Hypophosphatemia, Hyperphosphaturia, and Bisphosphonate Treatment Are Associated With Survival Beyond Infancy in Generalized Arterial Calcification of Infancy

Frank Rutsch, Petra Böyer, Yvonne Nitschke, Nico Ruf, Bettina Lorenz-Depierieux, Tanja Wittkampf, Gabriele Weissen-Plenz, Rudolf-Josef Fischer, Zulf Mughal, John W. Gregory, Justin H. Davies, Chantal Loirat, Tim M. Strom, Dirk Schnabel, Peter Nürnberg, Robert Terkeltaub and the GACI Study Group

Circ Cardiovasc Genet. 2008;1:133-140; originally published online December 9, 2008;
doi: 10.1161/CIRCGENETICS.108.797704
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1942-325X. Online ISSN: 1942-3268

Hypophosphatemia, Hyperphosphaturia, and Bisphosphonate Treatment Are Associated With Survival Beyond Infancy in Generalized Arterial Calcification of Infancy

Frank Rutsch, MD; Petra Böyer, MS; Yvonne Nitschke, MS, Nico Ruf, PhD; Bettina Lorenz-Depierieux, PhD; Tanja Wittkampf, PhD; Gabriele Weissen-Plenz, PhD; Rudolf-Josef Fischer, MD, PhD; Zulf Mughal, MBChB, FRCPCH, DCH; John W. Gregory, MD; Justin H. Davies, MD, FRCPCH, MRCP; Chantal Loirat, MD; Tim M. Strom, PhD; Dirk Schnabel, MD; Peter Nürnberg, PhD; Robert Terkeltaub, MD; the GACI Study Group

Background—Generalized arterial calcification of infancy has been reported to be frequently lethal, and the efficiency of any therapy, including bisphosphonates, is unknown. A phosphate-poor diet markedly increases survival of NPP1 null mice, a model of generalized arterial calcification of infancy.

Methods and Results—We performed a multicenter genetic study and retrospective observational analysis of 55 subjects affected by generalized arterial calcification of infancy to identify prognostic factors. Nineteen (34%) patients survived the critical period of infancy. In all 8 surviving patients tested, hypophosphatemia due to reduced renal tubular phosphate reabsorption developed during childhood. Eleven of 17 (65%) patients treated with bisphosphonates survived. Of 26 patients who survived their first day of life and were not treated with bisphosphonates only 8 (31%) patients survived beyond infancy. Forty different homozygous or compound heterozygous mutations, including 16 novel mutations in *ENPP1*, were found in 41 (75%) of the 55 patients. Twenty-nine (71%) of these 41 patients died in infancy (median, 30 days). Seven of the 14 (50%) patients without *ENPP1* mutations died in infancy (median, 9 days). When present on both alleles, the mutation p.P305T was associated with death in infancy in all 5 cases; otherwise, no clear genotype-phenotype correlation was seen.

Conclusion—*ENPP1* coding region mutations are associated with generalized arterial calcification of infancy in ≈75% of subjects. Except for the p.P305T mutation, which was universally lethal when present on both alleles, the identified *ENPP1* mutations per se have no discernable effect on survival. However, survival seems to be associated with hypophosphatemia linked with hyperphosphaturia and also with bisphosphonate treatment. **(*Circ Cardiovasc Genet*. 2008;1:133-140.)**

Key Words: genetics ■ mortality ■ pediatrics ■ prognosis ■ survival

Generalized arterial calcification of infancy (GACI, MIM#208000) is a rare autosomal recessive disorder, reported to date in ≈180 individuals. Calcification of large- and medium-sized arteries and marked myointimal proliferation leading to arterial stenoses are characteristic vascular features of the GACI phenotype. An extravascular feature, foci of periarticular calcification, occurs in many of the affected subjects.[1,2] Initial signs of the disease may occur prenatally,[3] and most affected children die in early infancy from sequelae of vascular occlusion, typically myocardial

Received June 9, 2008; accepted October 15, 2008.

From the Department of General Pediatrics (F.R., P.B., Y.N., T.W.), University Children's Hospital, Münster, Germany; Laboratory of Developmental Genetics and Imprinting (N.R.), The Babraham Institute, Cambridge, United Kingdom; Institute of Human Genetics (B.L.-D., T.M.S.), Helmholtz Zentrum München, Munich, Germany; Institute of Human Genetics (B.L.-D., T.M.S.), Klinikum rechts der Isar, Technical University, Munich, Germany; Department of Cardiothoracic Surgery (G.W.-P.), University Hospital, Münster, Germany; Department of Medical Informatics and Biomathematics (R.-J.F.), Münster University Hospital, Münster, Germany; Department of Paediatrics (Z.M.), Saint Mary's Hospital for Women and Children, Manchester, United Kingdom; Department of Pediatric Endocrinology (J.W.G.), Wales School of Medicine, Cardiff University, Cardiff, United Kingdom; Department of Pediatric Endocrinology (J.H.D.), Southampton University Hospital, Southampton, United Kingdom; Department of Pediatric Nephrology (C.L.), Hôpital Robert Debré, Paris, France; Pediatric Endocrinology (D.S.), Otto Heubner Center, Charité, Berlin, Germany; Cologne Center for Genomics (P.N.), University of Cologne, Germany; and Department of Rheumatology Allergy/Immunology (R.T.), VA Medical Center, UCSD, San Diego, Calif.

The online-only Data Supplement is available at http://circgenetics.ahajournals.org/cgi/content/full/CIRCGENETICS.108.797704/DC1.

Correspondence to Dr Frank Rutsch, Klinik und Poliklinik für Kinder-und Jugendmedizin, Universitätsklinikum Münster, Albert-Schweitzer Strasse 33, D-48149 Münster, Germany. E-mail rutschf@mednet.uni-muenster.de

infarction or congestive heart failure due to hypertension.[1] Systemic deficiency of nucleotide pyrophosphatase (NPP1) activity (E.C. 3.6.1.9) leading to low serum and urine inorganic pyrophosphate (PP_i) levels has been identified as a diagnostic hallmark of the disease.[4,5] Deficient NPP1-catalyzed PP_i generation in GACI seems to be mediated by mutations in multiple exons of *ENPP1* (MIM*173335).[6] This gene, located on chromosome 6q22-q23, spans 83 kb of genomic DNA and contains 25 exons.

ENPP1 encodes a type II transmembrane glycoprotein ectoenzyme that forms homodimers of identical disulfide-bonded subunits.[7] NPP1 has an extracellular catalytic domain as well as somatomedin B-like and substrate-binding or substrate-specifying nuclease-like domains.[8] NPP1 regulates soft tissue calcification and bone and joint cartilage mineralization by generating PP_i, which not only serves as an essential physiological inhibitor of hydroxyapatite crystal growth[9] but also is a suppressor of chondrogenesis.[10] In artery smooth muscle cells, deficiencies of NPP1 (or of extracellular PP_i without NPP1 deficiency in *ank/ank* mice homozygous for functional inactivation of the PP_i transporter ANK) promote chondrogenic transdifferentiation in vivo and also in vitro under circumstances where excess of an inorganic phosphate (P_i) source is provided.[10,11] Although the pathophysiologic role of NPP1-mediated PP_i generation in GACI has come to light within recent years, the factors accounting for the variation of the GACI phenotype including the presence or absence of intracerebral artery calcification and periarticular calcification, early death in utero and long-term survival are not known.[12]

PP_i and P_i seem to have mutually antagonistic roles in tissue mineralization.[13] Significantly, either a phosphate-poor diet or crossbreeding with PHEX knockout mice to induce hypophosphatemia markedly decreased artery calcification and periarticular calcifications, and increased survival of $NPP1^{-/-}$ and *ank/ank* mice.[14] We previously reported a child of Turkish descent from a consanguineous marriage who manifested GACI and periarticular calcifications and was homozygous for the p.R774C mutation of *ENPP1* also detected on both alleles in his father.[6] Strikingly, the father was not affected by GACI, but suffered from severe hypophosphatemic rickets.[6] On the basis of this observation, we hypothesized that hypophosphatemia may inhibit potential pathological effects of deficient NPP1-mediated PP_i generation and may prevent humans from developing lethal pathological arterial calcification. Furthermore, within the last few years, bisphosphonates, which function in part as synthetic nonhydrolyzeable analogues of PP_i,[15,16] have been anecdotally reported to have varying degrees of success in the treatment of GACI.[17–19] However, it has been problematic that information on the clinical, as well as treatment and outcome features of the majority of cases of GACI comes from small case reports of one or a handful of patients.

Here, we describe a retrospective, multicenter study of 55 patients with GACI, by far the largest performed to date. In this study, we characterized subjects for *ENPP1* genotype, and assessed if *ENPP1* mutations, bisphosphonate therapy, and renal phosphate handling and serum phosphate levels (where specimens were available), were associated with survival beyond infancy.

Methods

Patients

Inclusion in the study was based on the clinical diagnosis of GACI and on the availability of DNA material for *ENPP1* mutation analysis. Patient history and clinical data were gathered through a standardized questionnaire, which was sent to the referring physician or geneticist. Diagnosis of GACI was based on the presence of cardiovascular symptoms associated with evidence of arterial calcification with or without periarticular calcification on x-ray or sonography in infancy,[1] or typical histology[20] (Figure 1). Diagnosis of GACI is exemplified in the following case report: The male infant (case 6 of our study) was born to consanguineous Turkish parents. The mother is a 20-year-old gravida II, para I, whose first pregnancy ended with a missed abortion. The father suffers from hypophosphatemic rickets since early childhood, presenting with genua vara and short stature. Pregnancy was complicated by macrosomia of the fetus and polyhydramnios. The infant was delivered by cesarian section because of fetal distress. Birth weight was 3070 g, length was 50 cm, and umbilical cord pH was 7.24. Because of respiratory distress, the infant was intubated and ventilated immediately after birth. Echocardiography at the first day of life revealed a small pericardial effusion and increased echogenicity of the walls of the pulmonary arteries, the aorta, the tricuspid valve, and the coronary arteries (Figure 1A and 1B). Sonography of the abdomen showed bright, hyperechogenic walls of the celiac trunc (Figure 1C), the superior mesenteric artery, and the renal arteries bilaterally. A chest X-ray showed cardiomegaly, prominent lung vessels, and periarticular calcifications of both shoulders (Figure 1E). Further radiographs demonstrated irregular calcifications of the left hip and spotted calcifications in the region of the carpal bones and the carpal joints (Figure 1D). Serum calcium (2.01 mmol/L) and serum P_i (1.5 mmol/L) levels were normal. On the basis of the signs of respiratory distress and pericardial effusion associated with the presence of arterial and periarticular calcifications, the diagnosis of GACI was established, and treatment with etidronate 15 mg/kg per day PO was started at the age of 2 weeks.

Fifty-five patients were included in our study after informed consent of the parents. The DNA from all patients was subjected to mutation analysis of *ENPP1*. The study protocol was approved by the Muenster University Hospital Ethical Committee and other participating institutional peer-review human subjects committees. Of the patients studied, 23 were part of an earlier reported study on the mutational spectrum of *ENPP1* mutations.[21]

ENPP1 Mutational Analysis

DNA was extracted from EDTA-blood after informed consent. In specific cases, DNA from blood samples was not available, because the patients were deceased before the onset of the study and no blood samples had been taken. In these families, the parents were screened for mutations, then DNA from the deceased child was extracted from formalin-fixed tissue blocks and was analyzed to confirm the segregation of the mutation. With a set of 24 primer pairs, we amplified all 25 exons and their flanking splice sites of *ENPP1* from genomic DNA by polymerase chain reaction (PCR). The PCR products were directly sequenced bidirectionally using an ABI 3730 DNA Analyzer and a BigDye Terminator v1.1 Cycle Sequencing Kit according to the manufacture's protocol (Applied Biosystems, Foster City, Calif). All primer sequences are available on request. Mutations were compared with the ENSEMBL polymorphism database.

Statistics

The Kaplan-Meier survival curve was calculated with SPSS software. The log-rank test was used to test equality of survival distributions for the different levels of therapy. The Wilcoxon test for paired samples was used when comparing serum phosphate levels and TmP/GFR levels with the respective reference values.

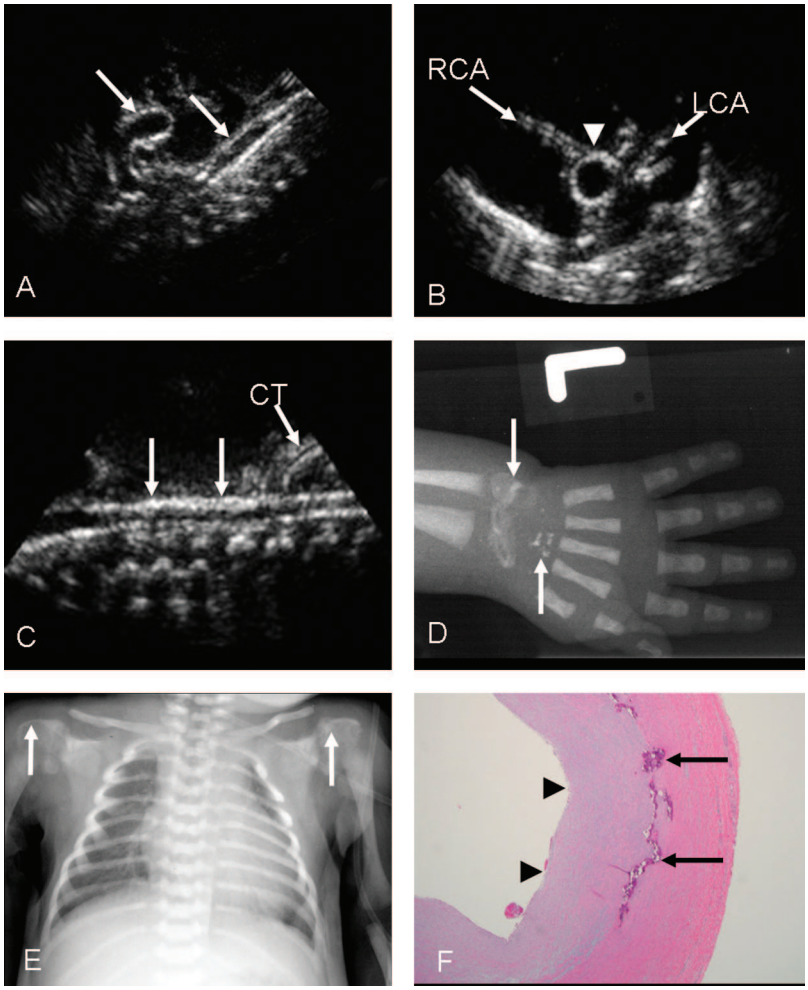

Figure 1. Characteristic manifestations of GACI. A through E, Patient 6 in his first week of age. A, Echocardiogram showing bright echogenic walls of the aortic arch and descending aorta (arrows), consistent with aortic calcifications. B, Increased echogenicity of the aortic root (arrowhead) and left (LCA) and right (RCA) coronary arteries (arrows). C, Increased echogenicity of abdominal aorta (arrows) and celiac trunc (CT) of the same patient. D, Periarticular calcifications (arrows) of the carpal joint and around carpal bones of the left hand. E, Chest x-ray showing moderate cardiomegaly and periarticular calcifications (arrows) of both shoulders. F, Cross-section through the aorta from patient 41, who died at the age of 6 weeks, showing calcification at the level of the internal elastic lamina (arrows) and marked intima proliferation (arrowheads) (hematoxylin–eosin, ×20).

Statement of Responsibility

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

The study cohort consisted of 55 patients with proven GACI (28 males and 27 females) of 45 unrelated families. Patients were included between 2001 and 2006. We included 1 dizygotic pair of twins and 4 monozygotic twins in our survey. The detailed clinical data on each individual patient are summarized in Supplemental Table 1 (supplemental material). Although 36 patients (65.5%) of our study cohort died in utero or in infancy, 19 patients survived beyond infancy.

Clinical Features of the GACI Cohort

In 28 of the 55 cases (51%), prenatal signs of the disease were detected (Supplemental Table 2), with fetal distress, polyhydramnios, and pericardial effusion reported most frequently. Thirty patients (55%) were delivered prematurely. Five patients (10%) died in utero and presented as stillbirth. Twenty-five patients (45%) presented symptoms immediately at birth. In the remaining 25 patients (45%), no obvious symptoms were noted immediately at birth. Three infants had appeared as asymptomatic until the age of 3 to 4 months, when they presented with failure to thrive, respiratory distress, arterial hypertension, or heart failure.

Presence of Arterial and Periarticular Calcifications

GACI was suspected during pregnancy in 6 (11%) cases, when early arterial calcifications were detected by sonography. Increased echogenicity of the great vessels was detected as early as in the 20th gestational week in a fetus. After birth, arterial calcifications were demonstrated predominantly in the aorta and in coronary arteries by imaging studies such as sonography and computed tomography (Supplemental Table 1). Additionally, autopsy, performed in 22 cases (40%), confirmed calcification of pulmonary and renal arteries in 15 deceased patients. In a subset of 16 patients (29%), periarticular calcifications were noted prenatally or in infancy (Table). Periarticular calcifications were present in surviving patients more frequently than in deceased patients.

ENPP1 Mutations in GACI Patients

In 41 (75%) of the patients studied, we detected homozygous or compound heterozygous mutations in *ENPP1*. In total, 40 different mutations were detected, including 30 missense mutations, 7 nonsense, and 3 splice site mutations (c.430+2T>C, c.565–2A>G, c.1164+2T>A). Mutation c.1164+2T>A leads to skipping of exon 11 causing the frameshift

Table. Sites of Calcifications Associated With Death and Survival in 55 GACI Patients

Site of Calcification	Death (n=36)	Survival (n=19)
Aorta	33 (92)	18 (95)
Coronary arteries	30 (83)	8 (42)
Pulmonary arteries	25 (69)	6 (32)
Renal arteries	17 (47)	5 (26)
Periarticular tissue	6 (17)	10 (53)

Data are presented as n (%).

P365fsX15.[21] The mutations were scattered over the whole coding region of the gene (Figure 2), but most concentrated in exons encoding the catalytic and the nuclease like domain. We detected 16 novel mutations (3 nonsense mutations, 11 missense mutations, and 2 splice site mutations; see Figure 2). In 14 (25%) cases, no *ENPP1* coding region mutations were found. These patients did not show any obvious difference regarding the distribution of the calcifications compared with the patients with proven *ENPP1* mutations (data not shown). Twenty-nine (71%) of the 41 *ENPP1* mutation positive patients died in infancy (median survival, 30 days), whereas 7 of the 14 (50%) patients without *ENPP1* mutations died in infancy (median survival, 9 days).

The mutation c.913C>A (p.P305T) in exon 8 was detected most frequently (Figure 2, insert). This mutation was present on both alleles in 5 unrelated patients, who all died in infancy. On the other hand, the homozygous mutation c.2320C>T (p.R774C) was associated with a relatively mild phenotype in 1 patient. This mutation was found on 6 alleles in 5 patients from unrelated 4 families of White origin. Apart from the 4 mutations c.1412A>G (p.Y471C), c.1709A>G (p.Y570C), c.2375A>G (p.N792S), and c.2713_2717delAAAGA (p.K905fsX15), which were present in the affected patients of 2 unrelated families, all other mutations were private mutations and presented only in single patients.

Course of GACI in the Study Cohort

Of the 55 patients in this cohort, 6 (11%) cases presented as stillborns and a total of 30 (55%) patients died within the first 6 months of life despite intensive care therapy, including ventilatory support. Death was attributed to congestive heart failure, persistent arterial hypertension, multiorgan failure, or myocardial infarction. After the age of 6 months, only 1 patient (case 40) died at the age of 7 months within the observation period (Figure 3A), the eldest living patients being 21 years old now.

Hypophosphatemia and Renal Phosphate Loss is Associated With Survival in GACI

Data on serum P_i levels were available from 13 of 19 surviving patients, additional data on maximal renal tubular phosphate reabsorption were available from 11 of 19 surviving patients with clinically proven GACI (Figure 4). In 8 of these patients, serum phosphate levels and TmP/GFR levels were measured beyond infancy. All these patients showed

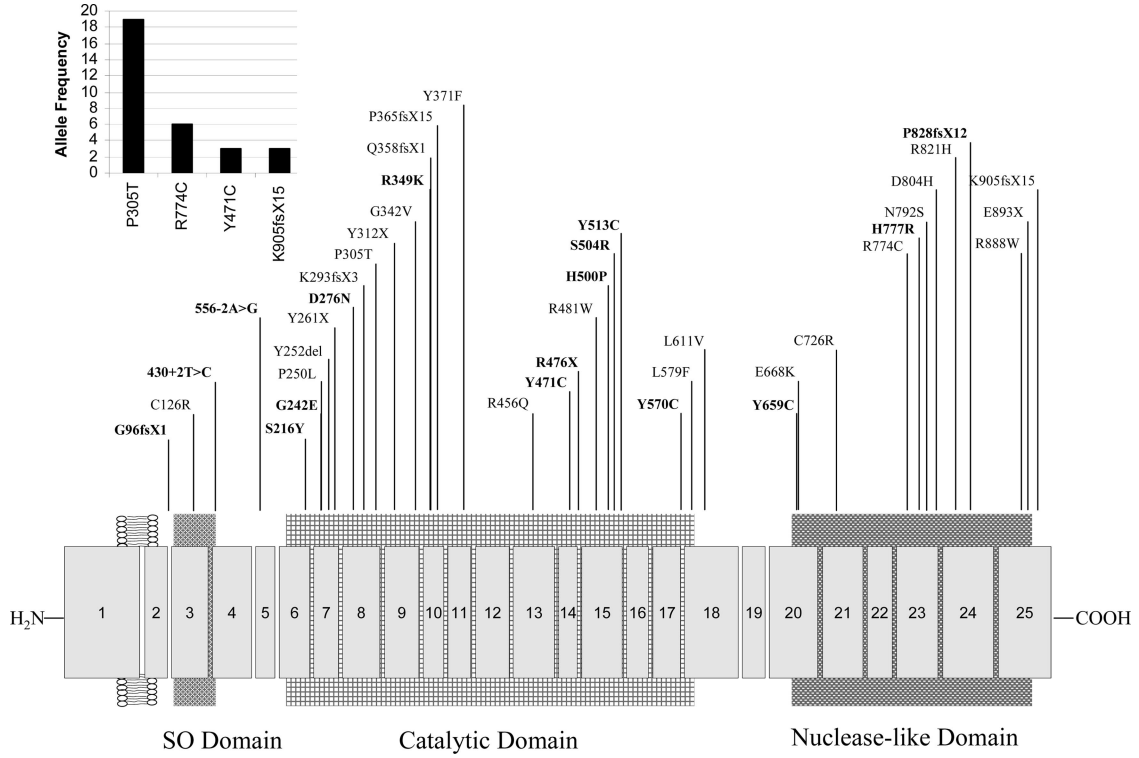

Figure 2. Schematic representation of the human *ENPP1* gene and protein with mutations identified in 55 GACI patients. Numbered boxes represent the 25 exons; patterned boxes represent functional domains. Novel mutations are shown in boldface. Insert at the left top of the figure shows allele frequency of most common *ENPP1* mutations in our study cohort. SO Domain indicates somatomedin B-like domain. *Splice site mutations c.430+2T>C and 556−2A>G result most likely in exon skipping and hence in frameshifts. †The mutation P365fsX15 was previously shown to result from skipping of exon 11 caused by the mutation c.1164+2T>A.[21]

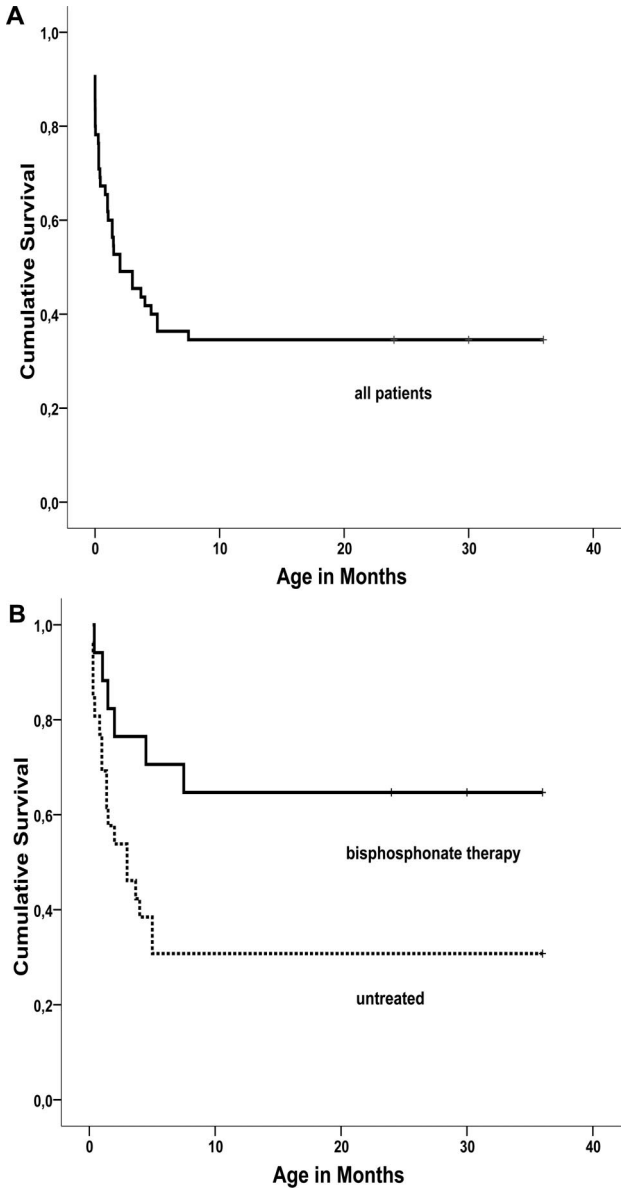

Figure 3. Cumulative survival in GACI patients of our study cohort. A, Overall survival in all 55 patients, including 12 patients who presented as stillbirths or died before the age of 1 day. B, Survival beyond infancy in 43 patients, who survived their first day of life, including 17 patients treated with bisphosphonates compared with 26 patients, who did not receive bisphosphonate therapy. Patients treated with bisphosphonates showed significantly increased likelihood of survival ($P=0.026$; log-rank test).

hypophosphatemia and hyperphosphaturia, which was noted first between the second and third year of life (Figure 4). In 4 of these children, the urine was also checked for the presence of microglobulinuria or hyperaminoaciduria, but these tests yielded normal results. Five patients (cases 8, 15, 16, 43, and 45) were supplemented with phosphate and calcitriol for signs of hypophosphatemic rickets becoming apparent between 8 months and 11 years of age, including bone pain, bowed femora, and short stature. In 1 of these patients (case 8), phosphate and calcitriol supplementation was associated with worsening of the arterial stenoses, therefore treatment for hypophosphatemic rickets was discontinued in this case.

In those patients with hyperphosphaturia, we amplified all 22 *PHEX* and 3 *FGF23* exons by PCR using intronic primers. PCR products were sequenced bidirectionally. No pathogenic *PHEX* or *FGF23* mutations were found. Intact FGF23 plasma levels were measured by ELISA in all patients with hyperphosphaturia and were found highly elevated in 2 patients at 1540 pg/mL and 3890 pg/mL, respectively, while on treatment with phosphate and calcitriol (normal range, 10 to 50 pg/mL).[25] After a 2-week cessation of calcitriol treatment, FGF23 levels were only moderately elevated in 1 patient (93 pg/mL; case 15), but still highly elevated in the other patient (560 pg/mL; case 45).

Bisphosphonate Treatment is Associated With Survival in GACI

In 17 patients, who survived their first day of life, therapy with bisphosphonates was instituted (Figure 3B), as etidronate (10 to 20 mg/kg body weight per day PO), pamidronate (0.1 mg/kg per week up to 5 mg/kg per day IV), clodronate or risedronate. Bisphosphonate treatment was associated with survival beyond infancy in 11 (65%) cases, whereas 18 of 26 (69%) patients not treated with bisphosphonates died in infancy (Figure 3B).

Discussion

GACI was initially held to be a universally fatal disease,[1] but within the last 20 years, anecdotal cases of survival beyond infancy have been reported.[26,27] Although bisphosphonate therapy has been advocated, it has remained unclear to what extent such therapy is effective, and predictors of disease outcome have not previously been defined. This study, albeit a cross-sectional analysis of subjects referred to a single international study group, rather than prospective analysis, clearly indicates GACI to not be an inevitably fatal condition. Specifically, whereas 36 patients died in utero or within the critical period of infancy, 19 patients survived beyond infancy and none of the survivors died within the observational period of 1 to 6 years.

Despite the limitations of the cross-sectional and retrospective analyses, several factors seemed to be predictive of a favorable prognosis and survival beyond infancy. First, with respect to sites of pathological calcifications, subjects who died in infancy had been reported to suffer from pulmonary, renal, and coronary involvement more frequently than surviving patients. However, this finding could have been biased by a higher detection rate of calcifications in these vessels in autopsy studies rather than in imaging studies performed in living patients. Taken this bias into account, one cannot conclude whether the mortality risk depends on specific sites of calcification or it is more related to the general degree of calcification.

GACI was observed to be caused by coding region mutations of *ENPP1* in 41 (≈75%) of the cases studied. We did not exclude *ENPP1* deletions or intronic mutations by our approach. Importantly, GACI survival was not associated with the presence or absence of *ENPP1* mutations per se, but the presence of the c.913C>A (p.P305T) mutation in exon 8

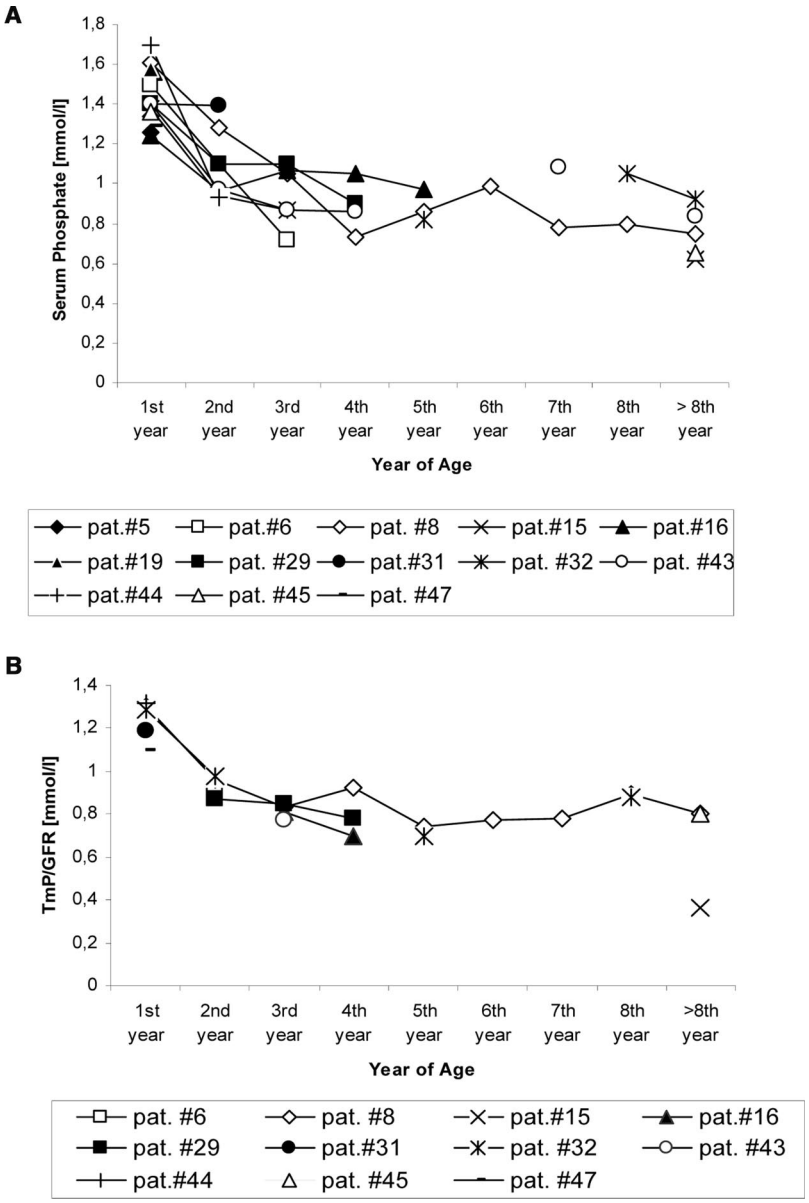

Figure 4. Serum phosphate levels and maximal renal tubular phosphate reabsorption in patients with GACI surviving beyond infancy. A, Serum phosphate levels available from 13 surviving patients. B, TmP/GFR levels available from 11 surviving patients. Normal serum phosphate levels in children between 1 and 3 years is 1.00 to 1.95 mmol/L, between 4 and 6 years is 1.05 to 1.80 mmol/L, and between 7 and 9 years is 0.95 to 1.75 mmol/L.[22] TmP/GFR was calculated according to Brodehl et al[23] using the formula $TmP/GFR = S_p - (U_p \times S_{crea})/U_{crea}$. Normal TmP/GFR in children between 2 and 15 years is 1.15 to 2.44 mmol/L.[24] The means of serum phosphate levels and the means of TmP/GFR levels of each surviving patient were significantly lower than the lowest reference values in patients older than 3 years of age ($P=0.031$ for serum phosphate and $P=0.004$ for TmP/GFR levels; Wilcoxon test for 2 paired samples).

on both alleles was always associated with death in infancy despite any treatment efforts. This mutation affects the catalytic region of the protein and is conserved across species. In our study cohort, the p.P305T mutation was the single most frequently detected mutation, present on 19 alleles of 14 patients (25%) from 10 families. The families carrying this mutation originated from an Anglo-American background, suggesting a common founder. Our results suggest the value of screening specifically for this mutation by PCR in clinical testing for GACI in the Anglo-American population. On the other hand, if present on both alleles, the mutation c.2320C>T (p.R774C) was associated with a relatively mild phenotype in 1 patient. This mutation affecting the nuclease-like domain of NPP1 was previously shown to be associated with residual enzyme activity.[6] Interestingly, the p.R774C mutation was also present on both alleles in the father of the proband. Although the proband was affected by GACI, the father did not present arterial calcifications in infancy, but suffered from severe hypophosphatemic rickets since early childhood.[6]

Stimulated by observations in 1 human kindred and by conclusive findings in NPP1 and extracellular PP_i-deficient mice,[28] for a protective/compensatory effect of hypophosphatemia for clinical phenotype of pathological soft tissue calcifications in GACI, we focused on available data on phosphate metabolism in 13 surviving GACI patients. In all surviving patients tested, serum P_i levels in infancy were normal, but we noted a decrease of serum P_i levels within the second year of life, which did not increase as subjects aged. Decrease of serum P_i levels was associated with a decrease of renal tubular phosphate reabsorption (TmP/GFR) in these patients. This effect was not caused by additional *PHEX* or *FGF23* mutations in these patients. However, in 2 patients, we detected elevated FGF23 levels (560 pg/mL and 93 pg/mL, respectively), which might at least partially mediate renal phosphate loss.[29]

Hypophosphatemic rickets was documented here in 5 survivors of GACI. Our collective findings suggest that clinical investigation of the application of a phosphate poor diet or a phosphate binding agent (eg, lanthanum carbonate, sevelamer hydrochloride) would be of interest with respect to early intervention in GACI. NPP1 is not universally expressed but is present in renal proximal tubule epithelial cells,[30] with unclear functional consequences. We speculate that NPP1 modulates renal proximal tubule epithelial cell function. In this context, NPP1 nucleotide pyrophosphatase activity (E.C. 3.6.1.9) modulates protein glycosylation and secretion (eg, IgA in plasma cells),[31] plays a major role in proteoglycans sulfation,[32] and modulates insulin receptor signaling.[33] Hence, deficient NPP1 expression in renal proximal tubule epithelial cells could modulate the function of these cells by PP_i-independent or PP_i-dependent means. With respect to the latter, PP_i seems to antagonize several functions of P_i and vice versa, including hydroxyapatite crystal growth in vitro, and architecture and chondrocyte differentiation of the endochondral growth plate in vivo, and pathological soft tissue calcification including arterial involvement.[13,14] In this context, it should be noted that tissue and serum levels of PP_i are in the low micromolar range, whereas serum P_i concentration is normally ≈2 mmol/L in humans and ≈8 mmol/L in mice.[14] Moreover, a rationale for bisphosphonate therapy becomes evident for GACI, because bisphosphonates function in part as nonhydrolyzeable analogues of PP_i.

In previous limited case reports, bisphosphonate treatment had variable success in GACI.[17,34] In the current study cohort, bisphosphonate therapy was associated with survival in 11 (65%) of 17 treated patients, whereas 69% of the patients not treated with bisphosphonates died. In any retrospective study, a comparison of these figures with respect to survival is limited, because the clinical phenotype of the untreated group was most likely more severe than in the treatment group. Also, several patients in this study died in utero or immediately after birth before diagnosis or treatment could be assigned. On the other hand, 7 patients receiving bisphosphonates died within infancy, and also, radiographic resolution of the calcifications did not prevent the subsequent development of arterial hypertension. Arterial hypertension might be caused by microcalcifications not visible on x-ray studies causing artery wall stiffness. However, we believe that bisphosphonates promote resolution of calcifications, but fail to alleviate the associated and often severe myointimal proliferation that plays a major role in vascular stenoses. Given that the extent of vascular occlusion has not appeared to grossly correlate with the extent of calcification in GACI in the literature,[26] and also in some patients included in our study (patients 8, 32, 33, and 40), direct therapeutic attention to this aspect of the disorder might improve outcome.

Among the limitations of this study was the inability to examine serum and urine PP_i levels or serum NPP1 protein levels and associated NPP1 enzyme specific activity, as well as affected tissue NPP1 mRNA and protein expression. We did not study other genes encoding mediators of NPP1 expression such as carminerin,[35] and regulators of PP_i levels such as ANKH,[14] or P_i levels such as TNAP,[13] or secondary determinants of PP_i effects on chondro-osseous differentiation of smooth muscle cells such as vanin-1 pantetheinase.[11] Nevertheless, the results of this relatively large GACI observational study indicated that the p.P305T mutation of *ENPP1* might serve as a potential tool for genotyping and prognosis. Furthermore, hyperphosphaturia and hypophosphatemia developed in some GACI subjects and were associated with survival beyond infancy, as also was bisphosphonate treatment. Further prospective, controlled studies of bisphosphonates and low phosphate dietary or phosphate binding treatment appear indicated for GACI.

Acknowledgments

The authors thank the patients and their families who participated in the study. The authors also thank Ulrike Botschen for expert technical assistance in *ENPP1* mutation analysis.

Sources of Funding

F.R. was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 492, subproject A12). R.T. was supported by the Veterans Administration Research Service and awards from the National Institutes of Health (HL077360, HL087252).

Disclosures

None.

References

1. Moran JJ. Idiopathic arterial calcification of infancy: a clinicopathologic study. *Pathol Annu*. 1975;10:393–417.
2. Rutsch F, Schauerte P, Kalhoff H, Petrarulo M, August C, Diekmann L. Low levels of urinary inorganic pyrophosphate indicating systemic pyrophosphate deficiency in a boy with idiopathic infantile arterial calcification. *Acta Paediatr*. 2000;89:1265–1269.
3. Spear R, Mack LA, Benedetti TJ, Cole RE. Idiopathic infantile arterial calcification, in utero diagnosis. *J Ultrasound Med*. 1990;9:473–476.
4. Rutsch F, Vaingankar S, Johnson KA, Goldfine I, Maddux B, Schauerte P, Kalhoff H, Sano K, Boisvert WA, Superti-Furga A, Terkeltaub R. PC-1 nucleoside triphosphate pyrophosphohydrolase deficiency in idiopathic infantile arterial calcification. *Am J Pathol*. 2001;158:543–554.
5. Whitehall J, Smith M, Altamirano L. Idiopathic infantile arterial calcification: sonographic findings. *J Clin Ultrasound*. 2003;31:497–501.
6. Rutsch F, Ruf N, Vaingankar S, Toliat M, Suk A, Höhne W, Schauer G, Lehmann M, Roscioli T, Schnabel D, Epplen JT, Knisely A, Superti-Furga A, McGill J, Filippone M, Sinaiko AR, Vallance H, Hinrichs B, Smith W, Ferre M, Terkeltaub R, Nürnberg P. Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. *Nature Genetics*. 2003;34:379–381.
7. van Driel IR, Goding JW. Plasma cell membrane glycoprotein PC-1. Primary structure deduced from cDNA clones. *J Biol Chem*. 1987;262:4882–4887.
8. Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C. Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit Rev Biochem Mol Biol*. 2000;35:393–432.
9. Johnson K, Goding J, Van Etten D, Sali A, Hu SI, Farley D, Krug H, Hessle L, Millán JL, Terkeltaub R. Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J Bone Miner Res*. 2003;18:994–1004.
10. Johnson K, Polewski M, van Etten D, Terkeltaub R. Chondrogenesis mediated by PP_i depletion promotes spontaneous aortic calcification in NPP1−/− mice. *Arterioscler Thromb Vasc Biol*. 2005;25:686–691.
11. Johnson KA, Yao W, Lane NE, Naquet P, Terkeltaub RA. Vanin-1 pantetheinase drives increased chondrogenic potential of mesenchymal precursors in ank/ank mice. *Am J Pathol*. 2008;172:440–453.
12. Ciana G, Trappan G, Bembi B, Benettoni A, Maso G, Zennaro F, Ruf N, Schnabel D, Rutsch F. Generalized arterial calcification of infancy: two siblings with prolonged survival. *Eur J Pediatr*. 2006;165:258–263.
13. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci U S A*. 2002;99:9445–9449.

14. Murshed M, Harmey D, Millán JL, McKee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev*. 2005;19: 1093–1104.
15. Evans JR, Robertson WG, Morgan DB, Fleisch H. Effects of pyrophosphate and diphosphonates on the dissolution of hydroxyapatites using a flow system. *Calcif Tissue Int*. 1980;31:153–159.
16. Terkeltaub RA. Inorganic pyrophosphate generation and disposition in pathophysiology. *Am J Physiol Cell Physiol*. 2001;281:C1–C11.
17. Meradji M, de Villeneuve VH, Huber J, de Bruijn WC, Pearse RG. Idiopathic arterial calcification in siblings: radiologic diagnosis and successful treatment. *J Pediatr*. 1978;92:401–405.
18. Stuart G, Wren C, Bain H. Idiopathic arterial calcification in two siblings: failure of treatment with diphosphonate. *Br Heart J*. 1990; 64:156–159.
19. Van Reempts PJ, Boven KJ, Spitaels SE, Roodhooft AM, Vercruyssen EL, Van Acker KJ. Idiopathic arterial calcification of infancy. *Calcif Tissue Int*. 1991;48:1–6.
20. Morton R. Idiopathic arterial calcification of infancy. *Histopathology*. 1978;2:423–432.
21. Ruf N, Uhlenberg B, Terkeltaub R, Nürnberg P, Rutsch F. The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy. *Hum Mutat*. 2005;25:98.
22. Soldin SJ. Pediatric reference ranges for phosphate on the Hitachi 747 analzyer. *Clin Chem*. 1997;42:198.
23. Brodehl J, Krause A, Hoyer PF. Assessment of maximal tubular phosphate reabsorption: comparison of direct measurement with the nomogram of Bijvoet. *Ped Nephrol*. 1988;2:183–189.
24. Payne RB. Renal tubular reabsorption of phosphate (TmP/GFR): indications and interpretation. *Ann Clin Biochem*. 1998;35:201–206.
25. Ito N, Fukumoto S, Takeuchi Y, Yasuda T, Hasegawa Y, Takemoto F, Tajima T, Dobashi K, Yamazaki Y, Yamashita T, Fujita T. Comparison of two assays for fibroblast growth factor (FGF)-23. *J Bone Miner Metab*. 2005;23:435–440.
26. Thiaville A, Smets A, Clercx A, Perlmutter N. Idiopathic infantile arterial calcification: a surviving patient with renal artery stenosis. *Pediatr Radiol*. 1994;24:506–508.
27. Thomas P, Chandra M, Kahn E, Mc Vicar M, Naidich J, LaCorte M. Idiopathic arterial calcification of infancy: a case with prolonged survival. *Pediatr Nephrol*. 1990;4:233–235.
28. Koshizuka Y, Ikegawa S, Sano M, Nakamura K, Nakamura Y. Isolation of novel mouse genes associated with ectopic ossification by differential display method using ttw, a mouse model for ectopic ossification. *Cytogenet Cell Genet*. 2001;94:163–168.
29. Yamazaki Y, Okazaki R, Shibata M, Hasegawa Y, Satoh K, Tajima T, Takeuchi Y, Fujita T, Nakahara K, Yamashita T, Fukumoto S. Increased circulatory level of biologically active full-length FGF-23 in patients with hypophosphatemic rickets/osteomalacia. *J Clin Endocrinol Metab*. 2002; 87:4957–4960.
30. Harahap AR, Goding JW. Distribution of the murine plasma cell antigen PC-1 in non-lymphoid tissues. *J Immunol*. 1988;141:2317–2320.
31. Rebbe NF, Hickman S. Modulation of nucleotide pyrophosphatase in plasmacytoma cells. *Biochem Biophys Res Commun*. 1991;175:637–644.
32. Harper GS, Hascall VC, Yanagishita M, Gahl WA. Proteoglycan synthesis in normal and Lowe syndrome fibroblasts. *J Biol Chem*. 1987;262: 5637–5643.
33. Belfiore A, Costantino A, Frasca F, Pandini G, Mineo R, Vigneri P, Maddux B, Goldfine ID, Vigneri R. Overexpression of membrane glycoprotein PC-1 in MDA-MB231 breast cancer cells is associated with inhibition of insulin receptor tyrosine kinase activity. *Mol Endocrinol*. 1996;10:1318–1326.
34. Bellah RD, Zawodniak L, Librizzi RJ, Harris MC. Idiopathic arterial calcification of infancy: prenatal and postnatal effects of therapy in an infant. *J Pediatr*. 1992;121:930–933.
35. Yamada T, Kawano H, Koshizuka Y, Fukuda T, Yoshimura K, Kamekura S, Saito T, Ikeda T, Kawasaki Y, Azuma Y, Ikegawa S, Hoshi K, Chung UI, Nakamura K, Kato S, Kawaguchi H. Carminerin contributes to chondrocyte calcification during endochondral ossification. *Nat Med*. 2006;12:665–670.

SUPPLEMENTAL MATERIAL

APPENDIX

The GACI Study Group consists of the following physicians and geneticists who recruited patients:

Abbasi, Afshan, Department of Neonatology, Mattel Children's Hospital at UCLA, Los Angeles, CA, USA

Chikarmane, Rashmi, Institute of Medical Genetics**,** St. Peter's University Hospital, New Brunswick, New Jersey, USA

Chitayat, David, Molecular Genetics Laboratory, Department of Paediatric Laboratory , The Hospital for Sick Children, Medicine, Toronto, Canada

Ciana, Giovanni, Neonatology Center, IRCCS "Burlo Garofolo", Trieste, Italy

Clark, Godfrey B, Paediatric Nephrology, King's College London, Great Britain

Ferre, Merry M., Prenatal Diagnostic Center, Carilion Center for Women and Children**,** Roanoke, VA, USA

Ferrero, Giovanni Battista, Department. of Pediatrics, University of Torino**,** Torino, Italy

Filippone, Marco, Dipartimento di Pediatria, Padova University Hospital, Padova, Italy

Fox, Michelle, Div. of Pediatrics, UCLA Medical Center, Los Angeles, CA, USA

Gardiner, Carol, Department of Clinical Genetics, Nottingham City Hospital, Nottingham, Great Britain

Gibson, James B. , Department of Pediatrics, Arkansas Children's Hospital , University of Arkansas for Medical Sciences, Little Rock, AR, USA

Gruskin, Daniel, Human Genetics and Pediatrics, Emory University School of Medicine**,** Decatur, Georgia, USA

Hinrichs, Bernd, Hamburg University Children's Hospital, Hamburg, Germany

Inwald, David, Paediatric Critical Care Medicine**,** St Mary's Hospital**,** London, Great Britain

Kahler, Stephen G., Division of Medical Genetics**,** Arkansas Children´s Hospital**,** Little Rock, AR, USA

Kimber-Foster, Judith, Center for Medicine and Prenatal Genetics, Brigham & Women's Hospital, Boston, MA, USA

Kivirikko, Sirpa, Department. of Clinical Genetics**,** Helsinki University Hospital, Helsinki**,** Finland

Librizzi, Ronald J , Virtua Antenatal Unit**,** Voorhees, NJ, USA

Lin, Shuan-Pei, Division of Genetics, Department of Pediatrics, MacKay Memorial Hospital, Taipei 10449,Taiwan

Lynch, Sally-Ann, National Center for Medical Genetics**,** OLHSC, Dublin, Ireland

Martinovic, Jelena, Unit of Fetal Pathology**,** Department of Histo-Embryology and Cytogenetics Hospital Necker-Enfants Malades, Paris, France

McGaughran, Julie, Queensland Clinical Genetics Service**.** Royal Children´s Hospital and Health District, Brisbane, Queensland, Australia

Newman, William, Academic and Regional Department of Clinical Genetics, St Mary's Hospital, Manchester, Great Britain

Quarrell, Oliver, Department of Clinical Genetics, Sheffield Children's Hospital, , Sheffield, Great Britain

Reardon, Willie, National Center for Medical Genetics**,** OLHSC, Dublin, Ireland

Roscioli, Tony, Department of Clinical and Molecular Genetics**,** Royal Prince Alfred Hospital**,** Sydney, Australia

Roux, Jean-Jacques, Service d'Anatomie et Cytologie Pathologiques. Centre Hospitalier, Chambery Cedex, France

Sinaiko, Alan R., Department of Pediatrics, University of MN Medical School**,** Minneapolis, Minnesota, USA

Smith, Wendy E., Division of Genetics, Pediatric Specialty Group, Barbara Bush Childrens' Hospital, Maine Medical, Portland, USA

Stavis, Robert, Neonatal ICU, Bryn Mawr Hospital, Bryn Mawr, PA, USA

Vallance, Hillary, Department of Pathology & Laboratory Medicine, Children's & Women's Health Centre of British Columbia, Vancouver, Canada

Van de Laar, Ingrid, Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands

Van Reempts, Patrick, Department of Neonatology, University Hospital Antwerp, Antwerp, Belgium

Wraige, Elizabeth, Paediatric Neurology, Guy's Hospital, London, Great Britain

Yee, Harris, Specialist in Medical Genetics**,** Calgary, Canada

Fam.	Pat.	Sex	Origin	affected siblings / non affected siblings	Arterial calcific-ations	Peri-articular calcific-ations	Bisphos-phonate treatment	Resolution of calcific-ations	Age at data collec-tion	Age at death	Hypophos-phatemia*	Hyper-phos-phaturia†	DNA change	Amino acid change	Ref.
1	1	m	Taiwan		a,c,p,r	knees	pamidronate	\	-	45 days	no	\	[1,025G>T] + [1,112A>T]	[G342V] + [Y371F]	1
	2	f	Taiwan	2/0	a,c,v	shoulder	no	\	4 years	-	\	\	[1,025G>T] + [1,112A>T]	[G342V] + [Y371F]	
2	3	m	America	1/0	a,c,r,v	no	no	\	-	stillbirth	\	\	[913C>A] + [913C>A]	[P305T] +[P305T]	2
3	4	f	GB / Nigeria	2/2	a,c,r	no	no	\	-	8 days	no	\	[1,831C>G + 2,002G>A] + [2375A>G]	[L611V + E668K] + [N792S]	3
	5	f	GB / Nigeria		a,c	no	pamidronate, risedronate	yes	2 years	-	no	\	[1,831C>G + 2,002G>A] + [2375A>G]	[L611V + E668K] + [N792S]	
4	6	m	Turkey	1/0	a,c,r	hip, shoulder, wrists	etidronate	yes	8 years	-	no	\	[2320C>T] + [2320C>T]	[R774C] + [R774C]	4
5	7	f	Canada	1/1	a,c,p	\	no	\	-	15 minutes	\	\	[913C>A] + [913C>A]	[P305T] + [P305T]	
6	8	m	Turkey	1/3	a,c	wrists, ankles	etidronate	yes	10 ½ years	-	yes	yes	[2,677G>T] + [2,677G>T]	[E893X] + [E893X]	4,5,6
7	9	f	Italy	1/0	a,c,p	soft tissues in right axilla	no	\	-	13 days	\	\	[1,367G>A] + [?]	[R456Q] + [2nd allele low]	4
8	10	m	Canada	1/0	a,c	no	etidronate	no	-	32 days	\	\	[376T>C] + [2,176T>C]	[C126R] + [C726R]	4
9	11	m	GB	1/0	a,c,p,r	no	\	\	-	1 day	\	\	[430+2 T>C + **2,330A>G**] + [**2,330A>G**]	[Splice-Site + **H777R**] + [**H777R**]	
10	12	m	Pakistan	2/3	a,c,p,r,v	no	no	\	-	5 hours	\	\	[2,410G>C] + [2,410G>C + 2,462G>A]	[D804H] + [D804H + R821H]	7
	13	f	Pakistan		d	no	no	\	-	30 minutes	\	\	[2,410G>C] + [2,410G>C + 2,462G>A]	[D804H] + [D804H + R821H]	
11	14	m	GB	2 (twins)/0	a,c,p	\	\	\	-	6 weeks	\	\	[749C>T] + [913C>A]	[P250L] + [P305T]	8
	15	f	GB		a,p	\	no	spontaneous	16 years	-	yes	yes	[749C>T] + [913C>A]	[P250L] + [P305T]	

12	16	f	India	1/0	a, c,p,r,v	shoulders, elbow, wrists, knees, ankles	pamidronate, etidronate, clodronate	yes	5 years	-	yes	yes	[753-755delATA] + [753-755delATA]	[Y252del] + [Y252del]	8, 9
13	17	f	Finland	1 / 0	c,r	\	\	\	-	4 months	\	\	[2,713-2,717delAAAGA] + [2,713-2,717delAAAGA]	[K905fsX15] + [K905fsX15]	
14	18	m	Ireland	1/0	a,p	no	no	no	-	9 days	\	\	[913C>A] + [913C>A]	[P305T] + [P305T]	
15	19	m	Ireland / Philippines	1/0	a	\	pamidronate	no	2 years	-	no	\	[**1,510A>C**] + [2,375A>G]	[**S504R**] + [N792S]	
16	20	f	America	1/0	a,p	no	\	\	-	25 days	\	\	[936T>G] + [2375A>G]	[Y312X] + [N792S]	10
17	21	f	GB	1/0	a,c,r	no	no	\	-	5 months	no	\	[913C>A] + [**1,426C>T**]	[P305T] + [**R476X**]	11
18	22	m	Ireland	1/2	a,c,r,v	no	pamidronate	\	-	4 ½ months	no	\	[913C>A] + [**1499A>C**]	[P305T] + [**H500P**]	
19	23	m	Sweden	1 / 1	d	\	\	\	-	1 month	\	\	[647C>A] + [**647C>A**]	[S216Y] + [**S216Y**]	
20	24	f	Germany	1 / 0	a,c,p	hips, great joints	no	\	-	2 months	\	\	[**288delG**] + [2,483-2,486insATTC]	[**G96fsX1**] + [P828fsX12]	
21	25	m	France	2 / 1	a,p	yes	no	\	-	stillbirth	\	\	[**826G>A**] + [1,412A>G]	[**D276N**] + [Y471C]	
	26	m	France		a,c,p	\	pamidronate etidronate	\	-	12 days	no	no	[**826G>A**] + [1,412A>G]	[**D276N**] + [Y471C]	
22	27	f	Italy	1 / 0	d	no	pamidronate	no	-	2 months	\	\	[**1,538A>G**] + [**1,976A>G**]	[**Y513C**] + [**Y659C**]	
23	28	m	GB	2/0	a,c,p	\	bisphosphonate	\	-	12 hours	\	\	[783C>G] + [878-879delAA]	[Y261X] + [K293fsX3]	8
	29	m	GB	2/0	a	no	no	\	5 1/2 years	-	yes	yes	[783C>G] + [878-879delAA]	[Y261X] + [K293fsX3]	
24	30	f	GB	1/0	d,c,p	no	\	\	-	16 weeks	\	\	[556-2A>G] + [913C>A]	[Splice-Site] + [P305T]	
25	31	m	Ireland	1/0	a,c,v	no	pamidronate	yes	2 1/2 years	-	no	\	[2,320C>T + 2,662C>T] + [2,375A>G]	[R774C + R888W] +[N792S]	

26	32	f	Italy / Germany-America	2 /0	a,p,v	shoulders, hip, knee	no	spontaneous	14 1/2 years	-	yes	yes	[913C>A] + [1,164+2T>A]	[P305T] +[P365fsX15]	12, 13
	33	f	Italy / Germany-America		a,v	no	\	spontaneous	6 years	-	no	no	[913C>A] + [1,164+2T>A]	[P305T] +[R364fsX15]	
27	34	f	German	1/3	a,c,r	\	\	\	-	1 month	\	\	[1,072-1,082delCAGCTTCCTAA] + [1,737G>C + 2,320C>T]	[Q358fsX1] + [L579F] +[R774C]	4
28	35	m	America	1/0	a,r,v	hip, elbows, wrists	\	\	3 years	-	\	\	[**1,046G>A**] + [**1,709A>G**]	[**R349K**] + [**Y570C**]	14
29	36	f	Australia	1/0	a,p	no	\	\	-	9 hours	no	\	[**1,412A>G**]+ [**1,709A>G**]	[**Y471C**] + [**Y570C**]	
30	37	f	America	2 (twins)/ 1	c,r	no	\	\	-	3 months	no	\	[913C>A] + [2,320C>T + 2,662C>T]	[P305T] + [R774C + R888W]	
31	38	m	America	2 /0 (prenatal diagnosis in 2nd child)	a,c	\	\	\	-	3 months	\	\	[**725G>A**] + [1,441C>T]	[**G242E**] + [R481W]	
32	39	f	America/ Ireland	2/3	d,p	no	no	\	-	1 hour	\	\	[913C>A] + [913C>A]	[P305T] + [P305T]	8, 15
	40	f	America/ Ireland		a,c,r,v	no	etidronate	yes	-	7 ½ months	no	\	[913C>A] + [913C>A]	[P305T] + [P305T]	16
33	41	m	America	1/0	a,c,p	no		\	-	6 weeks	\	\	[1,441C>T] + [2,713-2,717delAAAGA]	[R481W] +[K905fsX15]	4
34	42	f	America	1 / 2	d	\	no	\	-	9 days	\	\	-	-	4
35	43	m	Germany	1 / 1	v	hip, shoulder	\	\	15 years	-	yes	yes	-	-	
36	44	m	Turkey	1 / 1	a,c,r	wrists	etidronate	yes	4 years	-	no	\	-	-	17
37	45	m	Pakistan	2 / 3	a,p,v	hips, shoulders	no	spontaneous	17 years	-	yes	yes	-	-	
38	46	m	France	1 / 0	a,c,p	shoulder, hip	no	\	-	stillbirth	\	\	-	-	
39	47	m	Latin America	1 / 2	d	multiple sites	etdironate	yes	3 3/4 years	-	\	\	-	-	
40	48	f	Turkey	1(twin) / 2	a,p,v	no	?	\	-	9 days	\	\	-	-	
41	49	f	Turkey	1 / 2	a,p,v	shoulder, ankle	no	\	-	stillbirth	\	\	-	-	

42	50	f	Canada	1 / 0	a,c,p,r	no	no	\	-	6 ½ weeks	\	\	-	-	4, 18
43	51	m	America	2 (twins)/ 0	a,c,p	no	no	\	7 years	5 months	\	\	-	-	19
	52	m	America		a,p	no	no	spontaneous	-	-	\	\	-	-	
44	53	m	India	1 / 0	c	no	no	\	-	stillbirth	\	\	-	-	
45	54	f	Belgium	2 (twins) /0	a,v	no	etidronate	yes	21 years	-	no	\	-	-	4, 20
	55	f	Belgium		a,v	no	etidronate	yes	21 years	-	no	\	-	-	

Supplemental table 1. Clinical and mutational data on 55 individual patients with generalized arterial calcification of infancy.
m= male, f=female, a=aorta, c=coronary arteries, p=pulmonary artery, r=renal artery, v=heart valves, d= diverse arteries throughout the body. "\"= no data available.
Novel mutations are noted in bold face.
*Hypophosphatemia was diagnosed if serum phosphate levels were below the reference range (between 1 and 3 years: 1.00-1.95 mmol/l, between 4 and 6 years: 1.05-1.80 mmol/l, between 7 and 9 years: 0.95-1.75 mmol/l).[21]
†Hyperphosphaturia: TmP/GFR was calculated according to the formula $TmP/GFR = P_p - \frac{U_p \times P_{crea}}{U_{crea}}$.[22]
Hyperphosphaturia was diagnosed if TmP/GFR was below 1.15 mmol/l in patients between 1 and 12 years.[23]

Symptom	***Prenatal period***		***Neonatal period***		***Infancy***	
	Overall Death (n=36)	Overall Survival (n=19)	Overall Death (n=36)	Overall Survival (n=19)	Overall Death (n=36)	Overall Survival (n=19)
Pleural effusion	4 (11%)	0 (0%)	2 (5%)	0 (0%)	1 (3%)	0 (0%)
Ascites	5 (14%)	0 (0%)	2 (5%)	1 (5%)	1 (3%)	0 (0%)
Pericardial effusion	8 (22%)	3 (16%)	3 (8%)	2 (10,5%)	1 (3%)	0 (0%)
Fetal hydrops	7 (19%)	0 (0%)	3 (8%)	1 (5%)	/	/
Decreased fetal movements	3 (8%)	1 (5%)	/	/	/	/
Fetal distress	8 (22%)	6 (31,5%)	/	/	/	/
Polyhydramnios	10 (28%)	2 (10,5%)	/	/	/	/
Respiratory distress	/	/	15 (42%)	7 (37%)	2 (5,5%)	0 (0%)
Cardiac insufficiency	/	/	22 (61%)	8 (42%)	6 (17%)	0 (0%)
Arterial hypertension	/	/	10 (28%)	5 (26%)	2 (5,5%)	8 (42%)
Impalpable pulses	/	/	3 (8%)	4 (21%)	0 (0%)	1 (5%)

Supplemental table 2. Symptoms recorded in utero, within the neonatal period and later in infancy associated with death and survival in 55 patients with GACI. Prenatal symptoms were detected by sonography during pregnancy.

Supplemental References

1. Cheng KS, Chen MR, Ruf N, Lin SP, Rutsch F. Generalized arterial calcification of infancy: different clinical courses in two affected siblings. Am J Med Genet A. 2005; 136:210-213

2. Levine JC, Campbell J, Nadel A. Prenatal diagnosis of idiopathic infantile arterial calcification. Circulation. 2001;103:325-326

3. Whitehall J, Smith M, Altamirano L. Idiopathic infantile arterial calcification: sonographic findings. J Clin Ultrasound. 2003; 31:497-501.

4. Rutsch F, Ruf N, Vaingankar S, Toliat M, Suk A, Höhne W, Schauer G, Lehmann M, Roscioli T, Schnabel D, Epplen JT, Knisely A, Superti-Furga A, McGill J, Filippone M, Sinaiko AR, Vallance H, Hinrichs B, Smith W, Ferre M, Terkeltaub R, Nürnberg P. Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. Nature Genetics. 2003; 34:379-381

5. Rutsch F, Schauerte P, Kalhoff H, Petrarulo M, August C, Diekmann L. Low levels of urinary inorganic pyrophosphate indicating systemic pyrophosphate deficiency in a boy with idiopathic infantile arterial calcification. Acta Paed. 2000; 89: 1265-1269

6. Rutsch F, Vaingankar S, Johnson KA, Goldfine I, Maddux B, Schauerte P, Kalhoff H, Sano K, Boisvert WA, Superti-Furga A, Terkeltaub R. PC-1 Nucleoside Triphosphate Pyrophosphohydrolase Deficiency in Idiopathic Infantile Arterial Calcification. Am J Pathol. 2001;158: 543-554

7. Pashankar D, Moore. Test and teach. Number eighty three: Part 1. Idiopathic arterial calcification of infancy. Pathology. 1997;29:175-217.

8. Ruf N, Uhlenberg B, Terkeltaub R, Nürnberg P, Rutsch F. The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy. Hum Mutat**.** 2005;25:98

9. Azancot A, Diehl R, Dorgeret S, Sebag G, Baumann C, Vuillard E, Machado L, Luton D, Oury JF. Isolated pericardial effusion in the human fetus: a report of three cases. Prenat Diagn. 2003;23:193-197.

10. Knisely AS, Gannuch GM, Rossi A, Steinmann B, Superti-Furga A: The matrix GLA protein gene: two polymorphisms, but no pathogenic mutation, in a patient with idiopathic arterial calcification of infancy. Lab Invest. 1999; 80:37A

11. Inwald DP, Yen Ho S, Shepherd MN, Daubeney PE. Idiopathic infantile arterial calcification presenting as fatal hypertensive cardiomyopathy. Arch Dis Child. 2006;91:928.

12. Ciana G, Colonna F, Forleo V, Brizzi F, Benettoni A, de Vonderweid U. Idiopathic arterial calcification of infancy: effectiveness of prostaglandin infusion for treatment of secondary hypertension refractory to conventional therapy: case report. Pediatr Cardiol. 1997;18:67-71.

13. Ciana G, Trappan A, Bembi B, Benettoni A, Maso G, Zennaro F, Ruf N, Schnabel D, Rutsch F. Generalized arterial calcification of infancy: two siblings with prolonged survival. Eur J Pediatr. 2006; 165:258-263

14. Tran KH, Boechat MI . Idiopathic infantile arterial calcification: imaging evaluation and the usefulness of MR angiography. Pediatr Radiol. 2006;36:247-253

15. Van de Woestijne J, Kalchbrenner M, Librizzi R, Knisely AS. Idiopathic arterial calcification of infancy and hydrops fetalis associated with maternal cardiopilin antibody (Abstract). Ped Pathol. 1988; 8:675-676

16. Bellah RD, Zawodniak L, Librizzi RJ, Harris MC. Idiopathic arterial calcification of infancy: prenatal and postnatal effects of therapy in an infant. J Pediatr. 1992;121:930-933

17. van der Sluis IM, Boot AM, Vernooij M, Meradji M, Kroon AA. Idiopathic infantile arterial calcification: clinical presentation, therapy and long-term follow-up. Eur J Pediatr. 2006; 165:590-593

18. Glatz AC, Pawel BR, Hsu DT, Weinberg P, Chrisant MR. Idiopathic infantile arterial calcification: two case reports, a review of the literature and a role for cardiac transplantation. Pediatr Transplant. 2006;10:225-233.

19. Wax JR, Blackstone J, Pinette MG, Cartin A. Hepatic vascular calcification: an early second trimester sonographic feature of idiopathic infantile arterial calcinosis. Am J Obstet Gynecol. 2001; 185:1267-1268.

20. Van Reempts PJ, Boven KJ, Spitaels SE, Roodhooft AM, Vercruyssen EL, Van Acker KJ. Idiopathic arterial calcification of infancy. Calcif Tissue Int. 1991;48:1-6.

21. Soldin SJ. Pediatric reference ranges for phosphate on the Hitachi 747 analzyer. Clin Chem. 1997;42:198

22. Brodehl J, Krause A, Hoyer PF. Assessment of maximal tubular phosphate reabsorption: comparison of direct measurement with the nomogram of Bijvoet. Ped Nephrol. 1988; 2:183-189

23. Payne RB. Renal tubular reabsorption of phosphate (TmP/GFR): indications and interpretation. Ann Clin Biochem. 1998;35: 201-206