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Prospective phenotyping of long-term survivors of generalized arterial calcification of infancy (GACI)

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Purpose: Generalized arterial calcification of infancy (GACI), characterized by vascular calcifications that are often fatal shortly after birth, is usually caused by deficiency of ENPP1. A small fraction of GACI cases result from deficiency of ABCC6, a membrane transporter. The natural history of GACI survivors has not been established in a prospective fashion.

Methods: We performed deep phenotyping of 20 GACI survivors.

Results: Sixteen of 20 subjects presented with arterial calcifications, but only 5 had residual involvement at the time of evaluation. Individuals with ENPP1 deficiency either had hypophosphatemic rickets or were predicted to develop it by 14 years of age; 14/16 had elevated intact FGF23 levels (iFGF23). Blood phosphate levels correlated inversely with iFGF23. For ENPP1-deficient individuals, the lifetime risk of cervical spine fusion was 25%, that of hearing loss was 75%, and the main morbidity in adults was related to enthesis calcification. Four ENPP1-deficient individuals manifested classic skin or retinal findings of PXE. We estimated the minimal incidence of ENPP1 deficiency at ~1 in 200,000 pregnancies.

Conclusion: GACI appears to be more common than previously thought, with an expanding spectrum of overlapping phenotypes. The relationships among decreased ENPP1, increased iFGF23, and rickets could inform future therapies.

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INTRODUCTION

Generalized arterial calcification of infancy (GACI), an autosomal recessive disorder characterized by calcification of large- and medium-sized vessels, has a mortality of 55% within the first six months of life due to myocardial or cerebral infarctions.[1] Only ~200 patients have been reported.[2] In 67% of cases, GACI is caused by biallelic inactivating variants in *ENPP1* (ectonucleotide pyrophosphatase/phosphodiesterase 1),[3] which encodes an enzyme that cleaves adenosine triphosphate (ATP) into adenosine monophosphate (AMP) and inorganic pyrophosphate (PPi) at the cell surface. Vascular calcification is thought to occur as a result of a deficiency of PPi, the main inhibitor of physiological calcification. In 9% of cases, GACI results from biallelic inactivating variants in *ABCC6*, which encodes a plasma membrane transporter highly expressed in the liver;[4] biallelic variants in *ABCC6* typically cause pseudoxanthoma elasticum (PXE), a disorder characterized by calcification and fragmentation of elastic fibers in the skin (leading to coalescing papules in neck and flexures), retina (with peau d'orange, angioid streaks, and choroidal neovascularization), and cardiovascular system (with consequent adult-onset arterial

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calcification).[5] While the exact molecule transported by ABCC6 into the extracellular space is uncertain, one candidate is ATP, a substrate for ENPP1 and thus a source of plasma PPi. Accordingly, levels of PPi are decreased in affected humans and animal models of ABCC6 deficiency.[6] Further evidence of genetic heterogeneity in the pathogenesis of GACI is based on identification of affected individuals who lack variants in either *ENPP1* or *ABCC6*.[3]

In a previous study, 5 of 19 children who survived GACI beyond infancy developed hypophosphatemia.[1] Subsequently, ENPP1 deficiency was associated with autosomal recessive hypophosphatemic rickets type 2, or ARHR2; elevated or high-normal plasma intact FGF23 concentrations were documented and considered to be the cause of renal phosphate wasting in these patients.[7,8] Other complications (hearing loss,[9] PXE-like skin and retinal changes,[10] and cervical spine fusion[3,11]) have been described in case reports.

Here we report the results of clinical, laboratory, and molecular evaluations of 20 individuals with GACI. The results expand the phenotypic and mutational spectra of the disease and provide a basis for developing outcome measures for future clinical trials.

MATERIALS AND METHODS

Subjects

We enrolled 20 subjects aged 9 months to 58 years who had a clinical diagnosis of GACI plus at least one pathogenic variant in *ENPP1* or *ABCC6*.

Ethics statement

Affected individuals were co-enrolled in three institutional review board (IRB)–approved protocols at the National Institutes of Health (NIH): "Diagnosis and Treatment of patients with Inborn Errors of Metabolism and Other Genetic Disorders" (NCT00369421), "Study of People With generalized arterial calcification of infancy (GACI) or Autosomal Recessive Hypophosphatemic Rickets Type 2 (ARHR2)" (NCT03478839), and "Evaluation and Treatment of Bone and Mineral Disorders" (NCT00024804). All subjects or their guardians provided informed consent and, when appropriate, minors provided assent.

Data collection

All study participants underwent specialty consultations, echocardiograms, and computed tomography (CT) of the chest, abdomen, and pelvis with extension to extremities. Blood phosphate and alkaline phosphatase were measured on a Roche Cobas 6000 platform; parathyroid hormone was measured by electrochemiluminescence immunoassay on a Roche Cobas e601 analyzer; 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were measured by chemiluminescent immunoassay. Intact FGF23 (iFGF23) and C-terminal FGF23 were measured by enzyme-linked immunosorbent assay (ELISA) (Immutopics International, San Clemente, CA). Genomic DNA was extracted from peripheral blood mononuclear cells and sequenced by commercial laboratories.

Statistical analysis

Data were analyzed using descriptive statistics (conducted in SAS 9.4), with medians and ranges for continuous variables, and numbers and proportions for categorical variables. Kaplan–Meier curves were created with Prism version 6.0c (Graphpad Software Inc, La Jolla, CA). To model serum phosphate as a function of age, a generalized mixed model with an identity link function was used to account for unbalanced observations and intrasubject correlation using random intercepts and scaled identity covariance structure (IBM SPSS Statistics Subscription for Windows, IBM Corp., Armonk, NY, USA). Because serum phosphate concentrations vary with age in healthy children, we expressed phosphate values as *Z*-scores relative to published age-matched values.[12] A quadratic model was fitted using age and change in age as covariates.

Incidence calculation

The predicted incidence of ENPP1 deficiency was estimated by searching for all published *ENPP1* pathogenic variants. The Exome Aggregation Consortium (ExAC)[13] provided allele frequencies and allowed identification of variants predicted to damage ENPP1 function, but not reported in the literature before March 2017. ExAC was queried for canonical splice site, missense, frameshift, nonsense (stopgain), and stop loss variants. None of the cohorts or consortia in ExAC include patients ascertained for the presence of GACI, so we considered them to be unbiased with respect to variation in the *ENPP1* gene. Variants were excluded if (1) they had a minor allele frequency of >0.5%; (2) they were found in sites covered in fewer than 80% of individuals, as this may indicate a low-quality site; (3) they were only present in a noncanonical transcript; (4) they were in an untranslated region; or (5) they fell in the SMB2 domain, corresponding to amino acids 145–189, since variants in this domain are associated with a different phenotype, Cole disease.[14] Variants that passed these filters were evaluated for their potential to alter protein function by using in silico prediction models, i.e., PolyPhen-2,[15] SIFT,[16] and the Combined Annotation-Dependent Depletion (CADD) score.[17] Unpublished variants predicted to be benign by one or more in silico models were not considered for the calculation of incidence. For likely pathogenic variants, such as frameshift, stopgain (nonsense), and canonical splice site variants, only the CADD Phred-scaled score was provided.

The carrier frequency was calculated as the number of individuals having an *ENPP1* variant known or predicted to alter protein function, divided by the total number of individuals ascertained. The incidence of the disease was calculated based on the carrier frequency and/or the allele frequency using Hardy–Weinberg equilibrium.

RESULTS

Subjects

Twenty individuals (16 with biallelic *ENPP1* variants and 4 with *ABCC6* variants) survived infancy and underwent

extensive evaluations at the NIH Clinical Center at ages ranging from 9 months to 58 years. Their family pedigrees are presented in Fig. S1 and their clinical and molecular findings are described in Table 1. Five siblings of the 16 individuals in our cohort with *ENPP1* variants died within the first 5 months of life with findings typical of GACI. The location of each variant in the ENPP1 or ABCC6 protein is presented in Fig. S2; six novel variants in *ENPP1* are described. For the full cohort, the median ages of onset of symptoms and diagnosis were 2.5 and 23 days, respectively. Individuals with *ABCC6* variants did not differ from those with *ENPP1* variants with respect to these parameters.

Ectopic calcification

Representative histopathological findings of deceased individuals are shown (Fig. 1a–b). Sixteen of the 20 survivors initially had extensive arterial calcifications, most prominently involving the aorta and other large vessels (Fig. 1c), as well as the coronary arteries (Fig. 1d). The median age at first observation of calcification was 0.5 days, but at the latest NIH evaluation, the arterial calcification was no longer visible on imaging in all but five subjects (Fig. 2); these residual arterial calcifications were of minimal severity. Fifteen of the 16 individuals also exhibited organ calcifications, most frequently in the kidney and cardiac valves.

Joint calcifications, most often affecting the shoulders (Fig. 3a), hips, and wrists, occurred in half of patients and were first observed at a median age of 4 months (Fig. 2). By the time of the NIH evaluation, the calcifications had resolved in more than half of the involved joints. Cervical spine fusion was present in four survivors; patient 3 had fusions of C3–C4 and C5–C6 during infancy and patient 7 had fused laminae of C3–C6 at 7.6 years. Patient 8 exhibited fusion of cervical vertebral bodies and neural arches at 15 years and was diagnosed with Klippel–Feil syndrome (Fig. 3b). Patient 11 had fused C2–C3 and C4–C5 posterior vertebral bodies and articular processes.

Calcification of the tendons or ligaments at contact sites with bones (enthesis) was present in all three adults and was associated with local musculoskeletal pain. The calcification of patient 8 affected the common extensor origin of the right lateral epicondyle at 25 years of age. Patient 11 had calcification of the Achilles tendon, leading to unilateral spontaneous rupture at age 25. Patient 16 had ossification of the posterior longitudinal ligament at vertebrae C2–C3 and C3–C4 (Fig. 3c).

In general, the 16 individuals with *ENPP1* variants did not differ in the frequency or location of ectopic calcification compared with those having *ABCC6* variants.

Rickets/osteomalacia

Clinical rickets was diagnosed in children based on classic signs such as bowing, gait disturbance, metaphyseal flaring, etc., and/or metaphyseal irregularities on radiographs. In adults, osteomalacia was presumed based on a previous diagnosis of rickets during childhood and/or persistent hypophosphatemia in adulthood. There was no evidence of rickets/osteomalacia in the four patients with ABCC6 deficiency. By contrast, in individuals with ENPP1 deficiency, Kaplan–Meier analysis (Fig. 3g) estimated a 20% probability of developing rickets by 2 years of age, and a 100% probability of developing it by 13.6 years of age. In fact, 11 of the 16 ENPP1-deficient subjects had already developed hypophosphatemic rickets/osteomalacia at the time of NIH evaluation (Fig. 3d). Table 2[12,18,19] summarizes laboratory findings pertaining to mineral balance. Longitudinal blood phosphate data (a total of 131 observations, mean: 11/patient) were available for 12 subjects during childhood prior to onset of treatment. Age significantly influenced serum phosphate. At birth, the mean phosphate *Z*-score was 0, indicating serum phosphate equal to the normal population mean. However, after birth a sharp decline was observed (mean rate = −1.45 SD per year; 95% confidence interval [CI] = −1.90 to −1.00) that slowed over time (mean change in rate = 0.12 SD per year,[2] 95% CI = 0.06–0.18) with an average onset of hypophosphatemia (serum phosphate *Z*-score < −1.96) at 1.6 years of age (Fig. 3e). iFGF23 concentrations were frankly elevated (>50 pg/mL) in 14 of 16 patients with ENPP1 deficiency, and were significantly and inversely correlated with blood phosphate (Fig. 3f). The values of 1,25-dihydroxyvitamin D were inappropriately suppressed in patients with elevated iFGF23 concentrations, with a mean value of 57.4 pg/mL as compared with 120.3 pg/mL in those with normal iFGF23 values (two-tailed *P* value 0.014 by unpaired *t* test).

PXE

PXE-like complications appeared in four patients with *ENPP1* variants. Skin manifestations of PXE (Fig. 3h) were documented in two children and typical retinal findings were observed in two adults (peau d'orange) and one child (peau d'orange, optic nerve head drusen); this child also had PXE-like skin involvement. One patient with ENPP1 deficiency received a diagnosis of PXE at 43 years of age, after presenting with an acute retinal hemorrhage and angioid streaks (Fig. 3i). Signs of PXE were not seen in any of the ABCC6-deficient patients.

Other complications

Ten of 16 patients with ENPP1 deficiency manifested hearing loss (7 conductive; 3 mixed) at a median age of 3.7 years. The estimated probability of developing hearing loss was 20% by 2 years of age, 50% by 4 years, and 75% over a lifetime (Fig. 3j). Hearing loss was not observed in any of the ABCC6-deficient patients.

One subject (patient 10) presented in the neonatal period with cardiac failure due to multiple stenoses within the systemic vasculature, with no evidence of calcification of these vessels on CT. He was initially given a diagnosis of fibromuscular dysplasia (Figs. 1e and S3).

Hematochezia occurred in 3 of the 20 GACI survivors during the newborn period (patient 13), at 2.5 weeks (patient 4), and at 4 months of age (patient 10).

Table 1 Patient summary and clinical presentation of the full cohort.

Family	Patient	Pathogenic variants[a]		Age at presentation	Age at diagnosis			Hearing loss[b]	Age at last examination	Comorbidities
		cDNA	Protein		GACI	ARHR2	PXE			
ENPP1 (transcript: NM_006208.2) deficiency										
1	1	c.1441C>T **c.2312-5_2313del**	p.(Arg481Trp)	44 days	47 days	8 years 3 months		Conductive, 8 years 3 months	8 years 3 months	Hypertension
1	2	c.1441C>T **c.2312-5_2313del**	p.(Arg481Trp)	4 years		9 years 2 months		None	13 years 2 months	Hypertension
2	3	c.1438T>C c.2414G>T	p.(Cys480Arg) p.(Gly805Val)	8 months		4 years 4 months	4 years 7 months	Mixed, 3 years 10 months	8 years 11 months	Loeys–Dietz syndrome, chronic lung disease, hypertension
2	4	c.1438T>C c.2414G>T	p.(Cys480Arg) p.(Gly805Val)	Prenatally (38 wga)	Prenatally (38 wga)	3 years 4 months		None	4 years 5 months	None
3	5	**c.2735T>C** **3.4 kb deletion of exon 6 (delIVS5_IVS6)**	**p.(Leu912Ser)**	0 days	21 days			Conductive, 1 year 9 months	4 years 2 months	Neurological disorder (developmental delay)
4	6	c.1441C>T c.2713_2717del	p.(Arg481Trp) p.(Lys905Alafs*16)	Prenatally (20 wga)	Prenatally (20 wga)	8 months		Conductive, 4 years 1 month	5 years 1 month	None
5	7	c.1538A>G c.1538A>G	p.(Tyr513Cys) p.(Tyr513Cys)	2 years	2 years 8 months		2 years 2 months	Mixed, 1 year 4 months	7 years 11 months	Cerebrovascular disease, congestive heart failure, neurological disorder (cerebral palsy, developmental delay, epilepsy, hypotonia), hypertension
6	8	c.749C>T c.913C>A	p.(Pro250Leu) p.(Pro305Thr)	12 days	25 days	8 years 2 months	26 years 4 months	None	26 years 4 months	None
7	9	c.749C>T c.749C>T	p.(Pro250Leu) p.(Pro250Leu)	Prenatally (31 wga)	Prenatally (31 wga)	32 days		Conductive, 6 months	1 year 8 months	None (some developmental delay)
8	10	**c.1652A>G** c.2330A>G	**p.(Tyr551Cys)** p.(His777Arg)	10 days[c]				Conductive, 5 years 1 month	5 years 2 months	Brugada syndrome type 2, cerebrovascular disease (seizures), hypertension
9	11	c.1412A>G c.1442G>A	p.(Tyr471Cys) p.(Arg481Gln)	29 days	71 days	14 years 7 months		Conductive, 7 years 6 months	25 years 7 months	Hypertension
10	12	**c.1652A>G** c.1737G>C	**p.(Tyr551Cys)** p.(Leu579Phe)	0 days	4 months			None	2 years 1 month	Hypertension
11	13	c.913C>A c.1499A>C	p.(Pro305Thr) p.(His500Pro)	0 days	6 days	6 years 7 months		Mixed, 3 years	8 years 11 months	None
12	14	c.913C>A c.2662C>T	p.(Pro305Thr) p.(Arg888Trp)	Prenatally (32 wga)	1 days			None	9 months	Renal disease, hypertension
13	15	c.2320C>T c.2662C>T	p.(Arg774Cys) p.(Arg888Trp)	Prenatally (31 wga)	Prenatally (31 wga)	2 years 6 months		Conductive, 3 years 6 months	6 years 4 months	Phenylketonuria
14	16	**c.803**A>G **c.2596**G>A	**p.(Tyr268Cys)** **p.(Glu866Lys)**	4 years		56 years 2 months	43 years	None	58 years 3 months	Renal disease, diabetes mellitus, hypertension, hyperlipidemia
ABCC6 (transcript: NM_001171.5) deficiency										
15	17	c.3940C>T c.3940C>T	p.(Arg1314Trp) p.(Arg1314Trp)	Prenatally (16 wga)	1 day			None	9 years	Cerebrovascular disease (intrauterine stroke), renal disease, neurological disorder
15	18	c.3940C>T c.3940C>T	p.(Arg1314Trp) p.(Arg1314Trp)	5 days	6 days			None	5 years 10 months	Cerebrovascular disease, congestive heart failure
16	19	c.2861_286del **Deletion of exons 2–31**	p.(Phe954_Leu955del)	3 months	5 months			None	3 years 5 months	Congestive heart failure, developmental delay
17	20	c.1171A>G	p.(Arg391Gly)	0 days	15 days			None	11 years 9 months	None

ARHR2 autosomal recessive hypophosphatemic rickets type 2, *cDNA* complementary DNA, *GACI* generalized arterial calcification of infancy, *PXE* pseudoxanthoma elasticum, *wga* weeks' gestational age.
[a]Novel variants in bold.
[b]Type of hearing loss, age of onset specified.
[c]Patient presented with diffuse vascular stenosis/fibromuscular dysplasia shortly after birth, confirmed ENPP1 deficiency at 2 years of age.

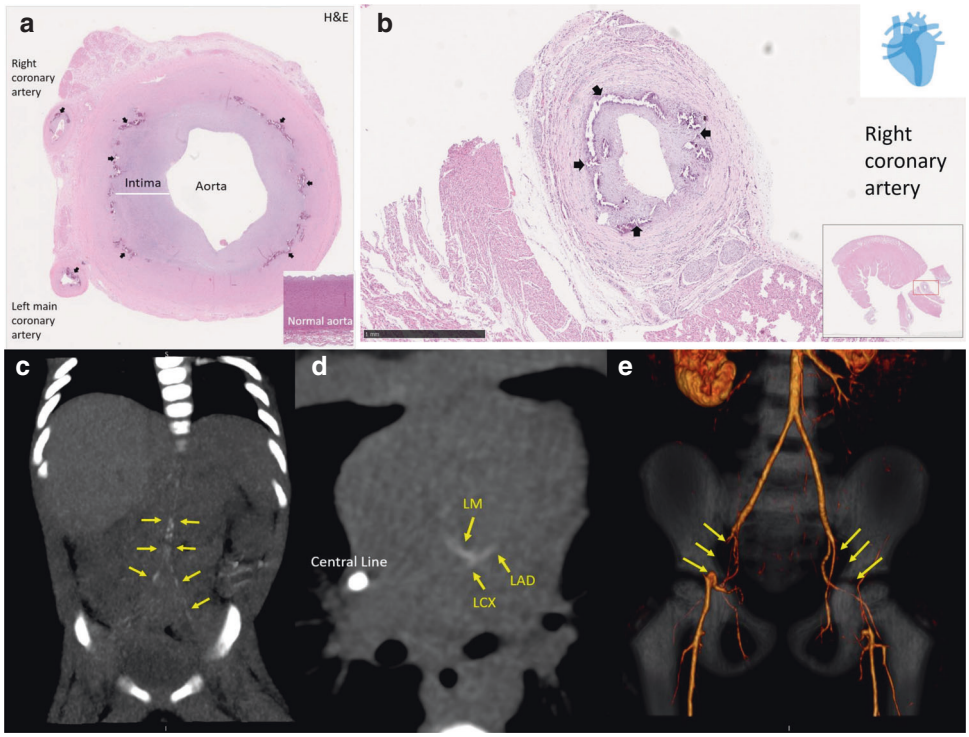

Fig. 1 Clinical presentation of ENPP1 deficiency. (**a**) Histopathology of the aorta of the first brother of patient 6 (deceased at 49 days), showing pronounced thickening of the tunica intima (indicated by white line, both in affected aorta and in the insert depicting a normal aorta) with consequent luminal narrowing, as well as internal elastic lamina distorted by dystrophic calcification (black arrows). (**b**) Histopathology of the heart of the second brother of patient 6 (deceased at 38 days), revealing deposition of calcium along the internal elastic lamina (disparate elastic fibers with severe degenerative changes separating the media from the intima), accompanied by fibrous thickening of the intima that results in luminal narrowing of the right coronary artery (hematoxylin and eosin [H&E]). (**c**) Coronal computed tomography (CT) of patient 6 at 4 weeks of life, showing calcification of the distal abdominal aorta and proximal bilateral iliac arteries (yellow arrows). (**d**) Axial CT scan of patient 6 at 4 weeks of life showing calcification (yellow arrows) of the left main (LM), left anterior descending (LAD) and left circumflex (LCX) coronary arteries. (**e**) Three-dimensional CT reconstruction of patient 10 at 5 years old revealing bilateral external iliac artery occlusion (yellow arrows) with prominent collaterals.

Four of 17 families (23%) experienced recurrent pregnancy loss involving 4–6 spontaneous abortions each (Fig. S1). Factor V Leiden was found in one of those families (family 4), but in the other families the etiology remained unexplained.

Treatment

Fifteen patients received some form of bisphosphonate; 12 received etidronate, 8 pamidronate, and 1 risedronate. The median age at initiation of bisphosphonate use was 32 days, with ten patients beginning treatment before 2 months of age (in four of those cases, within the first week of life). The mean length of treatment duration was 480 days (range: 60–835 days). Only one of the deceased siblings received bisphosphonates, from birth until the time of death at 1 month of age. Eight patients received oral phosphate supplementation and/or an active form of vitamin D for the management of hypophosphatemia; after at least 7 months of therapy, there was no significant worsening of vascular calcification by CT. The median age at initiation of rickets treatment was 5.5 years (range: 1.5 months–14.6 years). Medications for heart failure were used in 13 patients and antihypertensives were employed for 12 patients.

Phenotypic variability

In family 1, patient 2 presented in childhood with bone pain and deformities related to ARHR2, while her younger brother (patient 1) presented at 7 weeks with severe GACI leading to cardiac arrest, resuscitation, and extracorporeal membrane oxygenation. In family 2, patient 3 presented with periarticular calcifications of both shoulders in the absence of vascular calcification, while his younger sister (patient 4) had cardiovascular calcifications diagnosed in utero. In family 15, two siblings had one of the five most common missense variants associated with PXE,[20] a condition that typically does not present with vascular calcification until adulthood. Despite this, the older brother presented in utero with strokes leading to devastating neurologic sequelae, and the younger sister experienced neonatal stroke with residual contralateral paraparesis.

Incidence

Table S1 lists all *ENPP1* variants included and excluded from the calculation of the expected incidence of GACI. The allele counts for known and predicted pathogenic variants were 93 and 179, respectively, for a total of 272 over 121,412 alleles. The pathogenic allele frequency is thus 0.224%, or 1 in 446,

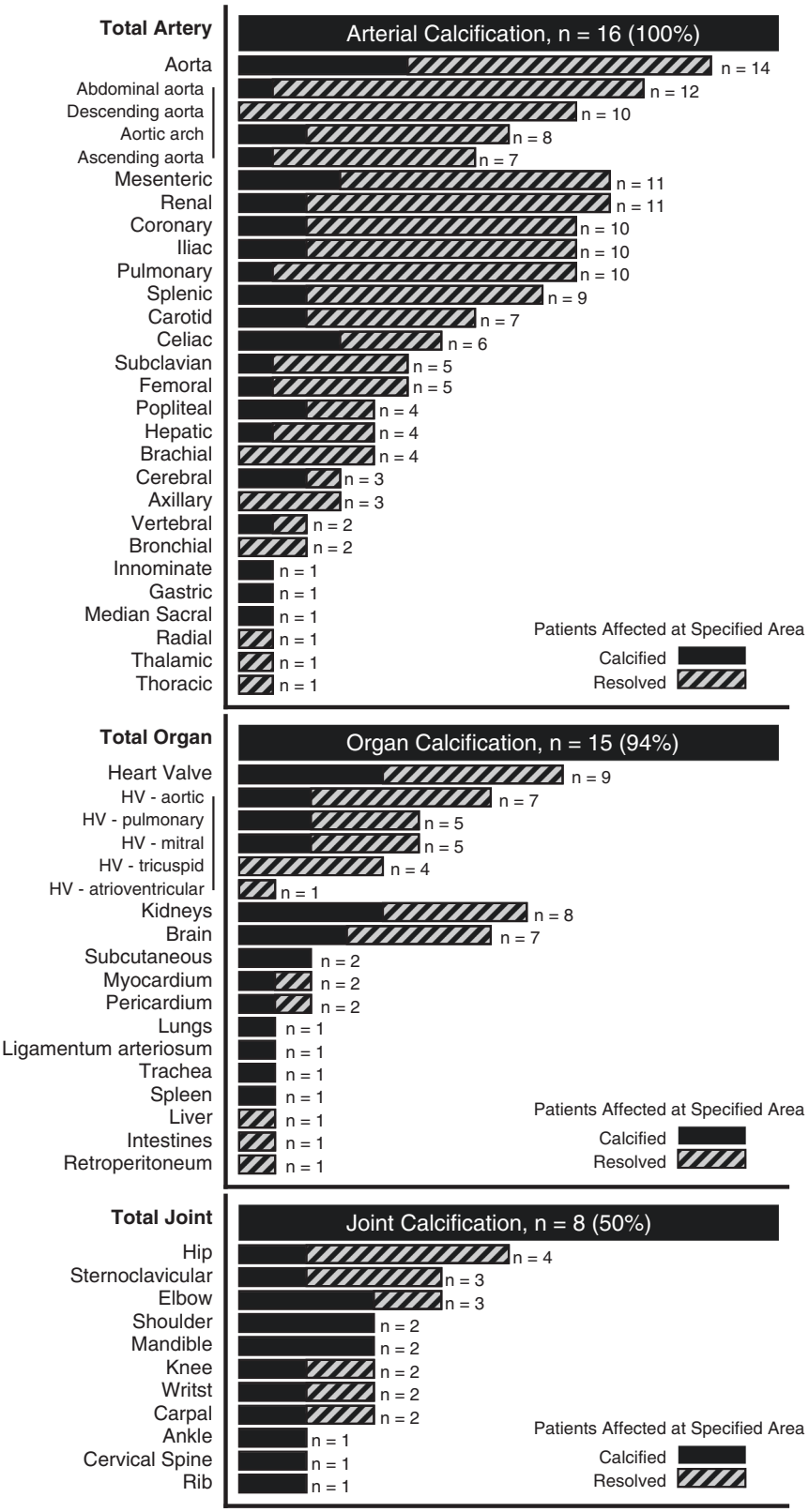

Fig. 2 Calcification of arteries, joints and organs in patients with generalized arterial calcification of infancy (GACI). The total length of each bar represents the frequency of calcification in affected patients, while the hatched bars represent the percentage of patients that showed resolution of calcification at last examination.

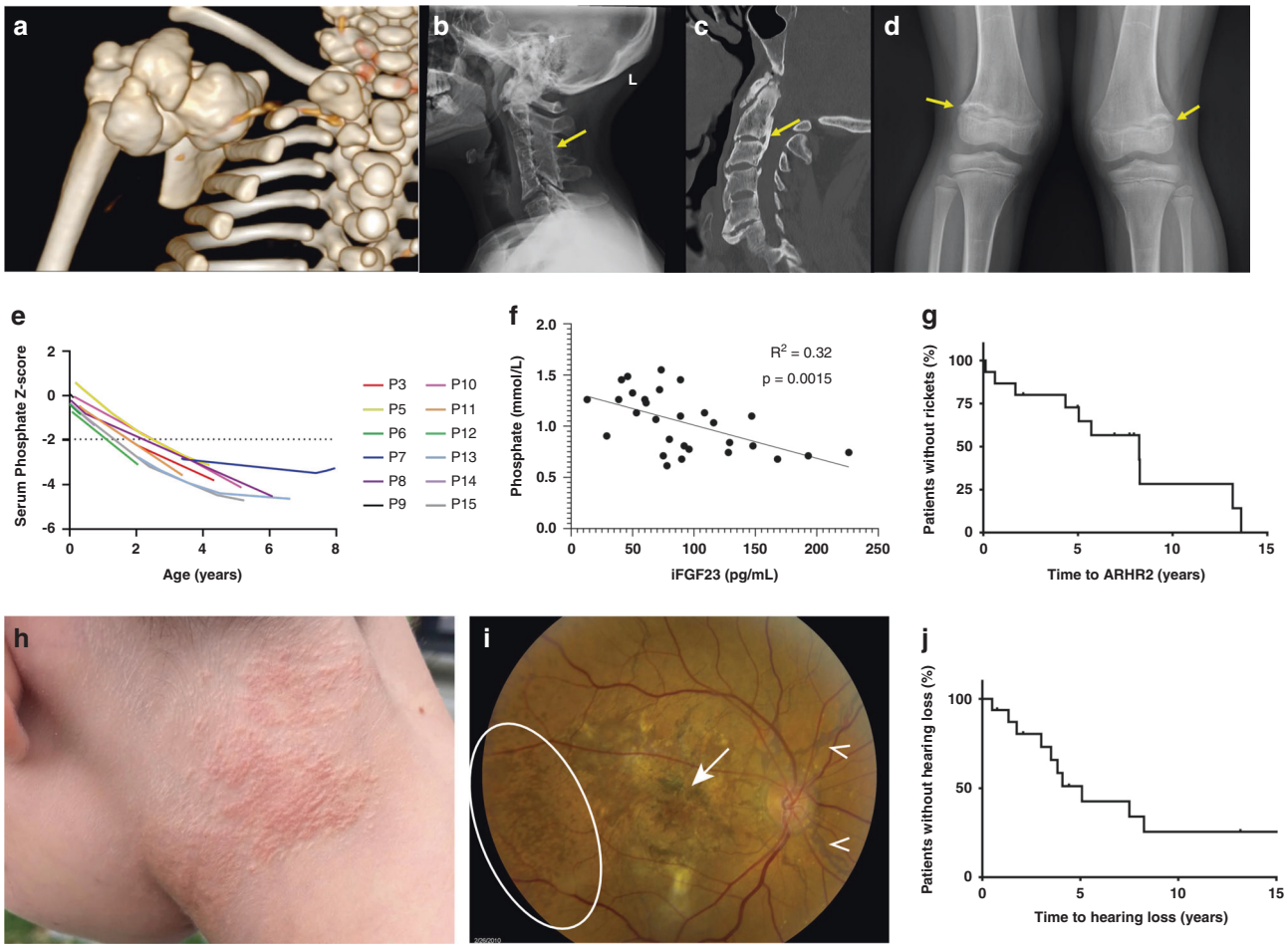

Fig. 3 Rickets in ENPP1 deficiency. (**a**) Three-dimensional computed tomography (CT) reconstruction showing periarticular calcification of the shoulder of patient 9 at 4 days of life. (**b**) Fusion of the C2–C3 and C4–C5 posterior vertebral bodies, articular processes, and laminae (patient 11, 25.5 years). (**c**) Calcification of the posterior longitudinal ligament enthesis (patient 16, 56.2 years). (**d**) Metaphyseal irregularities of the lateral distal femora as a result of untreated rickets (patient 13, 6.6 years). (**e**) Serum phosphate decreased with age; dashed line represents lower limit of normal (*Z*-score −1.96). (**f**) Correlation of serum phosphate and intact FGF23 levels. (**g**) Kaplan–Meier curve showing the probability of remaining free of hypophosphatemic rickets in the subpopulation of patients with generalized arterial calcification of infancy (GACI) due to *ENPP1* variants. (**h**) Neck skin in patient 3 at 8.9 years showing classical findings of pseudoxanthoma elasticum (PXE). (**i**) Fundus photography in patient 16 at 56 years of age, demonstrating known retinal complications of PXE, i.e., macular hemorrhage (arrows), angioid streaks (carets), and peau d'orange (oval). (**j**) Kaplan–Meier curve showing the probability of remaining free of hearing loss in the subpopulation of patients with GACI due to *ENPP1* variants. *ARHR2* autosomal recessive hypophosphatemic rickets type 2.

yielding a carrier frequency of 1 in 223 individuals in the general population and a predicted disease incidence of 1 in 199,244 pregnancies.

DISCUSSION

Our comprehensive evaluations of GACI survivors has revealed several significant new findings.

Rickets

The development of rickets in survivors of ENPP1-GACI appears universal by age 14 years. The inverse relationship between serum phosphate and iFGF23 and inappropriately normal 1,25-dihydroxyvitamin D suggest that the hypophosphatemia is FGF23-mediated.

Burosumab is a recently approved human monoclonal antibody against FGF23. Since rickets related to ENPP1 deficiency is FGF23-dependent, at least in principle one might consider the use of burosumab for the treatment of these patients. However, FGF23 suppresses alkaline phosphatase,[21] so that FGF23 inhibition might lead to alkaline phosphatase upregulation, which in turn would cause a further decrease in PPi. Thus, there is a theoretical concern that burosumab use could lead to worsening of ectopic calcifications in ENPP1-deficient patients.

Ectopic calcification

The presence or pathologic effects of ectopic calcification in GACI may not be fully appreciated. Because 80% of subjects showed evidence of very early-onset of arterial calcifications, cardiovascular calcification or intimal proliferation during fetal development could be responsible for the high frequency of recurrent pregnancy loss (≥4 miscarriages per family), i.e., 23%, compared with the general population rate of 1–2%.[22] Similarly, the hematochezia seen in 15% of GACI survivors

Table 2 Biochemical findings of the full cohort.

Family	Patient	Age at data collection	iFGF23	C-FGF23	Ionized calcium	Serum phosphate	Alkaline phosphatase	Parathyroid hormone	25-OH-Vit D	1,25-diOH-Vit D	TRP	TmP/GFR
Normal values (units):			≤50 (pg/mL)	≤230 (RU/mL)	1.15–1.27 (mmol/L)	Age dependent,[h] mmol/L (mg/dL)	Age dependent,[i] U/L (μkat/L)	10–65 ng/L	14–60 ng/mL (35–150 nmol/L)	25–45 pg/mL (60–108 pmol/L)	>85%	Age dependent,[i] mmol/L (mg/dL)
1	1	8 years, 3 months	75	176	1.24	0.71 (2.2)	544 (9.1)	28.7	32 (79.9)	37 (96.2)	85.9	0.61 (1.89)
	2	13 years, 2 months	90	180	1.19	0.68 (2.1)	301 (5.0)	62	13 (32.4)	61 (158.6)	83.9	0.57 (1.76)
2	3	4 years, 4 months	89	139	1.12	1.10 (3.4)	314 (5.2)	53.6	22 (54.9)	38 (98.8)	85.9	0.94 (2.91)
		4 years, 10 months[a]	**-**	**-**	**1.18**	**1.07 (3.3)**	**261 (4.4)**	**51.3**	**37 (92.4)**	**46 (119.6)**	**82.9**	**0.88 (2.72)**
		5 years, 11 months[a]	**-**	**-**	**-**	**0.94 (2.9)**	**302 (5.0)**	**42.3**	**32 (79.9)**	**22 (57.2)**	**84.9**	**0.75 (2.31)**
		6 years, 11 months[a]	**-**	**167**	**1.15**	**0.97 (3.0)**	**278 (4.6)**	**42.4**	**37 (92.4)**	**28 (72.8)**	**86.9**	**0.84 (2.60)**
	4	**1 years, 10 months[b]**	**46**	**177**	**1.20**	**1.49 (4.6)**	**204 (3.4)**	**63.5**	**18 (44.9)**	**152 (395.2)**	**90.7**	**1.47 (4.56)**
		2 years, 4 months[c]	**-**	**-**	**1.25**	**1.10 (3.4)**	**191 (3.2)**	**52**	**38 (94.8)**	**37 (96.2)**	**81.8**	**0.90 (2.79)**
		3 years, 5 months[c]	**-**	**-**	**-**	**1.07 (3.3)**	**322 (5.4)**	**29.5**	**40 (99.8)**	**42 (109.2)**	**87.8**	**0.94 (2.92)**
		4 years, 6 months[a]	**-**	**158**	**1.15**	**1.10 (3.4)**	**333 (5.6)**	**34.4**	**45 (112.3)**	**49 (127.4)**	**82.0**	**0.90 (2.79)**
3	5	1 year, 3 months	73	198	1.30	1.55 (4.8)	317 (5.3)	29.8	36 (89.9)	-	87.8	1.37 (4.25)
		2 years, 2 months	-	-	1.32	1.39 (4.3)	368 (6.1)	31.5	34 (84.9)	75 (195.0)	90.6	1.37 (4.25)
		4 years, 2 months	-	150	1.22	1.29 (4.0)	414 (6.9)	65.8	30 (74.9)	32 (83.2)	92.9	1.41 (4.35)
4	6	**4 months[d]**	**39**	**150**	**1.30**	**1.26 (3.9)**	**392 (6.5)**	**28.2**	**-**	**146 (379.6)**	**93.2**	**1.38 (4.28)**
		11 months[d]	**147**	**-**	**-**	**1.10 (3.4)**	**-**	**-**	**-**	**-**	**88.5**	**1.00 (3.10)**
		1 years, 3 months[d]	**109**	**253**	**0.91**	**1.13 (3.5)**	**513 (8.6)**	**72.7**	**42 (104.8)**	**-**	**83.3**	**0.94 (2.91)**
		1 years, 8 months[d]	**148**	**198**	**1.12**	**0.81 (2.5)**	**387 (6.5)**	**44**	**60 (149.8)**	**63 (163.8)**	**72.7**	**0.59 (1.82)**
		2 years, 1 months[d]	**80**	**-**	**0.70**	**0.87 (2.7)**	**453 (7.6)**	**42.7**	**40 (99.8)**	**96 (249.6)**	**90.0**	**0.84 (2.60)**
		3 years, 1 month[a]	**708**	**834**	**7.38**	**1.23 (3.8)**	**293 (4.9)**	**10.7**	**33 (82.4)**	**69 (179.4)**	**84.1**	**1.03 (3.20)**
		4 years, 1 month[a]	**-**	**630**	**5.55**	**1.07 (3.3)**	**335 (5.6)**	**18.7**	**43 (107.3)**	**41 (106.6)**	**66.3**	**0.71 (2.19)**
		5 years, 1 month[a]	**129**	**184**	**1.98**	**0.84 (2.6)**	**463 (7.7)**	**29.5**	**59 (147.3)**	**18 (46.8)**	**72.7**	**0.61 (1.89)**
5	7	7 years, 11 months	29	104	1.20	0.90 (2.8)	173 (2.9)	85.9	19 (47.4)	63 (163.8)	88.9	0.83 (2.58)
6	8	**26 years[e]**	**128**	**180**	**1.21**	**0.74 (2.3)**	**96 (1.6)**	**67.9**	**34 (84.9)**	**45 (117.0)**	**57.6**	**0.43 (1.32)**
7	9	**1 years, 8 months[f]**	**92**	**416**	**1.20**	**0.81 (2.5)**	**505 (8.4)**	**76.9**	**38 (94.8)**	**49 (127.4)**	**-**	**-**
8	10	5 years, 2 months	61	125	1.17	1.23 (3.8)	147 (2.5)	36.6	-	-	86.0	1.06 (3.27)
9	11	**21 years[a]**	**226**	**339**	**1.23**	**0.74 (2.3)**	**180 (3.0)**	**11.5**	**-**	**57 (148.2)**	**73.3**	**0.55 (1.69)**
		25 years[g]	**78**	**124**	**1.23**	**0.61 (1.9)**	**121 (2.0)**	**30.6**	**25 (62.4)**	**-**	**72.1**	**0.44 (1.37)**
10	12	2 years, 3 months	69	56	1.35	1.07 (3.3)	201 (3.4)	7.5	31 (77.4)	135 (351.0)	85.6	0.91 (2.83)
11	13	6 years, 7 months	116	447	1.28	1.03 (3.2)	349 (5.8)	62.6	18 (44.9)	29 (75.4)	78.3	0.81 (2.50)
		8 years, 3 months[a]	**-**	**-**	**1.23**	**1.07 (3.3)**	**322 (5.4)**	**26.3**	**23 (57.4)**	**58 (150.8)**	**64.1**	**0.68 (2.12)**
		8 years, 11 months[a]	**133**	**219**	**1.20**	**0.84 (2.6)**	**371 (6.2)**	**43.4**	**31 (77.4)**	**31 (80.6)**	**63.1**	**0.53 (1.64)**
12	14	9 months	72	217	-	1.36 (4.2)	568 (9.5)	42.3	35 (87.4)	65 (169.0)	-	-
13	15	5 years, 3 months	96	-	1.19	0.78 (2.4)	482 (8.0)	40.3	61 (152.3)	38 (98.8)	97.7	1.04 (3.22)

Table 2 continued

Family	Patient	Age at data collection	iFGF23	C-FGF23	Ionized calcium	Serum phosphate	Alkaline phosphatase	Parathyroid hormone	25-OH-Vit D	1,25-diOH-Vit D	TRP	TmP/GFR
		6 years, 4 months[a]	**-**	**112**	**1.22**	**0.71 (2.2)**	**511 (8.5)**	**22.2**	**62 (154.8)**	**70 (182.0)**	**94.4**	**0.82 (2.55)**
		7 years, 11 months[a]	**-**	**207**	**1.17**	**0.90 (2.8)**	**462 (7.7)**	**25.6**	**63 (157.2)**	**69 (179.4)**	**-**	**-**
	16	44 years	-	-	-	0.94 (2.9)	78 (1.3)	-	-	-	-	-
		56 years	168	329	1.17	0.68 (2.1)	114 (1.9)	61.5	21 (52.4)	77 (200.2)	82.3	0.77 (2.39)
15	17	8 years	50	113	1.21	1.32 (4.1)	91 (1.5)	26.7	36 (89.9)	146 (379.6)	97.2	1.74 (5.38)
	18	5 years, 10 months	89	123	1.25	1.45 (4.5)	269 (4.5)	28.2	23 (57.4)	116 (301.6)	92.2	1.53 (4.75)
16	19	2 years, 3 months	53	420	1.3	1.13 (3.5)	976 (16.3)	-	49 (122.3)	56 (145.6)	88.6	1.03 (3.19)
		3 years, 5 months	60	157	1.3	1.26 (3.9)	275 (4.6)	33.2	43 (107.3)	59 (153.4)	74.6	0.94 (2.91)
17	20	1 year, 3 months	-	-	-	2.07 (6.4)	452 (7.5)	10.2	-	-	-	-
		1 year, 6 months	-	-	-	1.87 (5.8)	419 (7.0)	12.2	-	-	-	-
		1 year, 10 months	-	-	-	1.84 (5.7)	327 (5.5)	8.8	27 (67.4)	31 (80.6)	-	-
		2 years, 4 months	-	-	-	1.20 (3.7)	357 (6.0)	15.7	20 (49.9)	57 (148.2)	-	-
		3 years, 7 months	-	-	-	1.42 (4.4)	282 (4.7)	23	57 (142.3)	64 (166.4)	99.7	2.10 (6.49)
		5 years	-	-	-	1.29 (4.0)	271 (4.5)	22	42 (104.8)	-	94.0	1.47 (4.56)
		6 years, 7 months	41	93	-	1.45 (4.5)	381 (6.4)	37.8	32 (79.9)	-	93.8	1.64 (5.07)
		10 years, 3 months	13	73	-	1.26 (3.9)	410 (6.8)	47.2	21 (52.4)	76 (197.6)	-	-
		12 years, 5 months	-	-	-	1.71 (5.3)	529 (8.8)	-	-	-	-	-

Bold values were measured while patients were taking medications as noted by superscripts a–g.
25-OH-vit D 25-hydroxyvitamin D, *1,25-diOH-vit D* 1,25-dihydroxyvitamin D, *iFGF23* intact FGF23, *C-FGF23* C-terminal FGF23, *TmP/GFR* tubular maximum reabsorption of phosphate to glomerular filtration rate, *TRP* tubular reabsorption of phosphate.
[a]Calcitriol and phosphate.
[b]Ergocalciferol.
[c]Ergocalciferol and phosphate.
[d]Cholecalciferol.
[e]Alfa-calcidol and phosphate.
[f]Cholecalciferol and phosphate.
[g]Calcitriol.
[h]Age-dependent reference range as per Lockitch et al.[13]
[i]Age-dependent reference range as per Estey et al.[18]
[j]Age-dependent reference range as per Stark et al.[19]

could be related to mesenteric ischemia; the frequency of cow's milk allergy—the most common cause of hematochezia in infancy—in the general population approximates 2%.[23]

Extravascular ectopic calcification also occurred commonly in the joints, organs, and other tissues of GACI survivors. Fusion of the cervical spine, reported only twice previously,[3,11] affected 25% of our ENPP1-GACI patients and involved mainly the posterior vertebral bodies and neural arches. Painful calcification of the attachments of tendons or ligaments was present in all three adults in our cohort, and appear to represent a late complication of GACI. Calcifications of the entheses were previously described in a 53-year-old woman[24] and 62-year-old woman[25] with ARHR2. In X-linked hypophosphatemia (XLH), the most common genetic form of FGF23-mediated hypophosphatemic rickets, calcification of the enthesis also develops with increasing age[26] and impairs quality of life.[27] The pathophysiology of enthesis calcification remains speculative, but it is likely directly related to FGF23 since it is present in other forms of FGF23-mediated hypophosphatemia[28] but absent from patients with *SLC34A3* variants, a genetic form of hypophosphatemic rickets not mediated by FGF23.[29] A mouse model of ENPP1 deficiency recapitulates the phenotype of calcification of fibrocartilage (present in entheses), tendons, and ligaments.[30]

Even the hearing loss of GACI might be attributable to calcification. A mouse model of ENPP1 deficiency develops progressive conductive hearing loss, with otitis media, aseptic effusion, fusion of malleus and incus, and thickening and overcalcification of the stapedial artery.[31] Although hearing loss was previously described in only four of more than 50 patients with confirmed ENPP1 deficiency,[9] the majority of our ENPP1-GACI patients exhibited hearing loss. Similar to the mouse model, our patients' hearing loss appeared to be progressive, since most patients passed a newborn hearing screen. Thus, we recommend audiologic assessment on an annual basis, as decreased hearing, when unaddressed, can affect school performance.

PXE-like changes

While it is widely recognized that individuals with biallelic *ABCC6* pathogenic variants can also manifest GACI, we found that patients with biallelic *ENPP1* variants can present with findings of PXE after surviving infancy. Angioid streaks, a typical retinal finding of PXE, were previously described in a 5-year-old child with ENPP1 deficiency,[3] but one of our patients developed retinal hemorrhage with subsequent macular scarring and legal blindness. The shared phenotypes associated with *ENPP1* and *ABCC6* variants suggest that modifying genes are in play.

Incidence

We estimate the incidence of ENPP1-GACI, based upon the frequency of pathogenic variants, as ~1 in 200,000. The disorder might be more common than previously thought and calls for greater recognition by obstetricians and neonatologists. The database we utilized accounts for diverse populations, including 55% non-Finnish European individuals, 13.6% South Asians, 9.5% individuals of Latino descent, 8.6% individuals of African or African American descent, 7.1% East Asians, and 5.4% persons of Finnish heritage;[13] notwithstanding, the incidence of GACI will vary depending on the specific population studied.

Genotype–phenotype correlation

Comparison of clinical and molecular findings in our cohort suggests significant phenotypic heterogeneity, even among siblings with identical genotypes. Five siblings of the 20 individuals who survived GACI as infants had severe enough disease to be fatal; four of the five deceased patients were born prior to the birth of the surviving sibling, which makes it possible that subsequent siblings survived due to earlier recognition and management of their disease. Despite this, surviving sibling pairs manifested substantial differences in the severity of their disease, arguing strongly against a genotype–phenotype correlation in GACI.

Unresolved issues

Despite the new understanding of some features of GACI, several issues remain unresolved. Is there a pathophysiological link between the resolution of ectopic calcification in GACI and the later development of hypophosphatemic rickets? It is known that PPi inhibits mineralization and inorganic phosphate (Pi) stimulates it. ENPP1 deficiency reduces PPi, and this deficit is exacerbated during the second half of pregnancy by increases in placental alkaline phosphatase, which cleaves PPi to Pi;[32] this drastically increases the Pi/PPi ratio in utero. After birth, however, two compensatory mechanisms ensue. First, the overriding influence of placental alkaline phosphatase vanishes, allowing an increase in PPi. Second, renal glomerular function matures postnatally, increasing Pi clearance.[33] This may promote resolution of vascular calcification and possibly contribute to survival in some. Later, excess FGF23 production further increases phosphate excretion, causing hypophosphatemia and rickets. However, the exact mechanism by which ENPP1 induces FGF23 excess, which may be beneficial to patients, remains unknown.

This study also highlights the controversy regarding therapy of ARHR2. It is reasonable to be concerned that treating GACI survivors with calcitriol and phosphorus might induce progression or recurrence of vascular calcification. In addition, phosphorus supplementation could lead to even higher concentrations of FGF23, an independent cardiovascular risk factor.[34] Moreover, although standard therapy of hypophosphatemic rickets apparently does not worsen the enthesopathy in XLH patients,[35] it can be associated with hyperparathyroidism[36] and exacerbate nephrocalcinosis.[37] Consequently, some GACI survivors remain untreated and experience bone pain, deformities, and short stature from their rickets. Hence, we previously reported one subject (patient 11 in the current cohort) in whom vascular calcification did not recur after years of vitamin D and

phosphate treatment for ARHR2,[38] and our current cohort includes another seven individuals who received judicious treatment of rickets without noticeable worsening of vascular calcification.

Another GACI mystery involves the relationship between ENPP1 and ABCC6 deficiencies, since the phenotypes of these two disorders overlap. Biochemically, ENPP1 deficiency results in reduced PPi and AMP, and the common occurrence of vascular calcification in ENPP1-GACI and in PXE has led to the interpretation that reduced PPi levels are responsible for the pathology of both diseases. However, AMP deficiency could be the pivotal parameter shared by ENPP1-GACI and PXE,[39] since AMP has protean biochemical effects. Specifically, intimal proliferation results from AMP or adenosine deficiency[40] and comprises part of the ENPP1-GACI phenotype. In fact, one of our patients (patient 10) with ENPP1 deficiency did not develop any vascular calcification, but did manifest multivessel narrowing and was diagnosed with pediatric fibromuscular dysplasia (FMD). Intimal fibroplasia is common in FMD, and affected individuals might benefit from *ENPP1* sequencing.

Strengths and limitations

The strengths of our study include the size of the cohort, its molecular characterization, and the uniform and comprehensive evaluation of patients in a prospective fashion. Nevertheless, we were unable to address whether bisphosphonates are helpful in GACI, since we evaluated only survivors of the disease. However, 5 of the 20 survivors did not receive bisphosphonates, suggesting that survival of bisphosphonate-treated patients may not be due to the treatment.

Conclusion

With a predicted incidence of 1 in 200,000 pregnancies, there should be 20 new cases of GACI every year in the United States alone. As new therapies are developed to treat GACI and ARHR2, additional prospective studies to elucidate the natural history of these disorders will help identify drug targets and outcome parameters for clinical trials.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-020-00983-0) contains supplementary material, which is available to authorized users.

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DISCLOSURE

C.R.F., R.I.G., W.A.G., and M.E.H. report a collaboration with Inozyme Pharma as part of a Cooperative Research and Development Agreement (CRADA). Inozyme is developing ENPP1 as therapy for ARHR2 and GACI. S.W. and K.M. are employees of ICON plc, a contract research organization. The other authors declare no conflicts of interest.

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