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INVITED REVIEW

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ABCC6 and pseudoxanthoma elasticum

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Abstract ABCC6 belongs to the adenosine triphosphate-binding cassette (ABC) gene subfamily C. This protein family is involved in a large variety of physiological processes, such as signal transduction, protein secretion, drug and antibiotic resistance, and antigen presentation [Kool et al. (1999) 59:175–182; Borst and Elferink (2002) 71:537–592]. ABCC6 is primarily and highly expressed in the liver and kidney [Kool et al. (1999) 59:175–182; Bergen et al. (2000) 25:228–2231]. The precise physiological function and natural substrate(s) transported by ABCC6 are unknown, but the protein may be involved in active transport of intracellular compounds to the extracellular environment [Kool et al. (1999) 59:175–182] [Scheffer et al. (2002) 82:515–518]. Recently, it was shown that loss of function mutations in ABCC6 cause pseudoxanthoma elasticum (PXE) [Bergen et al. (2000) 25:228–2231; Le Saux et al. (2000) 25:223–227]. PXE is an autosomal recessively inherited multi-organ disorder [Goodman et al. (1963) 42:297–334; Lebwohl et al. (1994) 30:103–107]. PXE is primarily associated with the accumulation of mineralized and fragmented elastic fibers of the connective tissue in the skin [Neldner (1988) 6:1–159], Bruch's membrane in the retina [Hu et al. (2003) 48:424–438], and vessel walls [Kornet et al. (2004) 30:1041–1048]. PXE patients usually have skin lesions and breaks in Bruch's membrane of the retina (angioid streaks). Also, a variety of cardiovascular complications has been observed [Hu et al. (2003) 48:424–438]. Recently, a mouse model for PXE was created by targeted disruption of *Abcc6* [Gorgels et al. (2005) 14:1763–1773; Klement et al. (2005) 25:8299–8310], which may be useful to elucidate the precise function of *Abcc6* and to develop experimental therapies.

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ABCC6 in man: structure, function and physiological substrates

Human *ABCC6*, formerly called MRP6, belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) gene subfamily C, together with *ABCC1-13* [7, 25]. In man, *ABCC6* consists of 31 exons spanning ~73 kb genomic DNA. The *ABCC6* mRNA is approximately 6 kb and has an open reading frame of 4.5 kb. The latter encodes a protein of 1503 aa. ABCC6 is composed of 17 transmembrane spanning domains 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 [9, 21]. The putative protein structure is presented in Fig. 1.

Two *ABCC6* pseudogenes have been identified, which are, respectively, homologous to exon 1 through intron 4, and exon 1 through intron 9 [8, 14].

The exact function and natural substrate(s) of ABCC6 are currently unknown. ABCC6 is highly homologous to ABCC1 (43% identity on amino acid level) [25]. For that reason, ABCC6 was classified as a multidrug resistance-associated protein. However, subsequently, Kool et al. [25] and Belinsky et al. [4] showed that the role of ABCC6 in drug resistance is limited to low-level resistance of a number of compounds, like etoposide, teniposide, doxorubicin, and daunorubicin. In vitro experiments showed that ABCC6 actively transports the glutathione S-conjugates

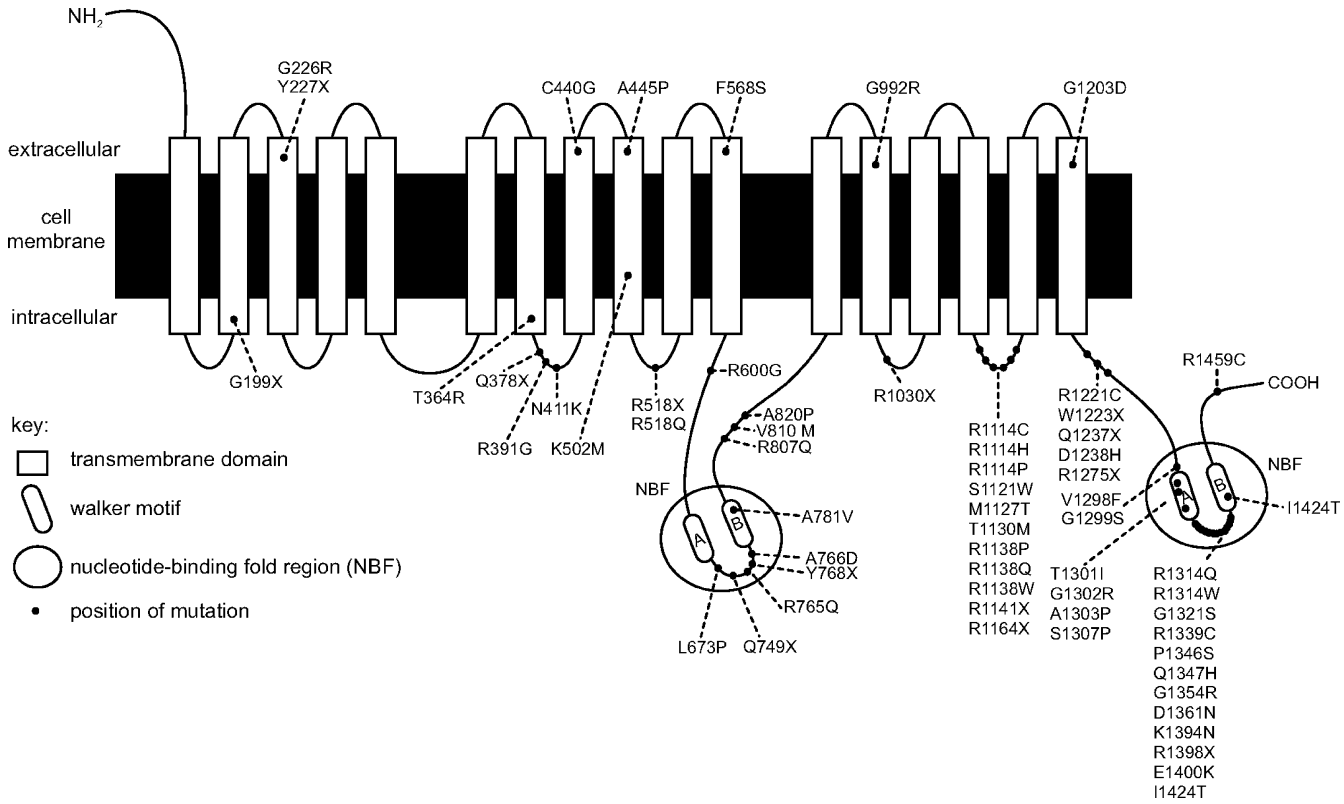

Fig. 1 Point mutations in *ABCC6* implicated in PXE. All missense and nonsense mutations described so far in the *ABCC6* gene implicated in PXE. Deletions, insertions, etc. associated with PXE are given in Table 1. Note the unequal distribution of mutations found, and the concentration of mutations in the functionally important NBF domains and the 8th intracellular loop

leukotriene C(4) and S-(2, 4-dinitrophenyl) glutathione, and the cyclopentapeptide BQ123 [4, 22, 32].

ABCC6 is highly expressed in human liver and kidneys (Fig. 2) [6, 25]. Low-expression levels of ABCC6 were detected in numerous other tissues, including skin, neural retina, and vessel walls [2, 6, 25]. Using several polyclonal antibodies, ABCC6 was localized to the basolateral side of human hepatocytes and to the basolateral membranes of the proximal kidney tubules. The latter suggests that ABCC6 transports intracellular biomolecules back into the blood [45].

ABCC6 mutations cause pseudoxanthoma elasticum (PXE)

Pseudoxanthoma elasticum

The gene implicated in PXE was initially localized to a subchromosomal segment of chromosome 16 [28, 46, 50], which contained both the *ABCC1* and *ABCC6* genes. Subsequently, several groups showed that loss of function mutations in *ABCC6* were responsible for PXE [5, 28, 29]. PXE is primarily an autosomal recessive disorder of the connective tissue, affecting the skin, eye, and the cardio-

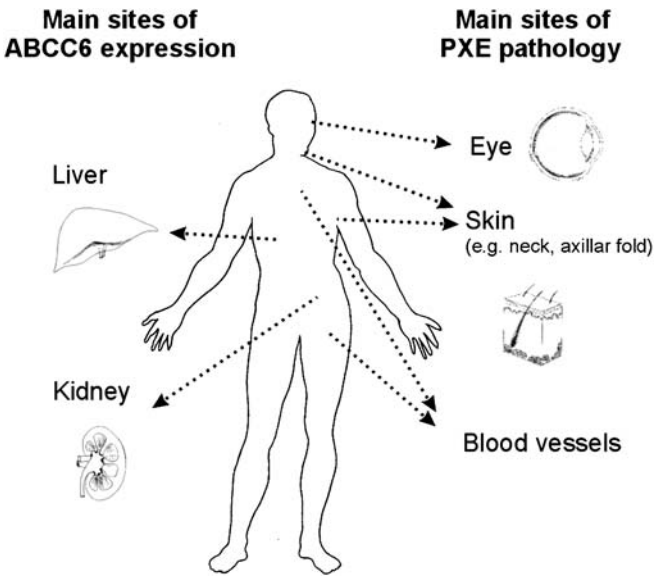

Fig. 2 ABCC6 expression and PXE pathology. The main sites of ABCC6 expression (liver and kidney) do not match the main sites of PXE pathology (skin, eye, and blood vessels). One possible explanation for these data is that PXE is a systemic disease caused by changes in blood composition that occur when the transport function of ABCC6 in liver and kidney is disturbed

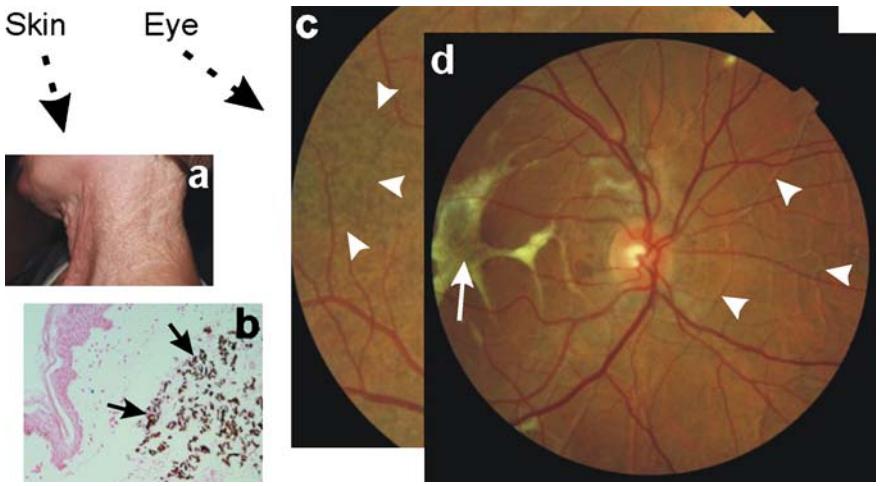

Fig. 3 Main sites of PXE pathology. Main sites of PXE pathology are skin, eye, and blood vessels. The skin shows yellowish papules, often starting in the neck (**a**). Histology reveals mineralization of elastic fibers in the dermis as shown in **b** by von Kossa staining (*brown color*, *arrows*). In the eye, inspection of the fundus frequently shows peau d'orange (subtle, mottled pigmentation, *arrowheads* in **c**), angioid streaks (*arrowheads* in **d**), and finally macular scarring as result of neovascularization (*arrow* in **d**)

vascular system (Fig. 2) [11, 37, 41, 42]. Pseudodominance occurs apparently frequently, due to high carrier frequency and consanguinity [41]. Also, a number of heterozygote carriers manifest mild symptoms of the disease [1]. PXE is, so far, incurable and appears to be present in all of the world's populations, with an estimated prevalence of at least 1: 70,000 persons [9, 41].

Histopathologically, progressive mineralization and fragmentation of elastic fibers in skin, Bruch's membrane in the retina, as well as vessel walls can be seen [11, 15, 37]. Mineralization of fragmented elastic fibers can be visualized using a Von Kossa stain [51]. The clinical expression of PXE is heterogeneous, with considerable variation in age of onset, progression, and severity, even within families [5, 20, 41]. Patients usually develop skin lesions and angioid streaks. Skin abnormalities frequently start at the side of the neck (Fig. 3) and in flexural areas such as armpits, antecubital and popliteal fossae, the inguinal region, and the periumbilical area. Skin lesions are usually ivory to yellowish-colored, raised papules of 1–3 mm. The papules may have a linear or reticular arrangement and may coalesce into plaques or larger confluent areas. In a number of PXE cases, the skin becomes wrinkled, redundant, and hangs in folds [21, 37]. Ocular disease eventually develops in all patients with PXE. The usual sequence of developing eye abnormalities is peau d'orange, angioid streaks, peripapillary atrophy, white glial tissue formation, and finally, subretinal neovascularization (Fig. 3). The latter results in disciform scarring of the macula which causes decreased visual acuity [21]. The most common cardiovascular complications in PXE are: diminished or absent peripheral vascular pulsations (in 25% of patients), early onset renovascular hypertension and echographic opacities due to calcification of arteries in kidneys, spleen, and pancreas (25%), arterial hypertension (22.5%), angina pectoris (19%), intermittent claudication (18%), and gastrointestinal hemorrhages (13%). In PXE patients, the compressibility of the carotid arterial wall was 44% higher than in control subjects, perhaps due to a higher amount of proteoglycans in the PXE vessel walls [9]. The PXE phenotype was recently extensively reviewed by Hu et al. [21].

Table 1 Deletions, insertions, and splice site mutations in ABCC6 causing PXE

Splice site mutations	
IVS8+2delTG	Intron 8
IVS13-29 T>A	Intron 13
IVS14-5 T>G	Intron 14
IVS17-12delTT	Intron 17
IVS18-2delAG	Intron 17
IVS21+1G>T	Intron 21
IVS25-3C>A	Intron 25
IVS26-1G>A	Intron 26
Insertions	
938_939insT	Exon 8
3544dupC	Exon 25
4220insAGAA	Exon 30
Deletions	
179_187del	Exon 2
179_195del	Exon 2
220_222del	Exon 3
960delC	Exon 8
1088_1120del1944_1966del	Exon 9 Exon 16
1995delG	Exon 16
2322delC	Exon 18
2542delG	Exon 19
3343_3345del3775delT	Exon 23 Exon 27
4104delC	Exon 29
4182delG	Exon 29
4318delA	Exon 30
del	Exon 15
del	Exons 23_29
del	*ABCC6*

Nonsense and missense mutations are given in Fig. 1

Mutations in *ABCC6* cause pseudoxanthoma elasticum

Mutations in *ABCC6* are implicated in pseudoxanthoma elasticum (PXE) [6, 27, 29, 44]. Currently, at least one *ABCC6* mutation is found in 90% of our patients with a clinically well-characterized PXE phenotype [19] (unpublished results). So far, approximately 100 different mutations have been reported in *ABCC6* (Fig. 1, Table 1) [9, 23, 35]. The most important sequence changes are missense (55%) and nonsense (15%) mutations, as well as small deletions (15%). The remainder of pathogenic variants consists of splicing errors, larger deletions, and a number of insertions. At least 30% of all mutations cause a frameshift and the introduction of a stopcodon, which leads to premature chain termination. Most pathogenic sequence changes replace evolutionary conserved residues [9, 20, 27].

Mutations associated with PXE occur in, virtually, every exon of *ABCC6*. The vast majority of mutations occur in cytoplasmatic domains and the carboxy-terminal end of the ABCC6 protein. Only sporadically, pathogenic variants were found in extracellular and transmembrane domains. Mutations especially target the NBF1 and NBF2 domains, and the 8th intracellular loop [20]. The latter distribution strongly supports the critical role of NBFs in ATP-driven transport. In addition, this distribution suggests that the 8th intracellular loop is functionally important, perhaps for ABCC6 substrate recognition [9, 20, 27].

Two *ABCC6* mutations, R1141X and del(ex23_29), occur very frequently, probably due to founder effects and genetic drift. R1141X accounts for up to 30% of mutations found in European populations [9, 18], but accounts for only 4% of the US PXE population. In contrast, *ABCC6* del (ex23_29) is present in 28% of the US population [9, 27], and in 4% of the European one. R1141X probably produces an instable *ABCC6* mRNA which is rapidly degraded by nonsense mediated RNA decay [18, 29]. Finally, R1141X may be associated with premature coronary artery disease [48].

ABCC6 and PXE in man: expression, protein distribution, pathology

Currently, the mainstream hypothesis is that PXE is a systemic disorder, caused by ABCC6 defects in the liver and kidney (Fig. 2) [49]. However, the alternative hypothesis is that PXE is a local, peripheral defect, and that ABCC6 defects play a direct role at the sites of pathology [38–40, 43]. Obviously, these hypotheses are not mutually exclusive.

How does *ABCC6* gene expression and protein localization in man relate to PXE pathogenesis? Reverse transcription polymerase chain reaction (RT-PCR), polymerase chain reaction Q-PCR, and immunohistochemistry studies all suggest that ABCC6 is highly expressed in the liver and kidney [6, 25, 45]. Interestingly though, neither organ is, apparently, the primary site of pathology in PXE. No liver pathology has been described in PXE. Kidney defects are apparently more common, because 25% of PXE patients get calcification of arteries in kidneys, spleen, and pancreas revealed by echographic opacities, resulting in renovascular hypertension [12, 17, 47]. However, even within the kidney, the sites of ABCC6 expression, and PXE pathology, apparently, do not match.

RT-PCR and immunohistochemistry with anti-ABCC6 polyclonal antibodies suggest that *ABCC6* is lowly expressed or absent at the main sites of PXE pathogenesis: skin, vessel wall, and RPE/Bruch's membrane in the eye [6, 45]. The latter data strongly favor a systemic disease origin hypothesis [49], although a pathogenic systemic-local interaction cannot be excluded. In a few tissues, like lungs, both *ABCC6* expression and PXE pathogenesis are absent [6, 25].

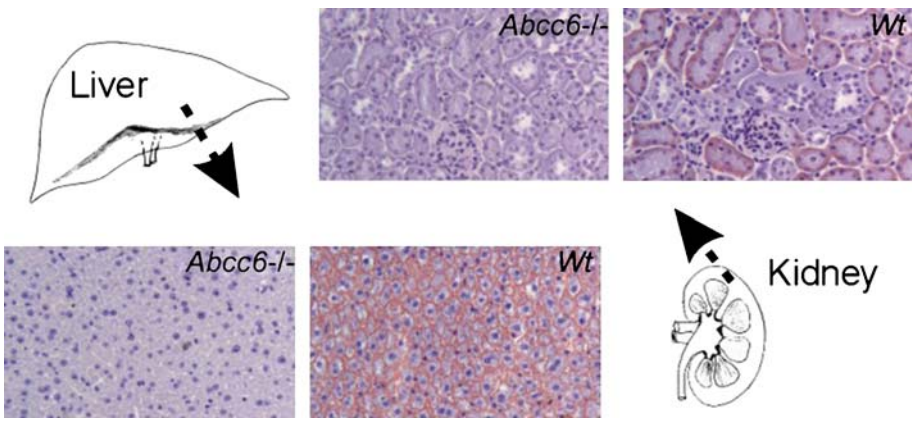

Fig. 4 Main sites of Abcc6 expression. Main sites of Abcc6 expression are the liver and kidney as is hereby illustrated in the mouse by immunohistochemistry employing Abcc6-specific monoclonal antibodies. Positive staining (*brown color*) is present at the basolateral membrane of hepatocytes in liver and at the basolateral membrane in the proximal tubules of the kidney of wild type mice (Wt). Tissues taken from *Abcc6*$^{-/-}$ mice are negative. Figure adapted from Gorgels et al. 2005 [16]

Abcc6 in mice: structure, function and physiological substrates

The mouse has been employed successfully as a model organism to study the function of several ABC transporters, such as ABCC1 [31, 52], ABCC3 [53], and ABCC7 (CFTR)[13]. The mouse genome contains homologues for at least 11 of the 13 human *ABCC* genes. *Abcc6* is evolutionarily well conserved between mouse and man. The mouse Abcc6 is, with 1,498 amino acids, only four amino acids shorter than human ABCC6. The identity at the amino acid level is 78%. The predicted topology is similar to that of human ABCC6 with 17 transmembrane domains and two intracellular NBFs. In the mouse genome, *Abcc6* and *Abcc1* are physically separated, located, respectively, on chromosomes 7 and 16. The transport characteristics of mouse Abcc6 have not been studied yet. As Abcc6 knock out mice were recently generated, suitable tissues for transport studies are now available.

Targeted disruption of *Abcc6* in the mouse: PXE-like signs

To clarify the function of Abcc6 and its relation with PXE, the *Abcc6* gene was disrupted in the mouse by gene targeting. Two Abcc6 knock out mouse models ($Abcc6^{-/-}$) were generated [16, 24]. The strategies used for disrupting *Abcc6* were quite similar and consisted of deleting the coding sequence of the first NBF. This should render any remaining gene product dysfunctional, as NBF regions are crucial for the transport function of ABC proteins. In addition, mutations in this region in man cause PXE (Fig. 1) [20, 27]. In both $Abcc6^{-/-}$ mouse models, the production of Abcc6 protein is apparently abolished, as judged from the absence of Abcc6 immunoreactivity in the liver (Fig. 4) [16, 24].

The phenotype of the $Abcc6^{-/-}$ mice was analyzed focusing on those tissues that are affected in PXE (skin, eye, and blood vessels). Necropsy of $Abcc6^{-/-}$ mice revealed pathology specifically in all these tissues. Blood vessel pathology was most prominent in the mouse. In young $Abcc6^{-/-}$ mice, calcification was observed in the wall of medium-sized arteries in the cortex of the kidney and in the capsule surrounding the sinuses of the vibrissae. It is currently not known why these vessel walls represent predilected sites of calcification in the Abcc6-deficient mouse. Calcification progresses with age and affects mice more than 12–15 months of age, also the aorta, coronary arteries, vena cava, as well as blood vessels in many of the tissues examined, such as muscle, tongue and adipose tissue. The calcium deposits are often found at or close to the elastic fibers in the vessel walls. In old $Abcc6^{-/-}$ mice, also the eye and skin become involved, showing calcification in Bruch's membrane of the eye and in the dermis. In conclusion, $Abcc6^{-/-}$ mice spontaneously develop a PXE-like pathology, which confirms the crucial role of Abcc6 in the etiology of PXE.

Abcc6 and PXE in mice: expression, protein distribution, pathology

The phenotypic resemblance between PXE patients and $Abcc6^{-/-}$ mice indicates that the $Abcc6^{-/-}$ mouse is a useful tool to study the functional role of Abcc6 (as a transporter) in PXE pathophysiology. An important question in this respect is whether Abcc6 is present locally at the pathogenic sites. Several studies have examined the tissue distribution of Abcc6 mRNA and protein in the mouse. Significant levels of mRNA have been repeatedly found in liver, kidney and small intestine [33, 34, 36]. At the protein level, Abcc6 immunoreactivity is readily demonstrated at the basolateral membranes of hepatocytes and basal membranes of the proximal tubules in the kidney (Fig. 4) [16]. There are also reports of a more widespread distribution of messenger and protein in the mouse [3]. High sensitive RNAse protection assays [3] and RT-PCR [6, 34] have detected small amounts of *Abcc6* transcripts in a variety of other tissues, including the sites of pathology. However, the biological significance of these low levels of mRNA remains questionable, especially because many of these messengers are *Abcc6* splice variants harboring premature stopcodons [34]. At the protein level, a recent study using tissues from $Abcc6^{-/-}$ mice as negative controls, failed to detect Abcc6 immunoreactivity at the pathogenic sites in skin, blood vessels and eye [16]. In summary, these expression studies did not support the local dysfunction of Abcc6 as the primary cause for PXE pathology. On the contrary, the data favor a systemic origin of PXE because the expression in liver, kidney, and small intestine put Abcc6 in a strategic position to control the blood values of its substrate. The nature of this substrate is currently not known. Analysis of the blood of $Abcc6^{-/-}$ mice revealed no aberrant concentration of minerals, such as calcium. However, $Abcc6^{-/-}$ mice do develop a reduction in plasma levels of high-density lipoprotein (HDL)-cholesterol, which suggests that Abcc6 may (also) be involved in lipid transport or metabolism.

Conclusions and perspectives

Five years ago, the discovery that PXE patients carry mutations in *ABCC6* came as a surprise because a relation between this membrane transporter with PXE was not immediately apparent [6, 29]. Since then, this relation has been further strengthened, e.g., by the detection of ABCC6 mutations in up to 90% of the PXE patients and the PXE-like phenotype in Abcc6 knock out mice [16, 24]. Currently, *ABCC6* is considered to be the only disease gene causing PXE.

Despite this increased certainty that ABCC6 mutations cause PXE, the function of ABCC6 and its role in PXE remain largely unresolved. Fortunately, Abcc6 knock out mice are now available and the close resemblance in phenotype with PXE patients suggest that they will be valuable for analysis of the role of Abcc6 in PXE.

Several questions need to be addressed. First, an important issue still is whether PXE is a systemic or local disease. This can be initially addressed by studying Abcc6 expression and localization of the protein in the mouse, with *Abcc6*$^{-/-}$ tissues as negative controls. However, it cannot be completely excluded that low and hardly detectable levels of Abcc6 may have physiological relevance. Therefore, functional studies are required for a more definitive answer. These studies may consist of reintroducing Abcc6 in a tissue-specific manner in the *Abcc6*$^{-/-}$ mouse.

The next urgent question is the identification of the substrate of ABCC6. Currently, hepatocytes are the most obvious cell type of choice for transport studies, because these cells express Abcc6 abundantly. Examination of the effect of *Abcc6* deficiency on gene expression in the liver may suggest candidate substrates. In addition, suggestions for candidate substrates may come from analyzing the blood of PXE and normal persons. The first analysis of blood samples of the *Abcc6*$^{-/-}$ mouse indicated that HDL-cholesterol values change in *Abcc6*$^{-/-}$ mice [16]. However, no significant changes in HDL-cholesterol have been observed so far in our PXE patients (unpublished results).

In case of a local disease, fibroblasts are the cell type of choice for studies to identify the substrate specificity of ABCC6. Indications for the substrate may be obtained by comparing physiology and gene expression of cultured fibroblasts from PXE patients and from healthy control persons. Possibly, toxicity assays can be developed using toxic agents such as saltpeter fertilizers, which upon contact cause PXE-like skin lesions [10].

The final challenge will be to unravel PXE etiology and to prevent or even cure the disease. However, many aspects of PXE pathogenesis are still unknown. For example, in the eye of PXE patients, fundus signs, such as white punched out lesions, comet-like tails, and peau d'orange occur frequently. The histological and pathological correlate of these features is unknown. In tissues that develop a PXE-like pathology in the *Abcc6*$^{-/-}$ mouse, the sequence of events can be analyzed in detail and we can try to intervene experimentally. In addition, the *Abcc6*$^{-/-}$ mouse models will enable us to test a variety of potential experimental therapies for PXE, ranging from dietary intervention to gene therapy.

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