ARTICLE

Generalized Arterial Calcification of Infancy and Pseudoxanthoma Elasticum Can Be Caused by Mutations in Either *ENPP1* or *ABCC6*

Yvonne Nitschke,[1,27] Geneviève Baujat,[2,27] Ulrike Botschen,[1] Tanja Wittkampf,[1] Marcel du Moulin,[1] Jacqueline Stella,[1] Martine Le Merrer,[2] Geneviève Guest,[3] Karen Lambot,[4] Marie-Frederique Tazarourte-Pinturier,[5] Nicolas Chassaing,[6,7] Olivier Roche,[8] Ilse Feenstra,[9] Karen Loechner,[10] Charu Deshpande,[11] Samuel J. Garber,[12] Rashmi Chikarmane,[13] Beat Steinmann,[14] Tatevik Shahinyan,[15] Loreto Martorell,[16] Justin Davies,[17] Wendy E. Smith,[18] Stephen G. Kahler,[19] Mignon McCulloch,[20] Elizabeth Wraige,[21] Lourdes Loidi,[22] Wolfgang Höhne,[23] Ludovic Martin,[24] Smaïl Hadj-Rabia,[25] Robert Terkeltaub,[26] and Frank Rutsch[1,*]

Spontaneous pathologic arterial calcifications in childhood can occur in generalized arterial calcification of infancy (GACI) or in pseudoxanthoma elasticum (PXE). GACI is associated with biallelic mutations in *ENPP1* in the majority of cases, whereas mutations in *ABCC6* are known to cause PXE. However, the genetic basis in subsets of both disease phenotypes remains elusive. We hypothesized that GACI and PXE are in a closely related spectrum of disease. We used a standardized questionnaire to retrospectively evaluate the phenotype of 92 probands with a clinical history of GACI. We obtained the *ENPP1* genotype by conventional sequencing. In those patients with less than two disease-causing *ENPP1* mutations, we sequenced *ABCC6*. We observed that three GACI patients who carried biallelic *ENPP1* mutations developed typical signs of PXE between 5 and 8 years of age; these signs included angioid streaks and pseudoxanthomatous skin lesions. In 28 patients, no disease-causing *ENPP1* mutation was found. In 14 of these patients, we detected pathogenic *ABCC6* mutations (biallelic mutations in eight patients, monoallelic mutations in six patients). Thus, *ABCC6* mutations account for a significant subset of GACI patients, and *ENPP1* mutations can also be associated with PXE lesions in school-aged children. Based on the considerable overlap of genotype and phenotype of GACI and PXE, both entities appear to reflect two ends of a clinical spectrum of ectopic calcification and other organ pathologies, rather than two distinct disorders. *ABCC6* and *ENPP1* mutations might lead to alterations of the same physiological pathways in tissues beyond the artery.

Introduction

Generalized arterial calcification of infancy (GACI [MIM 208000]) is a rare autosomal-recessive disorder characterized by calcification of the internal elastic lamina, fibrotic myointimal proliferation of muscular arteries, and resultant arterial stenosis.[1] Affected patients suffer from severe congestive cardiac failure, hypertension, and myocardial ischemia. In the past, few patients survived the neonatal period,[2–4] whereas more recently, patients treated with bisphosphonates have experienced a more favorable outcome.[5,6] Radiological studies reveal diffuse vascular and

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[1] Department of General Pediatrics, Münster University Children's Hospital, Albert-Schweitzer-Campus 1, Gbde. A1, D-48149 Münster, Germany; [2] Department of Genetics, Centre de reference des maladies osseuses constitutionnelles, Necker Hospital, Université Paris Descartes, 149 rue de Sèvres, 75743 Paris cedex 15, France; [3] Nephrology Unit, Necker Hospital, Université Paris Descartes, 149 rue de Sèvres, 75743 Paris cedex 15, Paris, France; [4] Department of Radiology, Necker Hospital, Université Paris Descartes, 149 rue de Sèvres, 75743 Paris cedex 15, Paris, France; [5] Pediatrics Unit, Centre Hospitalier de Montreuil, 56 Boulevard de la Boissiere, 93100 Montreuil, France; [6] Department of Medical Genetics, CHU Toulouse, F 31400 Toulouse, France; [7] EA-4555, Toulouse III Paul-Sabatier University, F 31400 Toulouse, France; [8] Department of Ophtalmology, Centre de Référence des Maladies Rares en Ophtalmologie, Necker Hospital, Université Paris Descartes, 149 rue de Sèvres, 75743 Paris cedex 15, Paris, France; [9] Department of Human Genetics, Radboud University Nijmegen Medical Centre, Geert Grooteplein 10, 6525 GA Nijmegen, The Netherlands; [10] NC Pediatric Osteoporosis Clinic, Pediatric Endocrine Unit, University of North Carolina Chapel Hill, 3341 Medical Biomolecular Research Building CB# 7039 103, Mason Farm Road, NC 27599, USA; [11] Department of Clinical Genetics, Guy's and St. Thomas' NHS Foundation Trust, 7th floor, Borough Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK; [12] Division of Neonatology, Department of Pediatrics, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, 34th St. and Civic Center Blvd., Philadelphia, PA 19104, USA; [13] Institute of Medical Genetics, St. Peter's University Hospital, 254 Easton Avenue, New Brunswick, NJ 08901, USA; [14] Division of Metabolism and Molecular Pediatrics, University Children's Hospital, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland; [15] Arabkir Joint Medical Centre, Mamikonyants St., 30 Building, 0014 Yerevan, Armenia; [16] Department of Genetics, Hospital Sant Joan de Déu, Calle de San Juan de Dios 2, 08950 Esplugues de Llobrega, Barcelona, Spain; [17] University of Wales College of Medicine Heath Park, CF4 4XN Cardiff, UK; [18] Division of Genetics, Maine Pediatric Specialty Group, Barbara Bush Children's Hospital, Maine Medical Center, 887 Congress Street, Suite 320, Portland, ME 04102, USA; [19] Division of Medical Genetics, Arkansas Children's Hospital, Slot 512-22, One Children's Way, Little Rock, AR 72202, USA; [20] Department of Pediatric Nephrology, Evelina Children's Hospital, Guy's and St. Thomas' NHS Foundation Trust, St. Thomas' Hospital, Westminster Bridge Road, London SE1 7EH, UK; [21] Department of Pediatric Neurology, Evelina Children's Hospital, Guy's and St. Thomas' NHS Foundation Trust, St. Thomas' Hospital, Westminster Bridge Road, London SE1 7EH, UK; [22] Unidad de Medicina Molecular, Fundación Pública Galega de Medicina Xenómica, Hospital Clínico Universitario, Choupana SN, E-15706 Santiago de Compostela, Spain; [23] Department of Biochemistry, Charité University Medicine, Oudenarder Strasse 16, Haus A, Aufgang 9, 13347 Berlin, Germany; [24] Department of Dermatology, Angers University Hospital, 4 rue Larrey, F-49933 Angers cedex 9, France; [25] Department of Dermatology, Centre de reference des Maladies Génétiques à Expression Cutanée, Necker Hospital, Université Paris V Descartes, 49 rue de Sèvres, 75743 Paris cedex 15, France; [26] Department of Rheumatology, Allergy and Immunology, VA Medical Center, University of California San Diego, 3350 La Jolla Village Drive, San Diego, CA 92161, USA

[27] These authors contributed equally to this work

*Correspondence: rutschf@ukmuenster.de

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periarticular soft-tissue calcifications. Some patients may also develop hypophosphatemic rickets, a presentation associated with a milder phenotype.[6–10] Inactivating mutations in *ENPP1* (MIM 173335), encoding ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1), have been identified as the underlying defect in about 75% of the cases of GACI.[6,11] NPP1 generates PP_i, a major physiologic inhibitor of calcification that exerts its effects by, among other things, inhibiting hydroxyapatite crystal growth.[12] Moreover, effects of NPP1 on adenosine metabolism also could modulate arterial calcification.[13]

Pseudoxanthoma elasticum (PXE [MIM 264800]) was first described in 1881,[14] and its prevalence is estimated to be 1 in 25,000.[15] It is known as an autosomal-recessive disorder, but autosomal-dominant inheritance has been proposed in rare PXE cases.[16,17] PXE is characterized by ectopic mineralization and fragmentation of elastic fibers of connective tissues, including skin, vascular walls, and the eyes.[18] The main dermatological features are yellowish papules or plaques of coalesced papules on the neck and in flexural areas that have typical histological abnormalities. The presence of fragmented basophilic elastic fibers in the upper and middle reticular dermis and calcifications of elastic fibers are characteristic histologic findings of this disorder. Cardiovascular involvement includes decreased peripheral pulses, vascular calcifications, and endocardial thickening.[19–21] Angioid streaks seen in fundoscopy reflect disruption of the so-called Bruch's membrane, which consists of elastic fibers between the pigmented retinal epithelium and the choroidea.[22] Additionally, PXE can manifest with gastrointestinal haemorrhage and abnormal tissue mineralization in different organs, including the liver, kidneys, spleen, breast, and testes.[23,24]

Classic PXE results from mutations in the *ABCC6* (ATP-binding cassette subfamily C number 6) gene (MIM 603234).[25] The ABCC6-transported substrate or substrates, which modulate arterial calcification and other phenotypic changes of PXE, are not known, and hepatic abnormalities that have effects on calcification-regulating plasma proteins such as fetuin have been suggested to at least partially mediate pathogenesis of PXE.[26] In this context, PXE-like findings have also been found in patients with β-thalassemia (MIM 613985)[27] and have been found to accompany deficiency of the vitamin-K-dependent clotting factors.[28] In 2007, mutations in *GGCX* (MIM 137167) were reported in several cases with PXE-like cutaneous lesions and deficiency of vitamin-K-dependent clotting factors.[29] *GGCX* encodes a gamma-glutamyl carboxylase essential for activation of hepatic coagulation factors and of the ectopic calcification inhibitor matrix gla protein (MGP).[29]

Most recently, our group reported on a family with two sons; the older son presented with PXE and mutations in *ABCC6*, and the younger one died of GACI at the age of 15 months.[30] Mutation analysis was not performed in the younger brother. However, retrospective analysis of the living family members was negative for mutations in *ENPP1*. On the basis of these observations, we hypothesized that GACI and PXE might be more closely related than previously thought. Here we report on three patients with GACI caused by biallelic mutations in *ENPP1*. These patients developed typical signs of PXE in childhood. We also present the results of the clinical and mutational analysis of 14 patients who have GACI but do not have disease-causing mutations in *ENPP1* and in whom *ABCC6* mutations were identified.

Patients and Methods

Patients

For this study, we used clinical data and DNA material from our international GACI registry.[6] This registry is an ongoing systematic collection of phenotypic and genotypic data from patients with the clinical diagnosis of GACI and currently contains data from 92 GACI patients of 85 unrelated families (Figure S1, available online). Diagnosis of GACI was based on the presence of cardiovascular symptoms associated with evidence of arterial calcifications with or without stenoses as seen on X-ray or ultrasound in infancy, periarticular calcifications detected on radiological studies, or typical histological findings. Patient history and clinical data were gathered through a standardized questionnaire, which was sent to the referring physician or geneticist. All patients in the registry were screened for mutations in *ENPP1* as part of the routine diagnostic analysis.

Clinical and mutational data on a subset of 55 of these patients have been published before.[6] The clinical course of GACI is exemplified by a case report on patients 1, 2, and 8 (Supplemental Data).

When available, clinical data on signs of PXE were evaluated (for detailed case reports, see Supplemental Data). PXE diagnostic criteria included characteristic skin involvement, characteristic histopathologic features of lesional skin,[31] and angioid streaks of the retina.[32]

The study protocol was approved by the Münster University Hospital Ethical Committee and other participating institutional peer-review human-subject committees. The parents of all subjects involved in this study gave informed written consent.

Mutation Analysis

Genomic DNA was extracted from whole blood. When blood was unavailable, patient DNA was extracted from formalin-fixed tissue blocks.

DNA from 92 patients of 85 unrelated families with clinically proven GACI was subjected to mutation analysis of *ENPP1*. Polymerase chain reaction (PCR) with 24 primer pairs was used for amplification of the 25 exons and the flanking splice sites in *ENPP1* (RefSeq accession number NG_008206.1), as previously described.[7] Primer sequences are available upon request. In those patients with less than two biallelic coding-region or splice-site mutations in *ENPP1*, *ABCC6* (RefSeq accession number NG_007558.2) mutation analysis was performed with previously described *ABCC6*-specific primers so that all 31 exons and the exon/intron boundaries would be amplified.[33,34] Previously reported sequences[35] were used for synthesis of intron-derived primers specific for PCR amplification of *ABCC6* exons 1–9. We investigated splice-site mutations and aberrant splicing by sequencing the ABCC6 cDNA. Primer sequences are available on request. PCR products were directly sequenced bidirectionally withan ABI 3730 Genetic Analyzer and a BigDye Terminator

Table 1. Clinical and Mutational Data of Patients with GACI and PXE Phenotype Carrying Mutations in *ENPP1*

Family	Patient	Sex	Geographic Origin	Consanguinity	Affected/ Nonaffected Siblings (GACI)	Arterial Calcifications	Periarticular Calcifications	Cardiovascular Complications	Additional Abnormalities	Age at Data Collection	PXE Features	Bisphosphonate Therapy	Histology	DNA Change in *ENPP1*	Amino Acid Change in ENPP1	Ref.
1	1	m	France	yes	1/0	a, c	hips, ankles, wrists, shoulders	hypertension in neonatal period	fusion of vertebral bodies C3-C5, hypophosphatemic rickets, stapedovestibular ankylosis leading to hearing loss, angiomatous skin lesions	9 years	pseudoxanthomatous lesions on the neck	clodronate p.o.	calcification of elastic fibers in the dermis of pseudoxanthomatous lesion	c.[1612G>C]; [1612G>C]	p.[Asp538His]; [Asp538His]	
2	2	f	France	no	1/0	endocardium, a, p	painful wrist calcifications in the neonatal period		hypophosphatemic rickets Stapedovestibular ankylosis leading to hearing loss	5 years	angioid streaks in the Bruch's membrane	no		c.[795+1G>A]; [1756G>A]	p.[altered splicing]; [Gly586Arg]	
3	3	m	Great Britain	no	2/1	a	no	left ventricular hypertrophy, arterial hypertension	"middle aortic syndrome," progressive hearing loss	9 years	pseudoxanthomatous periumbilical and cervical lesions	no	calcification of elastic fibers in the dermis of pseudoxanthomatous lesion	c.[783C>G]; [878_879delAA]	p.[Tyr261*]; [Lys293fs*4]	8

Abbreviations are as follows: A, aorta; c, coronary arteries; d, diverse arteries; p, pulmonary artery; and r, renal arteries.

v3.1 Cycle Sequencing Kit according to the protocol provided by the manufacturer (Applied Biosystems, Foster City, California, USA). Mutations were compared with the dbSNP, HGMD, and ENSEMBL polymorphism databases. The recurrent large deletion of exons 23–29 (c.2996_4208del) of *ABCC6* was screened by PCR with a previously described set of nested primers.[36] For segregation analysis, parental DNA was analyzed with the aim of excluding the occurrence of two mutations on the same allele.

Multiplex Ligation-Dependent Probe Amplification

The specific synthetic probe set for multiplex ligation-dependent probe amplification (MLPA) analysis of *ABCC6*, covering 23 of the 31 exons, was used (SALSA MLPA kit P092 ABCC6, MRC-Holland, Amsterdam, The Netherlands). The construction of the kit precludes the generation of signals from the *ABCC6* pseudogenes. MLPA reactions were performed with the reagents and recommendations of the *ABCC6* MLPA reagent kit and with 100 ng of genomic DNA. The PCR products were separated by capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The Peak ScannerTM Software v1.0 (Applied Biosystems, Foster City, CA, USA) was used for peak identification and fragment sizing. Data analysis was carried out according to the manufacturer's recommendations. All samples were tested in duplicate.

Splice-Site Prediction

For analysis of the splice-site mutation of patient #2, there was no RNA available. Thus, the software NetGene2 v. 2.4 (Center for Biological Sequence Analysis, Technical University of Denmark DTU) was used for in silico prediction of aberrant splicing.

Results

Cumulatively, we identified biallelic pathogenic mutations in *ENPP1* in 62 of 92 affected patients (data not shown). Three of these patients developed typical signs of PXE in childhood (Table 1). Of the remaining 30 GACI patients, 28 patients showed neither splice-site nor coding-region mutations in *ENPP1*, and 14 patients were found to carry *ABCC6* mutations (Figure S1). Two unrelated probands of our cohort carried only one pathogenic *ENPP1* mutation on one allele. Genomic DNA derived from these probands was also subjected to *ABCC6* mutation analysis. None of these probands carried a potentially pathogenic mutation in *ABCC6*.

Clinical Features of GACI Patients Who Have PXE and Carry Mutations in *ENPP1*

Of the 92 patients of our study cohort, three unrelated patients, two boys and one girl (patients 1, 2, and 3) who had generalized arterial calcification of infancy (Table 1; for detailed case reports on patients 1 and 2 see Supplemental Data) presented with clinical features of PXE in later childhood. In the first male patient (patient 1), who was born to consanguineous parents and presented with extensive calcifications of large and medium-sized arteries, the diagnosis of GACI was already established in the neonatal period and led to early bisphosphonate

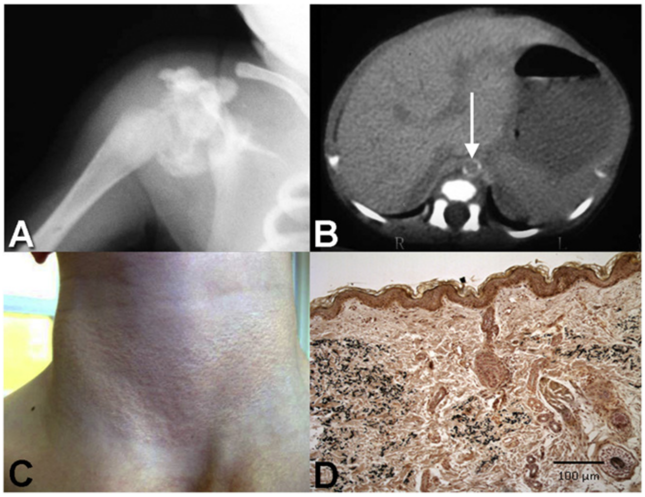

Figure 1. Manifestation of GACI and PXE Associated with *ENPP1* Mutations

Patient 1, who suffered from generalized arterial calcification in infancy. X-ray showing extensive peri-articular calcification of the right shoulder (A) and abdominal CT scan showing a ring-like calcification of the abdominal aorta (B, arrow) in the neonatal period. Yellowish papules, located on the frontal part of the neck when the patient was 9 years old, were suggestive of PXE (C), which was histologically proven by the presence of dermal calcium deposits associated with elastic fibers (D) (Von Kossa staining, ×10).

treatment. At the age of 8 yr, this patient developed pseudoxanthomatous skin lesions, which were proven histologically to reflect calcifications of elastic fibers in the dermis, as are typical for PXE (Figure 1). Also, the mother of this proband showed yellowish papules. The female patient (patient 2), who presented with painful periarticular calcifications in infancy, showed angioid retinal streaks suggestive of PXE at the age of 5 yr. At that time, widespread calcifications of the heart and the proximal great arteries were noted on computed tomography. She had not received bisphosphonate therapy. Interestingly, both patients developed hypophosphatemic rickets as well as deafness due to stapedo-vestibular ankylosis. Additionally, both patients showed capillary angiomas on different parts of the skin (Supplemental Data), which had not been described previously in patients with GACI or PXE. The third patient (patient 3) presented with left ventricular hypertrophy in the neonatal period. At 14 months of age, arterial hypertension was noted, and angiography revealed severe arterial stenosis of the celiac artery, the superior mesenteric artery, renal arteries, and both internal and external carotid arteries. Mutation analysis of *ENPP1* in this boy was performed when the mother had a second pregnancy, which resulted in a stillbirth at 31 weeks of gestation; the fetus showed fetal hydrops and aortic root calcification, suggestive of GACI. A detailed case report on the two siblings was recently published.[8] Most recently, starting at the age of 8 yr, the surviving boy developed pseudoxanthomatous skin lesions around his umbilicus and on his neck, and these were histologically proven to be typical PXE lesions.

Mutations in *ENPP1* in Three Patients with GACI and PXE

The homozygous missense mutation c.1612G>C (p.Asp538His) was detected in exon 16 of *ENPP1* in patient 1. Both parents were shown to be heterozygous carriers of the mutation. This amino acid change affects a conserved residue located within the catalytic domain of NPP1 and is therefore predicted to affect enzyme activity (Table 2 upper panel, Figure 2). In patient 2, the mutation c.795+1G>A was detected on the maternal allele, and c.1756G > A (p.Gly586Arg) was detected on the paternal allele. The mutation c.795+1G>A is located at the exon-intron boundary of exon 7 and is predicted to affect a splice donor site (NetGene2 v. 2.4, Center for Biological Sequence Analysis, Technical University of Denmark DTU) and therefore to lead to abnormal splicing. The mutation c.1756G>A (p.Gly586Arg) leads to a change of the conserved polar amino acid glycine to the polar charged amino acid arginine in the catalytic domain of the NPP1 protein and thus most likely affects NPP1 enzymatic activity. Patient 3 was compound heterozygous for the two nonsense mutations c.783C>G (p.Tyr261X) in exon 7 and c.878_879 delAA (p.Lys293fsX4) in exon 8,[8] both of which are predicted to lead to a severely truncated protein. Sequence analysis of the exons and flanking intronic regions of *ABCC6* in each of the three patients did not show any aberration.

Clinical Features of GACI Patients Carrying *ABCC6* Mutations

30 patients with GACI and with less than two coding regions or splice-site mutations in *ENPP1* were screened for mutations in *ABCC6*. Mutations in *ABCC6* were detected in 14 of these patients (Table 3). Homozygous mutations were found in two patients, compound heterozygous mutations in six, and in six patients only one mutation could be identified.[37,38]

The eight patients carrying biallelic *ABCC6* mutations were of different ethnic backgrounds and presented in early infancy with widespread calcifications of the aorta and medium-sized arteries, including coronary arteries and renal arteries (Table 3). Four of these patients (patients 4, 5, 7, and 11) were severely affected and died of myocardial infarction and cardiac failure within the first three months of life. Autopsy performed in one patient (patient 4) revealed the typical histological features consisting of calcification of the internal elastic lamina, myointimal proliferation in the coronary arteries, and consequent severe arterial stenosis (Figure 3D). Interestingly, in one patient, generalized arterial stenoses were demonstrated without any evidence of arterial calcification (patient #5). This patient died of myocardial infarction at the age of 8 weeks. Another patient (patient 8) presented with periarticular calcifications of the shoulder joints and the hip joints (Figures 3A and 3B). This patient also developed diffuse cerebral white matter disease leading to cystic encephalomalacia, which is quite uncommon in GACI.

Table 2. **Functional Consequences of Mutations in *ENPP1* and *ABCC6***

Exon	DNA Change	Amino Acid Change	Patient	Functional Consequences	References
ENPP1					
7	c.783C>G	p.Tyr261*	3	truncated protein	6, 8, 11
IVS7	c.795+1G>A	Loss of splice site	2	predicted loss of donor splice site, aberrant splicing	new
8	c.878_879delAA	p.Lys293fs*4	3	truncated protein	6, 8, 11
16	c.1612G>C	p.Asp538His	1	affects conserved aa in catalytic domain, negatively charged aa changed to neutral/positively charged aa	new
18	c.1756G>A	p.Gly586Arg	2	affects conserved aa in catalytic domain, polar aa changed to polar, positively charged aa	new
ABCC6					
4	c.450_451insC	p.Ala151Argfs*45	5	truncated protein	new
9	c.1064T>G	p.Leu355Arg	14	affects conserved aa in transmembrane domain, non-polar aa changed into polar, positively charged aa	25, 42
9	c.1171A>G	p.Arg391Gly	9, 15, 16	affects conserved aa in intracellular domain, polar, posively charged aa changed into polar aa	25, 42, 43
12	c.1552C>T	p.Arg518*	6, 10	truncated protein	25, 42
13	c.1769C>T	p.Ser590Phe	12	affects aa in transmembrane domain, conserved in bovine, chicken and fungus	new
IVS21	c.2787+1G>T	p.Arg929fs*1	4	truncated protein	25, 42
23	c.3105_3107delCTT[a]	p.Phe1036del	6	loss of one conserved aa in intracellular domain	25 (c.3106delTTT p.Phe1036del)
24	c.3340C>T	p.Arg1114Cys	13	affects conserved aa in intracellular domain, polar charged aa changed into non-polar aa, possible formation of incorrect disulfide bonds	25, 42, 44
24	c.3421C>T	p.Arg1141*	11	truncated protein	25, 33, 34, 47
26	c.3662G>A	p.Arg1221His	7	affects conserved aa in intracellular domain	42, 45
IVS26	c.3736-1G>A	p.Ala1246fs*26	4	truncated protein	25, 42, 47
28	c.3940C>T	p.Arg1314Trp	5, 8, 17	affects conserved aa in ATP-binding domain	25, 34, 42, 46
23-29	c.2996_4208del	p.Ile1000Trpfs*60	9	truncated protein	36

[a] The mutation p.Phe1036del is already known,[25] although the known DNA change is c.3106_3108 delTTT. The DNA change c.3105_3107delCTT leading to the same amino acid change, p.Phe1036del, is not described in the literature.

In six additional patients we were able to detect only one potentially pathogenic mutation in *ABCC6*. One of these patients was the recipient twin of a twin-to-twin transfusion syndrome. He died of myocardial ischemia at the age of 5 months, whereas the donor twin survived.[6,7,39,40] The other patients had a more favorable clinical course; for example, one female proband who presented with GACI[41] developed epilepsy at the age of 20 yr and was 31 yr old at the time of this study (Table 3, patient 14). Information on dermal elastorrhexis or retinal changes on this patient was not available.

Mutations in *ABCC6* in Patients with GACI

Thirteen different mutations in *ABCC6* were identified in patients with GACI. These included six missense mutations (c.1064T>G [p.Leu355Arg], c.1171A>G [p.Arg391Gly], c.1769C>T [p.Ser590Phe], c.3340C>T [p.Arg1114His], c.3662G>A [p.Arg1221His, and c.3940C>T [p.Arg1314Trp]), two nonsense mutations (c.1552C>T [p.Arg518*], c.3421C>T [p.Arg1141*]), one small deletion (c.3105_3107delCTT [p.Phe1036del]), one small insertion leading to a frameshift (c.450_451insC [p.Ala151Argfs*45), two splice-site mutations (c.2787+1G>T [p.Arg929fs*1], c.3736-1G>A [p.Ala1246fs*26]), and one large deletion (c.2996_4208del [p.Ile1000Trpfs*60]). Of these, 11 mutations have been previously described as disease-causing in patients with PXE (p.Leu355Arg,[25,42] p.Arg391Gly,[25,42,43] p.Arg1114His,[25,42,44] p.Arg1221His,[42,45] p.Arg1314Trp,[25,34,42,46] p.Arg518*,[25,42] p.Arg1141*,[25,33,34,47] p.Phe1036del,[25] p.Arg929fs*1,[25,42] p.Ala1246fs*26,[25,42,47]

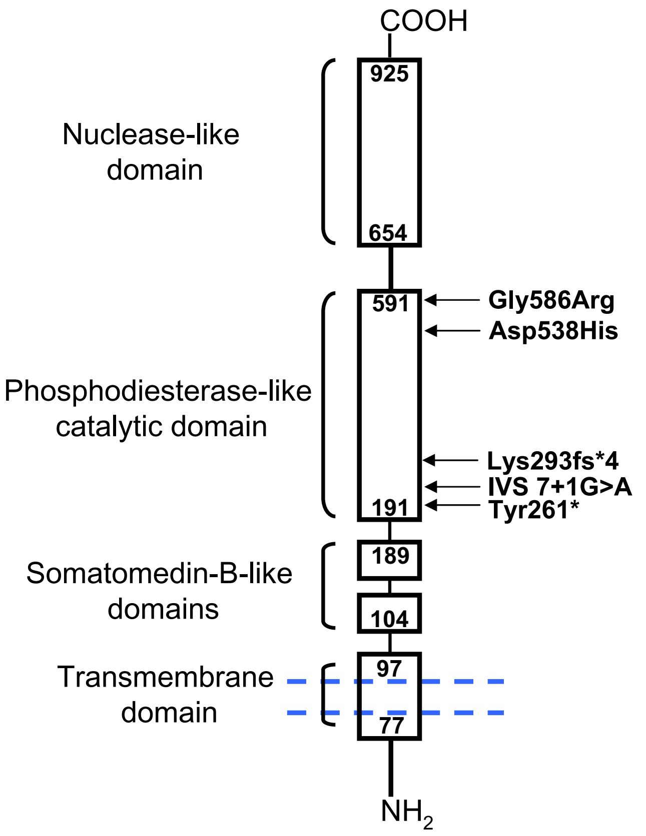

Figure 2. Schematic Representation of Human NPP1 Shows Mutations Identified in GACI Patients with PXE Features in the Current Study
Amino acid positions in functional domains according to spP22413 (SwissProt) = Q5T9R6 (UniProtKB/TREMBL) for human NPP1.

and p.Ile1000Trpfs*60[36]). Only four mutations, namely p.Arg391Gly, p.Arg518*, p.Arg1141*, and p.Arg1314Trp, were found in more than one patient; all other mutations occurred only once.

All mutations that led to amino acid exchanges involved amino acids located within cytosolic or transmembrane domains of ABCC6, reflecting the potential importance of these regions in terms of protein function. Localization of mutations in the ABCC6 protein is visualized in Figure 4. The missense mutations p.Leu355Arg, p.Arg391Gly, p.Ser590Phe, p.Arg1114His, and p.Arg1221His are predicted to result in alterations of the ABCC6 conformation. Some of these alterations might affect the function more than others, and the effect might be particularly strong at conserved positions. For the second ATP-binding domain, where the mutation p.Arg1314Trp of the current study is located, it is known that mutations are able to completely abolish the transport activity of the ABCC6 protein.[48] The missense mutations cause amino acid substitutions that lead to the introduction of a residue with different physical-chemical properties (Table 2, lower panel).

Multiple sequence alignment of ABCC6 across species showed changes in highly conserved amino acids, except for the mutation p.Ser590Phe, which is conserved in bovines, chicks, and fungus (Figure 5). The nonsense mutations (p.Arg518* and p.Arg1141*), the frameshift mutation (p.Ala151Argfs*45), and the large deletion of exons 23–29 (p.Ile1000Trpfs*60) are predicted to result in the production of a truncated protein. These polypeptides are devoid at least of an intact second ATP-binding domain and are therefore predicted to be nonfunctional.

It should be noted that the mutation p.Phe1036del has been described before in PXE patients as being caused by the DNA change c.3106_3108 delTTT.[25] The patient presented here, however, carried the mutation c.3105_3107delCTT, which led to the same in-frame deletion of phenylalanine at position 1036.

The splice-site mutations c.2787+1G>T and c.3736-1G>A have been predicted to alter splicing.[34,47] To determine the effect of these two mutations on the protein, we sequenced cDNA from our patients. Both splice-site mutations lead to a new splice site and aberrant splicing (Supplemental Data). This causes a frameshift and results in an abnormal and truncated protein.

Two disease-associated *ABCC6* mutations are presented here. The DNA change c.1769C>T (p.Ser590Phe) was found in a heterozygous state in a patient with only one mutation. It is located in the transmembrane domain of the protein. The DNA change c.450_451insC (p.Ala151Argfs*45) was also found in a heterozygous state in one patient. This small insertion leads to a frameshift and to generation of an abnormal and truncated protein. These two mutations were not present on 200 alleles from 100 individuals from corresponding ethnic backgrounds and could therefore be excluded as polymorphisms. Type and severity of the *ABCC6* mutations identified in our cohort of GACI patients did not differ from those previously found to be associated with PXE.

Discussion

PXE and GACI have been considered to be two distinct entities in the past and have been primarily linked to *ABCC6* and *ENPP1*, respectively. In the current study, we showed that biallelic mutations in *ABCC6* account for a substantial number of typical GACI cases, which involve typical disease manifestations such as widespread arterial calcifications, arterial stenosis, peri-articular calcifications, and, occasionally, hypophosphatemic rickets (patient 12). Additionally, in six patients with clinical GACI, we detected mono-allelic *ABCC6* mutations. These patients, from a clinical point of view, do not significantly differ from the patients carrying biallelic *ABCC6* mutations. Although MLPA failed to detect large deletions on the other allele in these patients, it is possible that the *ABCC6* mutation on the allele in trans in these patients was missed by our exon-based sequencing approach, e.g., mutations in regulatory untranslated regions of *ABCC6* could be present, which might influence transcription or translation. In this respect, the absence of mutations in either *ENPP1* or *ABCC6* in some of the patients might have been due to a failure to detect them with our approach.

Plomp et al. investigated the possibility of autosomal-dominant inheritance of PXE,[16] and the authors came to the conclusion that part of the phenotype in affected family members of individuals with PXE might be due to expression in heterozygous carriers and that autosomal-dominant inheritance is extremely rare. In this respect, it is still a matter of debate whether loss-of-function mutations on one allele are sufficient to cause a cardiovascular complication of the disease.[17] Digenic inheritance was alternatively suggested by Li et al., who detected the presence of heterozygous mutations in both *ABCC6* and *GGCX* in affected individuals with PXE.[49] However, a mutation in *ENPP1* in trans was ruled out in six patients of our study, whereas a copy-number variation in *ENPP1* or the presence of a larger deletion was not. Mutation analysis of *GGCX* was beyond the scope of our study and was therefore not performed in our cohort.

With the exception of *ABCC6* mutations c.450_451insC (p.Ala151Argfs*45) and c.1769C>T (p.Ser590Phe), reported here for the first time, all of the *ABCC6* mutations detected in our study cohort have already been described previously in typical PXE patients, who presented a much milder phenotype than our GACI patients.[25,34,36,42–47] In our study, of a population clearly selected for severe arterial calcification, we discovered that *ABCC6* mutations can be associated with a much more severe phenotype, including death in infancy from myocardial infarction, than was previously known. Interestingly, the *ABCC6* mutation p.Arg1141*, which was present on one allele in our cohort's patient 10, who presented with widespread arterial calcifications and arterial hypertension at the age of 3 yr, is present in 0.8% of certain populations and was reported to predispose individuals to premature coronary artery disease when it was present on one allele.[50] However, most recently, four large replication studies failed to ascertain this association.[51]

We conclude that the phenotypic spectrum of disease associated with *ABCC6* mutations is much broader than was previously assumed. In fact, we now show that the infantile phenotype of patients carrying *ABCC6* mutations can be indistinguishable from the phenotype associated with *ENPP1* mutations (Figure 6). Notably, this includes the presence of hypophosphatemic rickets, as evident in our cohort's patient 12, who carried the mutation p.Ser590Phe on one allele in *ABCC6*. Importantly, hypophosphatemic rickets has been found to be associated with mutations in *ENPP1*.[9,10]

The fact that the same *ABCC6* mutations can cause the severe GACI phenotype associated with myocardial infarction and death in early infancy in one patient and the relatively mild phenotype of PXE in another patient warrants further explanation. It is likely that mutations in modifying genes may play a role here. It would therefore be reasonable to screen GACI patients carrying *ABCC6* mutations for mutations in genes encoding other inhibitors of artery calcification.[52] Such genes might include *MGP* (MIM 154870), *TNFRSF11B* (MIM 602643), *Smad6* (MIM 602931), *CA2* (MIM 611492), *FBN1* (MIM 134797), *KL* (MIM 604824), *SPP1* (MIM 166490), *TIF1A* (MIM 603406) and *AHSG* (MIM 138680).

Three patients of our study cohort, who presented with GACI and carried biallelic *ENPP1* mutations, developed clinical signs of PXE, including angioid streaks and histologically proven calcifications of elastic skin fibers. Though fully penetrant, clinical findings of PXE are rarely present at birth, and skin findings only rarely become recognizable before the second or third decade of life.[18,23] Thus, given the poor prognosis of severe GACI, affected patients might die of the cardiovascular complications of the disease before they develop typical signs of PXE, and this might be the reason that no previous case of GACI has been described in the PXE literature. Accordingly, the number of patients showing PXE lesions would be low in any GACI cohort with individuals carrying *ENPP1* mutations. Also, more subtle PXE characteristics, including angioid streaks of the retina and peau d'orange skin lesions might frequently be overlooked in routine clinical examinations. As a limitation of the retrospective scope of our registry, characteristic signs and symptoms of PXE might have been overlooked in our cohort of surviving GACI patients. Hence, the true number of patients carrying *ENPP1* mutations and showing PXE lesions might be higher even in our cohort. On the basis of our observation of the development of PXE in individuals with GACI, we propose that all patients with GACI who survive the critical period of infancy[6] should carefully be investigated for typical skin and ophthalmologic PXE lesions later in life.

The fact that mutations in *ENPP1* and *ABCC6* manifest in overlapping clinical phenotypes suggest that the pathophysiologies of GACI and PXE are intertwined.

NPP1 is a major physiologic generator of extracellular PP_i, a potent inhibitor of hydroxyapatite crystal formation and growth.[12] Extracellular PP_i depletion caused by loss-of-function mutations in *ENPP1* is one of the driving forces leading to arterial and articular cartilage calcification in GACI and in the respective mouse model, the *ttw/ttw* mouse carrying a spontaneous nonsense mutation in *Enpp1*.[52] NPP1 expression, though relatively restricted, occurs in the liver, epithelium of the renal proximal tubule, salivary-gland epithelium, arterial wall,[53] chondrocytes, osteoblasts, mature plasma cells, and skin fibroblasts.[7] Decreased local expression of NPP1 at the sites of the observed ectopic calcifications is probably critical.[54] For example, deficiency of NPP1 in choroideal arteries might lead to the eye manifestations of PXE in our patients carrying *ENPP1* mutations, and NPP1 deficiency in dermal fibroblasts might lead to skin calcification in affected patients. Whether deficient NPP1 in the liver modulates circulating levels of calcification inhibitors beyond PP_i to

Table 3. Clinical and Mutational Data of Patients Who Have a GACI Phenotype and Carry Mutations in *ABCC6*

Family	Patient	Sex	Geographic Origin	Consanguinity	Affected/ Nonaffected Siblings	Arterial Calcifications	Periarticular Calcifications	Cardiovascular Complications	Additional Abnormalities	Bisphosphonate Therapy	Age at Data Collection	Age at death	Histology	DNA Change in *ABCC6*	Amino Acid Change in ABCC6	References
4	4	f	Canada	no	1/0	a,c,p,r,d	no	occlusion of right coronary artery, myocardial infarction,	tubular calcification in kidneys	no		6 $^{1}/_{2}$ weeks	autopsy: calcification of internal elastic laminae & elastic tissue in vessel wall, intima proliferation	c.[2787+1G>T]; [3736-1G>A]	p.[Arg929fs*1]; [Ala1246fs*26]	6 (patient 50), 11, 37
5	5	m	Afro-Caribia	no	1/0	no	no	generalized arterial stenosis; myocardial infarction; hypertension		no		8 weeks	no	c.[450_451insC]; [3940C>T]	p.[Ala151Argfs*45]; [Arg1314Trp]	
6	6	f	Armenia	no	1/0	a, c, r, d	\		oliguria; edema of ankles; failure to thrive	no	2 9/12 years		no	c.[1552C>T]; [3105_3107delCTT]	p.[Arg518*]; [Phe1036del]	
7	7	m	Spain	no	1/ 1	v,d	no	cardiac failure; hypertension	fetal hydrops; renal failure	no		3 months		c.[3662G>A]; [3662G>A]	p.[Arg1221His]; [Arg1221His]	
8	8	m	USA	no	1/1	a, p, c, r, d	stippled calcifications of proximal epiphyses of humeri, femora, pelvic cartilage, larynx and mandible	cardiac dysfunction, hypertension; respiratory insufficiency	diffuse white matter disease with cystic encephalomalacia, hyperbilirubinemia, anemia and thrombocytopenia	no	5 years		no	c.[3940C>T] ; [3940C>T]	p.[Arg1314Trp]; [Arg1314Trp]	
9	9	m	USA			d	no		hypoplastic kidney	short-term pamidronate	2 months		no	c.[1171A>G] ; [c.2996_4208del]	p.[Arg391Gly]; [Ile1000Trpfs*60]	
10	10	m	Spain	no	1/0	splenic arteries, pancreas, nephrocalcinosis	no	severe arterial hypertension, cardiomegaly	psychomotor retardation, abdominal distension	no	3 years			c.[1552C>T]; [3421C>T]	p.[Arg518X]; [Arg1141*]	

Table 3. *Continued*

Family	Patient	Sex	Geographic Origin	Consanguinity	Affected/ Nonaffected Siblings	Arterial Calcifications	Periarticular Calcifications	Cardiovascular Complications	Additional Abnormalities	Bisphosphonate Therapy	Age at Data Collection	Age at death	Histology	DNA Change in *ABCC6*	Amino Acid Change in ABCC6	References
11	11	f	France	no	1/2	c,d	no	severe arterial hypertension, cardiac failure		etidronate up to 30 mg/kg per day		6 weeks		c.[3421C>T]; [3940C>T]	p.[Arg1141*]; [Arg1314Trp]	
12	12	m	Pakistan	yes	2/3	a,p,v	hips, shoulders		hypophosphatemic rickets	no	17 years			c.[1769C>T];[?]	p.[Ser590Phe] ;[?]	6 (patient 45), 11
13	13	m	Latin America	no	1/2	d	multiple sites			etidronate	3 3/4 years			c.[3340C>T];[?]	p.[Arg1114Cys] ;[?]	6 (patient 47)
14	14	f	Netherlands	no	2/2	a, d			mentally retarded; developed epilepsia at 20 years	no	31 years		muscle biopsy: calcification & stenosis of perimyseal arterioles, degeneration of elastic fibres in vessel wall	c.[1064T>G];[?]	p.[Leu355Arg] ;[?]	41
15	15	m	USA	no	2 (twins)/ 0	a,c,p, cerebral vessels	no	cardiac ischemia; respiratory insufficiency; hypertension	recipient of twin-to-twin transfusion	no		5 months		c.[1171A>G];[?]	p.[Arg391Gly] ;[?]	6 (patient 51), 7, 39, 40
15	16	m	USA	no	2 (twins)/0	a,p	no		donor of twin to twin transfusion	no	7 years			c.[1171A>G];[?]	p.[Arg391Gly];[?]	6 (patient 52), 7, 39, 40
16	17	f	South Africa	no	1/0	a, spleen, pancreas, nephrocalcinosis	no	failure to thrive, arterial hypertension, cardiac failure	onset of symptoms at 2 1/2 years	no	3 years			c.[3940C>T];[?]	p.[Arg1314Trp] ;[?]	38

Abbreviations are as follows: A, aorta; c, coronary arteries; d, diverse arteries; p, pulmonary artery; and r, renal arteries.

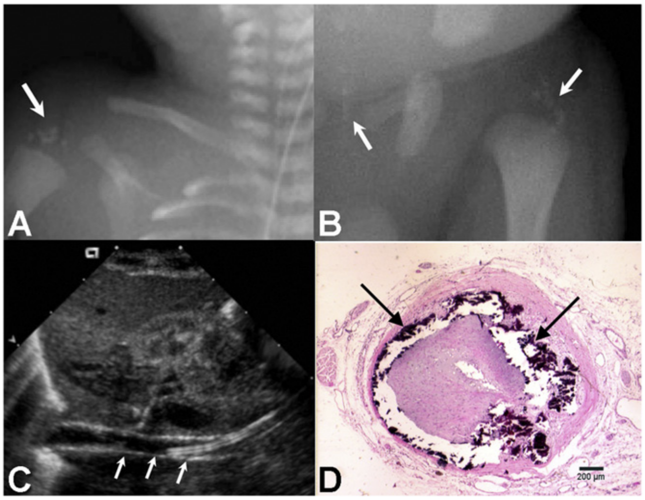

Figure 3. Manifestation of GACI Associated with *ABCC6* Mutations
Patient 8, who presented with cyanosis, respiratory distress, and truncal edema on the first day of life. (A) X-ray showing stippled periarticular calcifications of the right shoulder (arrow) and (B) the pelvic cartilage (left arrow) and the left hip joint (right arrow). (C) Abdominal ultrasound showing increased echogenicity of the aortic wall (arrows). (D) A cross-section of a coronary artery from patient 4, who died of myocardial infarction at the age of 6 weeks, shows disruption of the calcified internal elastic lamina (arrows) and massive intima proliferation (HE staining, ×2.5). An asterisk indicates the intraarterial vascular catheter.

contribute to widespread dystrophic calcifications in GACI and PXE remains to be determined. Bisphosphonates, which have been used as synthetic analogs of PP_i to suppress arterial calcification in GACI patients,[6] failed to protect an individual (patient 1 in our cohort) carrying *ENPP1* mutations from developing PXE lesions later in childhood. However, one may speculate that prolonged bisphosphonate therapy might have been beneficial for this individual.

ABCC6 encodes MRP6 (ABCC6), a transmembrane protein primarily expressed in the liver and a member of the ATP-binding cassette (ABC) transporter family. It has been proposed that absence of ABCC6 activity, primarily in the liver, results in the deficiency of circulating factors and thereby causes deficiency of physiological inhibition of artery calcification even when calcium and phosphate homeostasis are normal.[42] This hypothesis is supported by the finding that when muzzle skin from wild-type mice was grafted onto the back of $Abcc6^{-/-}$ mice, mineralization of the vibrissae was observed.[55] Furthermore, serum from PXE patients, when added to tissue-culture medium of fibroblasts, altered the expression of elastin.[56] Also, it was hypothesized that reduced γ-carboxylation of MGP is a major pathogenic factor contributing to the increased arterial mineralization in PXE because reduced γ-glutamyl carboxylation of MGP was demonstrated in the serum, in the liver, and in various calcified tissues of $Abcc6^{-/-}$ mice[57] as well as in human PXE skin lesions.[30,58] It was therefore postulated that ABCC6 participates in transmembrane transport and redistribution of vitamin KH2, an obligatory co-factor of γ-glutamyl carboxylase,[59,60] especially when conjugated to glutathione. However, the finding that supraphysiological doses of vitamin K, an inducer of γ-glutamyl carboxylation of MGP, failed to compensate for increased tissue mineralization in $Abcc6^{-/-}$ mice[61,62] did not substantiate this hypothesis.

Interestingly, mutations in *GGCX,* encoding γ-glutamyl carboxylase, cause a much milder phenotype

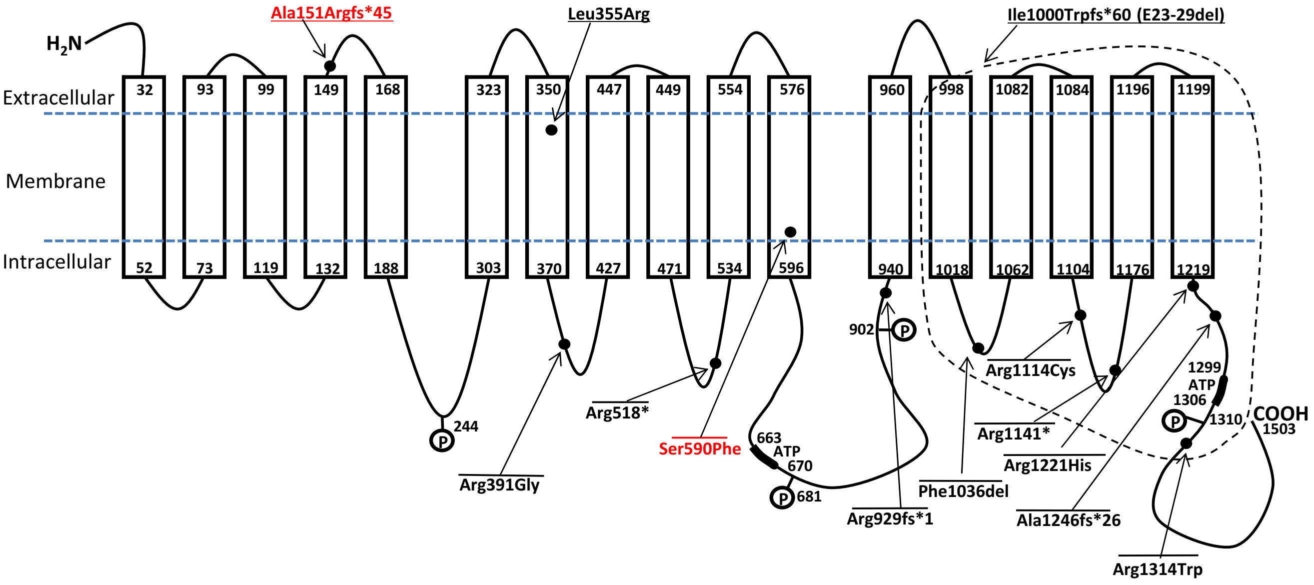

Figure 4. Schematic Representation of Human ABCC6 Shows Mutations Identified in GACI Patients in the Current Study
A darkened line indicates an ATP binding motif, a circled P indicates a putative phosphoserine, filled-in circles indicate sites of mutations for patients described in this paper, and a dashed line indicates a multi-exon deletion. Abbreviations are as follows: fs, frameshift; *, chain termination; del, deletion. Transmembrane helix positions are according to sequence accession number O95255 for human ABCC6.

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                        +(Ala151Argfs*45)              +(Leu355Arg)                   +(Arg391Gly)                   +(Arg518*)
Human       ..--CFVLPATNAAQQASGAGFQ..161   ..PAWKGYLLAVLMFLSACLQTL..365   ..LRSAITGLVYRKVLALSSGSK..401   ..GAFLDRVLGIR-GQELGALRT..527
Mouse       ..--CCILPGINTVQQASAGNLR..167   ..SAWTGWLLAVLMFAAACLQTL..353   ..LRTAITGLVYRKVLVLSSGSR..399   ..HAFLERLLHIR-GQELSALKT..525
Bovin       ..LLCALAILRSKIMTALKEDAR..166   ..PEWQGYFYTALLFISACLQTL..379   ..IKTAVIGAVYRKALVITNAAR..415   ..LAFKDKVLAIR-QEELKVLKK..541
Chick       ..LLCATVIFRSKIMLALNTDTE..166   ..PNWQGYFYTGLLFVCACLQTL..378   ..LKTAIVGVIYRKALVITNSAR..414   ..LAFREKVLEIR-QKELKVLKK..540
Frog        ..TVCAIIPFRSKVMASARQGQV..165   ..PSWWGFCIAVLMFLTSLVQTL..373   ..LRSAITGIIYRKSLVITNSAK..409   ..PSFAQKVLEIR-NKELNILKK..535
Fish        ..VVCAVFQFQTLLREALSQG-I..168   ..YAWTGYLYAVLLVLVAFVQSV..395   ..VRTAIMAAVYKKALVVSNDSR..431   ..TSFEAQVQEIR-EKELKVMRK..557
Roundworm   ..VVCGVPELRYYITGRLYKEYE..168   ..PMWIGVSIALLMFLSSLLQSM..352   ..IRSVLTSAVYTKTLNLSNEAR..388   ..KSMEKMVLEVR-EKEIRVLKK..514
Mosquito    ..MFFGIIQLRTELQNKELRGDV..161   ..PGWQGVMITFGLFATSLLIAL..362   ..IRTGLISGIYRKALRISSSAK..398   ..KSFQDTILEVR-DKEIGILKK..524
Fungus      ..TIAYGVKLRSLVSQKAYQDQL..168   ..PVISGVAIALAMFLVSVTQTI..361   ..VKSALTAMIYTKSLRLSSEGR..397   ..TAFMNKLSHIRNDLELNTLRK..524
Slime mould ..---------------------..21    ..SILKGILLCCLLCLCVLGQSI..169   ..VRGALAAKIFEKTLKLSNASR..205   ..NFFINKIDGQR-KQELKNIFL..332
                                                 *      :       ::          ::  : . :: * * ::. .:                :   :   *   *:

                        +(Ser590Phe)                   +(Arg929fsX1)                  +(Ile1000Trpfs*60)             +(Phe1036del)
Human       ..AQAFLPFSIHSLVQARVSFDR..600   ..PAGKDSIQYGRVKATVHLAYL..941   ..IFGLLGCLQAIGLFASMAAVL..1010  ..WDVVRSPISFFERTPIGHLLN..1046
Mouse       ..AQAFLPFSVHCIVQARVSFDR..598   ..TAEEDSVRYGRVKITIYLSYL..936   ..VFGLLGCLQAIGLFASMAAVF..1025  ..WDVARSPIGFFERTPVGNLLN..1041
Bovin       ..PLNILPMVISSIVQASVSLKR..615   ..LVEADKAQTGQVKLSVYWDYM..968   ..VYGALGISQGITVFGYSMAVS..1037  ..HNVLRSPISFFERTPSGNLVN..1073
Chick       ..PLNILPMVISSIVEASVSLKR..612   ..LTEADTAKTGRVKATVYWEYM..963   ..VYGALGISQGIAVFGYSMAVS..1032  ..HNVLRSPMSFFERTPSGNLVS..1068
Frog        ..PLNMLPQVISNLAQASVSIKR..606   ..LYQTETTETGRVKMTVFWQYM..969   ..VYAALGILQGLLVMTSSFSLA..1038  ..DNKMHTPQSFYDTTPIGRIIN..1074
Fish        ..PLAMLPQLISIMVQTSVSKKR..627   ..LIEKEMMETGRVKFSVYLQYL..991   ..VFGALGLAQGFLVFFGTILLA..1064  ..TNILKVPMMFFDTTPSGRIVN..1100
Roundworm   ..PLAVFAMVFSQAVQCSASNTR..585   ..LIEKEAVETGKVKFEVYMSYF..966   ..IYAVLGMGQATSVCAASIIMA..1040  ..ENIMRSPMAFFDVTPLGRILN..1076
Mosquito    ..PLGWLPMMVTFAMQAWVSVKR..593   ..LIEKEESATGAVTLAVYLKYT..938   ..VYGALGGIQSIALFISSVALG..1007  ..ESSMKMPMSFFDTTPLGRIIN..1043
Fungus      ..PLSILPMVITSIIEASVAVRR..597   ..KQTQETSQQGKVKWSVYGEYA..965   ..IYLAWGLGSSILVILQNLILW..1033  ..FSIFRSPMSFFETTPSGRILN..1070
Slime mould ..PLIRLPYDIFKAIGLIPSVKR..404   ..LLVKEDKNEGEVEFNVYKKYF..770   ..-YYLLIFGTFVVILMIRILLL..836   ..KSVTYASCRFFDTNPSGRILN..873
              .   :.  .        :  *               :    * *   :.  *            :          :      :             .    .  *:: .* *.::.

                        +(Arg1114Cys)                  +(Arg1141*)                    +(Arg1221His)                       +(Ala1246fs*26)
Human       ..LYVVSSCQLRRLESASYSSVC..1124  ..FQGSTVVRAFRTQAPFVAQNN..1151  ..QVTQTLQWVVRNWTDLENSIVSVERMQDYAWTPKE-----------APWRLP..1251
Mouse       ..LYVATSCQLRRLESARYSSVC..1119  ..FQGSLVVRAFRAQASFTAQHD..1146  ..QVTQTLQWVVRSWTDLENSMVAVERVQDYARIPKE-----------APWRLP..1246
Bovin       ..FYVASSRQLKRLESVSRSPVY..1151  ..LLGVSVIRAFEEQERFIRQSD..1178  ..QVTTYLNWLVRMSSEMETNIVAVERLKEYSETEKE-----------APWQIQ..1278
Chick       ..FYVATSRQLKRLESVSRSPVY..1146  ..LLGVSVIRAFEEQKRFIKQND..1173  ..QITAYLNWLVRMTSDLETNIVAVERVKEYAEMEKE-----------AEWSIE..1273
Frog        ..FYVATSRQLKRLESVSRSPIY..1152  ..ITGASIIRAYGRQNSFIVLSD..1179  ..QVTMSLNWMVRMTSDLETNIVAVERVKEYAENETE-----------APWHIE..1279
Fish        ..FYVATSRQLRRLDSVSRSPIY..1178  ..VSGLSVIRAYGHQDRFLKHNE..1205  ..NVTQTLNWLVRMTSELETNIVAVERVREYAEIQNE-----------APW-VT..1304
Roundworm   ..FYVSTSRQLKRLESASRSPIY..1148  ..ISGQSTIRAYNEQMRFTRESE..1148  ..NITQTLNWAVRMTSELETNIVAVERINEYTITPTE-----------DVWQKG..1248
Mosquito    ..VYIATSRQLKRLESVTRSPIY..1121  ..IQGASSIRAYGVVDKFIRESQ..1175  ..QISATLSFMVRMTAEVETNIVAVERLEEYTVLPRE-----------GNNS-Q..1276
Fungus      ..YYLRTSRELKRLDSVTRSPIY..1148  ..LGGISTIRGYRQENRFALENE..1175  ..QITQSLNWIVRQTVEVETNIVSVERVLEYANLPSE-----------APDVIF..1278
Slime mould ..LYRPSARELNRWESITVSPIF..951   ..YNGLLTIRTYKQESRFIKEMF..978   ..SLNGYLSWGIRRIVDLEVKMNSFQRIQSYIEIPKEGNKLVSTNSNEVDNHTI..1087
               *  :: :*.* :*   *.:                *   :* :     *                .:.  *.: :*   ::* .: :.:*: .*     *

                             +(Arg1314Trp)
Human       ..GRTGAGKSSLASGLLRLQEAAEGGIW..1324
Mouse       ..GRTGAGKSSLAWGLLRLQEAAEGNIW..1319
Bovine      ..GRTGAGKSSLTLGLFRIKESAEGEII..1351
Chicken     ..GRTGAGKSSLTLGLFRINEAAEGEII..1346
Frog        ..GRTGAGKSSMTLCLFRILEPAEGIVK..1352
Fish        ..GRTGAGKSSLTNCLFRIVEAADGRIL..1377
Roundworm   ..GRTGAGKSSLTLALFRIIEADGGCIE..1348
Mosquito    ..GRTGAGKSSLTLGLFRIVEAAGGQIV..1319
Fungus      ..GRTGAGKSSLTLALFRIIEAAGGSIS..1351
Slime mould ..GRTGAGKTTIASSLFRMVECSKGLIL..1160
              *******::::  *:*: *   * :
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Figure 5. Multiple Alignment around the Mutation Sites of ABCC6 Sequences from Different Organisms

Human, *Homo sapiens* (O95255); mouse, *Mus musculus* (Q9R1S7); bovine, *Bos taurus* (Q8HXQ5); chick, *Gallus gallus* (Q5F364); frog, Western claw frog, *Xenopus tropicalis* (A9JRK6); fish, Zebrafish, *Danio rerio* (Q6PH26); roundworm, *Caenorhabditis elegans* (Q9N2N3); mosquito, *Culex quinquefasciatus* (B0W537); fungus, *Aspergillus fumigatus* (Q4WUC5); and slime mold, *Dictyostelium discoideum* (Q8T6H3). +, mutant position; fs, frameshift mutation; SM, splice mutation; E, exon; *****, chain termination; del, deleted residue(s); underlined, ATP-binding motif.

with respect to dystrophic calcifications than do *ABCC6* mutations (Figure 6).[29] Although calcifications of the skin and peau d'orange changes of the retina have been reported in patients carrying *GGCX* mutations, retinal changes were relatively mild, and arterial calcifications have not been demonstrated in these patients so far.[29] It has been proposed that *GGCX* mutations lead to decreased activation of the Gla proteins MGP and osteocalcin,[29] which are known inhibitors of calcification. With respect to the severe phenotype associated with *ABCC6* mutations in the present study, it is possible that the decrease that *GGCX* mutations cause in γ-glutamyl carboxylation of calcification inhibitors might not be sufficient to trigger artery calcification in vivo and that additional factors apart from decreased activation of MGP are principal mediators in ABCC6-related dystrophic calcification.

Early studies by Ilias et al. have proven a role of ABCC6 in the transport of glutathione-conjugated substrates in vitro,[48] but the physiological substrate of this ABC transporter is still elusive. Most recently, mutations in *NT5E*, encoding CD73, a nucleotidase that generates adenosine from extracellular adenosine monophosphate, were shown to lead to a phenotype consisting of arterial calcifications of the lower extremities and distal joint calcifications (ACDC).[13] In their study, the authors identified adenosine as a potent inhibitor of tissue-nonspecific alkaline phosphatase (TNAP), whereas adenosine supplementation reversed the increase in TNAP activity and mineralization in CD73-deficient cells. On the basis of the clinical and histological similarities of the ACDC phenotype with that of PXE, Markello et al. postulated that ABCC6 might be a transporter for adenosine.[63] However, in our view, the clinical overlap of the ACDC and PXE phenotypes is less evident, and this view is in line with that of other authors.[64] Here, we establish that mutations in *ENPP1* and *ABCC6* can cause overlapping phenotypes (Figure 6), suggesting that NPP1 and ABCC6 might share common

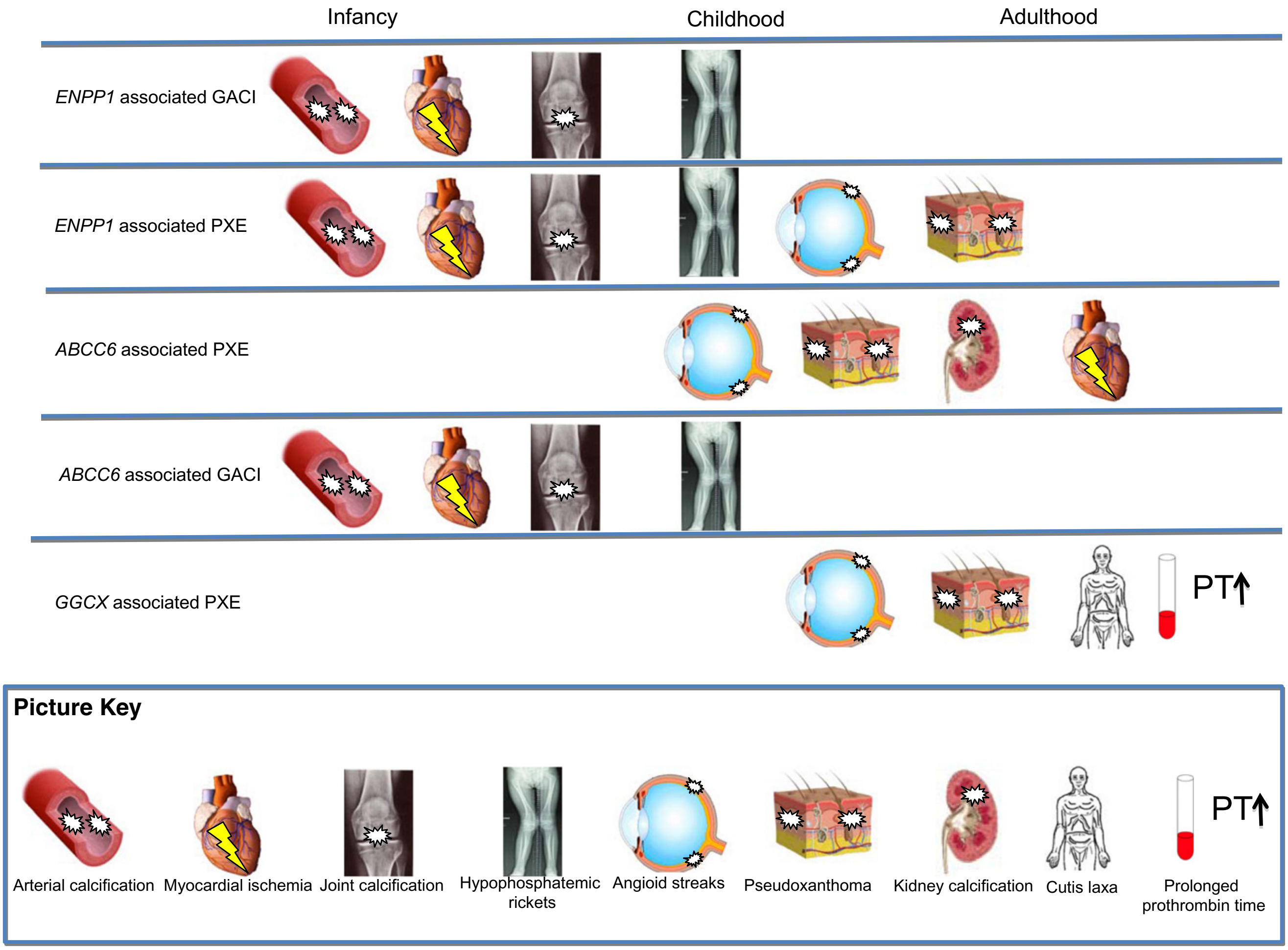

Figure 6. Spectrum of Clinical Manifestations and Affected Tissues Associated with Mutations in *ABCC6, ENPP1,* and *GGCX*
Mutations of either *ABCC6* or *ENPP1* can cause the severe phenotype of generalized arterial calcification of infancy, which frequently leads to death within the first year of life. Although mutations in *ENPP1* can also cause PXE-like skin lesions and angioid streaks of the retina in children who have GACI and survive the critical period of infancy, mutations in *ABCC6* can also manifest later in life with the "classic PXE" phenotype. Mutations in *GGCX* are associated with a PXE-like phenotype associated with mild retinopathy, skin calcifications, severe cutis laxa, and deficiency of vitamin-K-dependent clotting factors. Hypophosphatemic rickets has been observed frequently in patients with *ENPP1* mutations but here was observed only in one proband carrying a mutation in *ABCC6* on one allele.

downstream physiological pathways. *ABCC6* mRNA expression is abundant in the liver and kidneys, but it has also been observed in PXE-affected tissues, including the vessel wall.[33] Accordingly, we have detected ABCC6 in the aorta from C57BL/6 mice (Supplemental Data), and it is possible that local ABCC6 defects at multiple sites in the body result in the PXE phenotype. However, our findings cannot fully exclude the possibility that deficiency of a circulating factor generated by the liver is the driving force of dystrophic calcification in ABCC6 deficiency.

More study, including functional genomics, and identification of the elusive physiologic substrate(s) of the *ABCC6* transporter are warranted in future studies if we are to ascertain the factor(s) causing GACI in patients with *ABCC6* mutations. On the basis of the observed phenotypic overlap with GACI caused by *ENPP1* mutations, it is likely that this factor is a member of the same functional network in which NPP1 suppresses artery calcification. In this network, PP_i and P_i metabolism and adenosine signaling are intimately linked, and degradation of the artery calcification inhibitor PP_i and generation of the critical artery calcification promoter P_i at sites of fibrillar type I collagen expression plays a substantial role in driving artery calcification.[52,54]

Summary

Studying a unique and relatively large cohort of subjects and kindreds selected for severe, early-onset arterial calcification, we have discovered pathogenic monoallelic and biallelic mutations in *ABCC6*, previously associated with PXE, in a subset of patients with GACI. Conversely, we show that *ENPP1*-mutation-carrying patients, who presented with generalized arterial calcification of infancy, can develop typical signs of PXE, including pseudoxanthomatous skin lesions and angioid streaks of the retina.

The fact that even monoallelic mutations in *ABCC6* were associated with the severe phenotype of generalized arterial calcification cannot fully be explained on the basis of autosomal-recessive inheritance of PXE disease and may suggest an unexpectedly wide phenotypic heterogeneity ranging from mere skin calcifications and ocular findings to severe artery calcification in early childhood. However, mutations of other disease-modifying genes cannot yet be ruled out. The fact that mutations in both *ABCC6* and *ENPP1* can be associated with overlapping phenotypic features furthermore provides evidence that deficiency of either one of these molecules leads to alterations in the same functional network. Determining the substrate(s) of the ABCC6 transporter that promotes development of GACI and better understanding how NPP1 and its enzymatic products modulate skin and eye tissue homeostasis will require further study.

Supplemental Data

Supplemental Data include three case reports and five figures.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
SwissProt, UniProtKB/TREMBL, http://www.ebi.ac.uk/uniprot/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
The Human Gene Mutation Database, http://www.hgmd.org/
Ensembl Genome browser, http://www.ensembl.org/index.html/

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Note Added in Proof

After this manuscript had gone into final preparation for print, collaborators including Gary S. Gottesman, Center for Metabolic Bone Disease and Molecular Research, Shriners Hospital for Children, St. Louis, USA, detected the bi-allelic mutation c.653A>T (p.Asp218Val) in *ENPP1* in patient 13 of our cohort. We then confirmed the presence of this mutation in patient 13.