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ARTICLE

Phenotypic implications of pathogenic variant types in Pompe disease

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Abstract

Newborn screening and therapies for Pompe disease (glycogen storage disease type II, acid maltase deficiency) will continue to expand in the future. It is thus important to determine whether enzyme activity or type of pathogenic genetic variant in *GAA* can best predict phenotypic severity, particularly the presence of infantile-onset Pompe disease (IOPD) versus late-onset Pompe disease (LOPD). We performed a retrospective analysis of 23 participants with genetically-confirmed cases of Pompe disease. The following data were collected: clinical details including presence or absence of cardiomyopathy, enzyme activity levels, and features of *GAA* variants including exon versus intron location and splice site versus non-splice site. Several combinations of *GAA* variant types for individual participants had significant associations with disease subtype, cardiomyopathy, age at diagnosis, gross motor function scale (GMFS), and stability of body weight. The presence of at least one splice site variant (c.546 G > C/p.T182 = , c.1076–22 T > G, c.2646 + 2 T > A, and the classic c.−32–13T > G variant) was associated with LOPD, while the presence of non-splice site variants on both alleles was associated with IOPD. Enzyme activity levels in isolation were not sufficient to predict disease subtype or other major clinical features. To extend the findings of prior studies, we found that multiple types of splice site variants beyond the classic c.−32–13T > G variant are often associated with a milder phenotype. Enzyme activity levels continue to have utility for supporting the diagnosis when the genetic variants are ambiguous. It is important for newly diagnosed patients with Pompe disease to have complete genetic, cardiac, and neurological evaluations.

Introduction

Pompe disease (glycogen storage disease type II, acid maltase deficiency) is an autosomal recessive progressive metabolic myopathy caused by acid α-glucosidase deficiency due to pathogenic variants in *GAA*, located on chromosome 17q25.2-q25.3 and containing 19 coding exons and 1 non-coding exon [1]. Johannes Cassianus Pompe's report of a 7-month-old girl affected by cardiomyopathy in 1932 is the first case recognized to be the eponymous disease due to his key discovery of glycogen accumulation in the heart and skeletal muscles, as well as the liver [2]. Acid α-glucosidase deficiency was first determined to be the cause of Pompe disease in 1963 [3]. Loss of acid α-glucosidase enzyme activity results in progressive accumulation of glycogen in lysosomes, particularly in skeletal, cardiac and smooth muscle.

There are two major clinical phenotypes of Pompe disease: the originally described infantile-onset Pompe disease (IOPD) and late-onset Pompe disease (LOPD), which is

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characterized by onset of proximal muscle weakness after infancy, sparing of the heart, and frequent respiratory complications [4, 5]. The diagnosis was traditionally confirmed by acid α-glucosidase enzyme activity assays performed on blood leukocytes (including dried blood spots [6]), fibroblasts, or skeletal muscle, and more recently by genetic testing for *GAA* variants. Disease severity has been thought to correlate to some degree with the severity of pathogenic variants and inversely with residual acid α-glucosidase activity levels. IOPD was believed to be associated with residual enzyme activity levels <10% [7], while LOPD has been associated with a wide range of enzyme activity levels that do not appear to correlate well with phenotypic features [8]. Importantly, genetic testing has become widely available in a number of countries and is a key part of diagnosis and disease management.

Recombinant human acid α-glucosidase (rhGAA, alglucosidase alfa) enzyme replacement for Pompe disease was approved by the US Food and Drug Administration and the European Medicines Agency in 2006. Enzyme replacement therapy provides improvement in cardiac mass in IOPD [9–11] and stabilizes pulmonary function for LOPD [12, 13]. As newborn screening for Pompe disease becomes more widely adopted around the world [14–16] and newer therapies are developed, the question of how promptly to begin treatment upon diagnosis of neonates has become more urgent. Acid α-glucosidase enzyme activity levels are not sufficient in isolation to help with this decision. To address this matter, we have analyzed demographic, clinical, biochemical, and genetic data on a cohort of 23 participants with Pompe disease, with the goal of determining whether specific genotypes can predict phenotypic severity.

Materials and methods

Participants

Retrospective data collection was conducted under a protocol approved by the Institutional Review Board at the University of Florida. Participants were ascertained from three sources: an ongoing observational research study, interventional research studies, and clinical care settings, all at University of Florida Health/Shands Hospital. The inclusion criterion was a diagnosis of Pompe disease confirmed with biallelic pathogenic variants in *GAA*. The cohort was collected from clinical or research encounters between January 1st, 2010 and April 24th, 2015. Selected patients included in this study were previously published in a study of immunomodulation [17], a gene therapy study [18], and a study of respiratory function [19]; none of these prior publications included the detailed clinical and genetic analysis presented here and thus there is no meaningful overlap in data presentation.

Clinical data and phenotype classification

Basic phenotype data collection included the following domains: gender, ethnicity, age of onset, macroglossia, presence of hepatosplenomegaly, skeletal muscle weakness, baseline cardiac status, and deep tendon reflexes. Due to inconsistency of precise ages of onset in some cases, the age of clinical diagnosis was recorded rather than age of onset. Subjects were classified as having IOPD if they had onset in the first year of life, cardiac hypertrophy, and rapid progression when not on enzyme replacement therapy, or as LOPD if they had onset after the first birthday and proximal muscle weakness with sparing of the heart. Complications recorded included level of respiratory assistance, history of respiratory failure, obstructive sleep apnea (OSA), hypertrophic cardiomyopathy, ambulatory status, scoliosis, joint contractures, gastronomy tube presence and weight issues.

Laboratory and genetic data

Serum creatine kinase (CK) levels (standard reference range 10–120 IU/L), alanine aminotransferase (ALT) levels (standard reference range 10–40 IU/L), aspartate aminotransferase (AST) levels (standard reference range 7–56 IU/L), acid α-glucosidase activity, and *GAA* genetic data were collected. Acid α-glucosidase activity was determined in leukocytes and in cultured skin fibroblasts according to standard procedures, and was reported as pmol/punch/hr. As the enzyme testing was performed in various laboratories with differing reference ranges, the percentage of the lower limit of normal was calculated for each acid α-glucosidase activity measurement so that results could be compared across the entire cohort. Definite abnormal activity was defined as <30% of the lower limit of normal. Variant information was obtained from genetic test reports generated by commercial diagnostic laboratories and a research laboratory at the University of Florida. In the latter setting, genetic analysis was initiated via amplification of all 20 exons (19 coding and 1 noncoding) and flanking intronic sequences of *GAA* from purified genomic DNA. Amplicons were purified using Montage PCR filter units (EMD Millipore, Burlington, Massachusetts), then sequenced using the Model 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts) using the same primers. Sequences were analyzed using Clone Manager Professional Suite version 8 (Sci Ed Software, Westminster, Colorado). Nucleotide variants were annotated according to Gen Bank accession number NM_000152.5, with the coding region

Table 1 Demographics, physical examination features, and complications

		Overall ($n = 23$)		Mutation & GAA activity ($n = 18$)	
	Overall	IOPD	LOPD	IOPD	LOPD
(n)	23	6	17	6	12
Sex: Male	10 (43.5)	3 (50.0)	7 (41.2)	3 (50.0)	5 (41.7)
Age at Diagnosis	18.8 ± 19.1	0.3 ± 0.2	25.4 ± 18.1	0.3 ± 0.2	23.6 ± 17.7
Age at last Exam	24.5 ± 19.4	5.5 ± 3.4	31.2 ± 18.2	5.5 ± 3.4	28.2 ± 17.7
CK (IU/L)[a]	926.9 ± 750.8 [$n = 19$]	998.7 ± 593.1 [$n = 6$]	893.8 ± 833.8 [$n = 13$]	998.7 ± 593.1 [$n = 6$]	851.3 ± 824.7 [$n = 11$]
AST (IU/L)[a]	190.7 ± 116.2 [$n = 18$]	238.5 ± 122.4 [$n = 6$]	166.8 ± 110.5 [$n = 12$]	238.5 ± 122.4 [$n = 6$]	174.7 ± 110.2 [$n = 9$]
ALT (IU/L)[a]	149.4 ± 98.6 [$n = 18$]	132.2 ± 33.2 [$n = 6$]	158.0 ± 119.5 [$n = 12$]	132.2 ± 33.2 [$n = 6$]	169.6 ± 132.2 [$n = 9$]
Cardiomyopathy	6 (26.1)	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)
Macroglossia	1 (4.4)	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)
Hepatosplenomegaly	4 (17.4)	2 (33.3)	2 (11.8)	2 (33.3)	2 (16.7)
Scoliosis	4 (17.4)	3 (50.0)	1 (5.9)	3 (50.0)	1 (8.3)
Joint contractures	3 (13.0)	2 (33.3)	1 (5.9)	2 (33.3)	0 (0.0)
Gross motor function scale					
Level I: Normal	13 (56.5)	2 (33.3)	11 (64.7)	2 (33.3)	9 (75.0)
Level II	3 (13.0)	0 (0.0)	3 (17.7)	0 (0.0)	2 (16.7)
Level III	1 (4.4)	0 (0.0)	1 (5.9)	0 (0.0)	0 (0.0)
Level IV	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Level V: Complete Dependence	6 (26.1)	4 (66.7)	2 (11.8)	4 (66.7)	1 (8.3)
Respiratory					
Tracheostomy	4 (17.4)	3 (50.0)	1 (5.9)	3 (50.0)	1 (8.3)
Nightly BIPAP	5 (21.7)	1 (16.7)	4 (23.5)	1 (16.7)	3 (25.0)
Nightly CPAP	1 (4.4)	0 (0.0)	1 (5.9)	0 (0.0)	0 (0.0)
No ventilator assistance	13 (56.5)	2 (33.3)	11 (64.7)	2 (33.3)	8 (66.7)
Obstructive sleep apnea	13 (56.5)	5 (83.3)	8 (47.1)	5 (83.3)	6 (50.0)
G-Tube dependence	8 (34.8)	5 (83.3)	3 (17.7)	5 (83.3)	2 (16.7)
Deep tendon reflexes[a]	[$n = 18$]	[$n = 5$]	[$n = 13$]	[$n = 5$]	[$n = 10$]
Absent	9 (50.0)	3 (60.0)	6 (46.2)	3 (60.0)	4 (40.0)
Trace/Decreased	5 (27.8)	1 (20.0)	4 (30.8)	1 (20.0)	3 (30.0)
Intact	4 (22.2)	1 (20.0)	3 (23.1)	1 (20.0)	3 (30.0)
Non stable body weight	7 (30.4)	6 (100.0)	1 (5.9)	6 (100.0)	1 (8.3)

$N = 23$: Overall cohort with at minimum mutation data ($n = 18$ (78.3%) of the 23 have mutation data and GAA Activity)

[a]Deep Tendon Reflexes, CK, ALT, AST are not primary outcomes and thus missingness permitted; *n* reported for each item

chromosome 17 located from 80,101,556 to 80,119,881 on the forward strand [GRCh38:CM000679.2]. Variants were described according to the guidelines of the Human Genome Variation Society [20]. *GAA* variant types were classified as exonic, intronic, and then more specifically as frameshift, nonsense, deletion, splice site, and missense.

Statistical analysis

Variables were summarized using descriptive statistics, including mean, standard deviation (SD), median, ranges, percentages and/or frequencies. As clinical data were not comprehensive in all participants, gaps are noted within descriptive measures. Available genetic variants were noted and categorized by variant type and location, and acid α-glucosidease activity was subdivided into quartiles. P values ≤ 0.05 were determined to be statistically significant. *GAA* variant types and GAA enzyme activity were examined for associations with the global phenotypes IOPD and LOPD, in addition to various clinical complications of Pompe Disease. Mantel–Haenszel Chi-Square Exact statistics were used to evaluate associations between variant location and categorical data, and one-way ANOVA Welch Tests were performed for continuous measures with non-equal variances. To determine whether increasing GAA enzyme activity levels were associated with phenotype, Mantel–Haenszel Chi-Square Exact statistics were again performed for categorical data. A test for linear trend was conducted for continuous measures. SAS 9.4 (Cary, NC) was used for for data analysis and management.

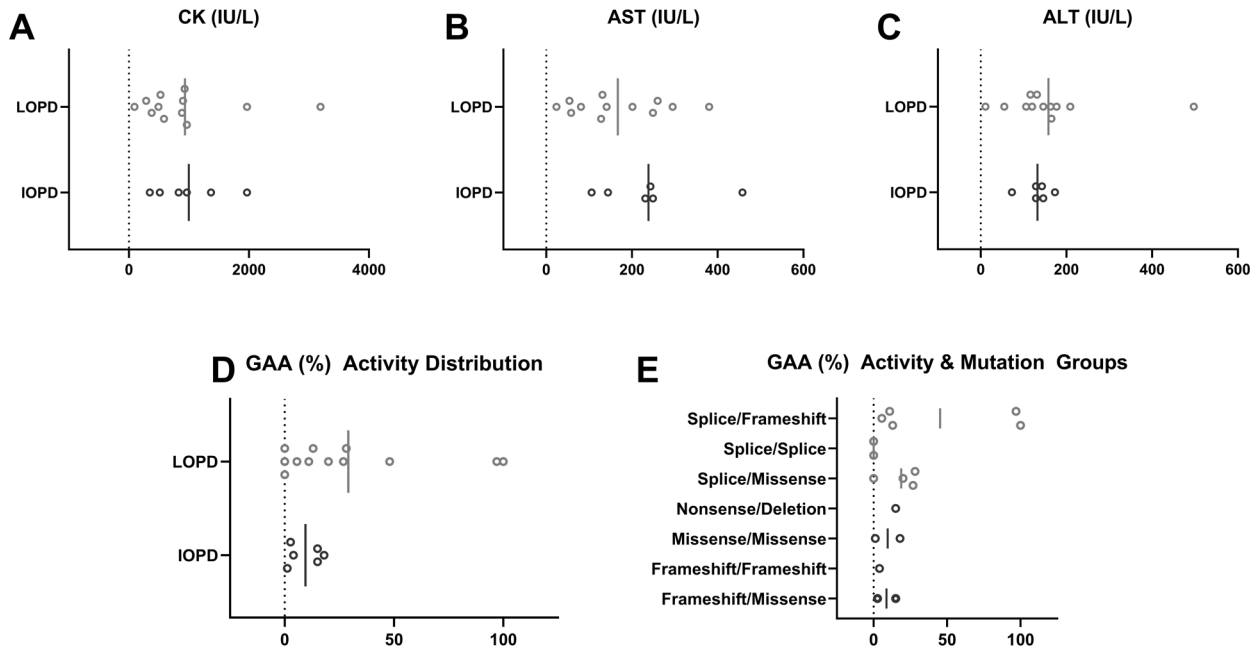

Fig. 1 Plots of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and acid α-glucosidase (GAA) levels with vertical bars representing the mean values. **A** Plot of serum CK levels (standard reference range 10–120 IU/L) separated by IOPD versus LOPD phenotypes. **B** Plot of serum AST levels (standard reference range 7–56 IU/L), separated by IOPD versus LOPD phenotypes. **C** Plot of serum ALT levels (standard reference range 10–40 IU/L) separated by IOPD versus LOPD phenotypes. **D** Plot of GAA enzyme activity levels separated by IOPD versus LOPD phenotypes. **E** Plot of GAA enzyme levels separated by different pathogenic variant types

Results

Participants

Of 62 participants who were ascertained as having a clinical diagnosis of Pompe disease, 23 had sufficient clinical, genetic, and enzymatic data for study inclusion. Of these, 6 were classified as having IOPD and 17 were classified as having LOPD. All 23 participants had relevant genetic data available for analysis, of whom 18 had documented levels of GAA enzymatic activity.

IOPD

Table 1 lists demographic and clinical data for the six participants with IOPD. The mean age at diagnosis was 0.3 years (SD: 0.2). All six had clinically significant cardiac hypertrophy and received enzyme replacement therapy. For the IOPD cohort at the last examination, one participant walked with assistance, one was able to crawl, while the other four neither walked nor crawled. All six were receiving enzyme replacement therapy and were alive at the last known contact. All IOPD participants had elevated CK levels at diagnosis (mean ± SD 998.7 ± 593.1 IU/L), elevated AST levels (mean ± SD 238.5 ± 122.4 IU/L), and elevated ALT levels (mean ± SD 132.2 ± 33.2 IU/L) (Fig. 1A–C). Acid α-glucosidase activity was measured for all six IOPD participants; all values were <20% of the lower limit of normal (Table 2, Fig. 1D). Variant information was available on all six participants with IOPD (Table 3). All variants were frameshift, nonsense, missense, or deletion changes, and all thus impacted various coding regions.

LOPD

Table 1 lists demographic and clinical data for the 17 LOPD participants. The mean age at diagnosis was 25.4 years (SD: 18.1). Two participants were diagnosed early due to incidental findings, one at 4 months of age after an evaluation for recurrent emesis and vomiting that began at 3 months of age, accompanied by persistent mild elevations in transaminase and CK levels, and another at 2 years after an evaluation for incidentally discovered transaminase elevations. The second participant had no cardiomyopathy or respiratory complications at diagnosis but required gastrostomy and was started on enzyme replacement therapy. All LOPD participants were alive at the time of data collection, and none of the 17 had clinically significant cardiomyopathy. Among LOPD participants with data available, CK levels were elevated in most cases (mean ± SD 893.8 ± 833.8 IU/L). AST levels were elevated in most cases (mean ± SD 166.8 ± 110.5 IU/L), along with ALT levels (mean ± SD 158.0 ± 119.5 IU/L) (Fig. 1A–C). Acid α-glucosidase activity, noted in Table 2, ranged from 0 to

Table 2 Acid α-glucosidase enzyme activity stratifications versus phenotypic features

GAA range:	Overall	0–9%	10–19%	20–29%	≥30%
Patients (n)	18	7 (38.9)	5 (27.8)	3 (16.7)	3 (16.7)
IOPD	6 (33.3)	3 (42.9)	3 (60.0)	0 (0.0)	0 (0.0)
Sex: Male	8 (44.4)	2 (28.6)	3 (60.0)	1 (33.3)	2 (66.7)
Age at diagnosis	15.8 ± 18.2	7.8 ± 9.3	13.2 ± 21.5	21.4 ± 18.7	33.3 ± 22.8
Age at last exam	20.6 ± 10.1	13.0 ± 8.4	18.2 ± 21.7	28.0 ± 20.8	35.0 ± 24.2
CK (IU/L)[a]	903.3 ± 735.1 [$n = 17$]	1114.7 ± 1088.8 [$n = 6$]	1049.2 ± 532.8 [$n = 5$]	624.7 ± 268.2 [$n = 3$]	516.0 ± 436.5 [$n = 3$]
AST (IU/L)[a]	200.2 ± 115.5 [$n = 15$]	190.8 ± 103.9 [$n = 6$]	265.2 ± 119.8 [$n = 5$]	129.5 ± 101.1 [$n = 2$]	136.5 ± 159.1 [$n = 2$]
ALT (IU/L)[a]	154.6 ± 103.6 [$n = 15$]	187.5 ± 154.6 [$n = 6$]	151.2 ± 22.7 [$n = 5$]	140.5 ± 34.6 [$n = 2$]	78.5 ± 95.5 [$n = 2$]
Cardiomyopathy	6 (33.3)	3 (42.9)	3 (60.0)	0 (0.0)	0 (0.0)
Macroglossia	1 (5.6)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)
Hepatosplenomegaly	4 (22.2)	2 (28.6)	2 (40.0)	0 (0.0)	0 (0.0)
Scoliosis	4 (22.2)	1 (14.3)	2 (40.0)	0 (0.0)	1 (33.3)
Joint contractures	2 (11.1)	1 (14.3)	1 (20.0)	0 (0.0)	0 (0.0)
Gross motor function scale					
Level I: Normal	11 (61.1)	3 (42.9)	2 (40.0)	3 (100.0)	3 (100.0)
Level II	2 (11.1)	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)
Level III	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Level IV	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Level V: Complete Dependence	5 (27.8)	2 (28.6)	3 (60.0)	0 (0.0)	0 (0.0)
Respiratory					
Tracheostomy	4 (22.2)	2 (28.6)	2 (40.0)	0 (0.0)	0 (0.0)
Nightly BIPAP	4 (22.2)	2 (28.6)	0 (0.0)	2 (66.6)	0 (0.0)
Nightly CPAP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No Ventilator Assistance	10 (55.6)	3 (42.9)	3 (60.0)	1 (33.3)	3 (100.0)
Obstructive Sleep Apnea	11 (61.1)	5 (71.4)	3 (60.0)	2 (66.7)	1 (33.3)
G-Tube Dependence	7 (38.9)	3 (42.9)	3 (60.0)	1 (33.3)	0 (0.0)
Deep tendon reflexes[a]	[$n = 15$]	[$n = 6$]	[$n = 5$]	[$n = 2$]	[$n = 2$]
Absent	7 (46.7)	3 (50.0)	1 (20.0)	1 (50.0)	2 (100.0)
Trace/Decreased	4 (26.7)	1 (16.7)	2 (40.0)	1 (50.0)	0 (0.0)
Intact	4 (26.7)	2 (33.3)	2 (40.0)	0 (0.0)	0 (0.0)
Non Stable Body Weight	7 (38.9)	3 (42.9)	4 (80.0)	0 (0.0)	0 (0.0)

GAA range:	Overall	0–9%	10+ %
Patients (n)	18	7 (38.9)	11 (61.1)
IOPD	6 (33.3)	3 (42.9)	3 (27.3)
Sex: Male	8 (44.4)	2 (28.6)	6 (54.6)
Age at Diagnosis	15.8 ± 18.2	7.8 ± 9.3	20.9 ± 20.9
Age at Last Exam	20.6 ± 10.1	13.0 ± 8.4	25.4 ± 21.2
CK (IU/L)	903.3 ± 735.1 [$n = 17$]	1114.7 ± 1088.8 [$n = 6$]	788.0 ± 479.9 [$n = 11$]
AST (IU/L)	200.2 ± 115.5 [$n = 15$]	190.8 ± 103.9 [$n = 6$]	206.4 ± 128.4 [$n = 9$]
ALT (IU/L)	154.6 ± 103.6 [$n = 15$]	187.5 ± 154.6 [$n = 6$]	132.7 ± 50.1 [$n = 9$]
Cardiomyopathy	6 (33.3)	3 (42.9)	3 (27.3)
Macroglossia	1 (5.6)	0 (0.0)	1 (9.1)
Hepatosplenomegaly	4 (22.2)	2 (28.6)	2 (18.2)
Scoliosis	4 (22.2)	1 (14.3)	3 (27.3)
Joint Contractures	2 (11.1)	1 (14.3)	1 (9.1)
Gross motor function scale			
Level I: Normal	11 (61.1)	3 (42.9)	8 (72.7)
Level II	2 (11.1)	2 (28.6)	0 (0.0)
Level III	0 (0.0)	0 (0.0)	0 (0.0)
Level IV	0 (0.0)	0 (0.0)	0 (0.0)
Level V: Complete Dependence	5 (27.8)	2 (28.6)	3 (27.3)

Table 2 (continued)

GAA range:	Overall	0–9%	10+ %
Respiratory			
Tracheostomy	4 (22.2)	2 (28.6)	2 (18.2)
Nightly BIPAP	4 (22.2)	2 (28.6)	2 (18.2)
Nightly CPAP	0 (0.0)	0 (0.0)	0 (0.0)
No ventilator assistance	10 (55.6)	3 (42.9)	7 (63.6)
Obstructive sleep apnea	11 (61.1)	5 (71.4)	6 (54.6)
G-Tube dependence	7 (38.9)	3 (42.9)	4 (36.4)
Deep tendon reflexes	[$n = 15$]	[$n = 6$]	[$n = 9$]
Absent	7 (46.7)	3 (50.0)	4 (44.4)
Trace/Decreased	4 (26.7)	1 (16.7)	3 (33.3)
Intact	4 (26.7)	2 (33.3)	2 (22.2)
Non stable body weight	7 (38.9)	3 (42.9)	4 (36.4)

Statistics: EXACT MHCHI and test for linear trend conducted, no statistical significance found for any metrics

[a]Deep Tendon Reflexes, CK, ALT, AST are not primary outcomes and thus missingness permitted; *n* reported for each item

100% in the 12 with available data; enzyme activity was above 30% of the lower limit of normal in three participants; the other 9 had activity below the normal range (Fig. 1D). For all participants, though not statistically significant, descriptive statistics suggest a positive trend between GAA activity and age at diagnosis. Individual variant information is noted in Table 3. The most common LOPD-associated variant was c.−32–13T > G in intron 1, being present in 12/17 participants, including the 4-month-old, each time paired with a different variant on the other allele.

Enzyme levels versus phenotypic features

A stratification of acid α-glucosidase activity levels versus phenotypic features is presented in Table 2 and Fig. 1D. We grouped enzyme levels into four categories: 0–9% activity level for what has traditionally been thought to be consistent with IOPD, 10–19% for early or childhood onset LOPD, 20–29% for LOPD, and ≥30% which is generally regarded as normal. We also compared subjects with acid α-glucosidase activity of <10% to those with ≥10% enzyme activity. Using either method, a clear threshold of acid α-glucosidase activity that could distinguish IOPD versus LOPD could not be identified due to significant overlap in activity levels between the two groups. A test for linear trend did not identify statistically significant associations of enzyme activity levels with age, CK levels, AST levels, or ALT levels. Exact Mantel–Haenszel Chi-Square tests did not identify a statistically significant association between enzyme activity levels and the presence of cardiomyopathy or IOPD.

Genotype-phenotype analysis

Distributions of variant types versus clinical features are listed in Table 4. Enzyme activity levels were not distinct enough to predict mutation type (Fig. 1E). A key genetic question for Pompe disease is whether specific variant types are associated with IOPD versus LOPD. An Exact Mantel–Hasenszel chi-square test to examine the relation between variant location (exon-exon, exon-intron, intron-intron) and the likelihood of cardiomyopathy/IOPD demonstrated, as expected, a significant association (p value: 0.0010) (Table 4). Similar results were obtained with respect to variant type versus Gross Motor Function Scale (GMFS) (p value: 0.0344), and non-stable body weight (p value: 0.0043). No associations were found between variant location and the presence of hepatosplenomegaly, macroglossia, scoliosis, joint contractures, need for repiratory support, presence of OSA, gastronomy tube dependence, or deep tendon reflexes. The following variants were associated with normal or near-normal enzyme activity: c.−32–13T > G in conjunction with c.281_282delCT (p.Pro49Argfs*50) and c.−32–13T > G in conjunction with c.258dupC (p.Asn87Glnfs*9) (Table 3). The c.−32–13T > G splice site variant is, of course, known as a mild pathogenic variant. Normal GAA enzyme activity has been reported in as many as 10% of certain cohorts of Pompe disease, and discordant GAA enzyme activity in various tissues has also been reported [21–26].

Discussion

Several of the pathogenic variants in our cohort have been noted to be common in various populations: c.−32–13T > G [27, 28], c.525delT [27, 28], and the deletion of exon 18 [27]. A number of prior articles have documented that the classic c.−32–13T > G variant is typically associated with LOPD in numerous populations [27–34], though there may be a range of outcomes within that category [35, 36]. The c.525delT variant and exon 18 deletion may be associated

Table 3 Individual patient genetic and enzyme activity data

Case	Type	Allele 1	Allele 2	Amino acid change(s)	Mutation type	Coding/non-coding	GAA activity (%)
1	IOPD	c.1396delG	c.1705dupT	p.Val466Phefs*11/ p.Tyr569Leufs*67	Frameshift/Frameshift	Exon 9/Exon 11	4
2	IOPD	c.925 G > A	c.925 G > A	p.Gly309Arg/p.Gly309Arg	Missense/Missense	Exon 5/Exon 5	1.2
3	IOPD	c.1548 G > A	c.2481_2646del	p.Try516*/p.Gly828_Asn882del	Nonsense/Deletion	Exon 10/Exon 18	15
4	IOPD	c.1000 G > T	c.1211 A > T	p.Gly334Cys/p.Asp404Val	Missense/Missense	Exon 6/ Exon 8	18
5	IOPD	c.1933G > A	c.2501_2502del	p.Asp645Asn/p.Thr834Argfs*49	Missense/Frameshift	Exon 14/Exon 18	15
6	IOPD	c.525delT	c.1448 G > T	p.Glu176Argfs*45/p.Gly483Val	Frameshift/Missense	Exon 2/Exon 10	2.7
7	LOPD	c.1076–22 T > G	c.1841C > A	noncoding/p.Thr614Lys	Splice Site/Missense	Intron 6/Exon 13	.
8	LOPD	c.−32–13T > G	c.925 G > A	noncoding/p.Gly309Arg	Splice Site/Missense	Intron 1/Exon 5	26.86
9	LOPD	c.−32–13T > G	c.925 G > A	noncoding/p.Gly309Arg	Splice Site/Missense	Intron 1/Exon 5	28.2
10	LOPD	c.−32–13T > G	c.525delT	noncoding/p.Glu176Argfs*45	Splice Site/Frameshift	Intron 1/Exon 2	.
11	LOPD	c.525delT	c.1076–22 T > G	p.Glu176Argfs*45/noncoding	Frameshift/Splice Site	Exon 2/Intron 6	5.6
12	LOPD	c.525delT	c.1076–22 T > G	p.Glu176Argfs*45/noncoding	Frameshift/Splice Site	Exon 2/Intron 6	11
13	LOPD	c.−32–13T > G	c.1441 T > C	noncoding/p.Trp481Arg	Splice Site/Missense	Intron 1 /Exon 10	.
14	LOPD	c.525delT	c.1076–22 T > G	p.Glu176Argfs*45/noncoding	Frameshift/Splice Site	Exon 2/Intron 6	13
15	LOPD	c.−32–13T > G	c.2646 + 2 T > A	noncoding/noncoding	Splice Site/Splice Site	Intron 1/ Intron 18	0
16	LOPD	c.546 G > C	c.546 G > C	p.Thr182=/p.Thr182=	Splice Site/Splice Site	Exon 2 /Exon 2	0
17	LOPD	c.−32–13T > G	c.307 T > G	noncoding/p.Cys103Gly	Splice Site/Missense	Intron 1/Exon2	0
18	LOPD	c.−32–13T > G	c.1525 C > T	noncoding/p.Gln509*	Splice Site/Nonsense	Intron1/Exon 10	.
19	LOPD	c.−32–13T > G	c.281_282delCT	noncoding/ p.Pro49Argfs*50	Splice Site/Frameshift	Intron 1/Exon 2	100
20	LOPD	c.−32–13T > G	c.1115 A > T	noncoding/p.His372Leu	Splice Site/Missense	Intron 1/Exon 7	20
21	LOPD	c.−32–13T > G	Deletion exon 18	noncoding/deletion	Splice Site/Deletion	Intron 1/Exon 18	48
22	LOPD	c.−32–13T > G	c.258dupC	noncoding/p.Asn87Glnfs*9	Splice Site/Frameshift	Intron 1/Exon 2	97
23	LOPD	c.−32–13T > G	c.2646 + 2 T > A	noncoding/noncoding	Splice Site/Splice Site	Intron 1/Intron 18	.

Table 4 Distribution of mutation types versus phenotypic features

	Overall	Exon-Exon	Exon-Intron	Intron-Intron	*p* value
Patients (n)	23	7 (30.4)	14 (60.9)	2 (8.7)	**<0.0001**
IOPD	6 (26.1)	6 (85.7)	0 (0.0)	0 (0.0)	**<0.0001**
Sex: Male	10 (43.5)	4 (57.1)	5 (35.7)	1 (50.0)	0.6255
Age at Diagnosis	18.8 ± 19.1	1.4 ± 2.9	29.3 ± 17.5	6.5 ± 6.4	**0.0365**
Age at Last Exam	24.5 ± 19.4	6.8 ± 4.8	35.1 ± 17.6	11.5 ± 0.7	**0.0006**
CK (IU/L)[a]	926.9 ± 750.8 [*n* = 19]	1312.3 ± 990.8 [*n* = 7]	587.1 ± 293.8 [*n* = 11]	[b]	0.1035
AST (IU/L)[a]	190.7 ± 116.2 [*n* = 18]	258.7 ± 123.8 [*n* = 7]	132.7 ± 82.2 [*n* = 10]	[b]	**0.0412**
ALT (IU/L)[a]	149.4 ± 98.6 [*n* = 18]	184.3 ± 141.2 [*n* = 7]	119.0 ± 51.9 [*n* = 10]	[b]	0.2497
Cardiomyopathy	6 (26.1)	6 (85.7)	0 (0.0)	0 (0.0)	**<0.0001**
Macroglossia	1 (4.4)	1 (14.3)	0 (0.0)	0 (0.0)	0.3913
Hepatosplenomegaly	4 (17.4)	2 (28.6)	1 (7.1)	1 (50.0)	0.7123
Scoliosis	4 (17.4)	3 (42.9)	1 (7.1)	0 (0.0)	0.0696
Joint contractures	3 (13.0)	2 (28.6)	0 (0.0)	1 (50.0)	0.5296
Gross motor function scale					
Level I: Normal	13 (56.5)	2 (28.6)	9 (64.3)	2 (100.0)	**0.0290**
Level II	3 (13.0)	1 (14.3)	2 (14.3)	0 (0.0)	
Level III	1 (4.4)	0 (0.0)	1 (7.1)	0 (0.0)	
Level IV	--	0 (0.0)	0 (0.0)	0 (0.0)	
Level V: complete dependence	6 (26.1)	4 (57.1)	2 (14.3)	0 (0.0)	
Respiratory					
Tracheostomy	4 (17.4)	3 (42.9)	1 (7.1)	0 (0.0)	0.1041
Nightly BIPAP	5 (21.7)	1 (14.3)	4 (28.6)	0 (0.0)	
Nightly CPAP	1 (4.4)	0 (0.0)	1 (7.1)	0 (0.0)	
No ventilator assistance	13 (56.5)	3 (42.9)	8 (57.1)	2 (100.0)	
Obstructive sleep apnea	13 (56.5)	5 (71.4)	7 (50.0)	1 (50.0)	0.4351
G-Tube dependence	8 (34.8)	5 (71.4)	3 (21.4)	0 (0.0)	**0.0234**
Deep tendon reflexes[a]	[*n* = 18]	[*n* = 6]	[*n* = 10]	[*n* = 2]	
Absent	9 (50.0)	3 (50.0)	5 (50.0)	1 (50.0)	0.9208
Trace/decreased	5 (27.8)	1 (16.7)	4 (40.0)	0 (0.0)	
Intact	4 (22.2)	2 (33.3)	1 (10.0)	1 (50.0)	
Non stable body weight	7 (30.4)	6 (85.7)	1 (7.1)	0 (0.0)	**0.0004**

Statistics: Mantel–Haenszel CHISQ EXACT scores = modridit, one-way ANOVA Welch Test for non-equal variances

Bold values indicate statistical significance $p < 0.05$

[a]Deep Tendon Reflexes, CK, ALT, AST are not primary outcomes and thus missingness permitted; *n* reported for each item

[b]Not reported: $n = 1$ patient

with either IOPD or LOPD, depending in part on what variant is on the other allele [27].

The most striking finding in our cohort is that this association can be extended further, with an array of different *GAA* splice site variants on at least one allele being found in participants with LOPD. In particular, the presence of at least one splice site variant (c.546 G > C, c.1076–22 T > G, c.2646 + 2 T > A, and the classic c.−32–13T > G variant) was more likely to be associated with LOPD. All of these variants have been previously reported [37], except participant 18 who had a nonsense variant c.1525 C > T in exon 10 that was paired with a c.−32–13T > G splice variant, associated with an LOPD phenotype. The c.546 G > A variant, similar to one of ours, has been documented to be a leaky splice site variant [38]. Overall, splice site variants are estimated to compose ~15% of pathogenic *GAA* variants

[37]. Our findings suggest that multiple splice site variants for *GAA* are "leaky", yielding enough residual enzyme expression to mitigate the impact of the disease. In contrast, the presence of exonic variants on both alleles, including missense and frameshift changes, was more likely to be associated with IOPD. A recent study also found a high frequency of missense variants in IOPD [7].

In the past, it was postulated that disease severity may correlate inversely with residual acid α-glucosidase activity. For IOPD, acid α-glucosidase activity levels do tend to be quite low [39]. Acid α-glucosidase activity levels tend to be higher in LOPD, but there are well-documented cases of LOPD associated with very low levels that are indistinguishable from those typical of IOPD [8, 40]. In concurrence with prior studies, enzyme activity levels in our cohort tended to be lower for IOPD versus LOPD, but several LOPD patients had activity levels that were in the same low range as IOPD. To take two extreme examples, one LOPD participant was diagnosed at age 13 years with 100% acid α-glucosidase activity (participant 19) while another LOPD participant was diagnosed at 26 years with 0% activity (participant 17). These results may reflect the inherent variability of enzyme activity testing depending on site and substrate [8, 22] but there are likely other yet to be understood processes modulating the clinical course in these patients.

Enzyme replacement therapy is now a standard therapy offered for patients diagnosed with Pompe disease. It is, however, not curative for either subtype [41, 42], with a persistent slowly progressive skeletal myopathy [42–44]. In addition, immune responses to the enzyme have restricted the ability to implement ongoing enzyme replacement in some patients [45]. Thus, newer gene therapy approaches are under active investigation using the adeno-associated viral (AAV) vector. Numerous pre-clinical AAV-based studies have shown promising results [46–51], including strategies to express *GAA* in the liver [52, 53]. An alternate approach uses a codon-optimized *GAAco* gene delivered via a lentiviral vector into hematopoietic stem cells, which are in turn transplanted into $Gaa^{-/-}$ mice; this approach could theoretically be used to develop an autologous gene-stem cell therapy [54]. A human study demonstrated safety with intradiaphragmatic delivery of rAAV1-CMV-*hGAA* in a cohort of children [55, 56], and studies of efficacy are underway.

To capitalize on currently available enzyme replacement therapy and expected new therapeutic approvals in the coming years, newborn screening for Pompe disease has become more widely implemented in multiple countries [14–16]. The traditional technique is to assess acid α-glucosidase levels on dried blood spots obtained at birth, via tandem mass spectrometry, fluorometry, or microfluidics combined with fluorometry [57]. As is evident from the data in our series, such enzyme-based assays are not always accurate, nor can the enzyme activity levels predict IOPD versus LOPD with certainty. Thus, diagnostic accuracy would be improved by introducing genetic analysis as the foundational technique for Pompe disease newborn screening.

Cardiomyopathy was a cardinal feature of the first reported case of Pompe disease [2], and continues to be a hallmark of IOPD. In our cohort, the presence or absence of cardiomyopathy, which can be determined quickly based on physical examination and echocardiography, was an accurate predictor of IOPD versus LOPD, with a perfect correlation. This mirrors findings in the literature, as cardiac manifestations in LOPD tend to be rare and mild [58, 59]. Once an infant is diagnosed by newborn screening, or an asymptomatic or minimally symptomatic individual is diagnosed at a later age, a cardiac evaluation is urgent, not only to screen for a potential medical emergency, but also to help predict the phenotypic subtype and thus the urgency of initiating enzyme replacement therapy. Enzyme replacement therapy is indicated for both IOPD and LOPD, but initiation is more urgent for the former. Accurate diagnosis and subtype assignments will also become more important with the likelihood that new molecular therapies for Pompe disease will become clinically available in the years to come.

Limitations of our study include a smaller sample size for the IOPD subtype, along with a sparsity of individuals with certain variant types, as noted above. Given the retrospective nature of our investigations, we were not able to collect all relevant data. Specifically, acid α-glucosidase enzyme activity data were not available on a few affected individuals, either due to incomplete clinical evaluations or incomplete documentation. The tissue sources for enzyme assays were not uniform for all participants (leukocytes versus skin fibroblasts). Another limitation of the cross-sectional study design is the inability to account for disease progression. As such, conclusions regarding the association of enzyme activity or genotype with hepatosplenomegaly, scoliosis, joint contractures, GMFS, respiratory status, G-Tube dependence, DTR and non-stable body weight are restricted.

With respect to overall diagnostic accuracy, genetic analysis is typically superior to acid α-glucosidase activity levels. In our cohort, the presence of at least one splice site variant is predictive of LOPD, but due to limitations of sample size, it would be premature to generalize this conclusion. The most accurate diagnostic information can be derived by genetic testing even if acid α-glucosidase deficiency has been documented, followed by rapid cardiac evaluation for those with pathogenic variants, especially those with apparent IOPD phenotypes. Assessment of acid α-glucosidase activity levels will continue to have

utility in cases with equivocal genetic findings or atypical phenotypes.

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Compliance with ethical standards

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