Genotype-Phenotype correlation of 17 cases of Pompe Disease in Spanish Patients and Identification of 4 Novel Mutations

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Research

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Abstract

Background: Pompe disease (PD) is an autosomal recessive metabolic disorder caused by mutations in the acid α-glucosidase gene (*GAA*) that produces defects in the lysosomal acid α-1,4-glucosidase. We aimed to identify genetic variations and clinical features in Spanish subjects to establish genotype-phenotype correlation.

Methods: A total of 2709 subjects who showed symptoms or susceptible signs of PD were enrolled in this observational study. Enzymatic activity was detected by fluorometric techniques and the genetic study was carried out using Next-Generation Sequencing.

Results: Fourteen different variants from seventeen patients were identified, four of whom had not been described in the literature previously, including a homozygous variant. In all of them α-glucosidase activity was decreased. Muscle weakness, respiratory distress, exercise intolerance, hypotonia, dysphagia and myalgia were commonly observed in patients.

Conclusions: This study report four new mutations that contribute to the mutation spectrum of the GAA gene. We confirm that patients in Spain have a characteristic profile of a European population, with c.-32-13T> G being the most prevalent variant. Furthermore, it was confirmed that the c.236_246delCCACACAGTGC mutation in homozygosis is associated with early disease and a worse prognosis.

Background

Pompe disease (PD) is an autosomal recessive metabolic disorder caused by mutations in the acid alpha-glucosidase gene (GAA) that produces biochemical defects in the lysosomal acid alpha 1,4-glucosidase. The deficient activity of the enzyme leads to lysosomal accumulation of glycogen in all tissues, specially skeletal muscle. PD is a disorder that manifests a clinical spectrum that varies regarding the age of onset, the rate of disease progression, and the degree of organ involvement; and in general, there is an inverse correlation between the severity of the disease and the level of residual enzyme activity. Because of the variation in the phenotypes, PD is classified into infantile-onset Pompe disease (IOPD) and late-onset Pompe disease (LOPD). IOPD is characterized by onset during the first year of life with hypertrophic cardiomyopathy, generalized muscle weakness, hepatomegaly and respiratory dysfunction. LOPD is characterized by onset in childhood or adulthood and the presence of slow progressive muscle weakness, cardiomyopathy and respiratory distress. The incidence of the disease varies in different ethnic groups and for different clinical forms. It was reported that the incidence of PD in Caucasian is 1:100000 and 1:60000 in IOPD and LOPD respectively [1]

The GAA gene (OMIN 606800) is situated on chromosome 17 and contains 20 exons and 19 introns extended over a distance of 20Kb. The first exon is noncoding and the beginning of the start codon is at position 33 of exon 2. To date, over 560 variants have been described in the *GAA* gene. Most of the variants described are marked as pathological and some of them have uncertain significance. Some mutation are widely found in certain populations. For instance, the intronic variant c.-32-13T > G is the most common in Caucasian individuals [2] In Asian population the most common variants are c.1935C > A and c.2238G > C in Taiwanese and Chinese individuals [3], c.1316T > A and c.1857C > G in Korean individuals and c.2560C > T is the most frequent in Afro-American individuals [4]

We report here an observational study as a result of a biochemical and genetic analysis of subjects suggestive of PD. We analyzed clinical manifestations, GAA activity and GAA mutations of Spanish patients with Pompe disease to establish genotype-phenotype correlation.

Materials And Methods

Design of the study and patients

In this observational study, clinical and biochemical aspects and GAA gene sequence in a large cohort of patients from different Spanish hospitals were analyzed.

Patients included either had a family member with PD or presented more than one sign or symptom associated to PD: generalized muscle weakness, CK elevations, exercise intolerance or pain, hipotonia, hypertrophic cardiomyopathy, respiratory distress, dysphagia, dyspnoea. Enzyme activity in DBS [Dried blood spots] was measured for each patient. Patients with decreased enzyme activity in DBS underwent lymphocyte determination to confirm the enzyme diagnosis. The sequencing of the *GAA* gene was performed in the patients who showed low enzyme activity in DBS and lymphocytes or who had a family member with PD.

Informed consent was signed by all patients and the study was approved by the Ethics and Research Committee of the Virgen Macarena and Virgen del Rocío University Hospital (Code: 0826-N-15).

BioChemical analysis

The determination was carried out according to the technique described by Chamoles et al. [5]. GAA activity was measured in DBS samples or isolated lymphocytes using 4-Methylumbelliferyl-a-D-glucopyranoside as sustrate and acarbose as inhibitor of competing enzymes at pH 4 [6, 7]

A standard curve of 4-methylumbelliferon allow to establish a relationship between the intensity of the fluorescence and the enzymatic activity as umol/L/h in DBS [cut off < 0,75 umol/L/h] and nmol/min/mg protein in lympohocytes (cut off < 0,15 nmol/min/mg protein).

Molecular and bioinformatics analysis

Genomic DNA was isolated from whole blood by standard procedures using *MagNA Pure Compact Nucleic Acid Isolation Kit I. (Roche Diagnostics, Basle, Switzerland)*. Genetic study was carried out by Next-generation sequencing (NGS). All coding regions and classical splicing sites of GAA gene were amplified using a custom design kit for Ion AmpliSeq in a S5 Ion Torrent Platform. The reads were aligned to Genome Reference Consortium Human Build 37 GRCh37. The limitations of the technique include non-detection in the intronic regions of the gene, nor highly repeated regions or other structural variants as inversions, translocations, large insertions or deletions.

Obtained sequences were compared with the GAA reference sequence NM_000152.3 to identify genetic variants. All of them had a minimum read depth of 20x. Single nucleotide changes, insertions or delections were compared to the online genome databases ClinVar and Human Gene Mutation Database (HGMD) open access and to the Erasmus MC University Medical Center Rotterdam.

After analyzing the numerous variants obtained, those with a frequency greater than 1% in the general population were discarded according to the polymorphism database [http://www.ncbi.nlm.nih.gov/projects/SNP/]. Variants that were described as benign, probably benign or polymorphisms in databases were not further researched. Novel missense mutation effects were determined using in silico analysis by Mutation Taster [http://www.mutationtaster.org] and Polyphen2 software programs [http://genetics.bwh.harvard.edu/pph2]. Novel nonsense mutation that generates a premature stop codon upstream of another known disease causing nonsense mutation or that affects the active protein center were assessed pathogenic.

Results

From August 2016 to December 2019 were tested 2637 samples of 1343 males and 1294 females [mean age 45.16 years, SD 20.77, range 0–98]. Patients with positive screening in DBS (activity < 0,75 µmol/L/h) are asked for a sample of lymphocytes to measure the enzyme activity and confirm the diagnosis (activity < 0.15 nmol/min/mg protein). The *GAA* sequence was performed in all patients whose enzyme activity measured in DBS and lymphocytes was decreased (Fig. 1).

Enzymatic GAA activity

Of the 2637 patients studied, 2520 (95,56%) showed normal α-glucosidase activity in DBS. The determination of the enzyme activity in lymphocytes was carried out in the 117 (4,44%) patients who showed low activity in DBS to confirm the enzyme diagnosis. The analysis of the enzyme activity in lymphocytes was normal in 93 (3,53% of total studied and 79,5% of 117 positives in screening) patients and a total of 24 (0,91% of total studied and 20,5 of 117 positives in screening) patients resulted with reduced activity and they were all sequenced.

GAA mutations

In relation to 24 sequenced, 17 subjects (#1 to #17), presented two variants compatible with disease: seven males and nine females with LOPD (mean age 36,07, SD 20,57, range 7–64) and a 2-day-old boy with IOPD. Subjects with two variants found will be called

“patients”. Subjects #18 and #19, a woman and a man respectively, showed a single variant in heterozygosis (#18 and #19) and in 5 subjects no genetic justification was found in this study. All the variants found in the molecular study were consulted in the bibliography and public databases and were subsequently analyzed using in silico tools if it was possible.

Of all the patients who showed two variants compatible with PD, 4 subjects were homozygous and the rest had two variants in heterozygosity. The clinical manifestations, biochemical analysis data and genotype of the 17 patients from 16 families are summarized in Table 1. Sixteen different variants were detected and the frequency in our population is shown in Table 2. The variants found including 10 missense mutations (11/36; 30.5%), one nonsense mutation (1/36; 2.8%), 2 frameships by deletions (4/36; 11.1%) and one frameship by insertion (2/36; 5.5%), 2 splicing variants (18/36; 50%).

Clinical manifestations

Among the 17 patients with PD included in the study, one patient had IOPD phenotype and 16 had LOPD phenotype. The most frequent symptoms and sings in LOPD were muscle weakness (62,5%), followed by high CPK serum values (37,5%) and respiratory distress (25%). Cardiomyopathy, exercise intolerance, hypotonia, dysphagia and myalgia were ascertained in 12.5% of patients. Patient #2, #3 and #6 were asymptomatic at the time of assessment. They were incorporated in the study because they had a member of the family with PD.

The only patient with IOPD, a male of two days of age (patient #1) presented at birth a hypertrophic cardiomyopathy and he died shortly after receiving the sample.

The mean of GAA activity in LOPD patients was 0.30 umol/L/h in DBS and 0.05 nmol/min/mg protein in the isolated lymphocytes. Enzyme activity in DBS of the only infantile-onset Pompe disease patient was 0.5 umol/L/h and lymphocyte measurement could not be performed due to the death of the patient.

Genotype-Phenotype correlations

Two mutations were the most frequent, contributing to 58% of the total alleles. The most common variant was **c.-32-13T > G**. It was detected in 15 patients (88.2%); 2 were homozygotes and 13 were heterozygotes. The next most frequent variant was **c.236_246delCCACACAGTGC** which was observed in two unrelated patients [5.8%]. One patient was homozygote who presented IOPD and one heterozygote who presented LOPD. The mutations **c.1328A > T** and **c.1396_1397insG** were identified in one homozygote and two heterozygotes patients respectively. The rest of the mutations (**c.281_282delCT; c.655G > A; c.875A > G; c.925G > A; c.1655T > C; c.2104C > T; c.2237G > C**) were detected once in each patient who presented the variant. The total number of mutations detected were analyzed by bibliography and in silico predictive tools [Table 3].

Four of the 14 different variants identified had not been reported previously and we considered them as likely pathogenic (**c.1328A > T, c.1831G > A, c.2819C > A, c.1889-1G > A**):

The missense variant **c.1328A > T** is located in exon 9 and it was found in homozygosis in patient #5. It results in a protein change and replaces aspartic acid with valine at codon 443 (**p.Asp443Val)**. All of in silico tools consulted predicted a damaging effect of the variant on the protein function. Mutation Taster consider it as disease causing and Polyphen-2 as probably damaging. Evolutionary conservation of amino acid and the position of the residue involved in the structure of the protein is showed in Fig. 3. This variant was observed in homozygosis in a seventeen woman of Pakistani origin who showed exercise intolerance, muscle weakness and high Creatine-Phosphokinase (CPK) serum values. She also had a muscle biopsy compatible with PD. Enzymatic activity measured in DBS was 0,37 umol/L/h (V.N:>0,75 umol/L/h) and 0,02 nmol/min/mg protein in lymphocytes.

The missense variant **c.1831G > A** is located in exon 9 and it was found in heterozygosis in patient #4. It produces a change in the protein and replaces glycine with serine at codon 611 **[p.Gly611Ser]**. The analysis with in silico predictive tools showed a probably damaging effect on the protein function. Mutation Taster consider it as disease causing and Polyphen-2 as probably damaging. Evolutionary conservation of amino acid and the position of the residue involved in the structure of the protein is showed in Fig. 3. The variant was found in heterozygosis in an eleven female (-32-13T > G + c.1831G > A) who presents cardiomyopathy, high CPK serum values and muscle weakness. Enzymatic activity measured in DBS was 0,16 umol/L/h [V.N:>0,75 umol/L/h] and 0.03 nmol/min/mg protein in lymphocytes.

The novel nonsense variant **c.2819C > A** is located in exon 20 and it was detected in heterozygosis in patient #9. It leads to a premature stop codon in protein synthesis [**p.Ser940Ter**]. Pathogenicity of the novel mutation was also predicted by in silico analysis. It was

classified as disease causing by Mutation Taster. The variant was found in a heterozygosis (c.-32-13T > G + c.2819C > A) in a forty-four female who presents hypertrophic cardiomyopathy and respiratory distress. Enzymatic activity measured in DBS was 0,34 umol/L/h (V.N:>0,75 umol/L/h).

The splicing variant **c.1889-1G > A** is located in intron 13 and it was observed in heterozigosis in patient #12. This variant affects the normal splicing process. Bioinformatics analysis were performed using Human Splicing Finder which reported that the variant disturbs the wild type acceptor site probably affecting the splicing. The other allele contained the mutation c.-32-13T > G, widely described as a pathogenic variant. Patient #12 is a fifty-two years old man who presents severe clinical manifestations: exercise intolerance, generalized muscle weakness and respiratory distress. Enzimatic GAA activity was also measured on DBS and lymphocites (0,48 umol/L/h and 0.01 nmol/min/mg protein respectively).

Variants detected in patients who showed a single variant in heterozygosis were **c.854C > G** and **c.2065G > A**. Both are missense variant and were not found in other patients. Variant c.854C > G is located in exon 4 and and it is detected in an asymptomatic female. Variant c.2065G > A is located in exon 15 in a male who presents muscle weakness and high level of CPK in serum. Both have been previously reported as pathological and in conflits of interpretation respectively.

Discussion

The proportion of patients with PD from the ones on suspection of PD in other similar studies in caucasic population was 0.29% [8] and 2.2% [9] In our population, we tested 2637 samples with suspection and a total of 17 new patients of PD were found (0.64%).

The median age at diagnosis in this set of patients was 38 years, this is in line with other authors who maintain that clinical manifestations in LOPD may present from the first decade to the seventh decade of life and the median age at diagnosis is 38 years [10, 11] In our population, three patients were asymptomatic [patients #2, #3 and #6]. All of them were included in the study because they had a family member with PD and they probably show no clinical symptoms because they are still too young. Patient #2 and patient #3 are sisters and they are seven and nine years old respectively and patient #6 is a nineteen year old female. The early diagnosis of LOPD in asymptomatic subjects is rare and it may be explained by the delay in the first clinical manifestations as described in previous studies 11 which shows the importance of family studies for preventive follow-up [12, 13]

The clinical symptoms in our cohort were similar to the classical findings in Pompe disease studies [14, 15]. We confirm that the most common symptom in LOPD is muscle weakness and it was present in all patients except the asymptomatic ones because they are early diagnosis prescribed for family study. Elevated CPK levels and respiratory distress were the next most frequent symptoms. Myalgia, dysphagia or hypotonia were less frequent symptoms in our population (12,5%). Patient #1 [two days of age], showed an hypertrophic cardiomyopathy, the most frequent manifestation in IOPD as reported the literature [16] PD presents a great clinical heterogeneity, even in patients with the same mutation. Therefore, the type and degree of manifestations of each individual could depend on the residual enzymatic activity and its interaction with other genetic or epigenetic factors.

In accordance with others studies, our results confirm that the mutations are distributed throughout the entire gene. [17, 18]. As published in the bibliography, the gene has three critical regions: exon 2, which includes start codon, exon 10 and 11 where the evolutionarily conserved catalytic site domain is contained, and exon 14 which includes a highly conserved region. Two variants of our study were detected in exon 2, none in exons 10, 11 or 14. The rest of the variants are distributed by almost all exons as shown in the table 2. Due to the NGS boom, it is expected that more variants of uncertain significance will be explained in the future.

Sequence analysis of the complete coding region of the *GAA* gene revealed 14 different variants from 17 patients including nine missense mutations (26.4%), one nonsense mutation (2.9%), three deletion or insertion (17.6%), eighteen splicing variants (52.9%).

Similar to others studies, the splice-site mutation **c.-32-13T > G** was the most frequent mutation found in our cohort. As is published in the literature, the intronic variant is the most common in Caucasian populations and it is present in 40–70% of the alleles in patients affected with PD [2]. In this study, it was seen in all patients except patient #1 and patient #5 (17 alleles, 50%). Patient #13 and patient #16 presented the variant in homozygosis. This variant is located in the 3´splice region and it causes aberrant splicing of the GAA gene. For this reason, the splicing variant c.-32-13T > G is considered pathogenic [19–21]

The next most frequent mutation present in our population was **c.236_246delCCACACAGTGC**. It was described by Palmer [22] in a patient who presented a severe infantile-onset Pompe disease. In concordance with the previous study, we encountered the delection in homozygosis in patient #1. Patient #1 had a sister with diagnosed PD who died at nine months of age and for whom we do not have

the results of the genetic study, the clinical information refers to parents as carriers of the disease. It was found too in heterozygosis in patient #10, a forty-six years old man. The presence of the homozygous variant could be established as providing a more serious effect or being indicative of a worse prognosis.

The mutation **c.1396_1397insG** was identified in two heterozygotes patients, they were asymptomatic sisters (patient #2 and #3) who showed the same genotype (c.-32-13T > G + c.1396_1397insG) very young to present the PD clinical symptoms (7 and 9 years old respectively). The variant is described as cause of PD creating a frame shift starting at codon Val466 and a stop codon in 39 position downstream [23]. The rest of the variants **(c.281_282delCT; c.655G > A; c.875A > G; c.925G > A; c.1655T > C; c.2104C > T; c.2237G > C)** had already been described in the literature as pathogenic were shown only once and, therefore, were less frequent in our population.

This study contributed to the identification of four new probably pathogenic variants which had not been described previously in the literature (**c.1328A > T; c.1831G > A; c.2819C > A; c.1889-1G > A**).

The substitution **c.1328A > T (p.Asp443Val)** was detected in exon 9 of patient #5. The missense variant produces a change in the protein and replaces aspartic acid with valine at codon 443. There are physicochemical differences between these amino acids. Acid aspartic is neutral and polar and valine is neutral and non-polar. This could modify the conformation of the protein and affect its function. Other missense variants have been reported as pathogenic in nearby codons [24, 25]. This findings suggests that this variant contributes to disease.

The missense variant **c.1831G > A** is located in exon 13 and it was shown in heterozygosis in patient #4. This substitution (**p.Gly611Ser**) replaces glycine with serine at codon 611. Glycine is non-polar and serine is polar. These physicochemical differences can alter the structure of the protein and could affect its function. On the other hand, it is the second variant discovered in this codon. The mutation c.1832G > A (p.Gly611Asp), which also change glycine for a polar amino acids, was described in previous study and was reported as pathogenic variant [26].

We detected the nonsense variant **c.2819C > A in** patient #9. This variant generates a slightly truncated protein **(p.Ser940Ter)**. It was assumed to be deleterious since the stop codons of other proteins were detected upstream of this and were known to result in a complete loss of enzyme activity. The variant c.2741delinsCAG [p.Gln944*fs30] produces a premature stop codon in aminoacid 944, was previously described by van Gelder [27]. in patients that did not present any activity of α-glucosidase. This leads us to think that a previous stop codon will also generate damage to the protein.

The splicing variant c.1889-1G > A in the intron 13 was detected in patient #11. As Anna [28] published, in general, mutations in the canonical acceptor and donor sites affect strongly conserved sequences that define exon-intron boundaries. Therefore, any variants in these canonical sequences might alter interaction between premRNA and proteins involved in the intron removal.

Conclusions

In this study fourteen genetic variants in *GAA* gene were identified, as cause of Pompe disease, including four new variants. This study confirms that patients in Spain have a characteristic profile of a European population, with c.-32-13T > G being the most prevalent variant. Furthermore, it was confirmed that the c.236_246delCCACACAGTGC mutation in homozygosity is associated with early disease and a worse prognosis. We propose to extend the genetic study in the 7 individuals without genetic justification using techniques that require the study of the intronic zones of the gene or alfa-glucosidase messenger RNA.

Our findings underscore the importance of early diagnosis and propose to accurate molecular analysis to improve genetic counseling in addition to enabling a better quality of life for patients.

Declarations

Ethics approval and consent to participate

Informed consent was signed by all patients and the study was approved by the Ethics and Research Committee of the Virgen Macarena and Virgen del Rocío University Hospital (Code: 0826-N-15).

Consent for publication

Not applicable

Availability of data and materials

No sé qué poner aquí

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Paula Hernández-Arevalo has written the article and has contributed to the diagnosis and analysis of the cases. Rocío Delarosa-Rodríguez has contributed to the diagnosis and analysis of the cases. Pilar Jiménez-Arriscado is the expert technician who realize all the genetic libraries. Antonio González-Meneses and Salvador García-Morillo have contributed to the recruitment of patients and the interpretation of clinical symptoms. José D. Santotoribio and Hada C. Macher are in charge of the molecular diagnostic laboratory and have reviewed all the diagnostic and results analysis processes for clinical validation and Juan M. Guerrero is the head of the department of clinical biochemistry and professor at the University of Seville, he is the main researcher of the laboratory projects and has supervised and approved the entire process.

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No se qué poner aquí

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Tables

Table 1. Clinical and biochemical analysis information of 17 patients of PD and 2 individuals with a single variant. F: female; M: male; B: biopsy; FM: Family member; A: asintomatic; 1: Cardiomyopathy; 2: HiperCKemia; 3: Muscle weakness; 4: Exercise intolerance; 5: Hipotoni; 6: Myalgia; 7: Dysphagia; 8: Respiratory distress; 9: Dyspnoea.

Subject	Age	Sex	Symptoms or Signs	GAA activity			
				DBS	Lymphocytes	Variant 1	Variant 2
				NR: >0,75 umol/Lh	NR: >0,15 nmol/min/mgprot		
#1	0	M	1 FM	0 .5	N/A	c.236_246delCCACACAGTGC	c.236_246delCCACACAGTGC
#2	7	F	A FM	0 .12	0,01	c.-32-13T>C	c.1396_1397insG
#3	9	F	A FM	0 .15	0,07	c.-32-13T>C	c.1396_1397insG
#4	11	F	1 2 3	0 .16	0,03	c.-32-13T>C	c.1831G>A
#5	17	F	2 3 4 B	0 .37	0,02	c.1328A>T	c.1328A>T
#6	19	M	A F	0 .37	0,05	c.-32-13T>C	c.281_282delCT
#7	31	M	23	0 .27	0,05	c.-32-13T>C	c.1655T>C
#8	32	F	23	0 .3	0,09	c.-32-13T>C	c.925G>A
#9	44	F	18	0 .34	N/A	c.-32-13T>C	c.2819C>A
#10	46	M	3 4 5	0 .36	0	c.-32-13T>C	c.236_246delCCACACAGTGC
#11	51	M	2 3 6 7	0 .21	0,04	c.-32-13T>C	c.2104C>T
#12	52	M	38	0 .48	0,01	c.-32-13T>C	c.1889-1G>A
#13	57	F	3 4 9	0 .37	0,01	c.-32-13T>C	c.2237G>C
#14	58	F	35	0 .33	0,1	c.-32-13T>C	c.-32-13T>C
#15	58	M	234	0 .31	0,11	c.-32-13T>C	c.655G>A
#16	59	H	2 3 8	0 .17	0,01	c.-32-13T>C	c.-32-13T>C
#17	64	M	2 3 6 7	0 .54	0,1	c.-32-13T>C	c.875A>G
#18	79	F	A FM	0 .65	0,11	c.854C>G	-
#19	63	M	2 3	0 .47	0,06	c.2065G>A	-

Table 2. Type and frequency of GAA variants of the patients.

	Nucleotide change	Effect on protein	Location	Frequency
	c.655G>A	p.Gly219Arg	Exon 3	1/36
	c.854C>G	p.Pro285Arg	Exon 4	1/36
	c.875A>G	p.Tyr292Cys	Exon 5	1/36
	c.925G>A	p.Gly309Arg	Exon 5	1/36
	c.1328A>T	p.Asp443Val	Exon 9	2/36
Missense	c.1655T>C	p.Leu552Pro	Exon 12	1/36
	c.1831G>A	p.Gly611Ser	Exon 13	1/36
	c.2104C>T	p.Arg702Cys	Exon 13	1/36
	c.2065G>A	p.Glu689Lys	Exon 15	1/36
	c.2237G>C	p.Trp746Ser	Exon 16	1/36
Nonsense	c.2819C>A	p.Ser940Ter	Exon 20	1/36
Delection or insertion	c.236_246delCCACACAGTGC	p.Pro79fs	Exon 2	3/36
	c.281_282delCT	p.Pro94fs	Exon 2	1/36
	c.1396_1397insG	p.Val466fs	Exon 9	2/36
Splicing variant	c.-32-13T>C	-	Intron 1	17/36
	c.1889-1G>A	-	Intron 13	1/36

Table 3. Analysis of variants by bibliography and in silico predictive tools.

	Nucleotide change	Effect on protein	ClinVar	Mutation Taster	PolyPhen-2	HGMD	Pompe database
Described mutations	c.-32-13T>C	-	Pathogenic	-	-	29	Potencially mild
	c.236_246delCCACACAGTGC	p.Pro79fs	Pathogenic	Disease causing	-	30	Very severe
	c.281_282delCT	p.Pro94fs	Likely pathogenic	Disease causing	-	-	-
	c.655G>A	p.Gly219Arg	Pathogenic	Disease causing	Probably damaging	31	Potencially less severe
	c.854C>G	p.Pro285Arg	Pathogenic	Disease causing	Probably damaging	32	Potencially mild
	c.875A>G	p.Tyr292Cys	Pathogenic	Disease causing	Probably damaging	33	Potencially mild
	c.925G>A	p.Gly309Arg	Pathogenic	Disease causing	Probably damaging	34	Potencially less severe
	c.1396_1397insG	p.Val466fs	Not described	Disease causing	-	-	-
	c.1655T>C	p.Leu552Pro	Pathogenic	Disease causing	Probably damaging	35	Potencially less severe
	c.2065G>A	p.Glu689Lys	Conflicts of interpretation	Polymorphism	Likely bening	36	-
	c.2104C>T	p.Arg702Cys	Pathogenic	Disease causing	Probably damaging	37, 38	Potencially less severe
	c.2237G>C	p.Trp746Ser	Not described	Disease causing	Probably damaging	39	Potencially less severe
Novel mutations	c.1328A>T	p.Asp443Val	Not described	Disease causing	Probably damaging	-	-
	c.1831G>A	p.Gly611Ser	Not described	Disease causing	Probably damaging	-	-
	c.1889-1G>A	-	Not described	Disease causing	-	-	-
	c.2819C>A	p.Ser940Ter	Not described	Disease causing	-	-	-

Figures

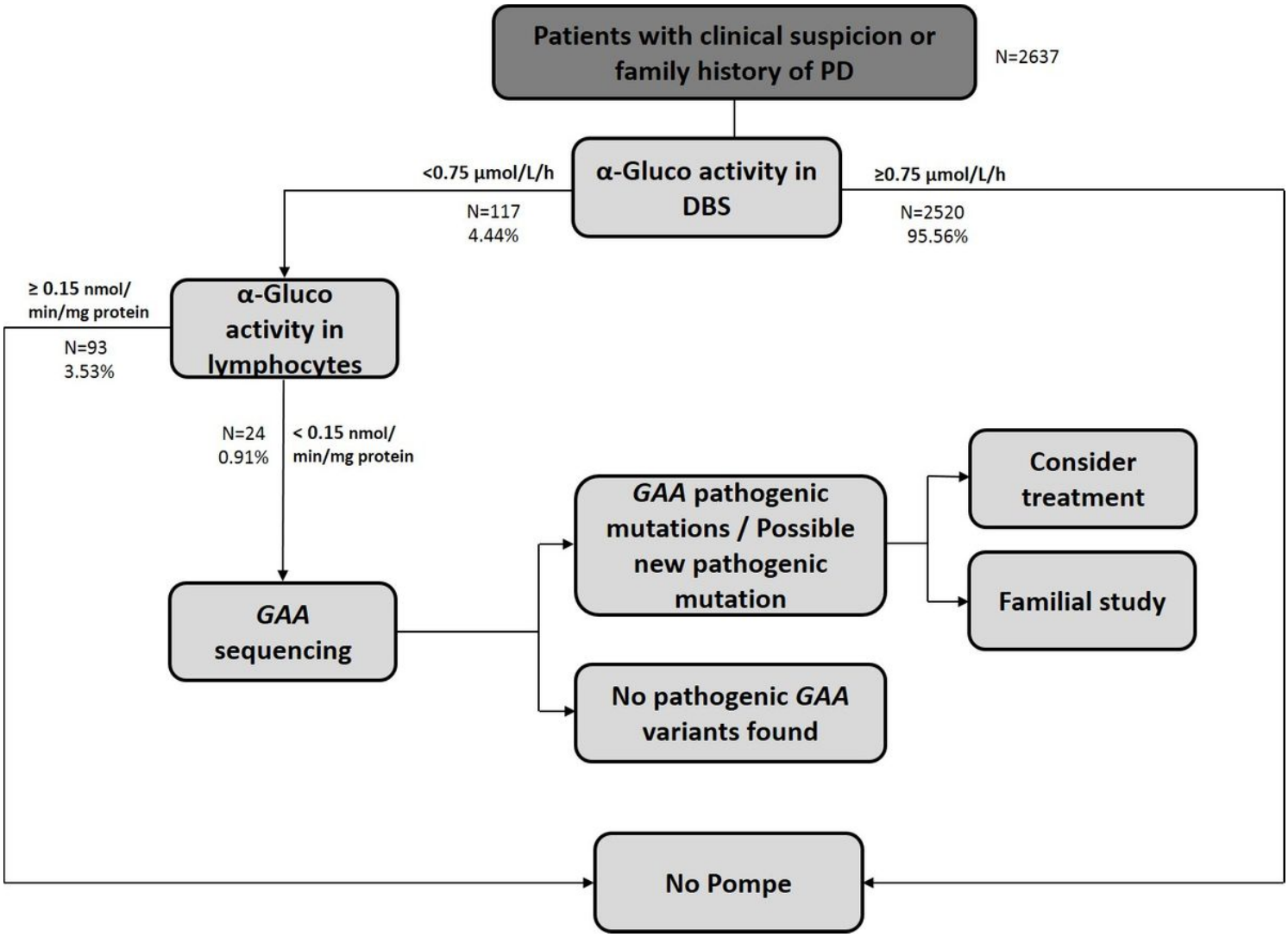

Figure 1

A description of PD screening protocol.

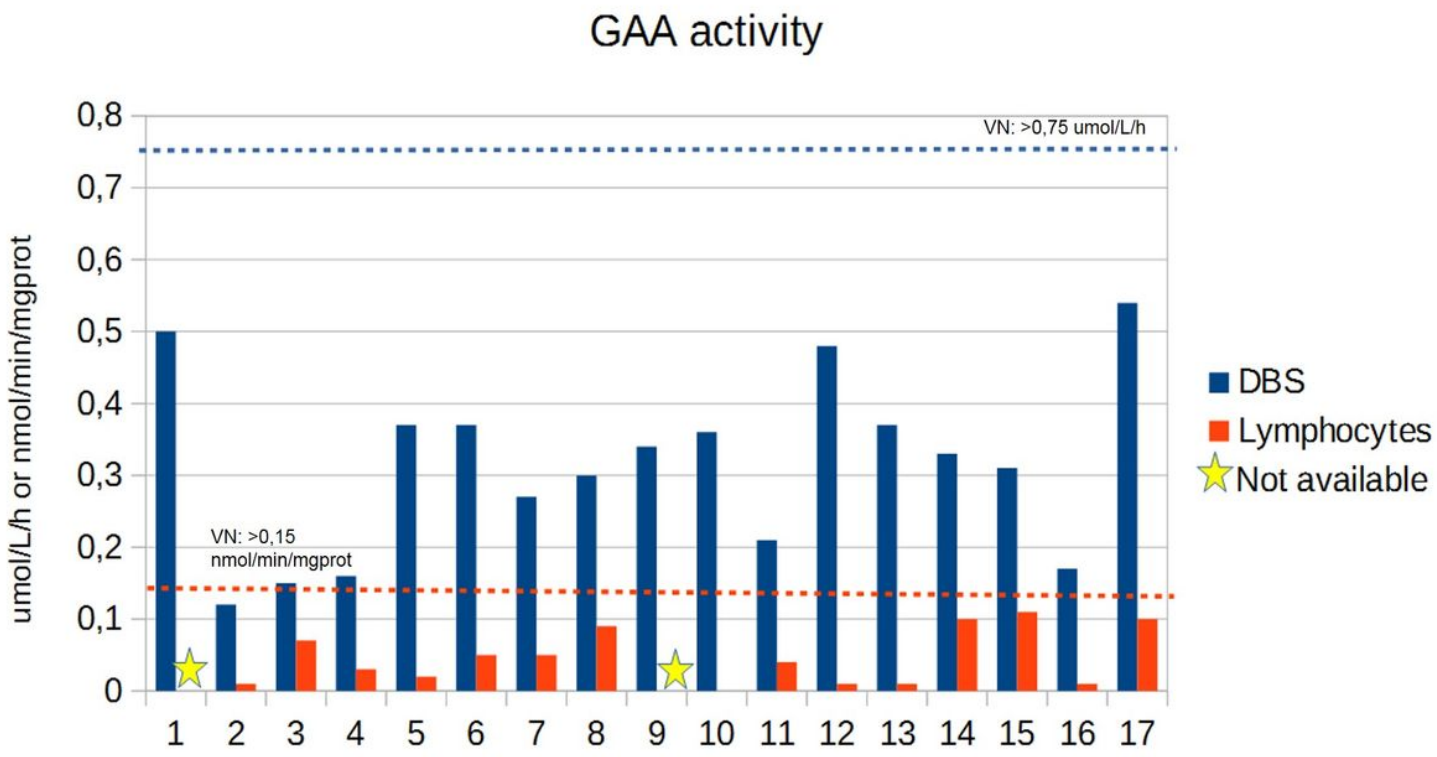

Figure 2

Enzyme activity values in DBS and lymphocytes in patients.

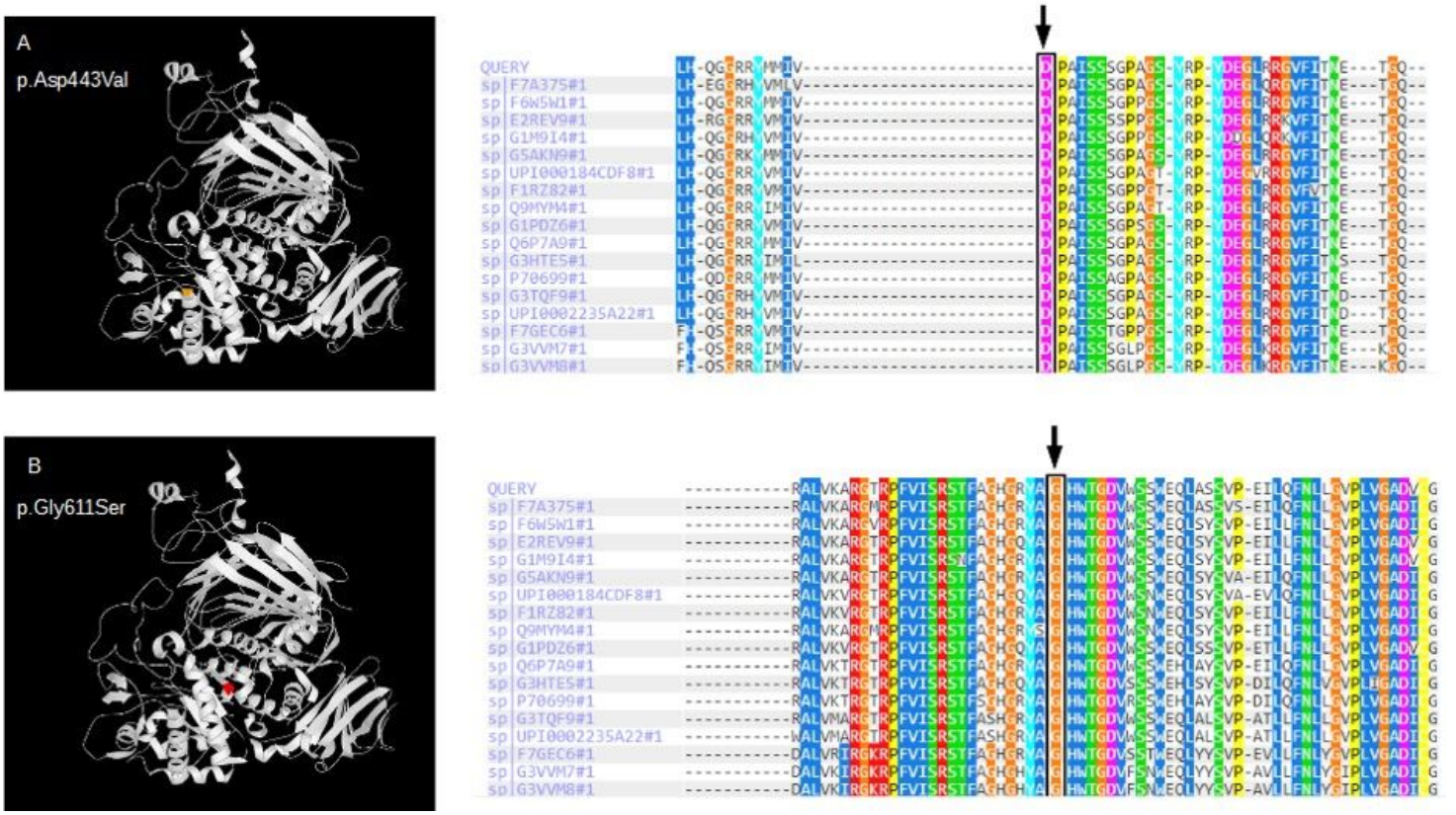

Figure 3

Evolutionary conservation of amino acids by missense mutation across different species and position of the residues involved in the structure of human GAA. A: variant c.1328A>T; B: variant c.1831G>A