Pseudoxanthoma Elasticum: Genetic Variations in Antioxidant Genes Are Risk Factors for Early Disease Onset

Ralf Zarbock,1 Doris Hendig,1 Christiane Szliska,2 Knut Kleesiek,1 and Christian Göttинг1*

Background: Pseudoxanthoma elasticum (PXE) is a rare hereditary disorder characterized by progressive calcification and fragmentation of elastic fibers in connective tissues. PXE is caused by mutations in the ABCC6 gene, which encodes the membrane transporter multidrug resistance-associated protein 6. Chronic oxidative stress was recently suggested to play a crucial role in the pathogenesis of the disease. Our aim was to investigate the association of PXE with genetic variation in genes coding for antioxidant enzymes.

Methods: We used restriction fragment length polymorphism and allele-specific PCR analyses to evaluate the distribution of single-nucleotide polymorphisms in the genes encoding catalase (CAT), superoxide dismutase 2 (SOD2), and glutathione peroxidase 1 (GPX1) in DNA samples from 117 German PXE patients and 117 healthy age- and sex-matched control individuals.

Results: The investigated genetic variants had previously been shown to affect the activities of these antioxidant enzymes. We found a correlation between genotype and age of disease onset for polymorphisms in CAT (c.-262C>T), SOD2 (c.47C>T), and GPX1 (c.593C>T). Furthermore, the age of disease onset was inversely correlated with the number of mutated alleles, indicating a cumulative effect on the time of disease onset [mean (SD) age of 40.9 (13.6) years, 32.4 (16.3) years, and 25.7 (15.9) years for carriers of 0, 1–2, and >2 mutated alleles, respectively; P = 0.03].

Conclusion: Our findings demonstrate that increased oxidative stress due to activity-affecting polymorphisms in genes encoding antioxidant enzymes leads to earlier PXE onset.

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Pseudoxanthoma elasticum (PXE) [Groenblad–Strandberg syndrome, Online Mendelian Inheritance in Man 177850 and 264800] is an autosomally inherited disorder that affects the skin, eyes, and cardiovascular system (1, 2) and is characterized by extensive connective tissue alterations, including progressive calcification and fragmentation of elastic fibers, and massive accumulation of proteoglycans in the extracellular matrix. PXE is caused by mutations in the ABCC64 gene (3–5). ABCC6 belongs to ATP-binding cassette transporter subfamily C and encodes a 165-kDa transmembrane protein termed multidrug resistance-associated protein (MRP)6. To date, more than 100 PXE-associated mutations have been identified in patients of different origins (4–10). The great majority of the mutations are located in the cytoplasmic C-terminal region of the MRP6 protein, close to the second Walker motif that is critical for ATP hydrolysis. These findings suggest that PXE is caused by impaired MRP6-transport activity (11). MRPs are involved in hepatic detoxification, drug distribution, and signal transport and carry out the ATP-dependent transport of a variety of compounds (12). MRP6 production is high in liver, kidney, and, to a lesser extent, tissues affected by PXE (13). On the basis of its high homology with the well-characterized MRP1 protein, an efflux transporter produced in the liver, and its

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Received February 27, 2007; accepted July 18, 2007.

Precededly published online at DOI: 10.1373/clinchem.2007.088211

Nonstandard abbreviations: PXE, pseudoxanthoma elasticum; MRP, multidrug resistance-associated protein; ROS, reactive oxygen species.

1 Human genes: ABCC6, ATP-binding cassette, subfamily C (CFTR/MRP), member 6; CAT, catalase; GPX1, glutathione peroxidase 1; SOD2, superoxide dismutase 2, mitochondrial.
localization in the basolateral membrane of hepatocytes and kidney proximal tubules, MRP6 has been supposed to export metabolites from these cells. Although MRP6 has been shown to transport the endothelin-1 receptor antagonist BQ123 and glutathione conjugates of organic anions in vitro (12), the precise physiological function of MRP6 and the molecules it transports in vivo remain unknown. The age of disease onset and the clinical expression of PXE symptoms are highly variable (14). Marked phenotypic variation has been observed in affected siblings bearing the same ABCC6 genotype (9). These findings led to the assumption that other genes and environmental factors might contribute to the expression and severity of PXE (15). Our group has already demonstrated that polymorphisms in genes encoding xylosyltransferases are associated with a severe disease course (16). Recently, PXE fibroblasts were shown to undergo mild chronic oxidative stress (17). Because many of the alterations observed in PXE could be explained by oxidative stress, which might therefore be an underlying pathologic mechanism of this disease, we speculated that genes associated with oxidative stress might be genetic cofactors in PXE.

Oxidative stress is characterized by an unphysiologically high production of reactive oxygen species (ROS) and has been implicated in numerous pathologic conditions and in aging (18). ROS play both deleterious and beneficial roles. On the one hand, they can cause severe damage to cellular macromolecules, including proteins, lipids, and DNA (19). On the other hand, ROS serve as important 2nd messengers for the induction of several genes in a variety of physiological and pathologic conditions (20). ROS activate cytoplasmic signal-transduction pathways related to growth, differentiation, and senescence, as well as those involved in the production and degradation of connective tissue (21, 22). The intracellular concentrations of ROS are regulated by the activities of a number of enzymes, including mitochondrial superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPX1), and catalase (CAT). These 3 enzymes share a common detoxification pathway in which SOD2 first catalyzes the dismutation of the superoxide anion to hydrogen peroxide and oxygen. The subsequent conversion of hydrogen peroxide to water and oxygen is catalyzed by CAT and GPX1, with the latter requiring reduced glutathione for its activity (23). Genetic polymorphisms can modify the activity of these enzymes and thus increase the oxidative burden on the cell. Various diseases have been reported to be associated with polymorphisms in antioxidant genes (18).

In the present study, we measured the genotype frequencies of CAT, GPX1, and SOD2 polymorphisms in PXE patients and healthy control individuals. We chose genetic variants known to modulate enzymatic activity and lead to increased oxidative burden to elucidate the role of oxidative stress in PXE.

**Materials and Methods**

**PATIENT CHARACTERISTICS**

The study cohort consisted of 117 German PXE patients from 117 nonconsanguineous families in which the PXE phenotype exhibited an apparently autosomal recessive or sporadic mode of inheritance. The diagnosis of PXE in all patients was consistent with the reported consensus criteria (24). The status of the PXE patients was established by the presence of ocular findings and dermal lesions and histologically confirmed by the observation of calcified elastic fibers in skin biopsy samples after von Kossa staining. The biopsy samples were taken from skin lesions. To minimize interobserver variability, a single medical specialist thoroughly questioned all participants in the study regarding their diseases, organ involvement, and family history. The 30 male PXE patients and the 87 female patients included in this study had mean (SD) ages of 49.2 (15.7) years and 42.9 (15.7) years, respectively. We used blood samples from 117 Westphalian blood donors (30 men, 87 women) as controls in the polymorphism analyses. The mean (SD) age of the control individuals was 44.1 (15.0) years. Table 1 summarizes the clinical characteristics of the patients and control individuals. The most common causative PXE mutations among these patients were the nonsense mutation c.3421C>T (p.R1141X), the splice site mutation c.2787 + 1G>T, and the large deletion Ex23_Ex29del. The allelic frequencies of these DNA alterations in the PXE patients were 28.6%, 5.1%, and 3.8%, respectively. The study was approved by the institutional review board; all patients gave their informed consent.

**COLLECTION OF DNA SAMPLES**

We used the QIAamp blood kit (Qiagen) according to the manufacturer’s instructions to extract genomic DNA from 200 µL of blood containing EDTA.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PXE patients (n = 117)</th>
<th>Blood donors (n = 117)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female sex, n</td>
<td>30/87</td>
<td>30/87</td>
</tr>
<tr>
<td>Age, years</td>
<td>44.5 (15.9)</td>
<td>44.1 (15.0)</td>
</tr>
<tr>
<td>Age at onset, years</td>
<td>30.5 (16.5)</td>
<td>NAb</td>
</tr>
<tr>
<td>Current smoking</td>
<td>28 (24%)</td>
<td>ND</td>
</tr>
<tr>
<td>Organ involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of organs</td>
<td>2.96 (1.51)</td>
<td>NA</td>
</tr>
<tr>
<td>Skin, n</td>
<td>106 (91%)</td>
<td>NA</td>
</tr>
<tr>
<td>Eyes, n</td>
<td>99 (85%)</td>
<td>NA</td>
</tr>
<tr>
<td>Cardiovascular tissue, n</td>
<td>37 (32%)</td>
<td>NA</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>36 (31%)</td>
<td>NA</td>
</tr>
<tr>
<td>Heart, n</td>
<td>17 (25%)</td>
<td>NA</td>
</tr>
<tr>
<td>Gastrointestinal tract, n</td>
<td>18 (15%)</td>
<td>NA</td>
</tr>
<tr>
<td>Kidney/urinary tract, n</td>
<td>13 (11%)</td>
<td>NA</td>
</tr>
<tr>
<td>Other, n</td>
<td>20 (17%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

aData for age and the number of organs are presented as the mean (SD).

bNA, Not applicable; ND, not determined.
RESTRICTION FRAGMENT LENGTH POLYMORPHISM
ANALYSIS OF POLYMORPHISMS
PCR primers were based on published sequences (GenBank accession nos. NT_009237.17, NT_022517.17, and NT_007422.13 for CAT, GPX1, and SOD2, respectively). We analyzed the CAT polymorphism c.−262C>T (rs1001179) with primers 5′-CTGATAACCCGGAGC- CCGCCTCTGGATGGATAT-3′ and 5′-CTAGGGACC- GCCAAAGTGGAGGCAATCG-3′. The first primer contains a mismatched base and creates a recognition sequence for EcoRV. The PCR was carried out in a 50-μL reaction volume containing approximately 65 ng genomic DNA, 25 pmol of each primer (Biomers), 1.5 U HotStar Taq DNA polymerase (Qiagen), and 0.25 mmol/L of each deoxynucleoside triphosphate (Promega) in PCR buffer (25 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl2, and 1 mmol/L β-mercaptoethanol). The PCR conditions were as follows: initial denaturation at 95 °C for 15 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 15 min. The 190-bp PCR product was digested at 37 °C overnight with 5 U EcoRV (New England Biolabs) and separated on a 1.5% agarose gel. The c.−262C allele yielded 157-bp and 33-bp fragments; the c.−262T allele remained undigested. We analyzed SOD2 polymorphism c.47C>T (rs1799725) with primers 5′-CCGCTACGGCTGAACCT-3′ and 5′-CTGACCTGCCGGCTGGTG-3′. The PCR mixture and amplification procedures were as described above except for an annealing temperature of 64 °C. The 256-bp PCR products were digested at 37 °C overnight with 5 U EcoRV (New England Biolabs) and separated on a 1.5% agarose gel. The c.−262C allele yielded 157-bp and 33-bp fragments; the c.−262T allele remained undigested. We analyzed GPX1 polymorphism c.47T (p.A16V) in the CAT promoter [32.8 (16.6) years, 28.3 (15.8) years, and 19.1 (12.9) years for the C/C, C/T, and T/T genotypes, respectively]. The difference in age of disease onset between the homozgyous carriers of the c.−262C and c.−262T alleles was statistically significant (P < 0.05; Fig. 1A). The age of disease onset was also significantly lower (P < 0.01) for the patients with the SOD2 C/T genotype than for individuals with the C/C genotype (Fig. 1C). There was also a tendency for disease onset to be earlier in homozygous carriers of the c.47T allele [36.8 (16.7) years, 26.4 (15.1) years, and 31.3 (16.6) years for C/C, C/T, and T/T genotypes, respectively]. No statistically significant difference in age of disease onset was detected between patients with the GPX1 C/C genotype and those with the C/T genotype [31.0 (16.2) years, 32.4 (16.9) years, and 16.8 (6.8) years for C/C, C/T, and T/T genotypes, respectively]; however, homozygous carriers of the c.593T allele had a significantly earlier age of disease compared to those patients homozygous for the wild-type allele or with heterozygous individuals (P < 0.05, and P < 0.01, respectively; Fig. 1B).

STATISTICAL ANALYSIS
We evaluated the data for departure from a gaussian distribution with the Kolmogorov–Smirnov test and used the Student t-test and the Mann–Whitney U-test for statistical analyses as appropriate. We used ANOVA to test for the significance of differences in age of disease onset among groups with different numbers of mutated alleles and used the χ2 test to examine whether genotype distributions departed from those expected under the Hardy–Weinberg equilibrium. P values <0.05 were considered statistically significant. SPSS 12.0 (SPSS) and GraphPad Prism 4.0 (GraphPad Software) software packages were used for statistical testing.

Results
The polymorphisms c.47C>T (p.A16V) in the SOD2 gene, c.593C>T (p.P198L) in the GPX1 gene, and c.−262C>T in the CAT gene were genotyped in the DNA samples from 117 German PXE patients and the 117 healthy control individuals. Table 2 presents the frequency distributions for the polymorphisms. The PXE patients and control individuals did not differ with respect to the genotype frequencies for any of the 3 polymorphisms. All of the genotype distributions showed no departures from the Hardy–Weinberg equilibrium.

Further analysis revealed an association of genotype with age of disease onset for all 3 of the investigated polymorphisms. Specifically, the age of disease onset was earlier in patients carrying at least 1 c.−262T allele in the CAT promoter [32.8 (16.6) years, 28.3 (15.8) years, and 19.1 (12.9) years for the C/C, C/T, and T/T genotypes, respectively]. The difference in age of disease onset between the homozgyous carriers of the c.−262C and c.−262T alleles was statistically significant (P < 0.05; Fig. 1A). The age of disease onset was also significantly lower (P < 0.01) for the patients with the SOD2 C/T genotype than for individuals with the C/C genotype (Fig. 1C). There was also a tendency for disease onset to be earlier in homozygous carriers of the c.47T allele [36.8 (16.7) years, 26.4 (15.1) years, and 31.3 (16.6) years for C/C, C/T, and T/T genotypes, respectively]. No statistically significant difference in age of disease onset was detected between patients with the GPX1 C/C genotype and those with the C/T genotype [31.0 (16.2) years, 32.4 (16.9) years, and 16.8 (6.8) years for C/C, C/T, and T/T genotypes, respectively]; however, homozygous carriers of the c.593T allele had a significantly earlier age of disease compared to patients homozygous for the wild-type allele or with heterozygous individuals (P < 0.05, and P < 0.01, respectively; Fig. 1B).

Table 2. Frequency distributions for SOD2 c.47C>T, GPX1 c.593C>T, and CAT c.−262C>T polymorphisms.a

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PXE patients</th>
<th>Blood donors</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>61.5% (72)</td>
<td>59.8% (70)</td>
<td>60.7% (142)</td>
<td>NS</td>
</tr>
<tr>
<td>C/T</td>
<td>32.5% (38)</td>
<td>34.2% (40)</td>
<td>33.3% (78)</td>
<td>NS</td>
</tr>
<tr>
<td>T/T</td>
<td>6.0% (7)</td>
<td>6.0% (7)</td>
<td>6.0% (14)</td>
<td>NS</td>
</tr>
<tr>
<td>GPX1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>45.3% (53)</td>
<td>49.6% (58)</td>
<td>47.4% (111)</td>
<td>NS</td>
</tr>
<tr>
<td>C/T</td>
<td>47.0% (55)</td>
<td>43.6% (51)</td>
<td>45.3% (106)</td>
<td>NS</td>
</tr>
<tr>
<td>T/T</td>
<td>7.7% (9)</td>
<td>6.8% (8)</td>
<td>7.3% (17)</td>
<td>NS</td>
</tr>
<tr>
<td>SOD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>26.5% (31)</td>
<td>28.2% (33)</td>
<td>27.4% (64)</td>
<td>NS</td>
</tr>
<tr>
<td>C/T</td>
<td>42.7% (50)</td>
<td>45.3% (53)</td>
<td>44.0% (103)</td>
<td>NS</td>
</tr>
<tr>
<td>T/T</td>
<td>30.8% (36)</td>
<td>26.5% (31)</td>
<td>28.6% (67)</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Genotype data are presented as percentage (n).

NS, Not statistically significant.
Age of disease onset was also associated with the number of mutated alleles for the 3 genes that an individual carried. The mean age of disease onset decreased with the number of mutated alleles [40.9 (13.6) years, 32.4 (16.3) years, and 25.7 (15.9) years for carriers of 0, 1–2, and >2 mutated alleles, respectively; \( P < 0.03 \) ] (Fig. 2).

Patients were divided into 2 groups according to the type of PXE-causative mutations in the \( ABCC6 \) gene. Genotype group A consisted of patients who were homozygous or compound heterozygous for mutations likely to produce either no MRP6 protein or no functional MRP6, whereas genotype group B consisted of patients with an MRP6 enzyme with reduced activity due to missense mutations. The mean age of disease onset was significantly lower in group A (Table 3), thus confirming results that we previously reported for a smaller cohort (25). In genotype group A, the effects of \( CAT \) and \( GPX1 \) genotype on the time of disease onset were more pronounced than in genotype group B. In the case of \( SOD2 \), the effect was stronger in genotype group B. The decrease in the age of disease onset associated with increased numbers of mutated alleles for genes encoding antioxidant enzymes was more apparent in genotype group A [42.0 (9.9) years, 26.5 (15.3) years, and 15.0 (6.1) years for carriers of 0, 1–2, and >2 mutated alleles, respectively; \( P = 0.04 \) ] than in genotype group B [40.4 (15.9) years, 33.9 (16.4) years, and 28.0 (16.5) years for carriers of 0, 1–2, and >2 mutated alleles, respectively; \( P = 0.14 \) ]. The \( ABCC6 \) genotype groups did not differ significantly with respect to the genotype frequencies for \( CAT \), \( GPX1 \), and \( SOD2 \).

**Discussion**

Cultured dermal fibroblasts from PXE patients show signs of chronic oxidative stress in vitro (17). On the basis of this finding, we speculated that the extent of the oxidative burden might influence disease onset and the clinical phenotype of PXE. Therefore, we analyzed sequence variants known to affect the activities of antioxidant enzymes and subsequent oxidative stress.

In the case of the \( SOD2 \) polymorphism p.A16V, we observed disease onset to be significantly earlier in patients heterozygous for the p.A16V allele. We also observed an earlier disease onset in homozygotes, although the difference did not reach statistical significance. The p.A16V polymorphism is located in the mitochondrial-targeting sequence and has been suggested to affect SOD2 transport into mitochondria (26). The p.16A allele has been associated with certain types of cancer (27, 28). Individuals carrying at least 1 p.16V allele were shown to
have higher enzyme activity in erythrocytes (23). In vitro studies have revealed a decreased enzyme activity for the GPX1 p.198L allele (29). An analysis of GPX1 enzyme activities in humans revealed lower activity in homoy-
gous male carriers of the p.198L allele, whereas all 3 GPX1
activities in humans revealed lower activity in homozy-
gous male carriers of this allele. The effect was even more
pronounced in homozygotes.

In summary, the genotypes associated with an altered
enzyme activity were associated with an earlier clinical
manifestation of PXE symptoms in all 3 of the polymor-
phisms investigated. All of these variant genotypes may
increase oxidative stress, particularly the intracellular
hydrogen peroxide concentration. When we combined
genotypes associated with higher hydrogen peroxide con-
centrations, we found the mean age of disease onset to
decrease as the number of mutations carried by an indi-
vidual increased. This result demonstrates a possible
cumulative effect of these mutations on the level of
oxidative stress. The effect was even more pronounced in
patients who were likely to have either no MRP6 protein
or no functional MRP6 because of nonsense, frameshift,
or splice site mutations, thus indicating a compound effect
of PXE genotype and variation in genes encoding antioxi-
dant enzymes.

Chronic oxidative stress could explain most of the
structural and biochemical alterations observed in PXE.
For the most relevant PXE lesions (i.e., alterations in
extracellular matrix components, degradation and miner-
alization of elastic fibers, and neovascularization), the
involvement of ROS has already been demonstrated
(22, 33–35). The actions of ROS seem to provide a unifying
pathologic mechanism for most of the features described
in PXE. The association of polymorphisms in antioxidant
genomes with disease onset that we have discovered in this
study supports the suggestion that ROS are involved in
PXE pathogenesis. Nevertheless, the connection between
MRP6 deficiency and oxidative stress remains unclear. A
cue in this context might be the observation that one of
the substrates transported by MRP6 in vitro is glutathione
conjugated to leukotriene C4 and N-ethylmaleimide, a
compound known to act as a radical quencher
(12, 36). MRP6 transport of free-radical scavengers such
as reduced glutathione conjugates might play a critical
role in protecting the extracellular milieu in the skin, eyes,

Table 3. Characteristics of the ABCC6 genotype groups.a

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Genotype group A (n = 24)b</th>
<th>Genotype group B (n = 93)c</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44.2 (16.6)</td>
<td>44.6 (15.8)</td>
<td>0.91</td>
</tr>
<tr>
<td>No. of affected organs</td>
<td>2.9 (1.5)</td>
<td>3.0 (1.5)</td>
<td>0.93</td>
</tr>
<tr>
<td>Age at onset, years</td>
<td>24.5 (14.6)</td>
<td>32.1 (16.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>CAT genotype, n</td>
<td>16/6/2</td>
<td>56/32/5</td>
<td>0.63</td>
</tr>
<tr>
<td>GPX1 genotype, n</td>
<td>9/11/4</td>
<td>44/44/5</td>
<td>0.17</td>
</tr>
<tr>
<td>SOD2 genotype, n</td>
<td>7/12/5</td>
<td>25/38/30</td>
<td>0.54</td>
</tr>
</tbody>
</table>

| Age at onset for CAT genotype, years | 28.6 (16.1) | 34.0 (16.8) | 0.26  |
| C/T                                    | 17.5 (4.6) | 30.5 (16.2) | 0.06  |
| T/T                                    | 12.5 (7.8) | 21.8 (14.3) | 0.44  |

| Age at onset for GPX1 genotype, years | 31.6 (17.3) | 30.7 (16.3) | 0.89  |
| C/T                                    | 22.8 (11.9) | 34.8 (17.0) | 0.03  |
| T/T                                    | 13.0 (5.9)  | 19.8 (6.5)  | 0.15  |

| Age at onset for SOD2 genotype, years | 28.0 (14.0) | 38.6 (17.0) | 0.15  |
| C/T                                    | 21.0 (12.3) | 28.1 (15.7) | 0.16  |
| T/T                                    | 27.8 (21.2) | 32.2 (16.2) | 0.60  |

a Data for age and number of affected organs are presented as the mean (SD).
b Patients who are (a) homozygous for PXE mutation p.R1141X, c.2787 + 1G>T, or c.4434delA; and (b) compound heterozygotes for 2 of the PXE mutations p.R1141X, c.2787 + 1G>T, Ex23_Ex29del, c.4182delG, p.Q378X, c.1995delG, p.E507X, and c.2835_2850del16. This subgroup contains PXE patients most likely to have either no MRP6 protein or no functional MRP6.
c PXE patients with (a) no PXE mutation, (b) 1 heterozygous PXE mutation, (c) 1 homozygous missense PXE mutation, or (d) 2 PXE mutations in a compound heterozygous state, of which at least 1 is a missense mutation. These patients are postulated to have MRP6 reduced activity.

a CAT, GPX1, and SOD2 genotype data are presented in the order C/C, C/T, T/T.
and vessel walls against oxidative stress (13, 37). In the absence of MRP6-transport activity, oxidative stress impairs the correct assembly and deposition of extracellular matrix polymers. This effect might be enhanced by increased ROS production because of activity-influencing polymorphisms in antioxidant genes and thereby cause an earlier disease onset. The combined effects on age of disease onset of activity-affected variants of genes encoding antioxidant enzymes and ABCC6 mutations likely to cause complete loss of MRP6-transport activity also suggest the involvement of MRP6 in the regulation of oxidative stress; however, these arguments will continue to be speculative as long as the physiological substrate of MRP6 remains unidentified.

The strong expression of ABCC6 in liver and kidney, organs not primarily affected by PXE, lead to the assumption that PXE is a systemic disease (38). An alternative hypothesis is that the disease is caused by local dysfunction of the protein within the connective tissue itself, e.g., in fibroblasts. This hypothesis is supported by the fact that PXE fibroblasts show an aberrant phenotype in vitro (39). The presence of oxidative stress in cultured PXE cells is also consistent with a local cause. If the lack of MRP6-transport activity for certain antioxidants were the cause of oxidative stress and subsequent impairment in the assembly of the extracellular matrix, one would expect even greater damage in tissues with high MRP6 concentrations, which is obviously not the case. One possible explanation is that other export pumps in the liver and kidneys but not in connective tissues compensate for the lack of MRP6-transport activity. MRP6 may also transport different substrates in different tissues, leading to various pathologic consequences.

In conclusion, our results show that increased oxidative stress due to genetic variants affecting the activities of antioxidant enzymes leads to an earlier onset of PXE. This finding supports the hypothesis that oxidative stress is involved in the development of disease symptoms. The genotypes of the genes we have investigated may serve as predictors of disease onset and may prove useful for building risk profiles for PXE patients and yet unaffected relatives.

Grant/funding support: This work was supported by a grant from the Stiftung für Pathobiochemie und Molekulare Diagnostik of the Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin. Financial disclosures: None declared.

Acknowledgments: We are grateful to all the PXE patients and their relatives, whose cooperation made this study possible. Furthermore, we thank Peter Hof, chairman of the Selbsthilfe für PXE Erkrankte Deutschlands e.V., and the members of the clinical ambulance for PXE at the Bethesda Hospital in Freudenberg, Germany. We thank Alexandra Adam and Marlen Ewald for excellent technical assistance and Sarah L. Kirkby for linguistic advice.

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