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

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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 \( \approx 75\% \) 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 \( \approx 180 \) 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. Initial signs of the disease may occur prenatally, and most affected children die in early infancy from sequelae of vascular occlusion, typically myocardial.
Infection or congestive heart failure due to hypertension. Systemic deficiency of nucleotide pyrophosphatase (NPP1) activity (E.C. 3.6.1.9) leading to low serum and urine inorganic pyrophosphate (PPi) levels has been identified as a diagnostic hallmark of the disease. Deficient NPP1-catalyzed PPi generation in GACI seems to be mediated by mutations in multiple exons of ENPP1 (MIM*173335). 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. NPP1 has an extracellular catalytic domain as well as somatotomin B-like and substrate-binding or substrate-specifying nuclease-like domains. NPP1 regulates soft tissue calcification and bone and joint cartilage mineralization by generating PPi, which not only serves as an essential physiological inhibitor of hydroxyapatite crystal growth but also is a suppressor of chondrogenesis. In artery smooth muscle cells, deficiencies of NPP1 (or of extracellular PPi, without NPP1 deficiency in ank/ank mice homozygous for functional inactivation of the PPi transporter ANK) promote chondrogenic transdifferentiation in vivo and also in vitro under circumstances where excess of an inorganic phosphate (Pi) source is provided. Although the pathophysiologic role of NPP1-mediated PPi 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.

PPi and Pi seem to have mutually antagonistic roles in tissue mineralization. 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. 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. Strikingly, the father was not affected by GACI, but suffered from severe hypophosphatemic rickets. On the basis of this observation, we hypothesized that hypophosphatemia may inhibit potential pathological effects of deficient NPP1-mediated PPi generation and may prevent humans from developing lethal pathological arterial calcification. Furthermore, within the last few years, bisphosphonates, which function in part as synthetic nonhydrolyzable analogues of PPi, have been anecdotally reported to have varying degrees of success in the treatment of GACI. 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, or typical histology (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 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 Pi (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.

ENPP1 Mutation 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.
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.

ENPPI Mutations in GACI Patients

In 41 (75%) of the patients studied, we detected homozygous or compound heterozygous mutations in ENPPI. 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.
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 Pi 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 TmPi/GFR levels were measured beyond infancy. All these patients showed

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

<table>
<thead>
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<th>Site of Calcification</th>
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<th>Survival (n=19)</th>
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<tr>
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<td>8 (42)</td>
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<tr>
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<td>6 (32)</td>
</tr>
<tr>
<td>Renal arteries</td>
<td>17 (47)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>Periarticular tissue</td>
<td>6 (17)</td>
<td>10 (53)</td>
</tr>
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</table>

Data are presented as n (%).

[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]
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). 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, but within the last 20 years, anecdotal cases of survival beyond infancy have been reported. 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.
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 PPi-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 Pi levels in infancy were normal, but we noted a decrease of serum Pi levels within the second year of life, which did not increase as subjects aged. Decrease of serum Pi 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

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 8 years is 1.05 to 1.80 mmol/L, and between 7 and 9 years is 0.95 to 1.75 mmol/L. TmP/GFR was calculated according to Brodehl et al23 using the formula TmP/GFR=S_p/U_p×S_crea/U_crea. Normal TmP/GFR in children between 2 and 15 years is 1.15 to 2.44 mmol/L. 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).
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, 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), plays a major role in proteoglycans sulfation, and modulates insulin receptor signaling. Hence, deficient NPP1 expression in renal proximal tubule epithelial cells could modulate the function of these cells by PPi-dependent or PPi-dependent means. With respect to the latter, PPi seems to antagonize several functions of P1 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. In this context, it should be noted that tissue and serum levels of PPi are in the low micromolar range, whereas serum P1 concentration is normally \( \approx 2 \) mmol/L in humans and \( \approx 8 \) mmol/L in mice. Moreover, a rationale for bisphosphonate therapy becomes evident for GACI, because bisphosphonates function in part as nonhydrolyzable analogues of PPi.

In previous limited case reports, bisphosphonate treatment had variable success in GACI. 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 bisphosphonate 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, 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 PPi, 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, and regulators of PPi, levels such as ANKH, or P1 levels such as TNAP, or secondary determinants of PPi effects on chondro-osseous differentiation of smooth muscle cells such as vanin-I pantetheinase. 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, hyperphosphatemia 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.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

APPENDIX

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Yee, Harris, Specialist in Medical Genetics, Calgary, Canada
<table>
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<th>Peri-articular calcificat-ions</th>
<th>Bisphosphonate treatment</th>
<th>Resolution of calcificat-ions</th>
<th>Age at data collection</th>
<th>Age at death</th>
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| 27 | 31 | m | Ireland | 1/0 | a,c,v | pamidronate | yes | 2 1/2 years | - | no | | | | [2,320C>T] + [2,662C>T] + [2,375A>G]

CICULATION AHA 2008/797704/R4
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**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)].

†Hyperphosphaturia: TmP/GFR was calculated according to the formula TmP/GFR = P_ratio x Pcrea/ Ucrea. Hyperphosphaturia was diagnosed if TmP/GFR was below 1.15 mmol/l in patients between 1 and 12 years.
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


