars: 1  $\mu$ m



Since the discovery of *ABCC6* as the PXE associated gene in 2000 (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000), molecular genetic testing have been rapidly developed and, although frequently limited to research laboratories or to highly specialized centres, they may represent an important diagnostic assessment. The techniques most frequently used are sequence analysis and mutation scanning, which are capable to detect missense, nonsense and frameshift mutations as well as small deletions and insertions. Testing strategies usually involve a first screening of exons in which a large number of mutations are located (i.e. exons 24 and 28), and, in case of negative results, the sequencing of the other coding regions. Moreover, since a 16.4 kb deletion involving exons 23-29 is another recurrent mutation, a specific deletion analysis can be required.

Analyses performed so far revealed that there is a considerable spectrum of genetic mutations (>300), with wide inter- and intra-familial phenotypic variations, and an extreme variability in terms of progression of the disease as well as of severity and extent of clinical manifestations. It must be reminded that PXE is a systemic disorder and therefore management of PXE requires coordinated input from a multidisciplinary team of specialists including dermatologist, primary care physician, ophthalmologist, cardiologist, vascular surgeon, plastic surgeon, genetics professional, and a nutritionist.

Moreover, once a diagnosis of PXE has been established, in order to delay and eventually manage ocular and cardiovascular complications, patients are encouraged to have clinical/instrumental examinations whose frequency may depend on the age of patient, age at diagnosis and severity of clinical manifestations. In particular, it is advisable to perform a complete dilated eye examination by a retinal specialist, particularly looking for *peau d'orange* and angioid streaks and baseline cardiovascular examination with periodic follow-up including: echocardiography, cardiac stress testing, and Doppler evaluation of peripheral vasculature.

At present, no specific treatment for PXE exists, in the sense that mineralization of elastic fibres cannot be delayed or reverted. Never the less, skin surgery has been successfully applied for cosmetic improvement (Ng et al., 1999), as well as peripheral and coronary arteries interventions in order to limit vascular complications (Donas et al., 2007; Shepherd & Rooke, 2003).

The only stage of the disease where therapy for ocular complications is possible and indicated is whenever a choroidal neovascularization (CNV) has developed (Georgalas et al., 2011). Traditional therapeutic options consist of laser photocoagulation (used to halt the progression of CNVs, although characterized by high rate of recurrence, visual loss, and central scotomas) (Pece et al., 1997) or of photodynamic therapy (PDT) (used to arrest the progression of CNVs, even though results appeared less encouraging then expected) (Heimann et al., 2005). Experimental surgical procedures, such as macular translocation or subfoveal CNV excision (Roth et al., 2005), appeared unsuccessful. More recently, taking advantage from the experience on macular degeneration, the intraocular injection of anti-angiogenic drugs, as avastin and lucentis, actually appeared to be the most effective therapeutic options for ocular complications (Verbraak, 2010).

In the absence of effective treatments specifically targeting pathways leading to ectopic calcifications, anecdotal reports can be found in the literature concerning the use of drugs or of nutritional supplements. For instance, it has been suggested that pentoxifylline and cilostazol may ameliorate intermittent claudication (Muir, 2009), however, controlled studies of these drugs in PXE patients have not been performed. Interestingly, the ARED (Age Related Eye Disease) study suggested that a regimen of antioxidant vitamins could be



beneficial in patients with macular degeneration. Given the similarities with PXE (i.e. ocular manifestations and altered redox balance) it is possible that the same recommendation can be valuable also for PXE (Lecerf & Desmettre, 2010), even though further investigations are required to support this hypothesis. By contrast, more promising perspectives, at least in the mouse model, seem to be represented by a diet supplemented with an excess of magnesium, although the mechanisms of reduced calcifications are still unknown (Gorgels et al., 2010; LaRusso et al., 2009) and the effects of long-term treatments with high doses of magnesium in humans have not been yet investigated.

## 7. PXE-like diseases

Even though no other phenotypes are known to be associated with mutations in the *ABCC6* gene, PXE-like clinical features, including aberrant mineralization of elastic fibres, have been reported in a number of apparently unrelated acquired and genetic clinical conditions (Neldner, 1988).

### 7.1 Acquired conditions

Among the acquired conditions, PXE-like cutaneous changes may be associated with multiple pregnancy, longstanding end-stage renal disease (Lewis et al., 2006), L-tryptophan induced eosinophilia myalgia syndrome (Mainetti et al., 1991), and amyloid elastosis (Sepp et al., 1990) as well as after D-penicillamine treatment, cutaneous exposure to calcium salts (Neldner & Martinez-Hernandez, 1979), and salpeter (Nielsen et al., 1978). In these cases, mineralization of skin may result from metabolic abnormalities affecting calcium and/or phosphate homeostasis or from direct deposition of mineral salts on collagen or elastic fibres. However, the pathomechanistic details and the role of predisposing genetic factors are unknown.

In papillary dermal elastolysis, white-yellow papules resembling PXE can be observed in aged women (60–80 years), although, in contrast to PXE, these lesions are histologically characterized by loss of elastin in the papillary dermis (Ohnishi et al., 1998).

Moreover, elastic fibres similar to those of PXE have been observed in the lesional skin of patients with a variety of inflammatory skin diseases in the absence of clinical evidence of PXE (Bowen et al., 2007), in calcific elastosis, lipedema, lipodermatosclerosis, granuloma annulare, lichen sclerosus, morphea profunda, erythema nodosum, septal panniculitis, basal cell carcinoma and fibrosing dermatitis (Bowen et al., 2007; Taylor et al., 2004).

Even though sporadically, ocular lesions similar to those typical of PXE, have also been reported in Paget's disease, Marfan's and Ehlers-Danlos syndromes (Gurwood & Mastrangelo, 1997) and calcifications of retina, retina vessels and the presence of osseous metaplasia have also been noted in patients with renal failure, Coats' disease, tuberous sclerosis and retinocytomas (Miller et al., 2004; Patel et al., 2002).

Finally it has to be mentioned that ectopic calcifications may occur in the vascular system during physiological aging, in atherosclerosis and hypercholesterolemia, hypertension, smoking, calcific aortic stenosis, Marfan syndrome, diabetes, renal failure and in smokers (Proudfoot & Shanahan, 2001). There are two types of calcifications that occur in arteries: the intimal calcification, characteristic of the atherosclerotic plaque and associated with cells and collagen, and the medial calcification (also known as Mönckeberg sclerosis) mainly associated with elastin. Patients with chronic kidney disease (CKD) frequently display both forms of calcification. Another form of vascular calcification also occurs nearly exclusively in CKD

patients: calciphylaxis or calcific uremic arteriolopathy. This is a disorder of medial calcification of the small arterioles of the skin, resulting in skin necrosis (Moe & Chen, 2003).

## 7.2 Genetic conditions

Unexpectedly, PXE-like cutaneous changes have also been found in approximately 20% of patients with beta-thalassemia (beta-thal) and sickle cell anaemia (SCD), that are well known severe congenital forms of anemia resulting from the deficient or altered synthesis of haemoglobin beta chains (Baccarani-Contri 2001; Fabbri et al., 2009). The first report suggesting a link between beta-thal and PXE was based on the observation that angioid streaks were actually present in both diseases (Aessopos et al., 1989). Subsequent studies confirmed that PXE-like syndrome in beta-thalassemias and SCD, although less severe, is histopathologically and clinically identical to inherited PXE consisting of indistinguishable cutaneous, ocular and vascular abnormalities due to elastic fibre calcification (Baccarani-Contri et al., 2001; Bercovitch & Terry, 2004; Hamlin et al., 2003) (Figure 9). In particular, it has been observed that beta-thal patients have calcifications of the posterior tibial artery (55%), typical skin lesions (20%), angioid streaks (52%) and that one or more of the three manifestations are actually present in 85% of the patients (Aessopos et al., 1998). Cardiovascular complications have been only sporadically observed, although they include intracranial haemorrhages, ischemic strokes, coronary arterial calcification complicated by unstable angina, myocardial infarction, mitral valve prolapse, valve calcification and leaflet thickening, pericardial thickening, renal artery calcification with arterial hypertension and peripheral arterial abnormalities complicated by gastric haemorrhage and intestinal infarcts (Aessopos et al., 1997,1998, 2001; Cianciulli et al., 2002; Farmakis et al., 2003).

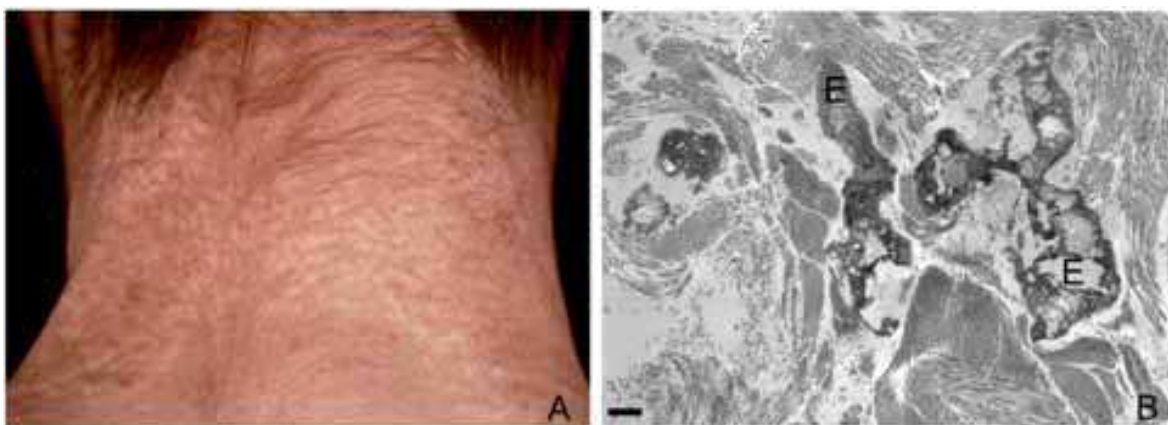


Fig. 9. PXE-like alterations in a patient affected by beta-thalassemia. In these patients, papules and skin folds in typical areas of the neck (A) are associated to mineralized elastic fibres (E) in the dermis, as visualized by transmission electron microscopy (B). Bar: 1 μm

A genetic link between beta-thalassemia or SCDs and PXE is unlikely. In first instance, no mutations in the *ABCC6* gene were found in a cohort of beta-thal patients (Hamlin et al., 2003), moreover, *ABCC6* as well as other genes encoding for elastin or elastin associated molecules (i.e. fibrillins 1 and 2, elastin-related glycoproteins and lysyl-oxidase) are located on chromosomes different from that of the  $\beta$ -globin gene (Ringpfeil et al., 2000) and family members who do not have a haemoglobinopathies fail to show any PXE stigmata (Aessopos et al., 1994).

Never the less, in a study by Martin and coworkers (2006), fifty PXE patients have been investigated with the aim to determine the incidence of haemoglobin abnormalities typical of thalassemia. No cases of beta thalassemia were diagnosed in this cohort of patients, however in 20% of cases a significant but isolated (i.e. without microcytic anemia) increase of haemoglobin A2 (HbA2) was observed. The severity of clinical manifestations, other than the extent of cutaneous involvement, appeared independent from levels of haemoglobin. Therefore, *ABCC6* plus beta-globin digenism was ruled out of the pathogenesis of PXE, but it could be hypothesized a functional epigenetic reaction between *ABCC6* and the beta-globin locus, even though reciprocal interactions are clearly unequal since the change in *ABCC6* transcription occurring during the course of beta thalassaemia is responsible for a PXE phenotype, while increased HbA2 during the course of PXE has no haematological clinical consequences.

Interestingly, it has been recently demonstrated that a mouse model of beta-thal (*Hbb(th3/+)*) exhibits a NF-E2-induced transcriptional down-regulation of liver *ABCC6* (Martin et al., 2011), even though there are no evidence for spontaneous calcifications. It has been therefore suggested that decreased expression of *mrp6* occurring later in life is probably insufficient to promote mineralization in the *Hbb(th3/+)* mouse C57BL/6J genetic background. However, these data may indicate that i) other factors, beside *ABCC6* expression are involved in the pathogenesis of calcifications, ii) responsive fibroblasts or other mesenchymal cells are required in order to modify connective tissue homeostasis, and iii) independently from the primary gene defect, common pathways may be involved in these disorders.

Within this context, it has been suggested that the elastic tissue injury in these patients may be the result of an oxidative process, induced by the combined and interactive effects of different factors (Aessopos et al., 1998; Garcia-Fernandez et al., 2008; Pasquali-Ronchetti et al., 2006). Plasma membrane microparticles, derived from the oxidative damage of red cell membranes by the effect of denatured hemoglobin products and free iron (Olivieri, 1999), as well as unbound fractions of hemoglobin and haem, which exceed the binding capacity of haptoglobin and hemopexin in the context of chronic hemolysis, have been considered to elicit inflammatory and oxidative reactions (Belcher et al., 2000; Gutteridge & Smith, 1988). The accumulated and prolonged effects of ROS/free radicals may result in disturbance of mesenchymal cell metabolism with structural deterioration of elastic fibres (Bunda et al., 2005). Accordingly, oxidative stress constitutes a potential acquired mechanism affecting the same molecular pathways, which are implicated in the pathogenesis of hereditary PXE.

The recent observation that a PXE-like phenotype can be observed in patients with pronounced deficiency of the vitamin K-dependent clotting factors raises the intriguing and exiting possibility that there might an additional pathway, independent of *ABCC6*, leading to the PXE phenotype (Vanakker et al. 2007).

Congenital deficiency of the vitamin K-dependent factors (VKCFD) is a rare bleeding disorder that can be caused either by mutation in the gamma-glutamyl carboxylase gene (*GGCX*) or in the vitamin K epoxide reductase complex (*VKORC*) (Oldenburg et al, 2000; Pauli et al, 1987). Moreover, acquired forms of the disorder can occur more frequently due to intestinal malabsorption of vitamin K (Djuric et al., 2007) or after prolonged treatments with vitamin K antagonists as warfarin (Palaniswamy et al., 2011). Vitamin K undergoes oxidation-reduction cycling within the endoplasmic reticulum, donating electrons to activate specific proteins via enzymatic gamma-carboxylation of glutamate groups before being enzymatically re-reduced (Figure 6).



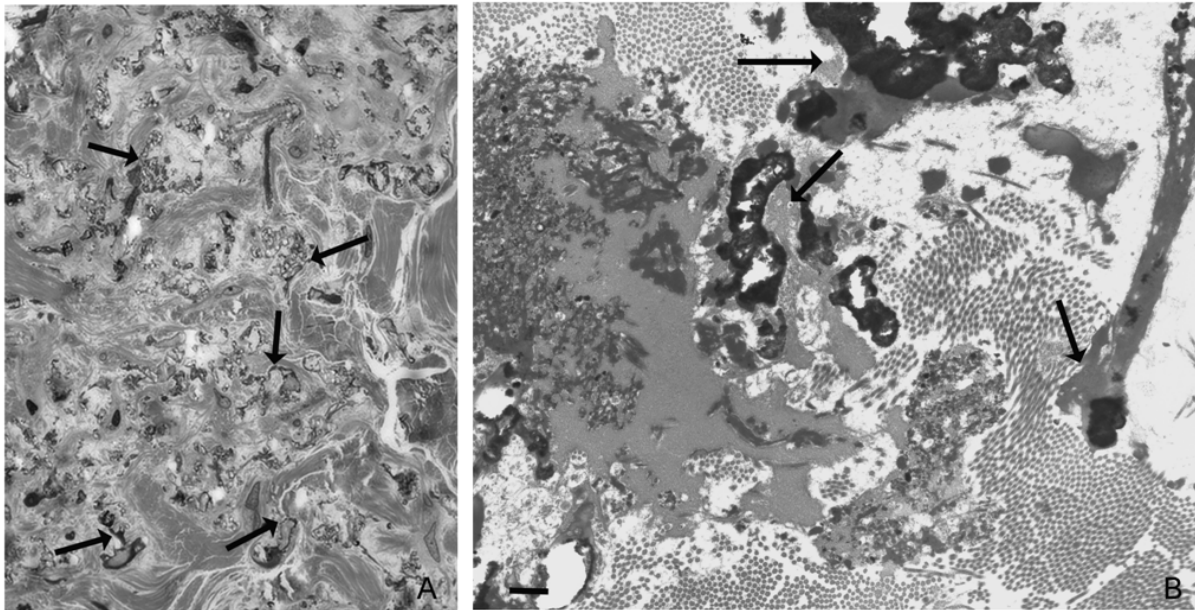


Fig. 10. Mineralized elastic fibres in a patient affected by VKFCD. A) Light and B) transmission electron microscopy of a skin biopsy. Heavy mineralized elastic fibres (arrows) can be seen. Bar: 1  $\mu$ m

In addition to coagulation factors (II, VII, IX, X, and prothrombin) vitamin K activates protein C and protein S, osteocalcin (OC), matrix Gla protein (MGP), periostin, Gas6, and other vitamin K-dependent (VKD) proteins that, within bones, support calcium homeostasis and the mineralization process, whereas in vessel walls, and possibly in other peripheral soft connective tissues, they inhibit calcification, favouring endothelial integrity, cell growth and tissue renewal (Kidd, 2010).

Clinical overlap of PXE and VKCFD was obvious from the skin manifestations of yellowish papules or leathery plaques, with dot-like depressions, angioid streaks and/or ocular *peau d'orange*, as well as fragmentation and calcification of elastic fibres in the dermis. Important phenotypic differences from PXE included much more severe skin laxity with involvement of the trunk and limbs with thick, leathery skin folds rather than confinement to flexural areas, and no decrease in visual acuity. By light microscopy, changes in the reticular dermis were identical to those typical of PXE, consisting in polymorphous, fragmented, and mineralized elastic fibres, as shown by von Kossa stain. At the ultrastructural level, however, elastin had a more fragmented and mottled appearance than that typically observed in PXE (Vanakker et al., 2007) (Figure 10). In the light of these observations, it has been demonstrated *in vitro* (Gheduzzi et al., 2007) and *in vivo* (Li et al., 2007) that PXE is characterized by low levels of carboxylated-Matrix Gla Protein (Gla-MGP), thus suggesting that these changes may play a role in the pathogenesis of PXE, as described in more details in paragraph 4.

## 8. Conclusions

Pseudoxanthoma elasticum (PXE) is a rare genetic disorder characterized by mineralization of elastic fibres within all connective tissues, although the most important clinical manifestations affect skin, eyes and the cardiovascular system. Despite the dramatic involvement of the extracellular matrix, the first attempts made by researchers to find out



the gene defect among those coding for matrix molecules failed and in 2000 three groups, independently, demonstrated that PXE is due to mutations in the *ABCC6* gene, that belongs to the ABC membrane transporters. To date the physiological substrate of this transporter is not known and still elusive are the pathogenetic mechanisms linking a defective cellular transporter, mainly expressed in liver and kidney, to ectopic calcification of connective tissues. This disease may therefore represent a very interesting example for investigating the complexity that regulates molecular pathways and the influence of metabolism on several organs/systems. Moreover, there is also evidence that similar endpoints (i.e. clinical and histological alterations) can be observed in some patients starting from gene defects different from *ABCC6* (i.e. beta-thalassemia, vitamin-K dependent coagulation deficiency). These data support the importance of using wide-spread technologies as transcriptomic or proteomic analyses to have a broader view of the molecular pathways that may be involved in the pathogenesis of elastic fibre calcification. Moreover recent findings in the literature highlights the role of polymorphisms in other genes that could be responsible for phenotypic changes and for a different severity of clinical manifestations in this monogenic disorder.

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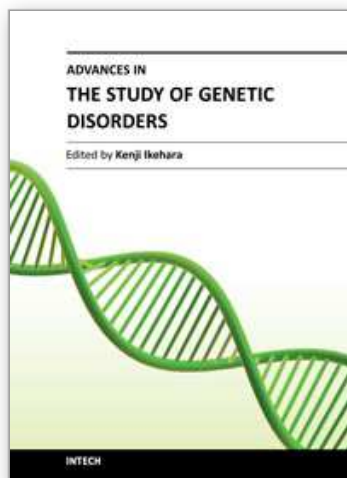
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## **Advances in the Study of Genetic Disorders**

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The studies on genetic disorders have been rapidly advancing in recent years as to be able to understand the reasons why genetic disorders are caused. The first Section of this volume provides readers with background and several methodologies for understanding genetic disorders. Genetic defects, diagnoses and treatments of the respective unifactorial and multifactorial genetic disorders are reviewed in the second and third Sections. Certainly, it is quite difficult or almost impossible to cure a genetic disorder fundamentally at the present time. However, our knowledge of genetic functions has rapidly accumulated since the double-stranded structure of DNA was discovered by Watson and Crick in 1956. Therefore, nowadays it is possible to understand the reasons why genetic disorders are caused. It is probable that the knowledge of genetic disorders described in this book will lead to the discovery of an epoch of new medical treatment and relieve human beings from the genetic disorders of the future.

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