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Genetic Screening of Anderson-Fabry Disease in Probands Referred From Multispecialty Clinics

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ABSTRACT

BACKGROUND Anderson-Fabry disease (AFD) is a rare X-linked lysosomal storage disease, caused by defects of the alpha-galactosidase A (*GLA*) gene. AFD can affect the heart, brain, kidney, eye, skin, peripheral nerves, and gastrointestinal tract. Cardiology (hypertrophic cardiomyopathy), neurology (cryptogenic stroke), and nephrology (end-stage renal failure) screening studies suggest the prevalence of *GLA* variants is 0.62%, with diagnosis confirmation in 0.12%.

OBJECTIVES This study sought to expand screening from these settings to include ophthalmology, dermatology, gastroenterology, internal medicine, pediatrics, and medical genetics to increase diagnostic yield and comprehensively evaluate organ involvement in AFD patients.

METHODS In a 10-year prospective multidisciplinary, multicenter study, we expanded clinical, genetic, and biochemical screening to consecutive patients enrolled from all aforementioned clinical settings. We tested the *GLA* gene and α-galactosidase A activity in plasma and leukocytes. Inclusion criteria comprised phenotypical traits and absence of male-to-male transmission. Screening was extended to relatives of probands harboring *GLA* mutations.

RESULTS Of 2,034 probands fulfilling inclusion criteria, 37 (1.8%) were carriers of *GLA* mutations. Cascade family screening identified 60 affected relatives; clinical data were available for 4 affected obligate carriers. Activity of α-galactosidase A in plasma and leukocytes was diagnostic in male subjects, but not in female subjects. Of the 101 family members harboring mutations, 86 were affected, 10 were young healthy carriers, and 5 refused clinical evaluation. In the 86 patients, involved organs or organ systems included the heart (69%), peripheral nerves (46%), kidney (45%), eye (37%), brain (34%), skin (32%), gastrointestinal tract (31%), and auditory system (19%). Globotriaosylceramide accumulated in organ-specific and non-organ-specific cells in atypical and classic variants, respectively.

CONCLUSIONS Screening probands with clinically suspected AFD significantly increased diagnostic yield. The heart was the organ most commonly involved, independent of the clinical setting in which the patient was first evaluated. (J Am Coll Cardiol 2016;68:1037–50) © 2016 by the American College of Cardiology Foundation.

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ABBREVIATIONS AND ACRONYMS

AFD = Anderson-Fabry disease

Gal = galactosidase A (enzyme)

Gb3 = globotriaosylceramide

HCM = hypertrophic cardiomyopathy

LV = left ventricle

LVH = left ventricular hypertrophy

PBMC = peripheral blood mononuclear cell(s)

TIA = transient ischemic attack

WML = white matter lesions

Anderson-Fabry disease (AFD) is an X-linked disorder caused by deficiency of the lysosomal enzyme α-galactosidase A (a-Gal) (1), resulting in intracellular accumulation of globotriaosylceramide (Gb3) and multiorgan system damage. AFD prevalence at birth ranges between 1 in 40,000 and 1 in 110,000, based on level of α-Gal activity (1–3). The diagnosis most often results from screening large series of patients with phenotypic traits commonly observed in AFD (4–6). In a recent systematic review of screening studies of high-risk populations, the overall prevalence of individuals with alpha-galactosidase A (*GLA*) gene variants was 0.62% (including genetic variants of unknown significance); prevalence of a definitive diagnosis was 0.12% (7).

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Often associated with a delayed diagnosis, AFD is otherwise characterized by variability in age of onset, phenotype (including the early, severe classic phenotypes as well as the later, more mild presentation) (3), and phenotype among carriers of the same mutations within families (3,8,9). Male subjects with the classic form of AFD and low or absent enzymatic activity develop early signs and symptoms in childhood or adolescence (acroparesthesias/neuropathic pain, angiokeratomas, gastrointestinal symptoms, corneal opacities) (10). Vascular complications, renal failure, thickening of the left ventricular (LV) walls mimicking sarcomeric hypertrophic cardiomyopathy (HCM), cryptogenic stroke, or transient ischemic attack (TIA) are features that may occur in adults (4–6,8). Patients with atypical variants may develop late-onset renal failure, left ventricular hypertrophy (LVH), cerebrovascular disease, or a combination thereof (3,11). Female subjects experience later onset of disease and, typically, exhibit heterogeneous and milder phenotypes (3,8,9,11). The residual enzyme activity in female heterozygotes may be normal, limiting the role of ascertaining α-Gal activity (12–14). Thus, genetic testing is necessary to confirm the diagnosis in female subjects. The integrated phenotypic and genetic diagnosis provides the basis for enzyme replacement therapy, though evidence-based clinical benefits when the heart, kidney, and brain are involved are limited by low numbers of randomized controlled trials (15).

Our study was designed to screen patients from all clinical settings pertinent to AFD to increase the yield of diagnoses of AFD in high-risk cohorts of patients presenting with different but relevant phenotypic traits (isolated or combined) and accurately describe organ involvement in patients/relatives (16).

METHODS

In 2004, we established a prospective multidisciplinary and multicenter study, evaluating patients with a high degree of suspicion for AFD presenting to cardiology, neurology, nephrology, pediatrics, ophthalmology, dermatology, gastroenterology, internal medicine, and genetics clinics. These patients underwent comprehensive clinical evaluation, genetic counseling, pathologic analysis, and cascade family screening. Plasma and leukocyte enzyme

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activity assays and genetic testing were centralized in 2 core labs, 1 for the former (overseen by M.M.) and 1 for the latter (overseen by E.Ar.). Clinical evaluation included the following: physical examination to assess overt traits such as "Fabry face" and angiokeratomas; cardiological work-up with visit, electrocardiography, echocardiography, and cardiac magnetic resonance imaging (when accepted and possible); neurologic evaluation with magnetic resonance imaging, according to the neurologist's indications; nephrology evaluation, and related biochemical and imaging investigation in patients with otherwise unexplained proteinuria, increased urinary albumin excretion, abnormal glomerular filtration rate, and estimated glomerular filtration rate, up to severe chronic renal failure; ophthalmologic evaluation for corneal deposits and other traits associated with AFD (e.g., retinal vessel tortuosity and cataracts); pediatric evaluation in children with acroparesthesia/neuropathic pain, gastrointestinal disturbances after exclusion of diseases such as celiac disease and inflammatory bowed disease; dermatologic visit with dermoscopy and biopsy, when needed or feasible, of skin lesions; and psychological support, when accepted, by patients or their relatives.

The project was approved by the ethical committees of all participating centers. Each specialist participating in the project proceeded with multidisciplinary evaluation upon identifying a tell-tale clinical trait suggestive of AFD. **Table 1** shows major inclusion criteria based on organ system (details in the Online Appendix). A web-assisted database was developed to collect clinical information. Family screening was offered to all relatives of probands, including clinical and imaging evaluation, as well as genetic testing and determination of enzyme level assay. We used the MOGE(S) (morphofunctional, organ involvement, genetic or familial, etiology, stage) classification system (16) for genotype/phenotype description.

GENETIC TESTS AND ASSAYS. We sequenced coding and flanking regions of the *GLA* gene (Online Appendix) and performed multiple ligation-dependent probe amplification in male patients who demonstrated low α-Gal activity in plasma or granulocytes but tested normal at sequencing. The analysis included intronic haplotypes potentially associated with decreased enzyme activity (17). The *IVS4+919 G>A* mutation that has been reported as common in late onset Chinese and Taiwanese AFD patients (18,19) was investigated by sequencing using specific primers. Mutations and variants of unknown significance have been defined according to guidelines (20); criteria are in the Online Appendix.

In 8 patients with LVH (>15 mm), we additionally sequenced the HCM gene panel to exclude the concomitant, coincidental presence of 2 different genetic diseases. In patients with cryptogenic stroke or TIA and carriers of the p.(Asp313Tyr) variant, we sequenced the mitochondrial deoxyribonucleic acid region encompassing *MT-TL1* and *MT-RNR2* genes commonly associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome mutations (21).

We assessed α-Gal activity in blood plasma and peripheral blood leukocytes using fluorimetry and 4-methylumbelliferyl-a-D-galactopyranoside as a substrate in the presence of N-acetylgalactosamine (Online Appendix). We generated standards for

TABLE 1 Clinical Criteria for Enrollment

Clinical Setting	n	Age (yrs)	Mutated Probands	Inclusion Criteria → Common to All Disciplines*
Neurology	1,323	48 (40–55)	17 (1.28)	Cryptogenic stroke, TIA, migraine with/without aura with imaging showing WML
Cardiology	473	46 (33–59)	8 (1.69)	HCM, concentric mild LVH, no LV outflow tract obstruction
Nephrology	72	51.5 (46–64)	2 (2.7)	Chronic renal failure; proteinuria, increased urinary albumin excretion
Ophthalmology	23	43 (35–51)	3 (13)	Noniatrogenic cornea verticillata, juvenile cataract, retinal vessel tortuosity
Pediatrics	41	13 (8.5–15)	3 (7.3)	Abdominal crises of pain with diarrhea after exclusion of CD and IBD; acroparesthesias/neuropathic pain, heat intolerance
Internal medicine/ gastroenterology	47	45 (36–53)	3 (6.3)	Gastrointestinal and multiorgan/tissue disturbances/involvement
Medical genetics	28	40 (39–53)	2 (7.1)	Multiorgan/tissue involvement suspected for AFD with evaluation of possible X-linked transmission in family pedigrees
Dermatology	27	45 (37–53)	0	Presence of angiokeratomas not exclusively located in typical "bathing suit" areas; labial and proximal nail fold telangiectasia

Values are n, median (interquartile range), or n (%). *Exclusion of most common causes of similar phenotypes and of probands from families with male-to-male transmission.
AFD = Anderson-Fabry disease; CD = Crohn's disease; HCM = hypertrophic cardiomyopathy; IBD = irritable bowel disease; LV = left ventricular; LVH = left ventricular hypertrophy; TIA = transient ischemic attack; WML = white matter lesions.

reference values of plasma and granulocyte α-Gal activity in a control group in accordance with the Standards for Reporting of Diagnostic Accuracy (22).

TISSUE BIOPSY AND IN VITRO CELLULAR STUDIES. We performed tissue biopsies to evaluate intracellular accumulation of Gb3. Anti-α-Gal and anti-Gb3 (CD77) antibodies were used for immunohistochemical studies, both by light and electron microscopy. Tissue biopsies were performed in mutation carriers with low α-Gal levels as follows: patients carrying novel mutations and patients with very mild LVH (≤13 mm) before deciding about enzyme replacement therapy administration; patients with symptoms such as severe gastrointestinal disturbances to exclude comorbidities; and patients with severe LVH to confirm Gb3 accumulation. In patients with predominant neurologic traits (stroke, TIA), we could not perform brain biopsy, and determination of organ involvement was imaging based. We therefore generated cell cultures from circulating peripheral blood mononuclear cells (PBMC) to test the presence of α-Gal and Gb3 (Online Appendix). In fact, recent guidelines for investigating causality of sequence variants in human disease indicate the need of experimental validation of the predicted damaging impact of candidate variants using assays of patient-derived tissue or well-established cell systems (23).

STATISTICAL ANALYSIS. Descriptive statistics were computed as median and interquartile range for continuous variables and as counts and percentages for categorical variables. We used Mann-Whitney *U* tests for continuous variables and Fisher and chi-square tests for categorical variables. A p value <0.05 was considered statistically significant. MEDCALC (version 14.10.2, MedCalc Software, Ostend, Belgium) was used for statistical evaluation. Receiver-operating characteristic analysis was used to find optimal thresholds for plasma and leukocytes in both male and female carriers versus noncarriers of mutations. Receiver-operating characteristic areas under the curve were calculated as a measure of diagnostic performance, with differences calculated and tested according to the methods of Hanley and McNeil (24). We used cutoff points that maximized both sensitivity and specificity (25).

RESULTS

We enrolled 2,034 consecutive probands who presented with 1 or more clinical traits typically associated with AFD, after excluding known causes of the same traits (**Table 1**, **Central Illustration**).

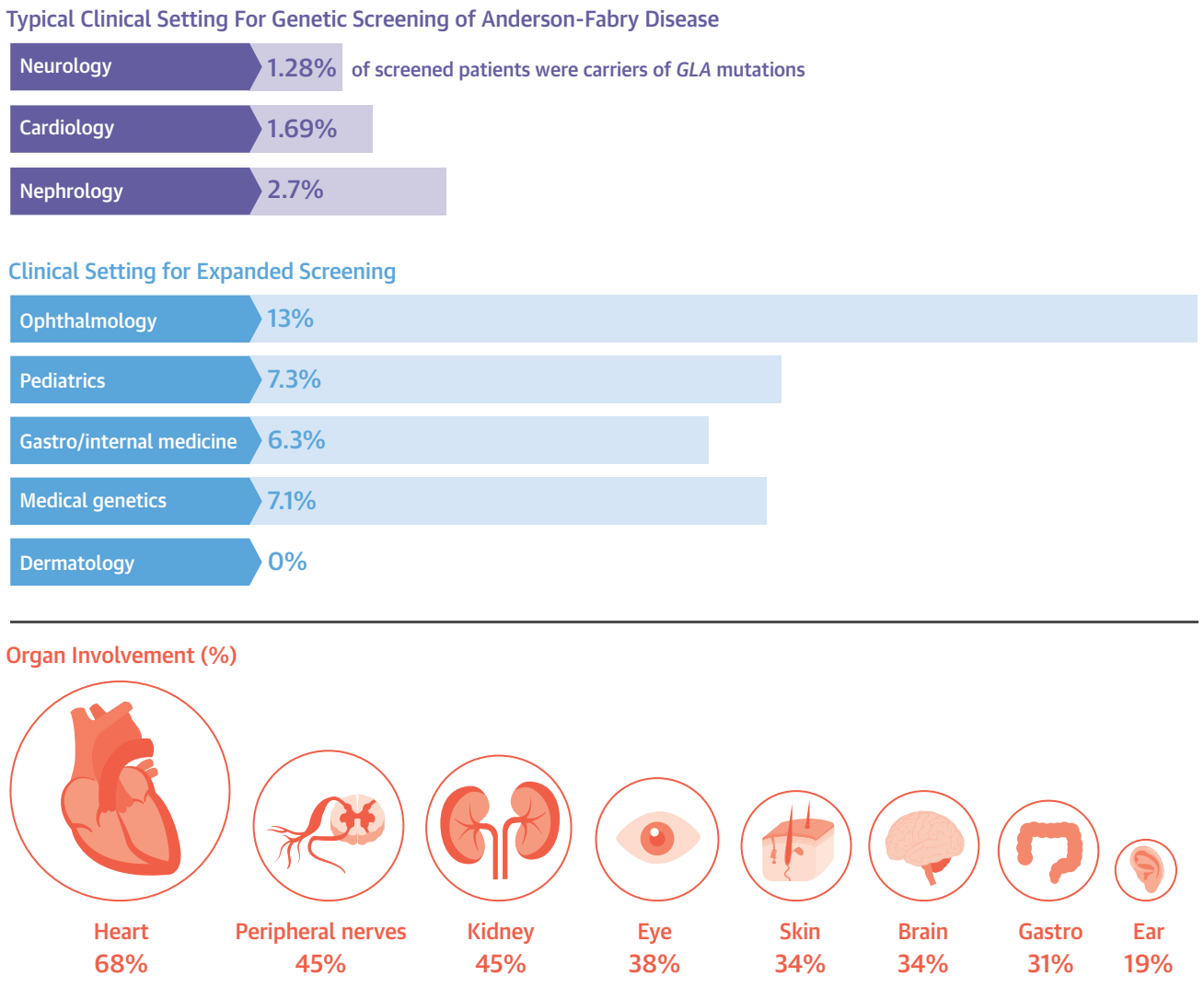
We identified *GLA* mutations in 37 of 2,034 probands (1.8%) including 19 male and 18 female patients (Online Table 1). Excluding the 8 proband carriers of the debated pseudodeficiency variant p.(Asp313Asn) and the carrier of the novel p.(Gln57Arg), the prevalence was 1.3%. Overall, 133 relatives of 37 probands underwent clinical and genetic screening, 60 of whom were mutation carriers. We obtained detailed clinical information for 4 obligate carriers. Multiple ligation-dependent probe amplification in 135 cases with plasma enzyme activity in the low to normal ranges did not show large gene rearrangements. Haplotypes I to IV that were reported as being associated with decreased enzyme activity levels (11) were found in 517 of 2,034 patients and did not show significant association with enzyme plasma levels when compared with cases without the same haplotypes (Online Table 2). Novel, unique intronic, synonymous, and missense variants are reported in Online Table 3. The Taiwanese-Chinese *IVS4+919 G>A* mutation (18,19) was not seen in our series. Sequencing of the HCM genes in 8 patients with LVH >15 mm identified known and novel genetic variants of unknown significance (Online Table 3), in presence of substantial tissue accumulation of Gb3 *GLA* p.(Phe113Leu), n = 1 (Online Figure 1); p.(Tyr184Asp), n = 1; and p.(Asn215Ser), n = 4], in absence of segregation of the HCM variant with the cardiac phenotype in the family [*GLA* p.(Gln57Arg)], and in a boy with classic AFD and carrier of the *GLA* p.(Phe337Ser).

Plasma α-Gal activity measurements were available in 1,501 consecutive probands (714 were female, 787 male) of the 2,034 genetically screened probands. Leukocyte α-Gal assay results were available in 865 probands (421 were female, 444 male). Plasma median values were similar in male and female subjects who did not carry *GLA* mutations (**Figures 1A and 1B**), lower and diagnostic in male carriers of mutations, but lower and nondiagnostic in female subjects with *GLA* mutations (Online Table 4). The specificity, sensitivity, and area under the curve are presented in **Figures 1C to 1F**.

ORGANS, TISSUES, AND CELLS. Organ system involvement was assessed in a multidisciplinary context. Application of the MOGE(S) system displayed the clinical profile (Online Table 5): 62 of 96 mutation carriers (65%) demonstrated multiple organ involvement; 23 (24%) had 1 organ involvement only; 1 (1%) complained of acroparesthesias; 10 (10%) were healthy carriers. For 5 of 101 mutation carriers, we were unable to perform multidisciplinary evaluation (**Table 2**).

We performed tissue biopsies in 17 patients (endomyocardial in 6, gastric in 1, skin in 10). Light and electron microscopy of the endomyocardial biopsy demonstrated intracellular Gb3 deposits in

CENTRAL ILLUSTRATION **Screening for AFD**



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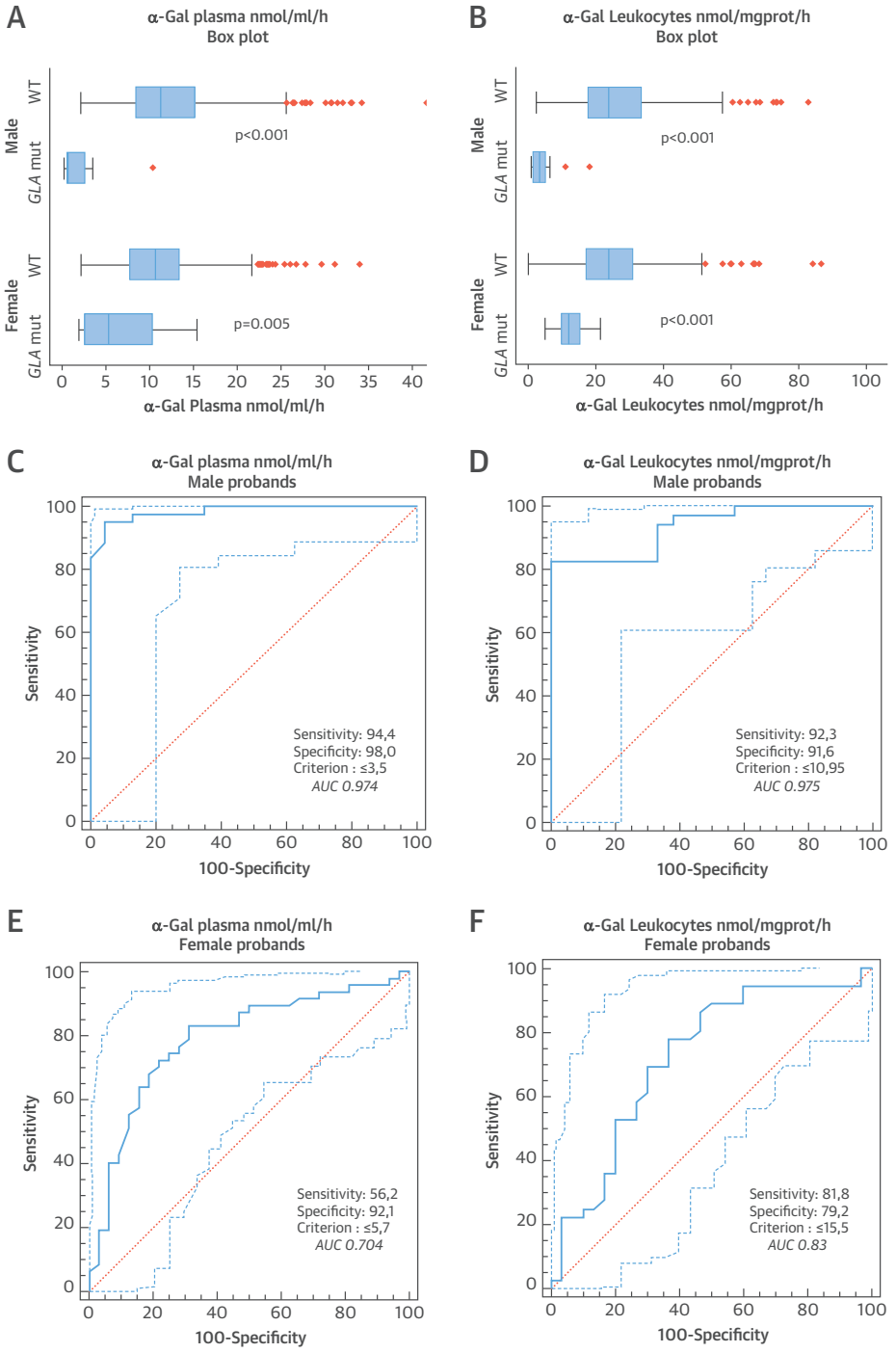
This study was designed to demonstrate that screening high-risk patients from multiple clinical settings could increase the diagnostic yield for Anderson-Fabry disease (AFD). Of the 8 clinical fields pertinent to AFD included, all produced positive cases except dermatology. In the 86 affected family members, the heart was the organ with highest involvement.

cardiac and classic AFD (**Figures 2A and 2B**). Immunohistochemistry with anti-Gb3 antibodies demonstrated specific immunostaining of the deposits (**Figures 2C and 2D**). In classic AFD, skin biopsies demonstrated Gb3 deposits in vascular and nonvascular smooth muscle cells, dermal fibroblasts, adipocytes, and endothelial cells (**Figures 3A to 3E**); myelin sheaths of sensitive neurons showed degenerative features (**Figure 3F**). However, skin biopsies failed to demonstrate significant Gb3 accumulation in carriers of *GLA* cardiac variants (Online Figures 2 and 3). Gastric biopsy in a carrier of the p.(Ser401X) mutation showed positive anti-Gb3 immunostain in vascular smooth muscle cells and endothelia as well as interstitial cells (**Figure 4**). Immunostain with anti-α-Gal and anti-CD77 antibodies demonstrated attenuated and normal expression of the enzyme in classic and atypical forms, respectively (**Figure 5A**), and Gb3 presence (**Figure 5B**) in cultured PBMC from patients with atypical variants.

The *GLA* p.(Asp313Tyr) pseudodeficiency allele was classified as a genetic variant of unknown

FIGURE 1 Plasma and Leukocytes Enzyme Activity in Probands



In assays of α-galactosidase A (α-Gal) plasma activity **(A)** in 1,501 tested probands and α-Gal leukocyte activity **(B)** in 865 probands, enzyme activity level in mutated probands was significantly lower than in nonmutated probands. Receiver-operating characteristic analysis in male subjects **(C)** and female subjects **(D)** established the cutoff values of α-Gal activity in plasma with better ratios between sensitivity and sensibility (3.5 nmol/ml/h in male subjects; 5.7 nmol/ml/h in female subjects). The specificity and sensitivity values confirmed enzyme activity to be a more reliable biomarker in male subjects. Receiver-operating characteristic analysis in male subjects **(E)** and female subjects **(F)** established the cutoff value of α-Gal activity in leukocytes with better ratios between sensitivity and sensibility (10.95 nmol/ml/h in male subjects; 15.5 nmol/ml/h in female subjects). AUC = area under the curve; *GLA* = alpha-galactosidase A gene; WT = nonmutated.

significance (Online Table 5). The 8 probands who carried this variant were included in the screening for brain involvement. Data regarding plasma enzyme activity in probands and relatives are in the Online Appendix. Of the 8 probands in this group, 6 had a stroke, 1 had a TIA, and 1 had migraine with aura, the latter 2 with magnetic resonance–verified white matter lesions (WML); 6 probands also showed additional traits. Of the 17 relatives with *GLA* p.(Arg313Tyr), 6 showed involvement of more than 1 organ, 4 showed involvement of 1 organ, 3 were healthy carriers, and 4 did not proceed with multidisciplinary evaluation. Six probands and 5 relatives were carriers of the mitochondrial deoxyribonucleic acid 3010G>A mutation in the MT-RNR2, a common single nucleotide polymorphism that is also associated with cyclic vomiting syndrome with migraine.

The *GLA* p.(Asn215Ser) cardiac variant was identified in 5 probands referred from cardiology in the setting of HCM. Carriers of this mutation showed low plasma enzyme activity (Online Appendix) and severe Gb3 accumulation in myocytes (**Figure 2**). Skin biopsies in 3 patients were noninformative, nonsignificant, or showed focal intracellular Gb3 accumulation similar to that observed in skin biopsies of carriers of the p.(Asp313Tyr) variant (Online Figure 3), respectively. Cultured peripheral mononuclear cells demonstrated normal enzyme expression and Gb3 accumulation in male and female subjects.

DISCUSSION

In this study, we found that genetic screening of high-risk populations selected in the context of multispecialty clinics provided an overall higher yield of diagnoses [1.8% and 1.3% excluding probands with p.(Asp313Tyr) and p.(Gln57Arg)] than that achieved by screening large series of patients with HCM, cryptogenic stroke, or end-stage renal failure (4–7,26–28). The incremental rate of detection of AFD in our series compared with prevalence data reported to date in selected cardiology, neurology, or nephrology settings was thus likely explained by the contribution of patient evaluations being performed in multiple clinical settings. Selection of patients without evidence of male-to-male transmission of the trait in the family might also have contributed. Ophthalmologic evaluation/involvement was found to be very important: 3 of 3 patients enrolled because of noniatrogenic cornea verticillata tested positive for *GLA* mutations, whereas 20 patients enrolled because of vessel tortuosity and/or cataracts tested negative.

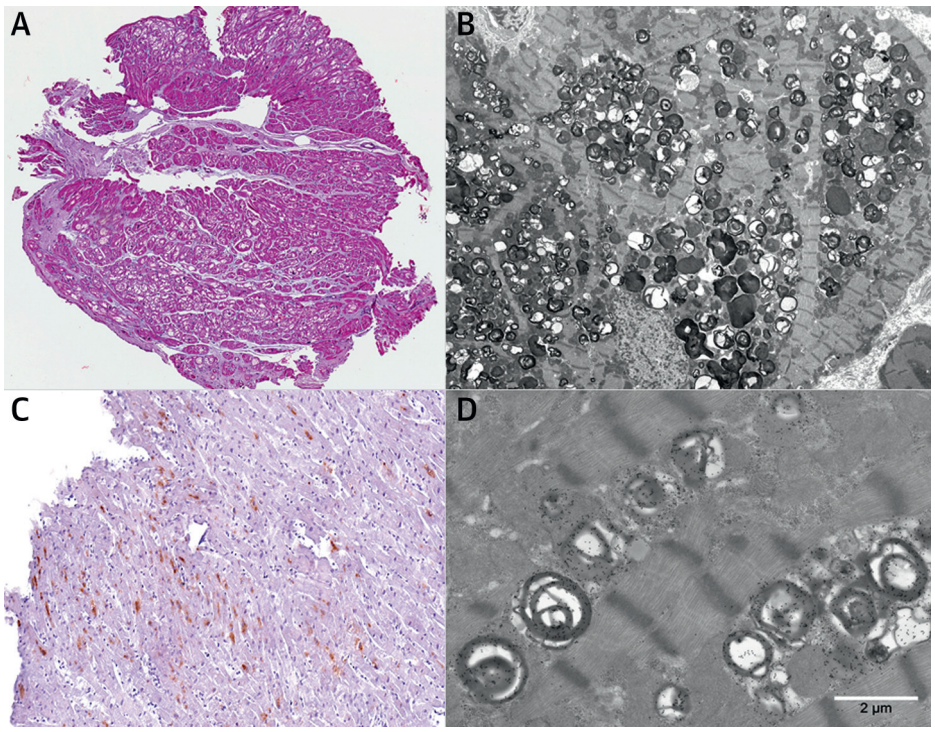
TABLE 2 Overall Organ and Tissue Involvement in Mutated Family Members

	Probands (n = 37)	Relatives (n = 64)*	Total (N = 101)
Clinically affected	37	49 (including 4 OC)	86
HC	0	10	10
Clinical data available	37	59	96
Nonmultidisciplinary evaluation		5 female subjects [4 p.(Asp313Tyr); 1 p.(Gln57Arg)]	5
Organ involvement†			
Heart	28	31	59
LVH >13 mm	24	19	43
With AVB	(9)	(2)	(11)
LVH 11–13 mm (early)	4	12	16
With AVB		(1)	(1)
No cardiac involvement or ECG signs only	9	28	37
Short PR interval as only marker (without LVH)	2	4	6
No involvement	7	24 (including 10 HC)	31
Kidney	20	19	39
Dialysis	3		3
Kidney transplantation	1		1
Severe CRF	2	6	8
Increased urinary albumin excretion, proteinuria, increased creatinine, decreased eGFR, increased GFR	14	13	27
Brain	23	6	29
Stroke	14	3	17
TIA	4	1	5
WML (imaging screening)	7	2	9
Migraine with aura + WML	1		1
Cerebral artery disease (dolichoectasia/aneurysm)	2	2	4
Gastroenteric system	11	16	27
Eye	15	17	32
Corneal deposits	14	17	31
Corneal deposits and retinal artery thrombosis	1		1
Skin (angiokeratomas)	16	12	28
Peripheral nerves (acroparesthesias)	18	22	40
Auditory system	9	7	16
Comorbidities			
HPN	10	15	25
HPN + diabetes	1		1
HPN + IHD		1	1
Patent foramen ovale	2		2
Phenylketonuria	1		1
Nephrectomy for hydronephrosis		1	1
Celiac disease + gastric lymphoma	1		1
Cancer (colorectal)	1		1
Cancer (tongue)		1	1
Rheumatoid arthritis	1		1
Acoustic neuroma	1		1
Dyslipidemia	2	1	3
Total comorbidities	20	19	39

*Number of relatives = 60 + 4 OC = 64. †Independently of the screening setting, in 86 patients with ≥1 trait of the disease (up to 7).

AVB = atrioventricular block; CRF = chronic renal failure; ECG = electrocardiogram; eGFR = estimated glomerular filtration rate; GFR = glomerular filtration rate; HC = healthy carrier; HPN = hypertension; IHD = ischemic heart disease; OC = obligate carriers; other abbreviations as in Table 1.

FIGURE 2 Endomyocardial Biopsies in Cardiac and Classic AFD

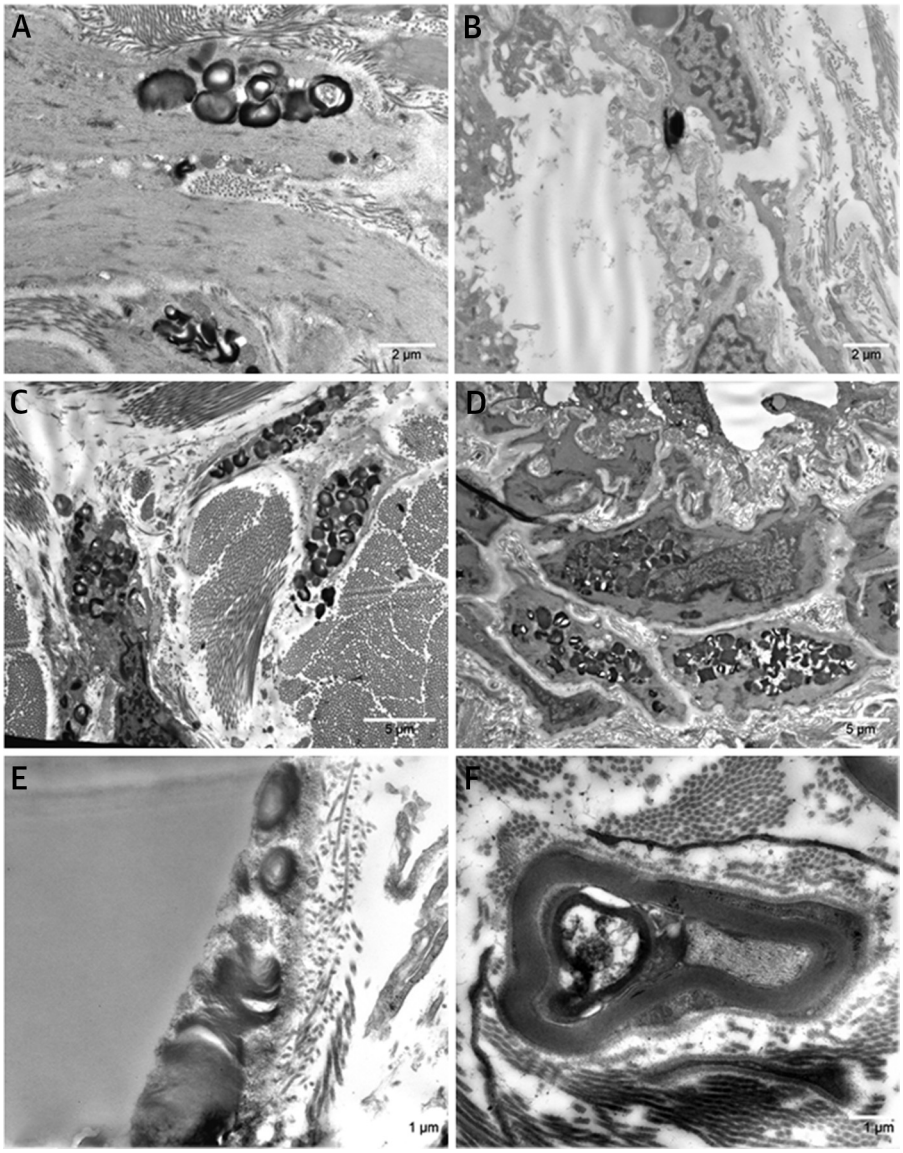


Mutations seen in endomyocardial biopsies from patients include the following: **(A)** a hematoxylin and eosin stain sample from a male carrier of the p.(Asn215Ser) mutation in which the optically empty myocytes constitute a typical light microscopy marker of intracellular storage; **(B)** electron micrograph showing typical osmiophilic bodies in the endomyocardial biopsies of a female carrier of the p.(Tyr184Asp) mutation; **(C)** light immuno-microscopy of EMB samples from a young male carrier's of the p.(Asn215Ser) mutation demonstrating early hypertrophic cardiomyopathy; and **(D)** electron immuno-microscopy of EMB sample from a male patient carrier of the p.(Phe113Leu). AFD = Anderson-Fabry disease; EMB = endomyocardial biopsy.

This supported evidence from other recent reports (29,30), in which noniatrogenic cornea verticillata was a highly specific clinical marker.

Whereas severity scores showed that heart, kidney, and brain involvement are the major prognostic determinants (31,32) in AFD, diagnostic scoring systems could better take advantage of objective markers of high diagnostic specificity, regardless of clinical relevance. Traits such as acroparesthesias (33) or abdominal crises (3) are subjectively described, and thus challenging to ascribe to AFD. Differential diagnosis in adults includes systemic amyloidosis with peripheral neuropathy in patients with cardiac and renal involvement. In children, the recurrence of abdominal crises and acroneuropathic pain triggered by heat or fever increases clinical suspicion. Concomitant clinically overt involvement of the heart, kidneys, and brain, which may contribute to the clinical suspicion, does not necessarily recur in all patients, especially those with cardiac, renal, or nervous *GLA* variants. Mitochondrial diseases, such as MELAS, might show concentric HCM that typically evolved through late ventricular dilation and dysfunction, renal failure that may necessitate hemodialysis, and recurrent cryptogenic stroke, but they might also demonstrate ocular traits that do not include corneal deposits. Diffuse angiokeratomas rarely bring a patient to clinical attention. Phenotype heterogeneity within families demonstrating classic forms of AFD or involvement of the same organ in those carrying atypical *GLA* variants might contribute to clinical suspicion. Overall, patients with AFD should be evaluated as a whole, and within the context of family history, independent of the predominant organ involved, a strategy requiring a disease-oriented diagnostic mindset. The MOGE(S) nosology was recently generated to precisely describe the genotype-phenotype in cardiomyopathies (16) (Online Table 5).

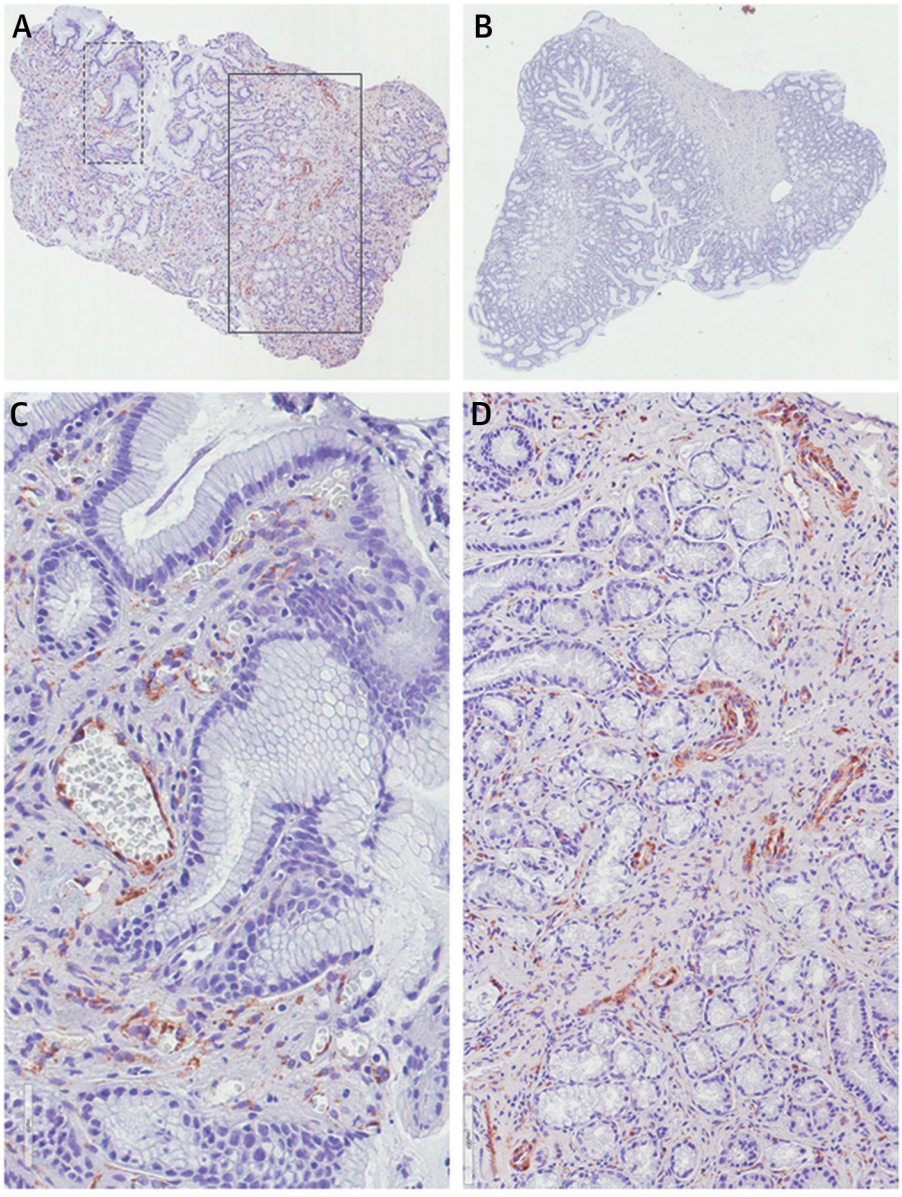
FIGURE 3 Skin Biopsy in AFD



Electron micrographs of a skin biopsy sample from a male patient carrier of the p.Ala292_Met296del mutation show globotriaosylceramide accumulation in **(A)** nonvascular smooth muscle cells; **(B)** endothelial cells; **(C)** dermal fibroblasts; **(D)** vascular smooth muscle cells; and **(E)** adipocytes. **(F)** This sensory cutaneous nerve shows degenerative features. AFD = Anderson-Fabry disease.

DIAGNOSIS OF AFD. Definitive diagnosis should rely on genetic testing, enzyme activity, and tissue studies demonstrating intracellular Gb3 accumulation. Absent pathological evaluation, interpretation of genetic testing results is supported by enzyme assays in male subjects and by imaging or functional tests sufficient to demonstrate tissue involvement otherwise. Confirmed pathologic mutations overall are sufficient for the diagnosis. For novel mutations or atypical or provisional variants, in silico analyses might contribute, but are not conclusive: a typical example is p.(Asn215Ser), which is benign in silico but associated with severe intramyocyte accumulation of Gb3; or conversely, p.(Asp313Tyr), which is damaging in silico but considered to be a genetic variant of unknown significance or single nucleotide polymorphism. Recently, p.(Arg118Cys), originally interpreted as pathologic, has been reported as a variant

FIGURE 4 Gastric Biopsy in AFD

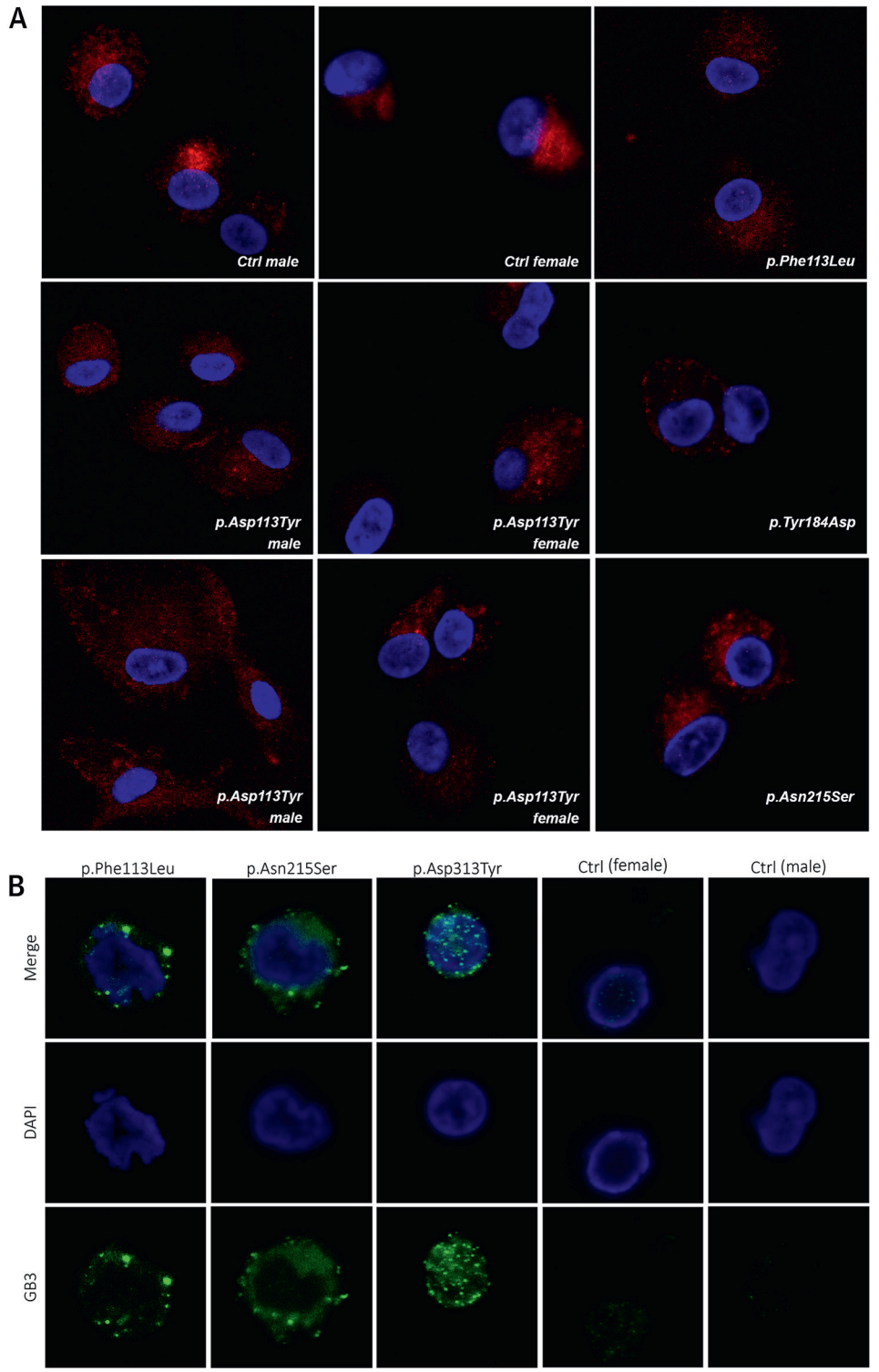


(A) Low magnification view of the gastric biopsy from a young male patient carrier of the p.(Ser401X); the patient complained of severe gastrointestinal symptoms, with epigastric pain crises, and acroneuropathic pain during febrile episodes. Antiglobotriaosylceramide immunostain of vascular smooth muscle cells and endothelia (insets: **dashed line** = **C**; **solid line** = **D**) as well as interstitial cells. **(B)** Negatively immunostained gastric biopsy of a patient with gastrointestinal symptoms and carrier of the p.(Asp313Tyr). AFD = Anderson-Fabry disease.

not causing an AFD phenotype (34). The pathogenic roles of p.(Ala143Thr), p.(Ser126Gly), p.(Arg112His), p.(Pro60Leu), and p.(Glu66Gln) have been questioned on the basis of tissue studies and Gb3 dosage (35-38). These examples highlight the need for integrated interpretation of genetic data based on precise evidence or reasons for exclusion. As an example, if the above-mentioned variants were confirmed as being nonpathogenic, published prevalence data would likely vary.

Tissue studies, when feasible, demonstrate Gb3 storage. Conventional light microscopy shows optically empty cells; ultrastructural hallmarks are the typical osmiophilic lamellar bodies; and

FIGURE 5 **In Vitro Study of AFD**



(A) Cultured peripheral blood mononuclear cells immunostained with anti-α-Gal antibody from patients carriers of the p.(Asn215Ser), p.(Phe113Leu), p.(Asp313Tyr) and p.Tyr184Asp mutation showed immunostaining similar to that of cells from control subjects (Ctrl). **(B)** In cultured peripheral blood mononuclear cells of 3 patient carriers of p.(Phe113Leu), p.(Arg215Ser), and p.(Asp313Tyr), **green** particles indicated anti-Gb3 immunoreactivity, which is highly represented in the 3 cell lines. DAPI = 4',6-diamidino-2-phenylindole; Gb3 = globotriaosylceramide; other abbreviations as in Figures 1 and 3.

immuno-light and electron microscopy demonstrate specific labeling with anti-Gb3 antibodies. Our results supported a tissue-specific, mutation-dependent "affinity" for Gb3 storage and contributed to explaining the cardiac, renal, and, probably, neurologic variants: in fact, Gb3 accumulated in myocytes but not in skin or vascular cells of patients who carry the cardiac variants [i.e., p.(Asn215Ser) and p.(Phe113Ser)]. However, classic, juvenile, and systemic forms have typically demonstrated involvement of interstitial and vascular smooth muscle cells [p.(Ala292_Met296del) in skin; p.(Ser401X) in gastric tissue] (**Figures 3 and 4**). Therefore, skin biopsy is useful in patients with classic AFD but useless in atypical variants (Online Figures 2 and 3). In cultured PBMC from carriers of atypical cardiac variants, the immunohistochemical expression of α-Gal was normal, confirming a functional impairment rather than decreased enzyme expression; the paradigmatic example is p.(Asn215Ser), which causes the loss of α-Gal glycosylation at site 3, which is essential for enzyme solubility (39).

In our experience, the issue of the pseudodeficiency allele p.(Asp313Tyr) requires further discussion: all 8 probands in our series were from neurologic settings; 7 also showed extraneurologic traits. Skin biopsies, similar to those in cardiac variants, were not diagnostically helpful. Brain biopsies are not possible, although imaging demonstrated tissue and vascular pathology. Autopsy or other pathologic evaluations do not exist. Family segregation studies (Online Figure 4) and in silico analysis do not help, based on both past in vitro studies demonstrating 60% of wild-type enzyme activity in vitro (40,41) and identification of 3 patients with a second *GLA* mutation (42,43). Based on our data, the p.(Asp313Tyr) variant recurs in patients presenting with cryptogenic stroke, TIA, WML identified by imaging, and migraine with aura associated with WML. Cultured PBMC from mutation carriers showed features similar to those from carriers of confirmed pathologic mutations. Evidence, both for (44–46) and against (41,43), was nonconclusive for unambiguous assignment of disease causality or contribution for this variant. In the large family reported by Lenders et al. (46), all 7 mutation carriers had WML but 2 noncarriers did not. Neurologists face the difficult role of managing these patients and excluding with certainty this variant's contribution to the complex neurological spectrum of signs/symptoms observed.

STUDY LIMITATIONS. Our study was designed more than 10 years ago and did not include data on Gb3 and Lyso-Gb3, which is emerging as a sensitive diagnostic marker. The different prevalence of traits mimicking cardiac, neurologic, and renal disease (seen in AFD) in the general population may help to explain the differences in numbers of cases enrolled per discipline.

CONCLUSIONS

Expanded screening of high-risk populations from the cardiology, neurology, and nephrology settings to ophthalmology, gastroenterology, internal medicine, pediatrics, dermatology, and medical genetics increased the yield of diagnosis of AFD. The heart was the most commonly involved organ, regardless of the clinical setting in which the patient was first evaluated. Traits contributing to the diagnosis differed from those impacting prognosis, including non-iatrogenic cornea verticillata. Pathologic analysis confirmed disease-causing mutations and also contributed to unraveling the role of novel and provisional variants identified in the past. The systematic annotation of key genotype-phenotype data, such as with the MOGE(S) nosology system, helped to collect precise clinical and genetic data for future studies.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: A rare, lysosomal disease, AFD affects multiple organ systems. Precise diagnosis is essential for the effective enzyme replacement therapy administration. The heart is the most commonly involved organ, regardless of the clinical setting in which the patient presents, but screening patients in ophthalmology, dermatology, gastroenterology, internal medicine, pediatrics, and medical genetics clinics in addition to high-risk patients in cardiology (HCM), neurology (cryptogenic stroke, TIA, and migraine), and nephrology (end-stage renal failure) clinics for defects in the *GLA* gene, increases the diagnostic yield.

TRANSLATIONAL OUTLOOK: Future AFD studies could benefit from use of the MOGE(S) nosology system to provide patient-specific description of multiorgan involvement, inheritance pattern, and the associated mutation.

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KEY WORDS α-Gal, biochemical, family screening, *GLA*, MOGE(S) classification, multidisciplinary evaluation

APPENDIX For supplemental material, please see the online version of this article.