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ORIGINAL COMMUNICATION

Pompe disease in a Brazilian series: clinical and molecular analyses with identification of nine new mutations

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Abstract Pompe disease (glycogen storage disease type II or acid maltase deficiency) is an inherited autosomal recessive deficiency of acid α-glucosidase (GAA), with predominant manifestations of skeletal muscle weakness. A broad range of studies have been published focusing on Pompe patients from different countries, but none from Brazil. We investigated 41 patients with either infantile-onset (21 cases) or late-onset (20 cases) disease by muscle pathology, enzyme activity and GAA gene mutation screening. Molecular analyses identified 71 mutant alleles

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from the probands, nine of which are novel (five missense mutations c.136T > G, c.650C > T, c.1456G > C, c.1834C > T, and c.1905C > A, a splice-site mutation c.1195-2A > G, two deletions c.18_25del and c.2185delC, and one nonsense mutation c.643G > T). Interestingly, the c.1905C > A variant was detected in four unrelated patients and may represent a common Brazilian Pompe mutation. The c.2560C > T severe mutation was frequent in our population suggesting a high prevalence in Brazil. Also, eight out of the 21 infantile-onset patients have two truncating mutations predicted to abrogate protein expression. Of the ten late-onset patients who do not carry the common late-onset intronic mutation c.-32-13T > G, five (from three separate families) carry the recently described intronic mutation, c.-32-3C > A, and one sibpair carries the novel missense mutation c.1781G > C in combination with known severe mutation c.1941C > G. The association of these variants (c.1781G > C and c.-32-3C > A) with late-onset disease suggests that they allow for some residual activity in these patients. Our findings help to characterize Pompe disease in Brazil and support the need for additional studies to define the wide clinical and pathological spectrum observed in this disease.

Keywords Acid α-glucosidase · Pompe disease · Glycogen storage disease type II · Acid maltase deficiency · Mutation analysis · Novel mutation

Introduction

Pompe disease, also known as acid maltase deficiency, or glycogen storage disease type II (GSDII), is an inherited autosomal recessive disease of glycogen metabolism resulting from a deficiency of the lysosomal enzyme acid 1–4 α-glucosidase (GAA) (EC.3.2.1.20). Deficiency of this enzyme occurs in all cell types, with the cardiac, skeletal and smooth muscle cells being the most markedly affected. The resulting accumulation of glycogen disrupts cellular architecture and contributes to progressive tissue damage [9]. The disease manifests as a clinical spectrum of severity ranging from severe infantile-onset disease to the milder late-onset disease. This spectrum comprises different ages of onset, rates of progression and extent of tissue involvement. The infantile form, with onset in the first few months of life, is characterized by severe hypotonia, progressive weakness, massive cardiomegaly, with variable hepatomegaly, and macroglossia. The infantile-onset disease is typically fatal before the age of 2 years due to cardiac failure from massive glycogen storage. Conversely, the late-onset forms, classically referred to as juvenile and adult onset, have symptoms that are generally limited to skeletal muscle, with a slowly progressive proximal myopathy and marked clinical involvement of respiratory muscles [9].

The human structural gene encoding GAA is located at chromosome 17q25.2–q25.3 and contains 20 exons, the first of which is non-coding [9, 10, 18, 26]. The GAA cDNA is over 3.6 kb in length, with 2,856 nucleotides of coding sequence, predicting a protein of 952 amino acids with a calculated molecular mass of 105 kDa for the non-glycosylated protein [11, 12, 19, 20]. The enzyme is synthesized as a 110-kDa glycoprotein precursor that matures into a multi-subunit complex through multiple proteolytic and carbohydrate moiety modifications [8, 21, 30]. To date, more than 289 different variations are listed in the Pompe disease mutation database (www.pompecenter.nl). Of these, 197 have been demonstrated to be pathogenic [17]. The most frequent mutation among late-onset GSDII patients is the leaky c.-32-13T > G, which gives rise to alternatively spliced transcripts, including a deletion of the first coding exon, but still allows for the production of a low amount of normally processed mRNA [12, 15, 27].

Pompe disease has been observed and reported in a number of different populations but not from Brazil. In order to characterize this population and compare it to others, we have performed a molecular analysis of 41 Brazilian patients with a deficiency of GAA activity with varying ages of disease onset. A total of 29 distinct mutations from these patients were identified, nine of which were novel.

Materials and methods

Patients and skeletal muscle biopsy

Forty-one patients with clinical features consistent with Pompe disease were included in this analysis. This study was approved by the local ethics committee, and informed consent was given by each patient or legal guardian. Twenty-one patients presented with the infantile-onset form (onset at birth or in the first 3 months of life, cardiomegaly, respiratory insufficiency), and 20 patients presented with the late-onset form (onset after 1 year of age, less severe or absence of cardiac involvement and slower progression) [4] (Table 1). Diagnoses were based on neurological examination, muscular biopsy findings from biceps brachialis performed in 32 patients, autopsy

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Table 1 Clinical profile of 41 Brazilian Pompe patients

Patient	Gender	Age		First symptoms	Disease classification	Survival time	Current age
		Onset	Diagnosis				
1	M	At birth	3 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	8 months	–
2	M	At birth	4 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	8 months	–
3	F	At birth	4 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	4 months	–
4	F	At birth	6 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	28 months	–
5	F	At birth	7 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	2 y2 months	–
6	M	At birth	7 months	Hypotonia, cardiomegaly, respiratory distress, dispnea	Infantile-onset	–	3 y5 months
7	M	At birth	8 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	8 months	–
8	F	At birth	9 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	4 months	–
9	M	At birth	1 year	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	16 months	–
10	F	At birth	4 months	Hypotonia, cardiomegaly, gastro-esophageal reflux	Infantile-onset	9 months	–
11	F	2 months	6 months	Hypotonia, cardiomegaly	Infantile-onset	–	33 months
12	M	3 months	8 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	9 months	–
13	F	At birth	45 days	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	23 months	–
14	F	2 months	5 months	Hypotonia, cardiomegaly, hepatomegaly	Infantile-onset	10 months	–
15	M	3 months	5 months	Hypotonia, cardiomegaly, hepatomegaly	Infantile-onset	22 months	–
16	M	At birth	2 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	–	1 year
17	M	At birth	5 months	Cardiomegaly, respiratory distress	Infantile-onset	5 months	–
18	F	2 months	5 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	5 months	–
19	M	At birth	5 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	9 months	–
20	M	1 months	6 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	9 months	–
21	M	At birth	4 months	Hypotonia, cardiomegaly, respiratory distress	Infantile-onset	5 months	–
22	M	18 months	21 months	Hypotonia, respiratory distress, scoliosis, limb girdle muscle weakness	Late-onset	–	9 years
23	F	2 years	10 years	Limb girdle muscle weakness, scoliosis	Late-onset	–	14 years
24	M	7 years	15 years	Limb girdle muscle weakness, rigid spine, respiratory distress	Late-onset	15 years	–
25	M	8 years	23 years	Fatigability	Late-onset	–	26 years
26	F	10 years	36 years	Limb girdle muscle weakness	Late-onset	–	51 years
27	M	10 years	33 years	Limb girdle muscle weakness	Late-onset	40 years	–
28	M	10 years	24 years	Limb girdle muscle weakness, rigid spine	Late-onset	–	27 years
29	F	10 years	15 years	Limb girdle muscle weakness	Late-onset	–	31 years
30	M	27 years	57 years	Limb girdle muscle weakness	Late-onset	–	61 years
31	F	28 years	41 years	Limb girdle muscle weakness	Late-onset	–	45 years
32	M	35 years	51 years	Limb girdle muscle weakness	Late-onset	–	56 years
33	M	27 years	33 years	Limb girdle muscle weakness	Late-onset	–	30 years
34	M	31 years	40 years	Limb girdle muscle weakness, respiratory distress	Late-onset	–	43 years
35	F	12 years	23 years	Limb girdle muscle weakness, respiratory distress	Late-onset	–	26 years
36	F	21 years	32 years	Limb girdle muscle weakness	Late-onset	–	32 years
37	M	35 years	43 years	Limb girdle muscle weakness	Late-onset	–	44 years
38	F	36 years	46 years	Limb girdle muscle weakness, respiratory distress	Late-onset	–	46 years
39	F	34 years	40 years	Limb girdle muscle weakness	Late-onset	–	42 years
40	M	14 years	27 years	Limb girdle muscle weakness, respiratory distress	Late-onset	–	42 years
41	M	15 years	36 years	Limb girdle muscle weakness	Late-onset	–	37 years

M male; *F* female

findings (two patients), paraffin-embedded cardiac biopsy (one patient) and dry-blood spot analysis (eight patients). The analysis of muscle biopsies by light microscopy was performed after H&E, modified Gomori, periodic acid Schiff (PAS) and acid phosphatase staining. Parents were genotyped when blood samples were available.

GAA mutation analysis

Genomic DNA was extracted from peripheral blood (34 patients), frozen muscle biopsies (five patients), a formalin fixed muscle biopsy (one patient) and skin fibroblasts cultured by standard methods (one patient). All samples had the coding exons (exons 2 through 20) as well as the flanking intron/exon junctions of the GAA gene (about 60 base pairs upstream and downstream of the coding exons) amplified by polymerase chain reaction (PCR). Due to its size, exon 2 was divided into three overlapping fragments, while adjacent exons separated by small introns, such as exons 4 and 5, 6 and 7, and 10 and 11, were amplified together in one single PCR fragment. A total of 18 sets of primers were designed to amplify a region extending from exon 2 to exon 20. The sequence of the primers and expected PCR product sizes are shown in Table 2. PCR reactions were performed in 25 µl reaction mixtures containing PCR buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, pH 9.0), 0.16 mM of each deoxynucleotide triphosphate, 0.2 µM of each primer, 1 U of Taq DNA polymerase (GE Healthcare, Piscataway, NJ) and 100 ng of DNA. The reaction mixture was incubated at 94°C for 5 min for denaturation, followed by 30 cycles at 94°C for 30 s, annealing at variable temperatures (Table 2) for 30 s, extension at 72°C for 30 s, and a final extension of 10 min at 72°C. PCR amplification was confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide. The exon 18 deletion mutation was analyzed by PCR using the primers in intron 16 and intron 18. PCR conditions were the same as described for the other exons, with a melting temperature of 68°C and extension of 1 min. When analyzed by agarose gel electrophoresis, a PCR product of 374 bp was detected in cases with the exon 18 deletion, in contrast to a predicted normal product of 899 bp. PCR amplified products were purified with GFX column (GE Healthcare) and sequenced on an ABI PRISM 3130 DNA Sequencer using BigDye v.3.1 (Applied Biosystem, Foster City, CA, USA) with the original forward and reverse primers used for PCR amplification.

Cloning was performed whenever direct sequencing of the PCR product was insufficient to clarify the alteration, usually when there was an insertion or deletion. PCR products were cloned using a pGEM-T cloning system (Promega, Madison, WI, USA), and transformation was carried out using chemically competent *E. coli* DH5-alpha. At least five clones were sequenced for each PCR product cloned.

Disease associated allelic variants that had not previously been described were analyzed by sequencing 100 normal alleles.

Mutation nomenclature

The mRNA reference sequence (RefSeq) for GAA is filed under accession number NM_000152 at GenBank (www.ncbi.nlm.nih.gov/RefSeq). The location of the mutations is indicated by the nucleotide number whereby the "A" nucleotide of the start ATG codon at position 442 of the RefSeq nucleotide constitutes the +1 numbering of the cDNA sequence, as well as the amino acid numbering as set forth by the acid alpha glucosidase preprotein sequence NP_000143. Mutation nomenclature follows guidelines set by the Human Genome Variation Society (www.hgvs.org/mutnomen/).

Results

We analyzed the complete mutation profile of the GAA gene in 41 Brazilian patients presenting with infantile- and late-onset forms of the disease (Table 1). Among these patients, we evaluated three sibling sets (patients 31 and 32; patients 33 and 34; and patients 37, 38 and 39). Additionally, we analyzed the mother (patient 26) and maternal aunt (patient 27) of patient 25. Molecular analyses of 72 unique alleles of probands (excluding siblings) identified mutations in 71 alleles comprised of 29 different mutations, nine of which are novel (missense mutations c.136T > C, c.650C > T, c.1456G > C, c.1834C > T, and c.1905C > A, a splice-site mutation c.1195-2A > G, two deletions c.18_25del, c.2185delC, and one nonsense mutation c.643G > T) (Table 3). The second mutation of patient 1 was not identified despite full GAA gene screening by sequencing. The nature of the sample, a paraffin-embedded myocardial biopsy, did not allow further investigation at the mRNA and protein level. In order to characterize the association of the missense mutations (p.Ser46Pro, p.Pro217Leu, p.Ala486Pro, p.His612Tyr, and p.Asn635Lys) and the disease, we screened a total of 50 normal Brazilian individuals. None of the alleles carried any of these alterations.

The most common mutation encountered in this study was the c.2560C > T mutation, found in 12 out of 72 alleles (16.7%) from seven infantile-onset patients and one late-onset sibpair. The latter, patients 31 and 32, presented in heterozygosity with the c.-32-13T > G mutation. Although they had the same genotype, the ages of onset (28 and 35 years old) and the muscle biopsy morphology were quite different suggesting extragenic influences on disease severity (Fig. 1).

The second most frequent mutation was the c.-32-13T > G mutation, followed by the c.-32-3C > A mutation, found in 10 out of 72 alleles (13.9%) and 9 out of 72

Table 2 Nucleotide sequences of the primers used for PCR amplification of GAA gene, location and PCR product size and annealing temperature

Exon	Location	Orientation	Primer sequence (5′-3′)	PCR product (bp)	Annealing temperature (°C)
	Intron 1	Sense	TTT GAG AGC CCC GTG AGT GC	273	65
	Exon 2	Antisense	TCC CTG CTG GTG AGC TGG GT		
2	Exon 2	Sense	CGA GAG CTG AGT GGC TCC TC	270	68
	Exon2	Antisense	GAA GAA GCA CCA GGG CTG CC		
	Exon 2	Sense	CCT GCA AAG CAG GGG CTG CA	267	64
	Intron 2	Antisense	ATG TCC ACG GGC ACC CTC TG		
3	Intron 2	Sense	GAC CTG ACC TGT CCT TGG CG	271	70
	Intron 3	Antisense	TCG CCC TCC CCA TCA TGC TG		
4 and 5	Intron 3	Sense	GTG CTC TCA GGC TCG TGT GG	464	66
	Intron 5	Antisense	GTC TCC AGG GCA GGC AGC AC		
6 and 7	Intron 5	Sense	GGT GCA GAG CCC TCC AAG TG	445	68
	Intron 7	Antisense	TCT GCT GGG GCC TGA GGA GA		
8	Intron 7	Sense	GTG AGT TGG GGT GGT GGC AG	285	68
	Intron 8	Antisense	GAG AAG GAG CCA CTG GGC AC		
9	Intron 8	Sense	CTC AGT TTT CCC CGT GGC TG	230	68
	Intron 9	Antisense	GCT GGA GGC CTC TGC TTT CT		
10 and 11	Intron 9	Sense	GCT CAG TGG GGC TTC CAT GC	449	68
	Intron 11	Antisense	TGA GGG TGC TAA GTC TCC CA		
12	Intron 11	Sense	GAG GAA GCT CCC TGG AAA CC	210	62
	Intron 12	Antisense	CTT GTA GGA CAG GCT GTG AG		
13	Intron 12	Sense	TGA CAG GGT TCC CGA GTG AC	255	64
	Intron 13	Antisense	GCC TCC CAT AGA GGC CCC CG		
14	Intron 13	Sense	CTG GCT CTG CTG CAG CAG CC	295	68
	Intron 14	Antisense	GCA TGG GGT GCT TCT CCA GC		
15	Intron 14	Sense	TGA GAA GTG CAG CTC TCC CG	309	68
	Intron 15	Antisense	AGG GCT GCC TGG CAG TTA CG		
16	Intron 15	Sense	GGG TGG GCA TAT GAG CCA GC	265	68
	Intron 16	Antisense	TGG GAG GGC TGC TCT GGT CT		
17	Intron 16	Sense	AGC GTG GTT CCT GAG GAC AG	249	68
	Intron 17	Antisense	CTG CAG TGT GCT GTC CAC AC		
18	Intron 17	Sense	AGG CCT CCACCT CCA CCA GG	293	68
	Intron 18	Antisense	CCA GGT CCC CTC ACC CCT TC		
19	Intron 18	Sense	AGC TGT CTG CTG ACA CCT CC	319	64
	Intron 19	Antisense	CCC AGC TAC CTC TGT TCC TG		
20	Intron 19	Sense	CTG GGG TCT CAC TGC TGC TG	175	68
	Intron 20	Antisense	CTG CTT CCC TGG GGA ACC AG		

alleles (12.5%), respectively; all of them from late-onset patients. The c.-32-13T > G [IVS1] mutation is commonly present in heterozygosity in late-onset patients. Patient 25 inherited the -32-13T > G mutation from his father (confirmed by paternal genotype) and the c.1927G > A mutation from his mother. Genotype analysis of the mother (patient 26) and her brother (patient 27) revealed that they are also heterozygous for the c.-32-13T > G and c.1927G > A mutations.

The c.-32-3C > A mutation was found in three probands who did not carry the c.-32-13T > G mutation. Three affected siblings (patients 37, 38 and 39) were homozygous for the c.-32-3C > A mutation and had the late-onset presentation of the disease. Both parents were heterozygous for the mutation. They are second cousins suggesting that the two alleles inherited by patients 37, 38, and 39 are identical by descent. Two late-onset patients (sibpair patients 33 and 34) are heterozygous for the missense

Table 3 Overview of genotypes encountered in 41 Brazilian Pompe patients

Patient	Genotype	
	Allele 1	Allele 2
1	**c.1195 -2A > G (r.spl?)**	n.d.
2	c.784G > A (p.Glu262Lys)	c.1561G > C (p.Glu521Gln)
3	**c.1905C > A (p.Asn635Lys)**	**c.1905C > A (p.Asn635Lys)**
4	c.2481+102_2646+31del (p.Gly828_Asn882del)	c.2481+102_2646+31del (p.Gly828_Asn882del)
5	c.2560C > T (p.Arg854X)	c.2560C > T (p.Arg854X)
6	c.1913G > T (p.Gly638Val)	c.1912G > T (p.Gly638Trp)
7	**c.1905C > A (p.Asn635Lys)**	**c.1905C > A (p.Asn635Lys)**
8	c.377G > A (p.Trp126X)	c.2501-2502delCA (p.Thr834Argfs48X)
9	c.1655T > C (p.Leu552Pro)	c.1941C > G (p.Cys647Trp)
10	c.2560C > T (p.Arg854X)	c.2560C > T (p.Arg854X)
11	c.2560C > T (p.Arg854X)	c.2501_2502delCA (p.Thr834Argfs48X)
12	**c.1834C > T (p.His612Tyr)**	c.2560C > T (p.Arg854X)
13	c.2608C > T (Arg870X)	c.2608C > T (Arg870X)
14	c.2560C > T (p.Arg854X)	c.2560C > T (p.Arg854X)
15	**c.2185delC (p.Thr729fsX8)**	**c.2185delC (p.Thr729fsX8)**
16	**c.650C > T (p.Pro217Leu)**	c.2481+102_2646+31 (p.828_Asn882del)
17	c.2481+102_2646+31del (p.Gly828_Asn882del)	c.2560C > T (p.Arg854X)
18	c.2501_2502delCA (p.Thr834Argfs48X)	c.2560C > T (p.Arg854X)
19	**c.18_25del (p.Pro6fsX34)**	**c.136T > G (p.Ser46Pro)**
20	c.236_246del (Pro79X)	c.1927G > A (p.Gly643Arg)
21	**c.1905C > A (p.Asn635Lys)**	c.2501_2502delCA (p.Thr834Argfs48X)
22	c.1655T > C (p.Leu552Pro)	c.1655T > C (p.Leu552Pro)
23	c.377G > A (p.Trp126X)	c.1655T > C (p.Leu552Pro)
24	c.-32-3C > A (r.spl?)	**c.1905C > A (p.Asn635Lys)**
25	c.-32-13T > G (leaky splice)	c.1927G > A (p.Gly643Arg)
26	c.-32-13T > G (leaky splice)	c.1927G > A (p.Gly643Arg)
27	c.-32-13T > G (leaky splice)	c.1927G > A (p.Gly643Arg)
28	c.-32-3C > A (r.spl?)	c.1447G > A (p.Gly483Arg)
29	c.-32-13T > G (leaky splice)	**c.634G > T (p.Glu212X)**
30	c.-32-13T > G (leaky splice)	c.2481+102_2646+31del (p.Gly828_Asn882del)
31	c.-32-13T > G (leaky splice)	c.2560C > T (p.Arg854X)
32	c.-32-13T > G (leaky splice)	c.2560C > T (p.Arg854X)
33	c.1781G > C (p.Arg594Pro)	c.1941C > G (p.Cys647Trp)
34	c.1781G > C (p.Arg594Pro)	c.1941C > G (p.Cys647Trp)
35	c.-32-3C > A (r.spl?)	c.2173C > T (p.Arg725Trp)
36	c.-32-13T > G (leaky splice)	**c.1456G > C (p.Ala486Pro)**
37	c.-32-3C > A (r.spl?)	c.-32-3C > A (r.spl?)
38	c.-32-3C > A (r.spl?)	c.-32-3C > A (r.spl?)
39	c.-32-3C > A (r.spl?)	c.-32-3C > A (r.spl?)
40	c.-32-13T > G (leaky splice)	c.525del (p.Gly176fsX45)
41	c.-32-13T > G (leaky splice)	c.1913G > T (p.Gly638Val)

Novel GAA mutations are indicated in *bold*

n.d. not determined

mutation c.1781G > C in combination with the severe mutation c.1941C > G. The two remaining late-onset patients (22 and 23) presented with disease at a very young age (1.5 and 2 years, respectively). Patient 22 is homozygous for c.1655T > C, and patient 23 is heterozygous for the c.377G > A and the c.1655T > C mutations.

The infantile-onset patients carry a range of different mutations with a large proportion homozygous or

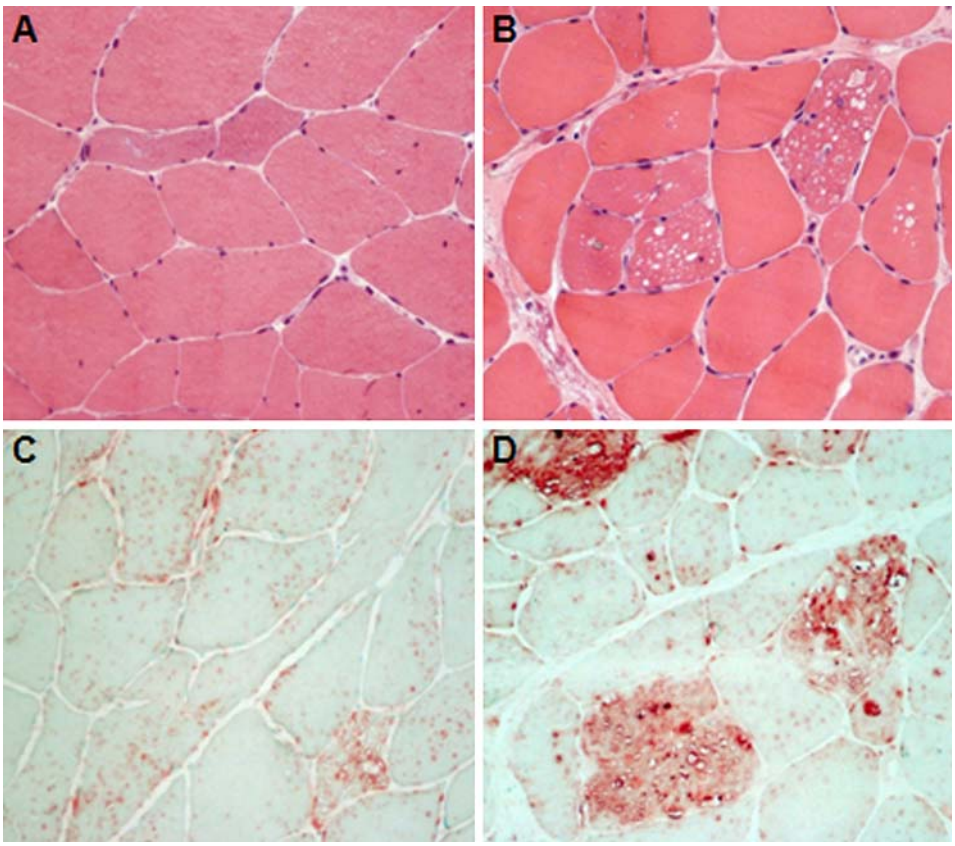

Fig. 1 Frozen sections of biceps brachial skeletal muscle of patients 31 (**a** and **c**) and 30 (**b** and **d**) with Pompe disease. **a** H&E, patient 31, late-onset form, showing less than 1% of vacuolated muscle fibers (200×). **b** H&E, patient 30, late-onset form, with 36% of vacuolated muscle fibers (200×). **c** Acid phosphatase from patient 31 (200×). **d** Acid phosphatase from patient 30 (200×)

heterozygous for two mutations predicted to abrogate protein expression (patients 5, 8, 10, 11, 13, 14, 15, and 18). The absence of maternal and paternal mutation status in patients 5, 10, 13, 14, and 15 prevent confirmation of biallelic inheritance of the identical mutation from both parents; thus, the possibility of an overlapping deletion cannot be ruled out.

The infantile-onset patients carry seven of the nine total novel mutations. One novel mutation observed in patient 1, c.1195-2A > G in intron 7, disrupts the conserved splice acceptor site at the junction of exon 8. The severity of this alteration is indicated by the rapid progression of disease in this patient with death at 8 months of age.

We also identified three allelic variants predicted to encode for truncated forms of GAA, two deletions c.18_25del, p.Pro6fsX36; and c.2185delC, p.Thr729fsX8, and one nonsense mutation c.643G > T, p.Glu212X. All are likely to abrogate protein expression.

Interestingly, one of the novel mutations, c.1905C > A, was detected in four non-related patients, three infantile-onset cases (patients 3, 7 and 21) for which it was found in homozygosity in two cases and one late-onset case (patient 24). This suggests that this mutation may be common in this population.

Coincidentally, patient 6 was found to have two different mutations in the same codon of exon 14. The presence of these mutations in distinct alleles was confirmed by genotyping the parents. One mutant allele was inherited from his mother (c.1912G > T) and the other from his father (c.1913G > T).

Discussion

We studied 41 Brazilian patients whom we diagnosed as having Pompe disease; 21 patients with infantile-onset and 20 with late-onset disease. For the 35 patients where biopsy material was available, examination of skeletal muscle using histological and histochemical analyses revealed abnormal lysosomal glycogen accumulation in muscle fibers. Additionally, Pompe disease was confirmed by abnormal GAA activity measurements by dry-blood spot analysis in eight patients. Genetic analysis of the GAA gene supports the diagnosis in all cases with the identification of two disease associated variants in 40 cases and one disease associated variant in one patient with limited sample availability.

One of the most striking finding in this population is the high number (9 of 36) of proband cases that were homozygous for a mutation. Seven of the nine patients with homozygous mutations presented with infantile-onset disease. Unfortunately, we did not have access to parental

DNA to confirm biallelic inheritance. Therefore, in order to confirm homozygosity and rule out intragenic deletions, we analyzed a series of polymorphisms across the GAA gene (data not shown). Only two of the patients (cases 5 and 13) were heterozygous for polymorphisms near the region of the detected mutation suggesting that an overlapping deletion on one allele was unlikely and that the two alleles in these patients were not identical by descent.

Patient 6 has two different missense mutations in the same codon of exon 14, confirmed to be in distinct alleles by inheritance from his mother (c.1912G > T) and from his father (c.1913G > T). Both mutations are predicted to be responsible for severe phenotypes by the replacement of glycine at codon 638 with valine and tryptophan, an amino acid residue that is highly conserved [29]. An homozygous patient for the c.1912G > T mutation presented with clinical onset at 2 months of age [25]. Functional analysis for the c.1912G > T mutation by Western blot and the protein expression in COS cells demonstrated low levels GAA synthesis and degradation of the enzyme precursor [28]. Despite unusual survival of patient 6, who is under enzyme replacement therapy (ERT) for 3 years, he had some improvement of cardiac function but no improvement of motor function, inability to move any segment of upper or lower limbs, dependence of BIPA 24 h/day and feeding by gastrectomy.

The novel mutation c.1905C > A [p.Asn635Lys] was found in homozygosity in two unrelated children with infantile-onset disease (patients 3 and 7) who survived for only 4 and 8 months, respectively, suggesting a severe phenotype due to this mutation. In addition, another unrelated child (patient 21), heterozygous for the c.1905C > A mutation and a deletion (c.2501_2502del), survived for 5 months. The c.1905C > A mutation alters amino acid 635 and occurs in a highly conserved region of the protein [14], strongly suggesting a deleterious effect on enzyme expression. Patient 4 was found to be homozygous for the common c.2481 + 102_2646 + 31del [p.Gly828_Asn882del, Del exon 18] mutation [13]. Homozygosity for this mutation is uncommon and may represent consanguinity; however, a comprehensive family history was not taken in this case nor for the other infantile-onset patients.

The mutation c.2560G > T [p.Arg854X] in exon 18, was observed in 16.7% (12 of 72) of the alleles studied. This mutation was detected in homozygosity in three infantile cases (patients 5, 10 and 14), and in heterozygosity in four other infantile cases (patients 11, 12, 17 and 18), and two affected adult siblings (patients 31 and 32). For the two adults, this mutation was observed in combination with the common intronic 1 mutation (c.-32-13T > G). All patients are of African descent, and it is most likely this is the origin of this mutation in Brazil. This is consistent with previous reports of this mutation having a higher incidence amongst Pompe patients of African origin [1, 2]. While this mutation has been described in a Spanish juvenile onset patient who inherited the mutation from his Dominican mother [5], it has not been reported with such a high frequency by others in Spain or South America [6, 7]. Despite the fact that patient 11 has two different alterations leading to protein truncation, c.2560G > T [p.Arg854X] and c.2501_2502del [p.Thr834Argfs48X] and first symptoms onset at 2 months of age, she is still alive, under ERT, being able to walk with support.

By way of comparison to other Pompe patient populations, the c.377G > A [p.Trp126X] mutation which has been observed as very common in the Argentinean Pompe patients of Italian origin [23], was only observed in two individuals. Another mutation, c.1655T > C [p.Leu552-Pro], which was initially classified as a severe mutation in a 7-year-old male with "late infantile" Pompe disease and was found to have very low residual activity after expression in COS cells [3], was observed in three individuals (patients 9, 22, and 23), one being homozygous (patient 22) for this mutation. All three of the current patients in our study presented with an age of onset ranging from at birth to 2 years of age (Table 1).

Of particular interest is the survival of patients 22 and 23, both of whom presented with disease early in life and are still alive, compared to patient 9 who died at 16 months of age. The c.1655T > C mutation has previously been described in Italian and Spanish Pompe patients [7, 25]. Pittis et al. [25] described the c.1655T > C as the second most frequent among Italian Pompe cases, and in vitro analysis demonstrated a response to chaperones [24], suggesting that this mutation may be associated with late-onset Pompe patients with onset in the first years of age. It is possible that other genetic or environmental factors modulate the severity and outcome in untreated patients with this mutation.

We also identified three allelic variants predicted to encode for truncated forms of GAA, two deletions c.18_25del, p.Pro6fsX36; and c.2185delC, p.Thr729fsX8, and one nonsense mutation c.643G > T, p.Glu212X. The mutant transcripts are likely to be targeted by nonsense-mediated decay based on their location within the gene [22]. Even the mutation c.2185delC, p.Thr729fsX8, found in patient 15, is likely to abrogate the expression as the patient presented with severe infantile-onset disease at 2 months of age and survived less than 2 years.

Five novel allelic variants described here encoded for missense mutations: c.136T > C, [p.Ser46Pro]; c.650C > T, [p.Pro217Leu]; c.1456G > C, [p.Ala486Pro]; c.1834C > T, [p.His612Tyr]; and c.1905C > A, [p.Asn635Lys]. As the effect of these variants on GAA activity and expression has not been studied, their significance to disease development remains unknown. Sequence analysis of 100

normal Brazilian GAA alleles did not reveal these mutations to be present in the normal individuals. This suggests that they are not common normal polymorphisms among our patient population.

Of the late-onset patients, 8 of 15 probands (53%) carried the common late-onset c.-32-13T > G (IVS1) mutation, whereas the remaining seven had combinations that lacked this mutation. The c.-32-13T > G mutation was originally identified in 68% of adult patients with ages at diagnosis ranging from 17 to 73 years [12]. When Kroos et al. [16] analyzed 98 adult compound heterozygotes with the c.-32-13T > G and another fully deleterious mutation, they observed a broad spectrum of age at onset of first symptoms manifestations, ranging from <1 to 78 years. The range of age of onset from 8 to 35 years among our patients is consistent with these findings and lends to the speculation that other genetic factors may play a role in the variability of onset and severity in cases carrying the c.-32-13T > G mutation [16].

We and others recently described the c.-32-3C > A mutation in intron 1 and c.1781G > C [p.Arg594Pro] mutation in exon 13 [17, 25]. Of interest, six of 20 late-onset cases (patients 24, 28 and 35, and siblings 37, 38 and 39) carry the c.-32-3C > A mutation. Patients 24, 28 and 35 presented as juvenile patients (ages 7, 10 and 12 years, respectively) while the three siblings who are homozygous for the mutation present with disease in their 30s. Although the ages of onset for the siblings are similar, the evolution and clinical symptoms need a longer follow-up, since only the older sister has presented with respiratory distress in addition to the limb girdle muscle weakness suggesting that the disease course may diverge. The diminished severity in the homozygous siblings as compared to patients 24 and 28 support the possibility of low residual activity from the mutant allele such that the presence of two copies may delay disease onset and rate of progression. It is also possible, however, that other factors within the mutant GAA allele or other modifier genes may contribute to the differences in age of onset. Interestingly, Pittis et al. [25] observed the c.-32-3C > A variant in an infantile-onset patient. They determined that the mutant allele leads to a deletion of 579 bp which would result in the loss of the first 182 amino acids of the protein (p.M1_T182). Thus, factors which control splice-site selection may contribute to the relative level of normal transcript the mutant allele is able to generate.

Analysis of our Brazilian Pompe patients showed that sometimes the nature of the mutation matched the phenotype within this group. When compared to other populations this was not always consistent. In many, the age of onset and the disease course were different in patients with the same genotype, as in the cases of relatives studied here.

In conclusion, this first study of Brazilian Pompe patients has been helpful in furthering the characterization of the mutations present in this population and has allowed us to compare our patients, their clinical presentation and severity to others with this disease. The remarkable heterogeneity in the mutational spectrum of Brazilian Pompe patients may reflect the ethnic diversity of our population and serve to aid in determining what other factors influence disease progression and outcome.

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