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## Alteration of Extracellular Nucleotide Metabolism in Pseudoxanthoma Elasticum

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### Abstract

Pseudoxanthoma elasticum (PXE) is a rare genetic condition primarily caused by hepatic ABCC6 transporter dysfunction. Most clinical manifestations of PXE are due to premature calcification of elastic fibers. However, the vascular impact of PXE is pleiotropic and remains ill defined. ABCC6 expression has recently been associated with cellular nucleotide export. We studied the impact of ABCC6 deficiency on blood levels of aden- osine triphosphate and related metabolites and on soluble nucleotidase activities in PXE patients and *Abcc6*<sup>-/-</sup> mice. In addition, we investigated the expression of genes encoding ectocellular purinergic signaling proteins in mouse liver and aorta. Plasma adenosine triphosphate and pyrophosphate levels were significantly reduced in PXE patients and in *Abcc6*<sup>-/-</sup> mice, whereas adenosine concentration was not modified. Moreover, 5'-nucleotidase/CD73 activity was increased in the serum of PXE patients and *Abcc6*<sup>-/-</sup> mice. Consistent with alterations of purinergic signaling, the expression of genes involved in purine and phosphate transport/metabolism was dramatically modified in *Abcc6*<sup>-/-</sup> mouse aorta, with much less impact on the liver. ABCC6 deficiency causes impaired vascular homeostasis and tissue perfusion. Our findings suggest that these alterations are linked to changes in extracellular nucleotide metabolism that are remote from the liver. This opens new perspectives for the understanding of PXE pathophysiology.

### INTRODUCTION

Pseudoxanthoma elasticum (PXE) (OMIM 264800) is an inherited multisystem disorder characterized by fragmentation (elastorrhaxis) and progressive calcification of the elastic

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#### CONFLICT OF INTEREST

The authors state no conflict of interest.

#### SUPPLEMENTARY MATERIAL

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fibers in the skin, Bruch's membrane of the retina, and the media layer of the arteries (Li et al., 2009; Neldner, 1988). Mutations causing PXE have been identified in *ABCC6*, which encodes an adenosine triphosphate (ATP)-binding cassette transporter that is primarily expressed in the liver and kidneys (Le Saux et al., 2000). These findings suggested that PXE is a metabolic disease displaying a silent "central" (liver) causative defect and "distal" (connective tissue) manifestations. It was then assumed that *ABCC6* transports anti-calcifying metabolite(s) from hepatocytes into the systemic circulation. In addition to this so-called *metabolic hypothesis*, a *cellular pathophysiological hypothesis* of PXE proposes that *ABCC6* is expressed at low levels in affected tissues, including dermal fibroblasts and vascular, immune, and nerve cells (Beck et al., 2003; Bergen et al., 2000; Matsuzaki et al., 2005).

Our understanding of the pathophysiology of inherited ectopic calcification evolved in the light of three rare diseases, namely, PXE, general arterial calcification of infancy due to *NPP1* deficiency (OMIM 208000), and arterial calcification due to *CD73* deficiency (ACDC, OMIM 211800). A recent breakthrough proposed that the *ABCC6* transporter contributes to cellular ATP export from hepatocytes. ATP serves as a substrate for *ENPP1* to generate pyrophosphate (PPi), a physiological anticalcifying molecule (Jansen et al., 2013) that opposes the effect of inorganic phosphate (Pi) on hydroxyapatite (Ca/Pi) crystal growth. Corroborating this hypothesis, deficiency in *ABCC6* or *ENPP1* results in ectopic calcifications and reduced levels of plasma PPi (Jansen et al., 2013)), and PPi treatment prevents mineralization in mouse models (Dedinszki et al., 2017; Pomozi et al., 2017). *ABCC6*-dependent general arterial calcification of infancy and *ENPP1*-dependent PXE phenotypes have been reported (Nitschke et al., 2012)), indicating (i) the central role of a PPi deficit in the pathophysiology of mineralization and (ii) the overlap of *ABCC6* and *ENPP1* functions. Finally, mutations in *NT5e*, which encodes the ecto 5'-nucleotidase adenosine (ADO)-generating enzyme, were associated with mineralization in ACDC, a phenotype with features overlapping with PXE and general arterial calcification of infancy. In ACDC, the calcification is due to insufficient ADO levels, which exert tonic repression of pro-calcifying/Pi-generating alkaline phosphatase (AP).

The redundancy in the clinical presentations of these diseases together sheds light on the central role of extracellular nucleotide phosphohydrolysis in the Pi/PPi ratio. However, little information on the exact molecular function of *ABCC6* has been identified.

Beside calcifications, other cardiovascular manifestations are frequent in PXE, including peripheral arterial disease, which represents a major determinant of morbidity associated with PXE; ischemic stroke (Pavlovic et al., 2005; van den Berg et al., 2000); premature arterial occlusion (Pingel et al., 2017)); and carotid malformations (Vasseur et al., 2011), Omarjee et al., submitted). Studies performed on the *Abcc6*<sup>-/-</sup> mouse model showed that *ABCC6* participates in cardiac protection in the context of dystrophic cardiac calcification (Meng et al., 2007), ischemia reperfusion injury (Mungrue et al., 2011), and cardiac fibrosis (Rau et al., 2015). Moreover, several studies proposed that *ABCC6* deficiency is associated with other genetic diseases, such as  $\beta$ -thalassemia (Aessopos et al., 1992; Martin et al., 2011) or general arterial calcification of infancy, as well as with acquired diseases such as

chronic kidney disease (Lau et al., 2014). Hence, *ABCC6* mutations cause a broad spectrum of cardiovascular manifestations that are not necessarily related to calcification.

Although ectopic calcifications seem to clearly be due to a PPi deficit, structural, ischemic, thrombotic, and other nonmineralizing manifestations associated with PXE may indeed be independent of the mineralization process and extracellular PPi levels. Therefore, we evaluated the global changes in extracellular purine metabolism by measuring the expression of genes implicated in nucleotide and phosphate metabolism; enzymatic activities; and circulating nucleotide, nucleoside, and PPi levels in both PXE patients and the *Abcc6*<sup>-/-</sup> mouse model. The changes we observed may cause alterations of purinergic signaling and showed molecular aspects of the pathomechanisms behind PXE that to our knowledge are unreported.

## RESULTS

### ABCC6 deficiency is associated with reduced plasma concentrations of adenine nucleotides and PPi

To evaluate circulating nucleotide and PPi concentrations in ABCC6 deficiency, we recruited PXE patients and age- and sex-matched healthy control individuals (Table 1). The characteristics of the PXE population, including the pathology severity (Phenodex) score (Legrand et al., 2017) and genetic mutations, as well as the sample used for the different assays, are presented in Supplementary Table S1 online. Together with a highly significant reduction in PPi concentrations in PXE patients ( $P = 0.0001$ ) (Figure 1a), corroborating the previous findings of Jansen et al. (2014), plasma ATP and adenosine diphosphate (ADP) concentrations were significantly reduced within the same range (2-fold) compared with those of healthy control individuals ( $P = 0.0005$  and  $P = 0.015$ , respectively) (Figure 1b and c). The deficit in PPi concentrations was corroborated in *Abcc6*<sup>-/-</sup> plasma mouse samples (Figure 1d), as previously described (Jansen et al., 2013), again with an equivalent 2-fold decrease in plasma ATP concentration ( $P = 0.008$ ) (Figure 1e). We did not observe a correlation between age and any of the quantified metabolites in the plasma of *Abcc6*<sup>-/-</sup> or wild-type (WT) mice (data not shown). ADO quantification requires a specific procedure to avoid active cellular uptake and catabolism (Saadjian et al., 2002). Because of the specificity of the procedure, the limited number of patients, and their low frequency of consultation, we were not able to evaluate ADO in PXE patients. We therefore measured adenosine monophosphate (AMP) and ADO in WT and *Abcc6*<sup>-/-</sup> mice. Both AMP and ADO were maintained in *Abcc6*<sup>-/-</sup> mice (Figure 1f and g), indicating that the extracellular ATP deficit does not affect ADO concentration.

### Alteration of soluble nucleotide metabolizing activities in ABCC6 deficiency

Soluble purine-converting enzymes are present in the bloodstream where they contribute, along with membrane-bound ecto-nucleotidases, to the metabolism of circulating nucleotides in the vasculature (Yegutkin et al., 2012). Here, we quantified the activities of several key enzymes—NTPDase1/CD39, 5'-nucleotidase/CD73, AP, adenylate kinase (AK), and NPP1—in the sera of PXE patients and *Abcc6*<sup>-/-</sup> mice. Although PXE patients displayed a slight up-regulation in serum AP activity, it did not reach statistical significance

( $P = 0.10$ ). PXE patients also showed increased 5'-nucleotidase/CD73 activity ( $P = 0.015$ , Figure 2b), but no change was found for the other soluble enzymes: NTPDase1/ADPase, AP, AK, and NPP1 (Figure 2a–e). The activity of 5'-nucleotidase/CD73 was also elevated in serum from *Abcc6*<sup>-/-</sup> mice ( $P = 0.030$ ), paralleling decreased NPP1 ( $P = 0.006$ ) activity, but not those of ADPase, AP, and AK (Figure 2f–j).

### Altered arterial expression of purinergic signaling partners in *Abcc6*<sup>-/-</sup> mice

To evaluate the impact of the PXE condition on hepatic (central) and arterial (distal) gene expression, we used a quantitative real-time PCR approach on liver and thoracic aorta from WT and *Abcc6*<sup>-/-</sup> mice. Because PXE symptoms develop with age, we compared 5-month-old (adult) and 24-month-old (senescent) animals. The mineralizing phenotype, a marker of PXE disease progression, was verified in old animals by measuring calcium accumulation in whiskers (Le Corre et al., 2012) and osteopontin (*Spp1*) arterial expression (see Supplementary Figure S1 online).

Gene expression levels showed important changes according to aging in the liver (76% of investigated genes, Figure 3a), but less in the aorta (22%, Figure 3b). By contrast, focusing on the impact of the *Abcc6*<sup>-/-</sup> genotype, we found a much more profound effect in aorta compared with liver. Bar graphs representative of the different gene expression patterns are shown in Figure 4a and b. Detailed statistical analysis according to the *Abcc6* genotype and aging relative to appropriate controls are shown in Supplementary Tables S3 (liver) and S4 (aorta) online. In the liver, a significant age/genotype interaction was found for four genes (*Atp5o*, *Atp5k*, *Opa1*, and *Enpp1*), showing their increased expression with aging in WT but not in *Abcc6*<sup>-/-</sup> mice (Figure 4a). In aorta, two genes displayed interaction (*Enpp1* and *P2rx7*). In this case, their overexpression occurred with aging only in *Abcc6*<sup>-/-</sup> mice, suggesting a consequence of the pathogenic process (Figure 4b).

Volcano plots illustrate the global impact of *Abcc6* deficiency on gene expressions independently of aging. It evidences both the importance (log expression relative to WT) and significance ( $-\log_{10}$   $P$ -value, two way analysis of variance) of the results (Figure 4c and d). Among 46 genes investigated in the liver, three (*Atp5l*, *Entpd1*, and *P2ry13*) were significantly overexpressed, whereas *Nt5e* was down-regulated in *Abcc6*<sup>-/-</sup> mice. Strikingly, in the thoracic aorta, 12 genes were overexpressed (*Adk*, *Enpp1*, *Slc20a1*, *Entpd1*, *Slc29a1*, *Ank*, *Nt5e*, *Sirt1*, *Prkaa2*, *P2rx7*, *Panx1*, and *Adora2a*) and two were down-regulated (*Atp5k* and *Atp5l*) in *Abcc6*<sup>-/-</sup> mice out of 36 genes investigated.

## DISCUSSION

Calcifications mostly caused by a lack of PPi (Zhao et al., 2017) represent the tip of the iceberg of extensive pathological processes affecting vascular homeostasis in PXE. We show in this study that PXE broadly affects intravascular nucleotide metabolism, thus representing a molecular pathway for nonmineralizing manifestations of PXE such as ischemia, thrombosis, carotid malformation, or systemic oxidative stress (Figure 5).

Extracellular nucleotides and ADO (a nucleoside) act as autocrine/paracrine messengers through P2 and P1 membrane receptor activation, respectively (Eltzschig et al., 2013). We

show here that PXE patients display a highly significant decrease in ATP and ADP concentrations, and we confirm a major drop in the plasmatic P<sub>Pi</sub> concentration. Important questions emerge from these observations. Although there are multiple sources of extracellular nucleotides (Praetorius and Leipziger, 2009), our results suggest that systemic concentrations of purines and P<sub>Pi</sub> largely depend on liver production and/or release through an ABCC6-dependent mechanism. Furthermore, reduced intravascular concentrations of ATP and ADP may alter inflammatory, thrombotic, and other cardiovascular manifestations linked to P<sub>2</sub> purinergic receptor activation (Eltzschig et al., 2013). Hence, altered P<sub>2</sub> receptor activation likely takes part in the pathophysiology of PXE and could underlie unexplained manifestations previously observed in *Abcc6*<sup>-/-</sup> mice, such as cardiomyocyte hypertrophy (Prunier et al., 2013), increased tone of resistant arteries (Kauffenstein et al., 2014), increased inflammatory infiltrate after ischemia reperfusion (Mungrue et al., 2011), and fibrosis (Rau et al., 2015). However, these changes appear to have no major influence on the life expectancy of *Abcc6*<sup>-/-</sup> animals (see Supplementary Figure S2 online). Finally, the data we present also suggest a potential link between ABCC6 function, extracellular purines, and mitochondrial ATP production. Pasquali-Ronchetti et al. (2006) previously showed that the mitochondrial membrane potential was higher in the fibroblasts of PXE patients, likely causing oxidative stress with no impact on intracellular adenine nucleotides. More recently, based on altered mitochondrial respiration in *Abcc6*<sup>-/-</sup> mouse tissues, it was proposed that the ABCC6 transporter was localized in mitochondria-associated membranes (Martin et al., 2012). The localization of ABCC6 in mitochondria-associated membranes was later disputed (Pomozi et al., 2013). However, the exploration of a possible link between ABCC6 and mitochondrial function and/or adenylate energy charge undoubtedly deserves further attention.

ADO, through activation of specific P<sub>1</sub> receptors, exerts anti-inflammatory, anti-thrombotic, and vasculoprotective action. A link between the ABCC6 transporter and ADO has indeed been evoked, because a 5' nucleotidase (ADO-generating enzyme) gene mutation in ACDC causes ectopic calcifications similar to those of PXE (St Hilaire et al., 2011). In this condition, inhibition of the P<sub>1</sub> receptor is responsible for AP overexpression and calcification. Because of the similarity between PXE and ACDC manifestations, it has been proposed that ABCC6 could transport ADO (Markello et al., 2011), which would consequently be reduced in PXE patients. However, in vitro studies did not confirm ADO transport through ABCC6 (Szabo et al., 2011). Because most extracellular ADO originates from adenine nucleotide phosphohydrolysis, one would expect its reduction in *Abcc6*<sup>-/-</sup> plasma, together with ATP and ADP concentrations. However, we found no significant reduction in ADO or its precursor AMP in *Abcc6*<sup>-/-</sup> mice plasma. These data corroborate a recent metabolomics study that reported a similar amount of AMP and xanthine, an ADO degradation product, in *Abcc6*<sup>-/-</sup> mice compared with WT animals (Rasmussen et al., 2016). The levels of uric acid, the end product of the purinergic catabolic chain, were no different in PXE patients than in healthy control individuals (data not shown), suggesting again that the adenylic nucleotide (ATP/ADP) deficit does not affect downstream hydrolysis products (i.e., AMP, ADO, xanthine, uric acid). These data notably suggest that PXE pathogenesis does not involve an ADO deficit.

Along with membrane-bound nucleotidases and other ectoenzymes, soluble purine-converting activities are constitutively present in the bloodstream, thereby representing an important auxiliary effector system for local regulation of purine levels in the vasculature (Yegutkin, 2008, 2014). Although the physiological relevance of coupling these activities to intravascular nucleotide homeostasis remains questionable, the activities of several key soluble nucleotidases may represent valuable biomarkers of disease, such as liver dysfunction, chronic hypoxia, atherosclerosis, and other cardiovascular diseases. Serum ATPase activity depends on ecto-NTPDase1/CD39, whereas AMPase activity is carried out by ecto-NT5E/CD73. Together, these enzymes sequentially hydrolyze ATP to ADO. Whereas CD39 ADPase activity was unchanged, 5'-nucleotidase was significantly increased in PXE patients and *Abcc6*<sup>-/-</sup> mice. Other soluble enzymes (AK and NPP) were down-regulated in these animals compared with WT littermates. The *Abcc6*<sup>-/-</sup> mouse recapitulates the phenotype of PXE patients (calcifications, ocular involvement, biology, etc.) and is considered as a good model. However, the latter changes, although showing modifications in extracellular purine metabolism, were not observed in humans and likely reflect the existence of species-specific differences.

An increase in CD73 AMPase activity could be responsible for enhanced AMP hydrolysis and normalized ADO plasma concentration despite reduced ATP and ADP levels in *Abcc6*<sup>-/-</sup> mice. Circulating 5'-nucleotidase activity is used as a biomarker for hepatic diseases (cholestasis), being complementary to but more specific than AP, which displays a broader expression in bone, placenta, and intestine. Of interest, soluble CD73 activity was recently shown to be positively correlated to hypoxemic conditions (Liu et al., 2016) and atherosclerosis progression in patients with peripheral arterial disease (Jalkanen et al., 2015). Hence, 5'-nucleotidase could be a useful biomarker in PXE. Although the possibility cannot be fully excluded, these changes are unlikely to be associated with liver dysfunction, which is absent in PXE patients. The potential of CD73 activity as a biomarker, in particular the correlation with vascular manifestations of PXE, warrants further testing.

Several in vitro studies using skin fibroblasts with inactivating *ABCC6* mutations (Boraldi et al., 2014b; Dabisch-Ruthe et al., 2014; Ziegler et al., 2017), or in cultured HepG2 cell lines with *ABCC6* knockdown (Miglionico et al., 2014), showed that *ABCC6* deficiency has an impact on the expression and activity of proteins that contribute to extra-cellular nucleotide hydrolysis and Pi and PPI homeostasis. These data indicated overall a ratio between inducers and inhibitors of PPI metabolism that is shifted toward the procalcifying state (up-regulation of *TNAP*, down-regulation of *NPP1* and *NT5E*) and, of note, suggested a transcriptomic impact of *ABCC6*.

To evaluate in vivo the local and distal impacts of *Abcc6* deficiency on purine and phosphate homeostasis-related gene expression, we used a transcriptomic approach that compared liver, an unaffected tissue expressing *Abcc6*, versus arteries, which are affected by the pathology but do not express *Abcc6*. We report that the expressions of 4 of the 46 investigated genes was modified in *Abcc6*<sup>-/-</sup> liver, whereas 14 of 36 genes were modified in arteries. These results point to a remote transcriptional impact of *ABCC6* deficiency, in agreement with the metabolic hypothesis of PXE (Li et al., 2009). In *Abcc6*<sup>-/-</sup> liver, the expressions of the mitochondrial complex *Atp5l*, the vascular ATPase *Entpd1* (CD39), and the *P2ry13* ADP



receptor were increased, and *Nt5e* was reduced, corroborating the effect of *ABCC6* silencing in HepG2 cells (Miglionico et al., 2014). To some extent, these limited changes are consistent with the absence of reported liver disease in PXE. However, altered lipid metabolism with a plausible liver origin has been reported in PXE patients and would be responsible for their increased tendency to develop atherosclerosis (Gorgels et al., 2005; Kuzaj et al., 2014; Pomozi et al., unpublished data; Wang et al., 2001). In this context, changes of *P2ry13* expression were significant. Because this receptor contributes to reverse cholesterol transport and biliary lipid secretions (Fabre et al., 2010), we hypothesize that alteration in its expression or function (because of insufficient ligand ADP) may contribute to reducing high-density lipoprotein and other lipid metabolism alterations in the PXE condition. Moreover, four genes (*Atp5o*, *Atp5k*, *Opa1*, and *Enpp1*) showed significant increased expression with aging in WT but not in *Abcc6*<sup>-/-</sup> mice. This may suggest that *Abcc6* loss of function abrogates the spontaneous induction in these genes, the pathophysiological impact remaining to be determined.

In the aorta of *Abcc6*<sup>-/-</sup> mice, 12 of 36 genes were significantly overexpressed, and 2 of 36 were down-regulated compared with those in WT animals. With our quantitative real-time PCR approach, we could not detect significant *Abcc6* expression in mouse aorta (see Supplementary Figure S3 online), in contrast to previous data (Beck et al., 2003; Bergen et al., 2000) and supporting the remote reprogramming of arterial gene expression rather than a local effect. Several inhibitors of arterial calcifications were over-expressed in *Abcc6*<sup>-/-</sup> aorta, including the intracellular-toextracellular PPi transporter Ank and the PPi-generating enzyme NPP1, which is consistent with the findings of studies conducted on PXE fibroblasts (Boraldi et al., 2014a) but contradicts the results of another study (Dabisch-Ruthe et al., 2014). We measured the overexpression of genes involved in nucleotide phosphohydrolysis (*Entpd1*, *Enpp1*) and in ADO local homeostasis. These genes encompassed molecules implicated in the generation (*Entpd1* and *Nt5e*), uptake (*Slc29a1/Ent1*), phosphorylation (*Adk*), and cellular effects (*Adora2A*) of ADO. These changes may reflect an adaptive process that preserves the local concentrations of PPi and ADO and facilitates purine salvage. Conversely, the procalcifying gene encoding the Pi transporter, *Slc20a1/Pit1*, was also overexpressed in *Abcc6*<sup>-/-</sup> arteries, likely modifying the pro-versus anti-calcifying ratio. We and other researchers previously reported that osteochondrogenic marker expression is increased in *Abcc6*<sup>-/-</sup> arteries with aging (Kauffenstein et al., 2014) or after dystrophic cardiac calcifications (Sowa et al., 2013). In this respect, the contribution of P1 and P2 receptors in these phenotypic changes is likely. Although it is difficult to say to what extent these changes contribute to PXE manifestations, these results reflect the existence of transcriptional feedback linking circulating levels of nucleotides/ADO and the vascular expression of the enzymatic machinery involved in their regulation. Expression patterns of two genes, *P2rx7* and *Enpp1*, displayed a marked overexpression associated with aging evidenced only in *Abcc6*<sup>-/-</sup> mice. Again, this may suggest that these changes are consequences of the pathological process rather than being a cause of it. Here, we show in vivo that *Enpp1* expression is tightly linked to *Abcc6* (in liver and arteries) as previously proposed in vitro (Boraldi et al., 2014a; Dabisch-Ruthe et al., 2014; Ziegler et al., 2017).

Finally, two genes involved in mitochondrial biogenesis (*Sirt1*) and energy sensing (*Prkaa2* protein kinase AMP-activated) were up-regulated, whereas the ATP synthase subunits *Atp5l*

and *Atp5k* were down-regulated. These results might indirectly suggest a link to mitochondrial function with potential alteration of energy production.

Overall, these molecular data suggest an alteration of specific gene expressions in *Abcc6*<sup>-/-</sup> mice, which nevertheless should be confirmed in human PXE.

In conclusion, we show here that ABCC6 loss of function has a profound impact on vascular purine metabolism through modified arterial gene expression, soluble nucleotidase activities, and, finally, circulating nucleotide levels. These results may bring some reconciliation between metabolic versus local influence in PXE etiology, because they associate modifications of both circulating factors and altered gene expression specifically in the affected tissue. Purinergic signaling alterations most likely contribute to thrombotic and low-grade inflammation associated with PXE, and the real impact of this disease on mitochondrial function and energy metabolism remains to be investigated. Moreover, the diagnostic and prognostic potential of 5'-nucleotidase in PXE warrants specific investigations. A deeper characterization of purinergic signaling alterations will open new perspectives in our understanding of the pathogenesis of PXE and might help identify new therapeutic targets.

## MATERIALS AND METHODS

Detailed Materials and Methods are available in the Supplementary Materials online.

### Patients

Human participants gave written informed consent that was approved by the institutional ethics committee. Phenodex score (Legrand et al., 2017) and carried mutations are presented in Supplementary Table S1.

### Animals

Animals were manipulated in accordance with the European Community standards on the care and use of laboratory animals. *Abcc6*<sup>-/-</sup> mice were generated as described (Gorgels et al., 2005).

### Quantification of ATP in plasma

Human blood was collected on EDTA anticoagulated tubes. ATP and ADP concentrations were determined by enzyme-coupled assay (Yegutkin et al., 2012).

### Quantification of PPI in plasma

PPI in human plasma was determined enzymatically as previously described (Jansen et al., 2013).

### Quantification of AMP and ADO in mouse plasma

Mouse blood was collected using a method adapted from (Saadjian et al., 2002). Plasma were deproteinized, extracted, and incubated with chloroacetaldehyde 1 mol/L H<sub>2</sub>PO<sub>4</sub> buffer



(pH 4). The resulting etheno-fluorescent nucleotides were separated with a C18 reverse phase for quantification.

### Measurement of soluble purinergic enzyme activities in serum

Soluble enzymes were measured in the serum according to the method previously described (Yegutkin et al., 2012). ENPP1 activity was determined by hydrolysis of the chromogenic substrate thymi-dine 5-monophosphate p-nitrophenyl ester (*p*-Nph-5-TMP; Sigma-Aldrich, Saint Quentin Fallavier, France). Alkaline phosphatase activity was measured using routine hospital procedures.

### Real-time quantitative PCR

Tissue RNA was extracted using the RNeasy micro kit and was used to synthesize cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed with Sybr Select Master Mix (Applied Biosystems, Foster City, CA) using a Light cycler 480 Real-Time PCR System (Roche, Basel, Switzerland). Primer sequences are shown in Supplementary Tables S2. Heat mapping was performed using the Multi-experiment Viewer (MeV) software (<http://mev.tm4.org>).

### Statistical analysis

Data are presented as mean  $\pm$  standard error of the mean. Statistical analysis was performed using GraphPad PRISM (La Jolla, CA). Differences between groups were assessed using unpaired *t*-test or two-way analysis of variance followed by Fisher least significant difference multiple comparison when indicated. *P*-values less than 0.05 were considered statistically significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations:

<b>ACDC</b>	arterial calcification due to CD73 deficiency
<b>ADO</b>	adenosine
<b>ADP</b>	adenosine diphosphate
<b>AK</b>	adenylate kinase
<b>AMP</b>	adenosine monophosphate

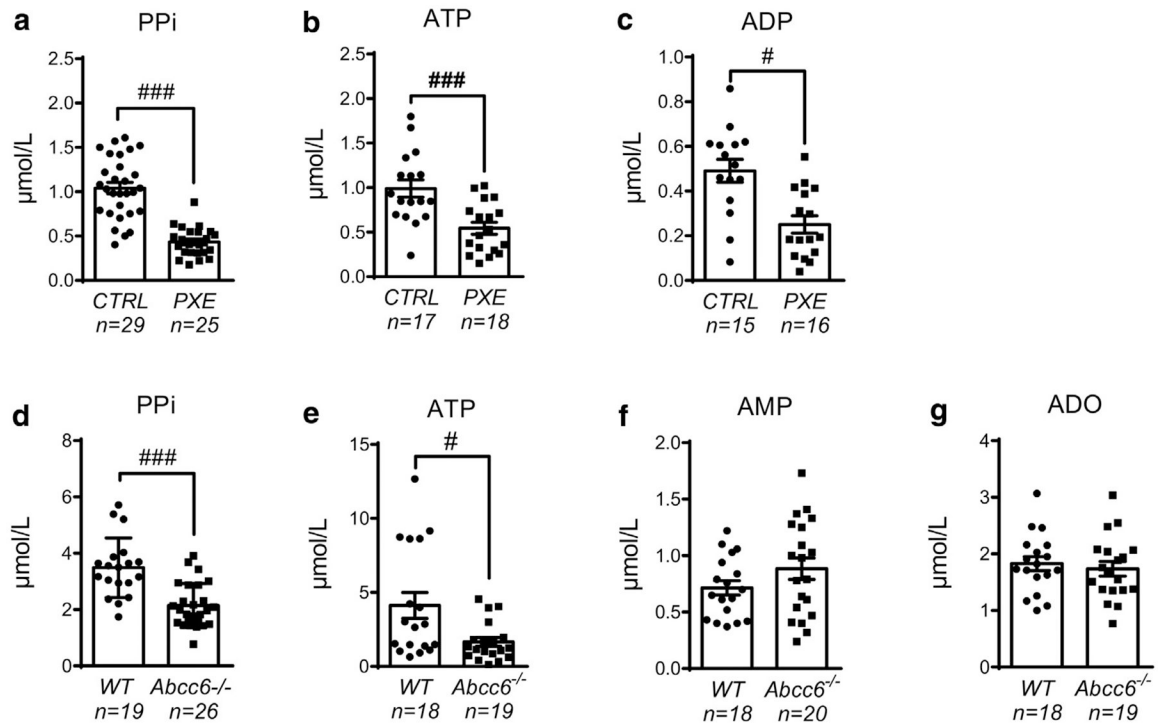
<b>AP</b>	alkaline phosphatase
<b>ATP</b>	adenosine triphosphate
<b>Pi</b>	inorganic phosphate
<b>PPi</b>	pyrophosphate
<b>PXE</b>	pseudoxanthoma elasticum
<b>WT</b>	wild type

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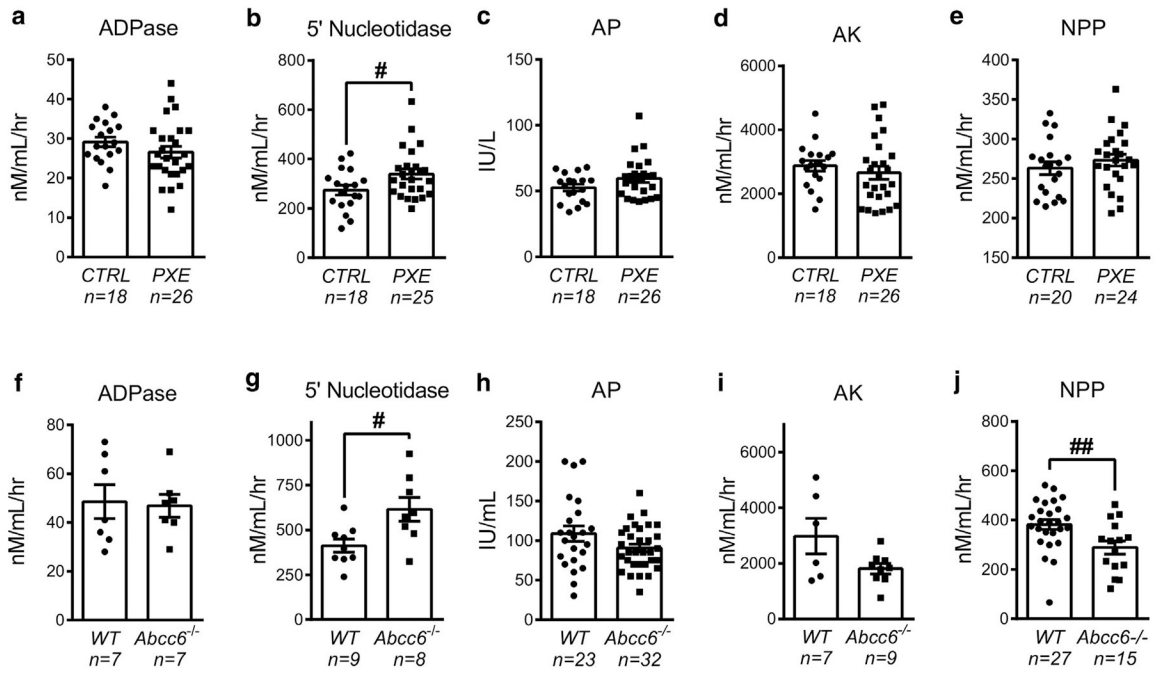
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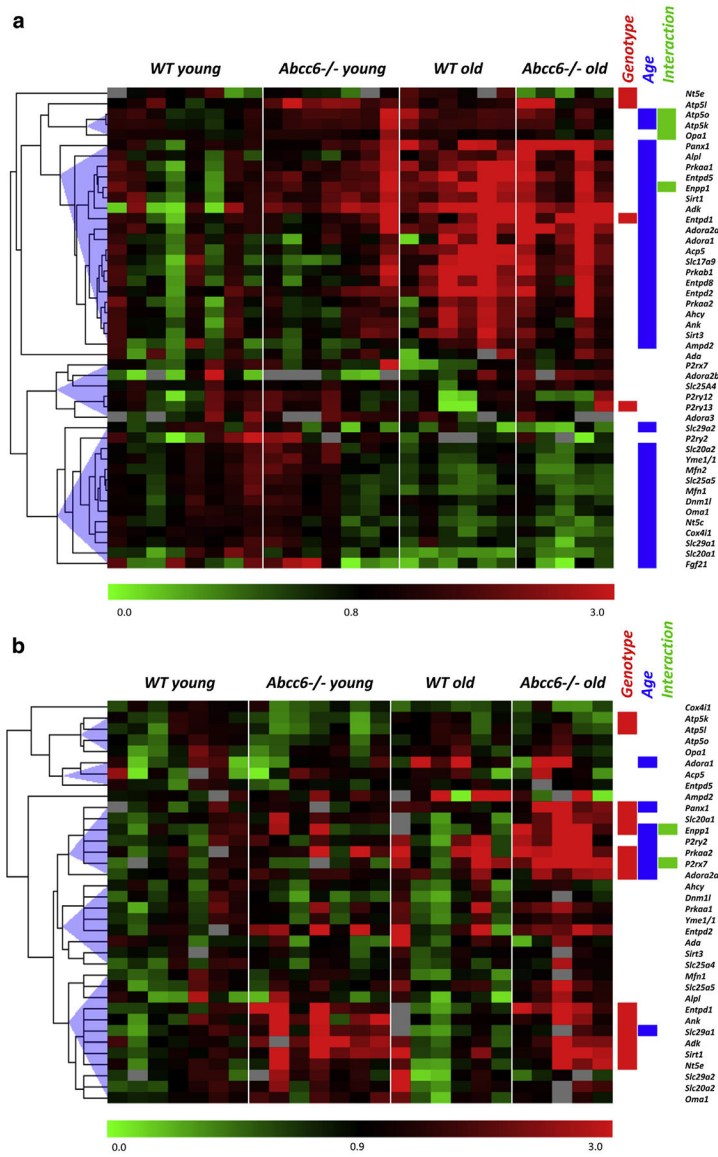
**Figure 1. Quantification of plasma adenine nucleotide, ADO, and PPI levels during ABCC6 deficiency.**

Concentrations of (a) PPI, (b) ATP, and (c) ADP were determined in blood plasma from healthy control (CTRL) and PXE individuals. (d) PlasmaPPI, (e) ATP, (f) AMP, and (g) adenosine were measured in WT and *Abcc6*<sup>-/-</sup> mice. Data represent the mean  $\pm$  standard error of the mean of the indicated number of plasma samples. #*P* < 0.05, ###*P* < 0.005. ADO, adenosine; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CTRL, control; PPI, pyrophosphate; PXE, pseudoxanthoma elasticum; WT, wild type.

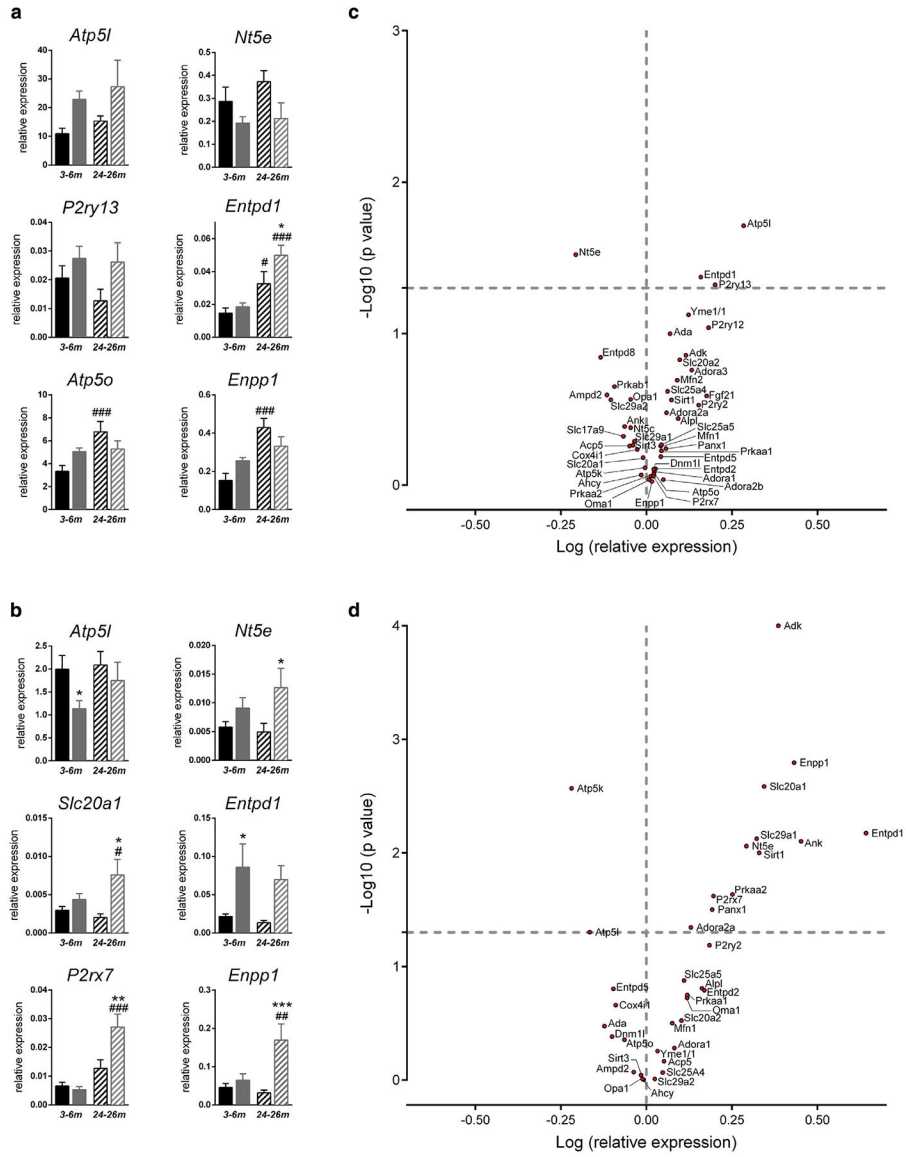


**Figure 2. Changes in soluble nucleotide-converting activities in PXE patients and *Abcc6*<sup>-/-</sup> mice.** The activities of key soluble enzymes, (a, f) NTPDase1/CD39 (ADPase), (b, g) ecto-5'-nucleotidase/CD73, (c, h) alkaline phosphatase (AP), (d, i) adenylate kinase (AK), and (e, j) nucleotide pyrophosphatase phosphodiesterase (NPP) were determined in (a–e) the serum of healthy control individuals and PXE patients and in (f–j) *Abcc6*<sup>-/-</sup> and WT mice. Data represent the mean ± standard error of the mean of the indicated number of serum samples. #*P* < 0.05; ##*P* < 0.01. CTRL, control; IU, international unit; nM/mL/hr, nano molar/milliliter/hour; PXE, pseudoxanthoma elasticum.

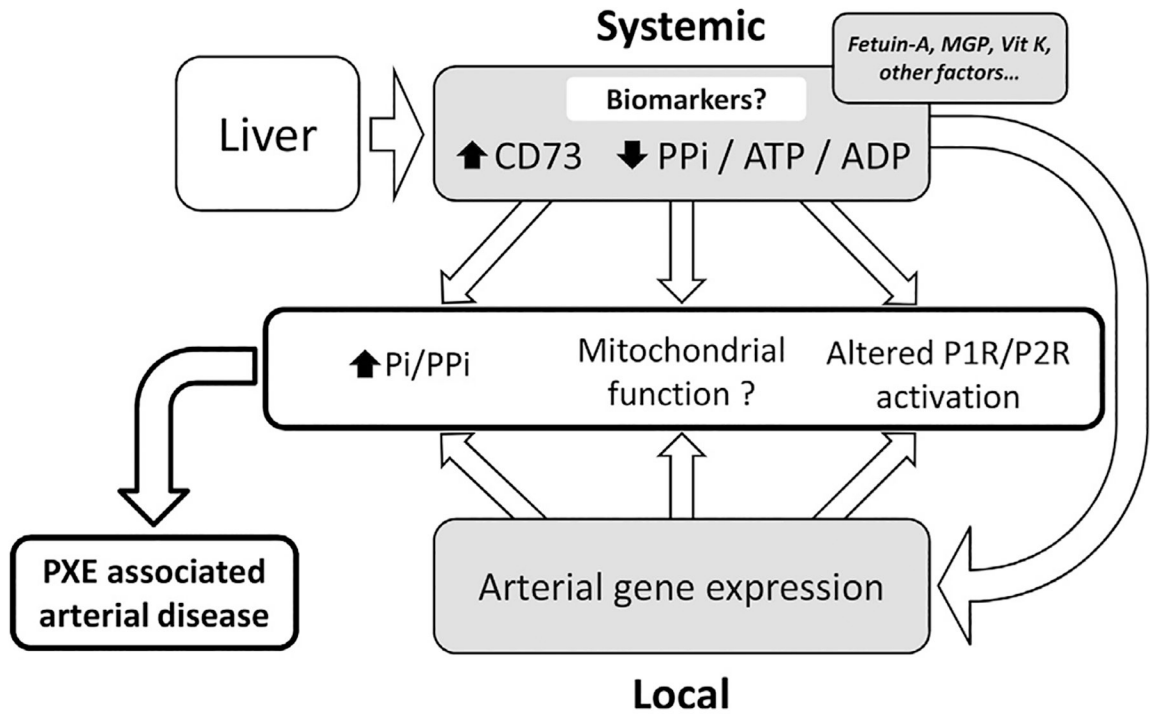




**Figure 3. Gene expression pattern in liver and aorta of *Abcc6*<sup>-/-</sup> mice.** Heat map of gene expression in the (a) liver and (b) aorta of young (5 months, n = 7) and old (24 months, n = 6, except for *Abcc6*<sup>-/-</sup> aorta, for which n = 5) *Abcc6*<sup>+/+</sup> and *Abcc6*<sup>-/-</sup> mice. Results represent color-coded expression relative to the control group (WT young). On the right are noted genes that are differently expressed (*P* < 0.05, two-way analysis of variance) according to genotype (red box) and to age (blue box) and the significance of the interaction between the two factors (green box). WT, wild type.



**Figure 4. Gene expression pattern in liver and aorta of *Abcc6*<sup>-/-</sup> mice.** Bar graphs illustrating representative gene expression pattern in young and old WT and *Abcc6*<sup>-/-</sup> mice in the (a) liver and (b) aorta. Genes represented were selected according to (i) the significant effect of *Abcc6* genotype and (ii) the significance of aging/genotype interaction. Black bars indicate WT mice, and gray bars indicate *Abcc6*<sup>-/-</sup> mice; solid fill indicates adult mice, and hatched fill indicates old mice. Significant differences according to age (#) or genotype (\*) (*P* < 0.05). Volcano plots showing the distribution of relative gene expression (log of fold changes) and *P*-values (log<sub>10</sub>) in (c) *Abcc6*<sup>-/-</sup> mice liver (n = 13) and (d) aorta (n = 13 WT and n = 12 *Abcc6*<sup>-/-</sup>). Data are the mean of five to seven values obtained from different animals. Values of log *P* above 1.3 (*P* < 0.05) are statistically significant. #*P* < 0.05; ###*P* < 0.001; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. WT, wild type.



**Figure 5. Proposal of a summary diagram for the pathophysiology of PXE.**

In the context of liver (central) ABCC6 deficiency, plasma levels of PPI and adenine nucleotides (ATP, ADP) are decreased and 5'-nucleotidase activity is increased each of these, representing potential disease biomarkers. ABCC6 deficiency is also associated with modifications of purine and phosphate gene expression in (remote) affected tissues like arteries. Both systemic and local alterations in purine and phosphate metabolism contribute to PXE-associated peripheral arterial disease. ADP, adenosine diphosphate; ATP, adenosine triphosphate; MGP, matrix gla protein; Pi, inorganic phosphate; PPI, pyrophosphate; PXE, pseudoxanthoma elasticum; Vit, vitamin.

**Table 1.**

## Patient Characteristics

<b>Subgroup</b>	<b>n</b>	<b>Age in Years, Mean <math>\pm</math> SD</b>
Patient characteristics for plasma ATP and ADP determination		
Control		
All	17	45 $\pm$ 11
Female	12	44 $\pm$ 11
Male	5	47 $\pm$ 9
PXE		
All	18	46 $\pm$ 13
Female	13	45 $\pm$ 14
Male	5	47 $\pm$ 9
Patient characteristics for plasma PPi determination		
Control		
All	29	38 $\pm$ 12
Female	20	38 $\pm$ 12
Male	9	42 $\pm$ 12
PXE		
All	25	43 $\pm$ 19
Female	15	46 $\pm$ 16
Male	10	39 $\pm$ 22
Patient characteristics for serum enzymatic activities determination		
Control		
All	20	46 $\pm$ 3
Female	15	45 $\pm$ 14
Male	5	47 $\pm$ 9
PXE		
All	26	44 $\pm$ 12
Female	19	43 $\pm$ 13
Male	7	46 $\pm$ 9

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; PPi, pyrophosphate; PXE, pseudoxanthoma elasticum; SD, standard deviation.