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STROKE Patients**

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Mutations of the *GLA* Gene in Young Patients With Stroke

The PORTYSTROKE Study—Screening Genetic Conditions in PORTuguese Young STROKE Patients

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Background and Purpose—Fabry disease is an X-linked monogenic disorder caused by mutations in the *GLA* gene. Recent data suggest that stroke in young adults may be associated with Fabry disease. We aimed to ascertain the prevalence of this disorder among young adult patients with stroke in Portugal by *GLA* genotyping.

Methods—During 1 year, all patients aged 18 to 55 years with first-ever stroke, who were admitted into any of 12 neurology hospital departments in Portugal, were prospectively enrolled (n=625). Ischemic stroke was classified according to Trial of Org 10172 in Acute Stroke Treatment criteria. Alpha-galactosidase activity was further assayed in all patients with *GLA* mutations.

Results—Four hundred ninety-three patients (mean age, 45.4 years; 61% male) underwent genetic analyses: 364 with ischemic stroke, 89 with intracerebral hemorrhage, 26 with subarachnoid hemorrhage, and 14 with cerebral venous thrombosis. Twelve patients had missense *GLA* mutations: 9 with ischemic stroke (p.R118C: n=4; p.D313Y: n=5), including 5 patients with an identified cause of stroke (cardiac embolism: n=2; small vessel disease: n=2; other cause: n=1), 2 with intracerebral hemorrhage (p.R118C: n=1; p.D313Y: n=1), and one with cerebral venous thrombosis (p.R118C: n=1). Leukocyte α -galactosidase activity was subnormal in the hemizygous males and subnormal or low-normal in the heterozygous females. Estimated prevalence of missense *GLA* mutations was 2.4% (95% CI, 1.3% to 4.1%).

Conclusions—Despite a low diagnostic yield, screening for *GLA* mutations should probably be considered in different types of stroke. Restricting investigation to patients with cryptogenic stroke may underestimate the true prevalence of Fabry disease in young patients with stroke. (*Stroke*. 2010;41:431-436.)

Key Words: gene ■ *GLA* ■ stroke ■ young

Stroke in young adults is an important cause of morbidity and mortality worldwide.^{1,2} In Portugal (population approximately 10 million), it is estimated that up to 3000 new cases occur every year in the population aged 18 to 55 years.³ Despite extensive investigation, the cause of stroke in young adults remains to be determined in up to 25% to 50% of cases.^{1,2}

Fabry disease (FD) is a rare X-linked lysosomal storage disorder associated with increased risk of stroke in young adults.⁴⁻⁷ The classical phenotype presents with early derma-

tologic, ophthalmologic, and peripheral nervous system involvement. Renal, cardiac, and cerebrovascular complications are major causes of morbidity and mortality in adults. Late-onset atypical phenotypes have also been reported.⁴ Because its clinical recognition requires a high index of suspicion, the diagnosis of FD is often delayed or missed.⁵ Early diagnosis became particularly important because enzyme replacement therapy may prevent the late renal and cardiac complications^{6,7} and seems to have a beneficial effect on abnormal cerebral blood flow.^{8,9}

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The underlying metabolic defect in FD is a deficiency of lysosomal α -galactosidase (α Gal) due to mutations in the α Gal gene locus (*GLA*) located at Xq22.^{4,10} Deficient α Gal activity ultimately leads to widespread intracellular accumulation of globotriaosylceramide and related neutral glycosphingolipids, including in vascular endothelial and smooth muscle cells.^{4,7} Mutations have been described throughout all 7 *GLA* exons and most families have private mutations.^{4,10} Typical for X-linked disorders, FD has a more variable and usually milder and later phenotypic expression in females.^{4–7}

A recent prospective study of cryptogenic stroke in young adults (n=721), using an α Gal enzyme assay for screening, suggested that up to 1.2% of patients with stroke <55 years of age might have FD.¹¹ However, the *GLA* mutations identified in these cases were never reported. Moreover, in a smaller retrospective study of similar patients (n=103), no *GLA* mutations were found in any of the subjects who had low enzyme activity.¹² The PORTUGUESE Young STROKE Patients (PORTYSTROKE) study was designed to screen for genetic disorders in young patients with stroke. We report the results of *GLA* mutational analysis as the screening approach to FD.

Methods

The PORTYSTROKE clinical protocol and the data collection and storage procedures were approved respectively by the ethics committee of each participating hospital and by the Portuguese authority for data protection. A computer database for online data entry was specifically developed and monitored by an independent clinical research organization. Individual patient records included demographic data, history of vascular risk factors, medical history, family history emphasizing the classical features of FD, and the clinical and brain imaging (CT or MRI) characteristics of stroke. Ischemic stroke (IS) was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.¹³ The molecular genetics laboratory was blinded as to the clinical and imaging data of each patient.

Patients

From November 1, 2006, through October 31, 2007, all patients aged 18 to 55 years presenting with first-ever stroke, admitted to neurology departments in any of 12 major Portuguese hospitals, were eligible for this study. Patient enrollment for genotyping required written informed consent.

Laboratory Methods

Venous blood samples for molecular genetic analyses were collected in tubes containing 5% edetate and mailed to the laboratory at room temperature. Genomic DNA (gDNA) samples were extracted from 1 mL of blood with the Citogene Blood Kit (Citomed, Lisbon, Portugal) and banked at 4°C. Leukocyte pellets were prepared from 5 mL of blood and frozen at –80°C until used for RNA extraction. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

Two partially overlapping cDNA fragments, together comprising 1371 nucleotides of the 1418-nucleotide sequence of *GLA* messenger RNA, were amplified from the RNA samples by reverse transcription–polymerase chain reaction (PCR). The cDNA reverse transcription-PCR amplicons contained a common sequence in their respective 3' and 5' terminals, corresponding to part of exon 4. When the cDNA could not be obtained, all 7 *GLA* exons and respective exon/intron boundaries were PCR-amplified from the gDNA samples. The reverse transcription-PCR and PCR products were purified and automatically sequenced in forward and reverse directions. Details of these laboratory methods have been formerly published.¹⁴ The reference *GLA* gDNA and cDNA nucleotide sequences, respectively GenBank Version X14448.1 and RefSeq Version NM_000169.2,

were obtained from the National Center for Biotechnology Information (Bethesda, Md; www.ncbi.nlm.nih.gov/entrez/). Mutations detected in the original *GLA* cDNA or gDNA analyses were confirmed in a duplicate PCR amplification of the corresponding exon using the stored gDNA samples.

A second blood sample for α Gal enzyme assays was obtained from all patients in whom a *GLA* mutation was identified. Plasma and leukocyte activities of α Gal were measured using a standard fluorometric method.¹⁵ Normal range values for α Gal enzyme activity were 6 to 19 nmol/h/mL in plasma and 36 to 80 nmol/h/mg in leukocytes.

In carriers of missense *GLA* mutations that were associated with subnormal enzyme activity, multiplex ligation-dependent probe amplification (SALSA MLPA kit P159 *GLA*; MRC-Holland, Amsterdam, The Netherlands) was additionally used to rule out the presence of coexisting gross gene deletions and/or duplications that might have been missed by gDNA or cDNA sequencing.

Control Population and Screening

To estimate the frequency in the general population of the missense *GLA* mutations found in the stroke patient cohort, we used banked gDNA samples of 130 (50 males) undergraduate medical students aged 20 to 25 years, of 40 fertile males, aged 25 to 35 years, and of 130 (95 males) volunteer bone marrow donors, aged 25 to 45 years. These control subjects were screened for the relevant *GLA* mutations (see “Results”) by restriction enzyme analysis. To this end, endonucleases *Hinf*I and *Hpy*188III were selected with the NEBcutter software (<http://tools.neb.com/NEBcutter2/>), respectively to identify alleles p.R118C and p.D313Y. *GLA* exons 2 and 6 were PCR-amplified as described¹⁴ and the digestion of the corresponding amplicons was carried out according to the instructions of the enzyme manufacturer (New England BioLabs, Hitchin, UK). In the restriction analyses, gDNA samples of p.R118C or p.D313Y heterozygous carriers from the patient cohort were used as positive controls.

Statistical Analyses

Parametric and nonparametric statistical analyses, as appropriate, were performed with the SPSS software, Version 15.0. The prevalence of *GLA* gene mutations among patients and the corresponding allele frequencies among healthy control subjects were estimated using 95% CIs.¹⁶ Demographic and clinical data were summarized using descriptive statistics. Comparisons of risk factors, personal background, and family history were done according to demographic characteristics (sex and age) and the presence or absence of *GLA* gene mutations. The relative prevalence of *GLA* mutations was compared for patients with or without evidence of small vessel disease (lacunar stroke or leukoencephalopathy) and according to the presence or absence of hypertension. The hypothesis that stroke in the posterior circulation might have been relatively more frequent in patients with missense *GLA* mutations^{7,17} was also specifically addressed. A significance level of <0.05 was assumed for all statistical tests.

Results

During the enrollment period, a total of 625 patients were eligible for inclusion. Of these, 15 refused consent, 21 died before inclusion, and 96 were lost for other reasons. Therefore, the study cohort comprised 493 patients (61% males) with a mean age of 45.4 years. The Table summarizes demographic and clinical features of the enrolled patients. IS occurred in 364 patients, intracerebral hemorrhage in 89, subarachnoid hemorrhage in 26, and cerebral venous thrombosis (CVT) in 14.

Screening for *GLA* mutations was based on cDNA sequence analysis in 68% of the cases. As detailed in the Figure, we identified one noncoding (g.1136C>T in the 5'-untranslated region of exon 1) and 2 missense *GLA* mutations

Table. Demographic and Clinical Characteristics of the Patient Cohort

	Total	<i>GLA</i> R118C	<i>GLA</i> D313Y	<i>GLA</i> (Total)
Study sample				
No. of patients	493	6	6	12
Male	300 (60.9)	3 (50.0)	4 (66.7)	7 (58.3)
Age, years	47.0	41.0	46.5	43.0
Risk factors				
Hypertension	233 (47.3)	4 (66.7)	1 (16.7)	5 (41.7)
Diabetes	74 (15.0)	0 (...)	1 (16.7)	1 (8.3)
Cardiac disease	54 (11.0)	1 (16.7)	1 (16.7)	2 (16.7)
Hyperlipidemia	222 (45.0)	4 (66.7)	1 (16.7)	5 (41.7)
Smoking	181 (36.7)	3 (50)	1 (16.7)	4 (33.3)
Alcohol	238 (48.3)	4 (66.7)	3 (50.0)	7 (58.3)
Oral contraception	87 (45.1)	2 (66.6)	0 (...)	2 (40.0)
Hormonal replacement	1 (0.52)	0 (...)	0 (...)	0 (...)
Median no.	2.0	2.5	1.0	2.0
History				
Cutaneous disease	25 (5.1)	1 (16.7)	0 (...)	1 (8.3)
Ophthalmologic disease	45 (9.1)	1 (16.7)	0 (...)	1 (8.3)
Renal disease	37 (7.5)	1 (16.7)	0 (...)	1 (8.3)
Neuropathic pain	7 (1.4)	0 (...)	0 (...)	0 (...)
Family history				
Stroke	215 (43.6)	2 (33.3)	2 (33.3)	4 (33.3)
Cardiac disease	179 (36.3)	3 (50.0)	3 (50.0)	6 (50.0)
Renal disease	48 (9.7)	1 (16.7)	0 (...)	1 (8.3)
Stroke				
Ischemic	364 (73.8)	4 (66.7)	5 (83.3)	9 (75.0)
Cardiac embolism	80	1	1	2
Large vessel disease	55
Small vessel disease	98	1	1	2
Other known cause	27	1	...	1
Unknown cause	104	1	3	4
Intracerebral hemorrhage	89 (18.1)	1 (16.7)	1 (16.7)	2 (16.7)
Subarachnoid hemorrhage	26 (5.3)	0 (...)	0 (...)	0 (...)
CVT	14 (2.8)	1 (16.7)	0 (...)	1 (8.3)
Leukoencephalopathy	76 (15.4)	1 (16.7)	2 (33.3)	3 (25.0)
Posterior circulation	101 (20.5)	4 (66.6)	2 (33.3)	6 (50.0)
Modified Rankin Scale >2	135 (27.4)	3 (50.0)	2 (33.3)	5 (41.7)

Numbers in parentheses are percentages.

(p.R118C in exon 2, p.D313Y, in exon 6) in 10 patients with IS (p.R118C: n=4, 2 males; p.D313Y: n=5, 3 males; g.1136C>T: n=1, male), in 2 males with intracerebral hemorrhage (p.R118C: n=1; p.D313Y: n=1), and in 2 females with CVT (p.R118C: n=1; g.1136C>T: n=1). The gDNA nucleotide changes corresponding to mutations p.R118C and p.D313Y were, respectively, g.5251C>T and g.10645G>T. MLPA analysis did not show gross gene deletions or duplications in any of these patients.

Because leukocyte α Gal activity was normal or above normal in patients with mutation g.1136C>T, their carriers were not included in prevalence estimates. Leukocyte α Gal

activity was subnormal in males and subnormal to low normal in females with p.R118C and p.D313Y mutations. The allelic frequencies of both the p.R118C and the p.D313Y mutations were 0.0087 (6 of 686; 95% CI: 0.0035 to 0.0194) and did not differ significantly between genders; none of these mutations was identified in more than 400 X chromosomes of healthy control subjects (95% CI: 0.0000 to 0.0111).

The combined prevalence of *GLA* missense mutations in the patient cohort was 2.4% (12 of 493; 95% CI: 1.4% to 4.3%) and showed no significant gender difference (2.3% in males versus 2.6% in females). The prevalence of each mutation was similar among the patients (1.2%) and did not differ significantly between genders. The combined prevalence of *GLA* missense mutations among patients with IS was 2.5% (9 of 364; 95% CI: 1.2% to 4.7%). The combined prevalence of *GLA* missense mutations among patients with cryptogenic IS was 3.9% (4 of 104; 95% CI: 1.2% to 9.8%), which did not significantly differ in comparison to patients with an identified cause of stroke.

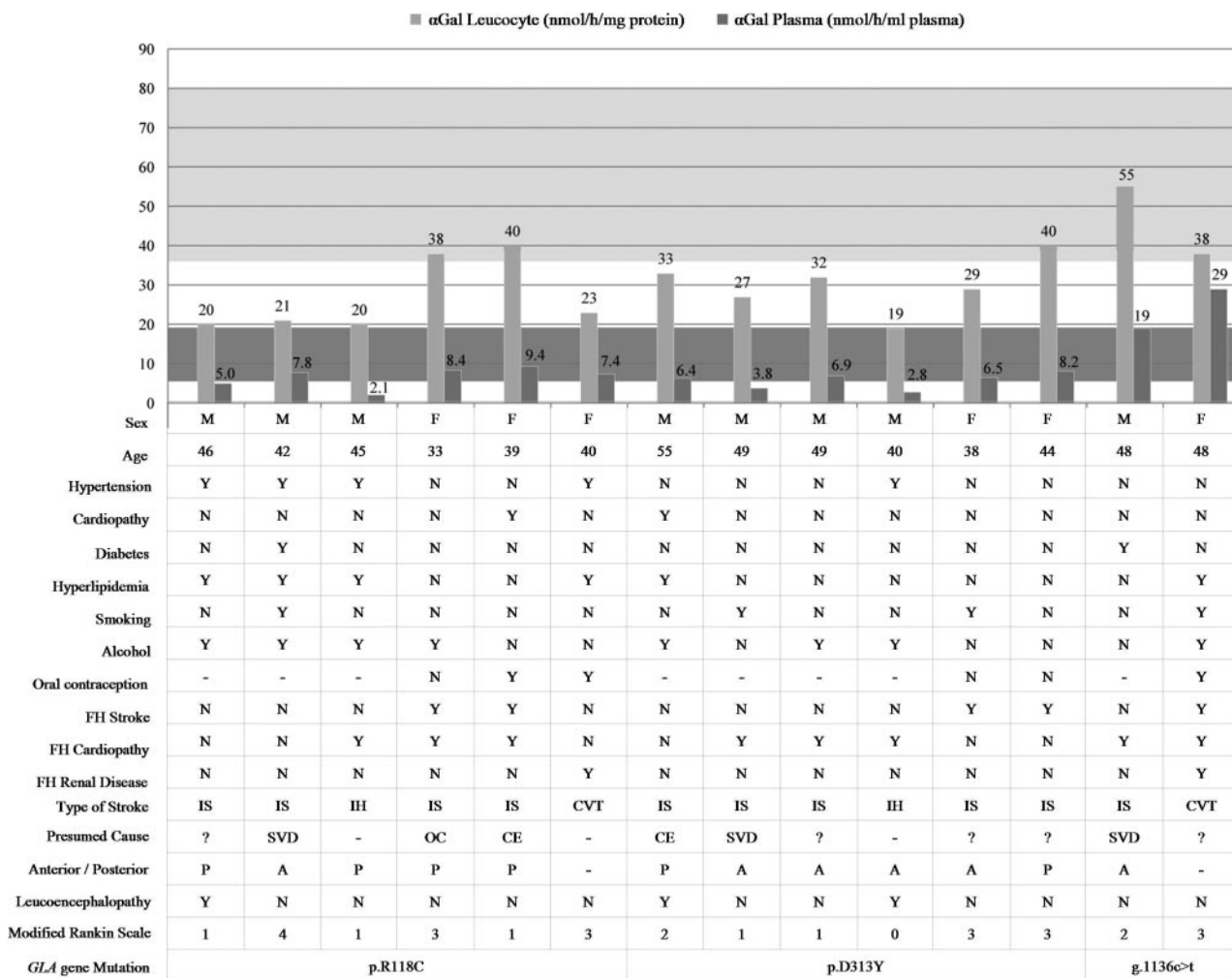
None of the patients carrying identical *GLA* mutations were of the same family. The type and median number of vascular risk factors, the characteristics of the stroke event, and the prevalence of classic features of FD in the medical history or family history did not significantly differ between patients with *GLA* mutation and those without.

Among patients with IS or intracerebral hemorrhage, the frequency of stroke in the posterior circulation was significantly higher in those carrying missense *GLA* mutations (Fisher exact test, $P<0.02$). This was particularly evident in patients with mutation p.R118C, of whom 80% (4 of 5) had a stroke event in the territory of posterior circulation.

In patients presenting with IS and brain imaging evidence of small vessel disease (lacunar stroke or leukoencephalopathy), the overall prevalence of missense *GLA* mutations was 4.6% (6 of 132; 95% CI: 1.9% to 9.8%), increasing to 7.0% (4 of 57; 95% CI: 2.3% to 17.2%) in patients without hypertension and to 12.5% (3 of 24; 95% CI: 3.5% to 31.8%) in normotensive patients with stroke in the posterior circulation.

Discussion

To our knowledge, this was the first study in which FD was prospectively screened in a large cohort of unselected young patients with stroke. We reasoned that not limiting eligibility to cryptogenic stroke might decrease selection bias and would be more appropriate to identify possible interactions between *GLA* mutations and environmental risk factors, particularly for populations with a very high prevalence of stroke, like the Portuguese. This was also the first time that mutational analysis was used to screen for FD in a large cohort with the same sensitivity for both genders, overcoming the limitations of α Gal activity assays to diagnose FD in females.⁵ Exon deletions and duplications as well as point mutations involving the last 16 amino acids of the α Gal protein might have been missed with our genotyping strategy. However, the gross defects are rare and have been described in patients with classic FD rather than in association with atypical phenotypes, and no pathogenic *GLA* point mutations have been reported in the last 15 amino acids of the enzyme (Human



(M: Male; F: Female; FH: Family History; IS: Ischemic stroke; IH: Intracerebral hemorrhage; CVT: Cerebral venous thrombosis; SVD: small vessel disease; OC: Other cause; CE: Cardioembolic; A: Anterior; P: Posterior; Y: Yes; N: No)

Figure. Alpha-galactosidase leukocyte and plasma enzyme activities in patients with missense *GLA* gene mutations. The normal ranges for leukocyte and plasma α -galactosidase activities are shadowed at the background. The clinical and brain imaging features of the stroke event and the relevant details of the history and of the family history of each patient are also summarized.

Gene Mutation Database, HGMD; www.hgmd.cf.ac.uk/ac/gene.php?gene=GLA, accessed October 1, 2009).

The prevalence of *GLA* missense mutations was 2.4% in the overall cohort and 3.8% in the subset of patients with cryptogenic stroke. These results are comparable to those of previous screening programs of atypical FD among patients with cryptogenic stroke¹¹ or with idiopathic left ventricular hypertrophy.^{18–21} Except for stroke in the posterior arterial territory, which was more frequent among patients with *GLA* mutations, there were no significant demographic and clinical differences between patients with *GLA* mutations and those without. Of note, reporting of personal or family history of possible complications of FD was no different in the 2 groups. The finding of *GLA* mutations in patients with intracerebral hemorrhage was not surprising, because these are a recognized cerebrovascular complication of FD,²² but CVT had never been reported in association with FD. Although a single case does not allow to conclude that α Gal deficiency is a risk factor for CVT, various abnormalities in cerebral blood flow and in the walls of intracranial vessel have been identified that are not exclusive of arterial sys-

tem^{7,17} and might contribute to a higher risk of CVT. In the large observational Fabry Registry database, the diagnosis of FD was established at later ages in patients who had stroke than in patients who did not.²² Moreover, most patients with stroke had not experienced renal or cardiac events before their first stroke. These data are in accordance with our own findings and suggest that the classical features of the disease may be absent or more subtle in these patients. Furthermore, the finding of *GLA* missense mutations in several patients who had a concurrent cause of stroke by TOAST criteria (Figure 1) suggests that screening of FD among patients with stroke should not be restricted to the cryptogenic cases. Evidence of small vessel disease (lacunar stroke or leucoencephalopathy) without hypertension in young patients with IS should prompt the search for FD, particularly when the stroke occurs in the posterior circulation.

None of our patients carried a *GLA* mutation unequivocally associated with the classical FD phenotype, but the 2 missense mutations that were identified have been previously reported in patients with FD and were not found in over 400 X chromosomes of healthy Portuguese control subjects.

However, given the 95% CI for their estimated prevalence in the general population, we cannot formally exclude that p.R118C and p.D313Y are low-frequency, missense single nucleotide polymorphisms, as previously suggested for the p.D313Y allele.²³

Mutation p.R118C was identified in a single case among 37 104 males enrolled in an Italian newborn screening program²⁴ but was not present in over 400 X chromosomes from normal control subjects.²⁴ However, in contrast to our findings, the neonate carrying this mutation had <5% residual enzyme activity. Experimental data suggest that mutation p.R118C behaves similarly to other known late-onset *GLA* mutations.²⁴ Mutation p.R118C has also been reported in a Portuguese family presenting with a mild clinical phenotype, including angiokeratomas in a young adult female²⁵ who had no histological evidence of globotriaosylceramide deposition in the kidney. Furthermore, the predominant association with stroke in the posterior circulation indirectly supports a pathogenic role for this mutation in cerebrovascular disease.

Mutation p.D313Y was first described in a patient with the classical FD phenotype,²⁶ but its pathogenic role has been subsequently challenged.^{23,27} Studies in transfected cells characterized the D313Y enzyme as a pH-sensitive variant associated with pseudodeficiency of α Gal activity in plasma.²³ Nonetheless, in recent screenings of FD among patients with idiopathic left ventricular hypertrophy, mutation p.D313Y was regarded as either pathogenic^{19,20} or nonpathogenic.²¹ Unfortunately, none of these studies provided histological evidence confirming or excluding the diagnosis of Fabry cardiomyopathy in such patients and the issue of whether mutation p.D313Y is pathogenic still remains open.

In our cohort, males with either the p.R118C or the p.D313Y mutation had comparable plasma α Gal activities (approximately 40% the normal mean) with average leukocyte activity, respectively, of approximately 35% and approximately 50% of the normal. Of note, plasma α Gal expression in several of these subjects was within the relatively high range observed in Japanese patients with a cardiac variant of FD.²⁸ Although α Gal expression was comparatively higher in leukocytes, the difference in the relative levels of α Gal activity in the leukocyte and plasma assays that we observed in p.D313Y hemizygous males was much lower than expected from available in vitro data.²³ In contrast to previous reports of classically affected patients with FD,^{23,27} none of our patients carried an additional missense *GLA* mutation in *cis* with mutation p.D313Y.

None of the clinical trials of enzyme replacement therapy with recombinant α Gal enrolled patients with substantial residual enzyme activity and there is no conclusive evidence that enzyme replacement therapy initiated in adulthood decreases the risk of stroke in patients with classic FD.⁷ Therefore, no recommendations regarding enzyme replacement therapy for the patients identified in this study can be issued at this time.

The pathogenic mechanisms of stroke in FD are uncertain.⁷ Our findings suggest that *GLA* mutations associated with relatively high residual α Gal activity may add to the risk of stroke in young adults, possibly by contributing to the underlying multifactorial pathogenesis rather than through a

classic Mendelian effect. Alternatively, it might be speculated that a late-onset cerebrovascular variant of FD is associated with relatively high levels of residual α Gal activity. These data need further confirmation in properly designed case-control and family segregation studies.

PORTYSTROKE Investigators (Listed by Participating Centers)

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