DATABASES

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

Extension of the Pompe mutation database by linking disease-associated variants to clinical severity

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Abstract

Pompe disease is an autosomal recessive lysosomal storage disorder caused by disease-associated variants in the acid alpha-glucosidase (*GAA*) gene. The current Pompe mutation database provides a severity rating of *GAA* variants based on in silico predictions and expression studies. Here, we extended the database with clinical information of reported phenotypes. We added additional in silico predictions for effects on splicing and protein function and for cross reactive immunologic material (CRIM) status, minor allele frequencies, and molecular analyses. We analyzed 867 patients and 562 *GAA* variants. Based on their combination with a *GAA* null allele (i.e., complete deficiency of GAA enzyme activity), 49% of the 422 disease-associated variants could be linked to classic infantile, childhood, or adult phenotypes. Predictions and immunoblot analyses identified 131 CRIM negative and 216 CRIM positive variants. While disease-associated missense variants were found throughout the GAA protein, they were enriched up to seven-fold in the catalytic site. Fifteen percent of disease-associated missense variants were predicted to affect splicing. This should be confirmed using splicing assays. Inclusion of clinical severity rating in the Pompe mutation database provides an invaluable tool for diagnosis, prognosis of disease progression, treatment regimens, and the future development of personalized medicine for Pompe disease.

KEYWORDS
cardiac and skeletal muscle disorder, genotype-phenotype relationship, glycogen storage disease type II, lysosomal storage disease, www.pompecenter.nl

1 | INTRODUCTION

Pompe disease or glycogen storage type II (MIM# 232300) is an autosomal recessive disorder caused by deficiency of acid α-glucosidase (GAA; NP_000143.2). Partial or complete GAA deficiency is caused by disease-associated sequence variants in the *GAA* gene, resulting in lysosomal glycogen accumulation in many cell types, with profound pathology in cardiac and skeletal muscle.

Pompe disease presents as a spectrum of phenotypes. Classic infantile patients have a rapidly progressing phenotype, with

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hypertrophic cardiomyopathy and general muscle weakness. Without therapy, these patients die within the first year of life. Patients with symptom onset at childhood or adulthood have a more slowly progressive disease, leading to mobility problems and respiratory difficulties, but generally without hypertrophic cardiomyopathy. Most of these patients will become ventilator and wheelchair dependent at some point in time (Gungor & Reuser, 2013; Reuser, Hirschhorn, & Kroos, 2018; Schoser et al., 2015).

The standard treatment for Pompe disease is enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA). In classic infantile Pompe disease, ERT reduces cardiac hypertrophy, improves survival, and helps to achieve important developmental milestones that were previously unmet (Chakrapani, Vellodi, Robinson, Jones, & Wraith, 2010; Kishnani et al., 2009; Van den Hout et al., 2000). Numerous studies in children and adults have shown improvement of muscle strength, stabilization of pulmonary function, decreased fatigue, and extended survival. However, there is variation in response and not all patients respond equally well (Broomfield et al., 2016; de Vries et al., 2012; Gungor et al., 2013; Gungor et al., 2016; Hahn et al., 2018; Kuperus et al., 2017; Papadopoulos et al., 2017; Regnery et al., 2012; Stepien, Whitby, Roberts, & Sharma, 2015; Strothotte et al., 2010; van Capelle et al., 2010; van der Ploeg et al., 2010).

Patients who do not produce detectable GAA protein are termed cross reactive immunologic material (CRIM) negative. In these patients, both alleles carry a CRIM negative variant, and the phenotype is per definition classic infantile. Whereas CRIM positive patients have at least one disease-associated GAA variant that allows expression of GAA protein, the protein produced is enzymatically inactive due to misfolding, defects in glycosylation, defects in intracellular routing to the lysosome, and so forth. The second allele in CRIM positive patients can contain either a CRIM positive or a CRIM negative GAA variant. CRIM positive patients who have no detectable GAA enzyme activity develop a classic infantile phenotype, whereas patients with a childhood or adult phenotype still retain some enzymatic activity and are therefore always CRIM positive. CRIM negative infants have a greater tendency to develop anti-GAA neutralizing antibodies compared to CRIM positive infants (Kishnani et al., 2010; van Gelder et al., 2016). Therefore, it is important to establish the CRIM status of GAA variants.

The rapidly expanding number of GAA sequence variants that have been discovered over the past 15 years has provided the basis for the establishment of the Pompe disease mutation database at www.pompevariantdatabase.nl. The present version of the Pompe mutation database lists all known GAA variants and provides a prediction of pathogenicity based on results obtained by in silico prediction algorithms, from the measured effect on GAA synthesis and enzymatic function when expressed in vitro, and from the type of variant. This approach resulted in a rating system with six different categories ranging from "very severe" to "nonpathogenic" (M. Kroos et al., 2008; M. A. Kroos et al., 2012). Here, we have extended the in silico predictions by analyzing all GAA variants using Alamut software for effects on RNA processing and protein function, and used these in

silico predictions as well as published data to report or predict CRIM status. We scrutinized the literature and report clinical phenotypes that are associated with GAA variants.

The results of our study described here have been included in the update of the Pompe mutation database, that will from now on be referred to as the "Pompe disease GAA variant database" under the link (www.pompevariantdatabase.nl). The improved database should help doctors, genetic counselors and scientists to better predict disease outcome in patients diagnosed with Pompe disease. As well as providing new insight into variant severity, it will also improve prediction of prognosis in newborn screening programs, and support decision-making on therapeutic intervention.

2 | METHODS

2.1 | Annotation of variants

Variant annotations and classification conform to recommendations of the human genome variation society (HGVS) according to update 2016 (den Dunnen et al., 2016). NM_000152.3 was used as reference sequence for GAA mRNA. Annotation of variants present in intronic regions were based on the LRG_673 reference sequence. Position c.1 represents the first nucleotide of the translation start codon ATG located in exon 2. NP_000143.2 was used for annotation of GAA protein variants.

2.2 | Minor allele frequency and reference SNP number

Minor allele frequency (MAF) was examined using dbSNP, Exac, Genome of the Netherlands (GoNL), ESP, and HGVD, with 1 January 2017 as last entry. The MAF reported in the database was based on the highest MAF score seen in any of the aforementioned databases. The link for the most recent information on MAF has been provided in the database. Reference SNP (RS) numbers, when available, are also provided.

2.3 | In silico prediction of pathogenicity

These were performed using Alamut software. Missense predictions were performed using Mutation Taster, SIFT, and Align GVGD.

2.4 | Splicing prediction

Splice variants were defined as intronic positions −1 and −2 relative to the canonical splice acceptor site, and intronic positions +1 and +2 relative to the canonical splice donor site. So far, all splice variants defined in this way had been listed as "very severe". To better evaluate variants' possible effect on pre-mRNA splicing, we re-evaluated all variants using Alamut® software and provided this information in the database (Algorithms used are listed in Table S1). Splice predictions were deemed to potentially affect splicing when at least two of the five algorithms predicted more than a 10% difference

in splice site strength as described previously (Bergsma et al., 2015). In some cases it was possible to use the literature to retrieve functional evidence of variants that affect splicing. These findings were added to the column "biochemical evidence of pathogenicity".

2.5 | Prediction of CRIM status

If CRIM status had been determined experimentally by endogenous protein (i.e., immunoblot) or RNA expression (i.e., RT-qPCR) analysis, this was indicated at the "variant info" page under "biochemical evidence of CRIM status" or "biochemical evidence of pathogenicity", respectively. We also predicted CRIM status based on the type of variant for all variants, and reported this in the database. Variants that caused a frame shift were predicted to be CRIM negative. Missense variants were predicted to be CRIM positive, provided that no effect on splicing was predicted. Known and predicted splice variants were classified with an "unknown" CRIM status, unless experimental evidence was reported on expression of in-frame mRNA transcripts. Please note that a) all prediction need to be confirmed experimentally. For example, it is known that certain variants that cause a premature stop codon can escape nonsense-mediated decay. In those cases, the actual CRIM status could be positive rather than negative. It is also known that splicing outcome is difficult to predict (e.g., see Bergsma et al., 2015). b) the CRIM status of a patient is determined by two disease-associated variants, and all patients with symptom onset at childhood or adulthood have some residual GAA enzyme activity. For this reason, these patients always have a CRIM positive status, caused by the presence of at least one CRIM positive variant.

2.6 | Patient classification and phenotypic information

We classified published patient information based on the criteria stated in Gungor and Reuser (2013). Patients were classified with classic infantile Pompe disease if they presented symptoms at or under 12 months of age, and had evident signs of a hypertrophic cardiomyopathy (cardiac enlargement visible on chest X-ray, evidence of hypertrophy by echography). Patients were classified with childhood Pompe disease if the age of symptom onset was before 18 years of age and evident hypertrophic cardiomyopathy was absent. Patients were classified with adult Pompe disease if the first symptoms presented at the age of 18 years or later. If specific clinical information was reported in the literature, it was included in the database as indicated. Since familial connections were not always clearly stated in publications, no familial connections were taken into account and all cases were reported independently. We did all we could to avoid duplicating patients who had been described in more than one publication. It nonetheless remains possible that some duplicate cases were not detected.

2.7 | Availability of the data

The open access Pompe disease GAA variant database is available at www.pompevariantdatabase.nl.

In addition, all variants and patient phenotypes associated with the variants are included in the Leiden Open Variation Database (LOVD) at www.LOVD.nl/GAA.

3 | RESULTS

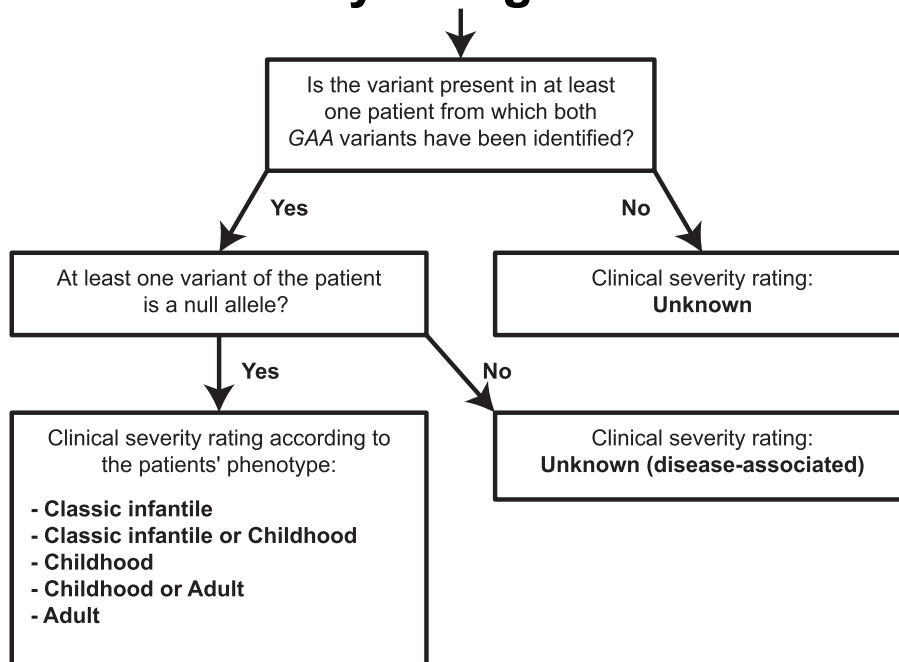### 3.1 | Overview of the updated Pompe database

A flow chart for the criteria used for clinical severity rating is given in Figure 1a. Clinical severity rating of variants was performed based on patients with two identified GAA variants, at least one of which was a null allele, which is defined as a GAA variant who's protein product lacks any detectable GAA enzyme activity. The classification of null alleles (listed in Table S2) was made based on their association with the classic infantile phenotype. In some cases data have been reported that are derived from site directed mutagenesis of GAA expression constructs, but no patients have been reported that have such variant. In those cases, the clinical phenotype was classified as "unknown". When patients were reported but the second GAA allele was not a null allele, was not reported or had an unknown severity, the clinical phenotype was classified as unknown (disease-associated). In those cases, the variant does cause Pompe disease because patients have been reported, and thus this variant is disease-associated. However the variant cannot be associated to a certain phenotype because the contribution of the second allele to the phenotype is unknown.

The updated open access Pompe disease GAA variant database is available at www.pompevariantdatabase.nl. The main access page contains the most essential information, including a) the location of the variant based on exon or intron number, b) the DNA, RNA and protein nomenclature according to the recommendations of the HGVS (version 2016) (den Dunnen et al., 2016), c) the predicted severity, d) the phenotype when combined with a null alelle, e) the CRIM status according to literature reports or based on prediction according to type of variant, and f) links to the variants and to the patients described in the literature. (Figure 1b).

The link to variants includes the type of variants, their predicted severity, the phenotype of variants when combined with a null allele, biochemical evidence and in silico prediction of (non-) pathogenicity, MAF, RS-number, biochemical evidence and in silico prediction of CRIM status, splicing prediction for all variants using different prediction algorithms, severity predictions for all missense variants, and the link to publications on the variant. The link to patients contains the clinical phenotype of patients found in the literature with that variant in combination with a second disease-associated variant, additional clinical symptoms, the patients' geographical location, and the link to publications in which the patient was reported. The extended database includes an analysis of all data contained in the Pompe disease mutation database. Eight new

(a)

Clinical severity rating of *GAA* variants

Is the variant present in at least one patient from which both *GAA* variants have been identified?

Yes — At least one variant of the patient is a null allele?

No — Clinical severity rating: **Unknown**

Yes — Clinical severity rating according to the patients' phenotype:
- **Classic infantile**
- **Classic infantile or Childhood**
- **Childhood**
- **Childhood or Adult**
- **Adult**

No — Clinical severity rating: **Unknown (disease-associated)**

(b)

Pompe Disease *GAA* Variant Database

FIGURE 1 The Pompe disease *GAA* variant database. (a) Flowchart for determining the clinical severity rating in the updated database. (b) Screenshot of the updated Pompe disease *GAA* variant database home page at www.pompevariantdatabase.nl

variants have been added since the last update in May 2016 (Table S3).

3.2 | Analysis of *GAA* variants based on clinical phenotype

Of the total number of 562 described variants, 422 are listed as disease-associated. We analyzed types of *GAA* variants according to their RNA and protein nomenclature (HGVS version 2016), and stratified these based on the clinical phenotype, being classic infantile, childhood, or adult (Figure 2). The variants present in a total of 867 patients were analyzed (Figure S2). In 49% of patients with Pompe disease reported in the mutation database (data not shown), the second *GAA* allele contained a null allele, and these patients were used for clinical severity rating of *GAA* variants. The reason for this is as follows. Usually, the second allele is a null allele, from which no residual GAA enzyme activity is produced. Any residual GAA enzyme activity is caused by the first allele. In this way, the disease-associated variant on the first allele can be rated. When the second allele would also have residual GAA enzyme activity, both alleles will contribute to the total residual GAA enzyme activity, and it would not be possible anymore to attribute the activity and the clinical severity rating to one of the two alleles. The remaining 140 of 562 *GAA* variants are classified as unknown (Table S4). The non-disease-associated variants include alleles such as the Caucasian variant c.271G>A (GAA2; MAF 0.02), and the Asian *GAA* variants c.1726G>A (MAF 0.0179) plus c.2065G>A (MAF 0.08797; Table S5). These variants have previously been shown to lower
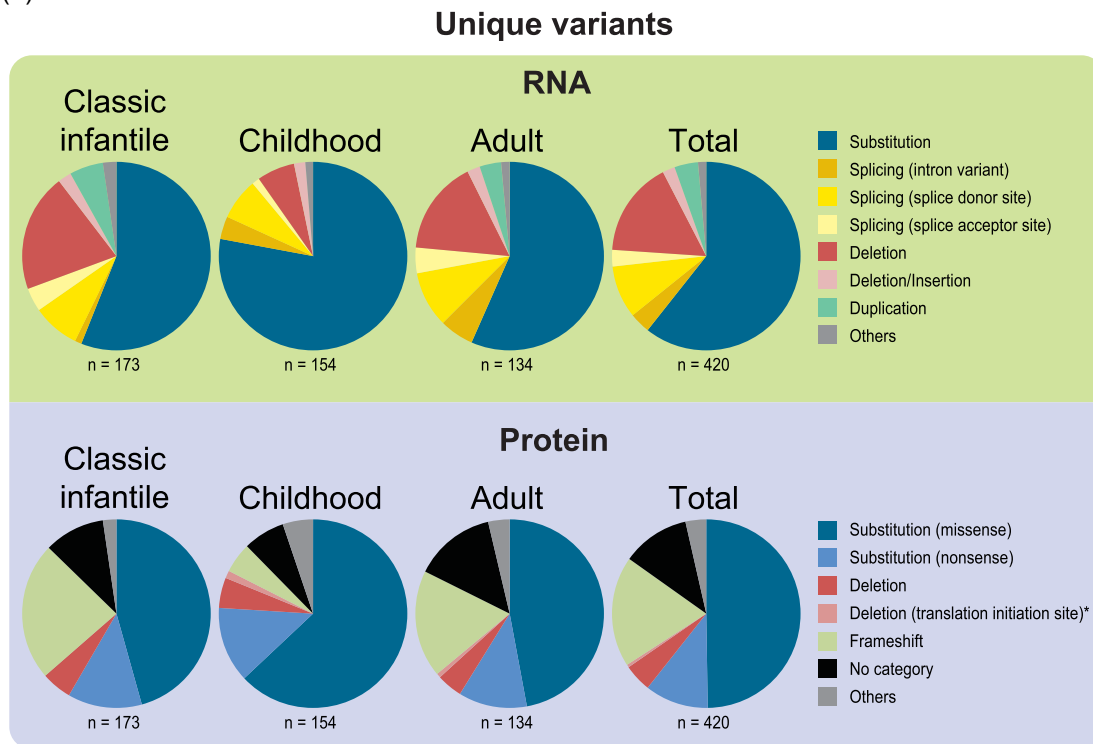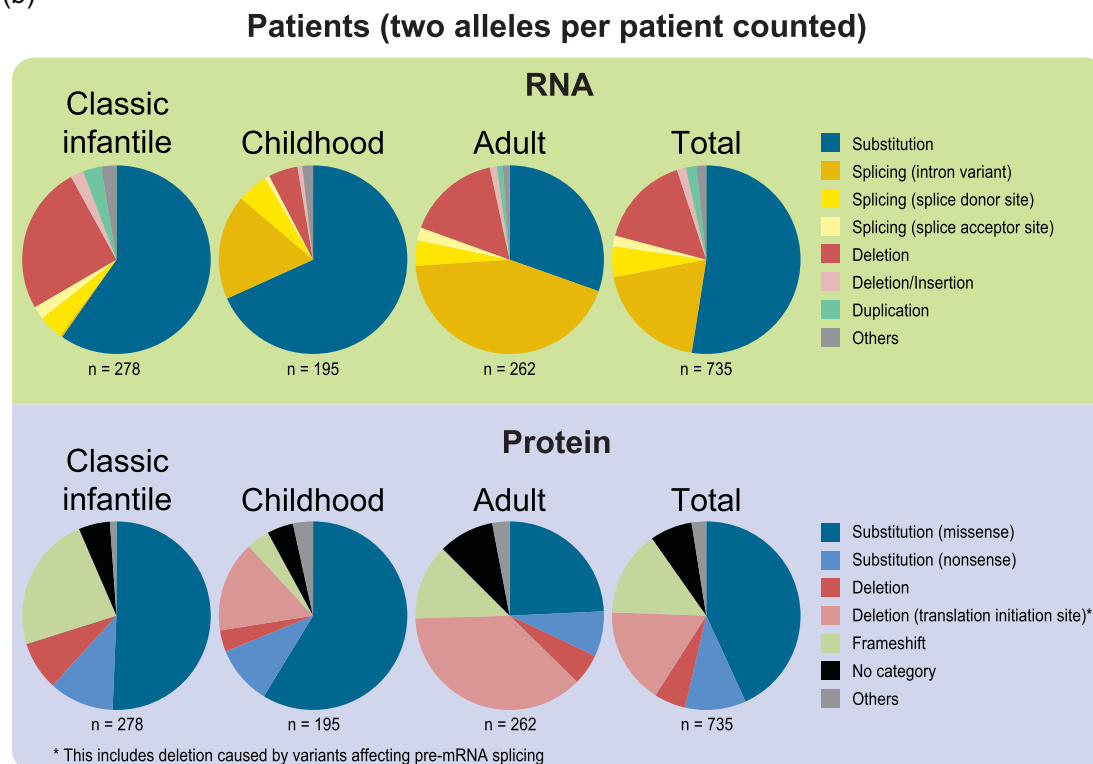
FIGURE 2 Frequencies of *GAA* variants and patients with specific *GAA* variants, stratified for disease severity. (a) Frequencies of unique variants, classified at the RNA level (upper panel) and protein level (lower panel). The consequences of variants that affect pre-mRNA splicing was only included in specific categories (e.g., deletion, insertion, frameshift) at the protein level if experimental evidence was available. A list of all included categories is shown in Table S6. (b) Frequencies of variant types in patients, classified at the RNA level (upper panel) and protein level (lower panel). Two *GAA* alleles per patient were counted. Please note that 1) only one *GAA* allele could be counted in patients with an unknown second *GAA* variant; and that 2) the allele was counted twice in patients with a homozygous genotype

GAA enzymatic activity to some extent, however, they cannot cause Pompe disease (M.A. Kroos et al., 2008; Shigeto et al., 2011; Swallow et al., 1989; van Diggelen et al., 2009).

First, we performed stratification of *GAA* variants. At the RNA level, substitutions were the predominant type of variants in all three phenotype groups. Other types of variants including splicing variants and deletions were present at a lower percentage without a clear trend relative to the phenotype. At the protein level, analysis of substitutions showed that the largest fraction was represented by missense variants, followed by nonsense variants, again without a clear trend relative to the phenotype. In 15% of cases, splicing prediction of disease-associated missense variants revealed possible effects on splicing (Figure S1). The total number of unique variants was similar between the three phenotype groups and ranged from 134 in adult to 154 in childhood and 173 in classic infantile patients.

A different result was obtained when the number of patients with a certain type of variant was plotted per disease onset group. The variants in all patients with a known phenotype were analyzed (Figure S2). We note that two *GAA* variants per patients were counted. A typical adult patient contains one null allele and one less severe allele, whereas a classic infantile patient contains two null alleles. In this case, the percentage of patients with a splicing variant (intron variant) increased from less than 1% in the classic infantile group to 18% in the childhood group and 44% in the adult group (Figure 2b). At the protein level, this effect was reflected at the level of the category deletion (translation initiation site), as the IVS1 variant causes skipping of the translation initiation site-containing exon 2. This effect can be attributed to the large number of childhood and adult Caucasian Pompe patients with the splicing (intron variant) variant c.-32-13T>G (IVS1). This variant leads to partial or complete skipping of exon 2 during *GAA* pre-mRNA splicing, which has been described previously (Bergsma et al., 2015; Boerkoel et al., 1995; Dardis et al., 2014; Huie, Chen, Brooks, Grix, & Hirschhorn, 1994; van der Wal, Bergsma, Pijnenburg, van der Ploeg, & Pijnappel, 2017; van der Wal, Bergsma, van Gestel et al., 2017).

3.3 | CRIM status of classic infantile variants

The CRIM status of a Pompe patient is determined by the combined effect of both *GAA* variants: a CRIM negative patient has by definition two CRIM negative variants. A CRIM positive patient has either one CRIM positive (and one CRIM negative) or two CRIM positive variants. Only for a limited number of variants, experimental evidence of CRIM status, either by immunoblot or by RT-PCR analyses, has been published. This resulted in 46 CRIM negative, and 15 CRIM positive variants (Figure 3a,b). At the RNA level, predominant categories for CRIM negative variants with experimental evidence were substitutions, splicing (splice donor site), deletions, and duplications (Figure 3a). At the protein level, most of these variants were substitutions (nonsense) or frameshifts, both of which cause premature translation initiation sites, whereas substitutions (missense) were absent (Figure 3b). For CRIM positive variants with experimental evidence, most variants were substitutions or

splicing variants. At the protein level, most variants were classified as substitutions (missense) or deletions. A small fraction of CRIM positive variants were frameshifts, suggesting that in these cases the nonsense-mediated decay pathway did not completely degrade the mRNA.

Prediction of CRIM status was performed on the basis of the variants' effects on the reading frame, but it should be noted that experimental validation is needed for these cases. 201 variants were predicted to be CRIM positive, and these were substitutions (missense) and deletions. Eighty-five *GAA* variants were predicted to be CRIM negative, the majority of which were frameshift and substitution (nonsense) variants. The total numbers of classified variants—that is those determined experimentally and those predicted—were 216 CRIM positive variants (62%) and 131 CRIM negative variants (38%).

3.4 | Distribution of disease-associated variants along the *GAA* gene

Figure S3 shows that disease-associated variants are distributed throughout the *GAA* RNA (Figure S3A) and protein (Figure S3B) with a slight enrichment in exons 4-16 relative to the flanking exons. A similar pattern was obtained when the distribution of variants was plotted along exons according to clinical severity (Figure S3C). For introns, disease-associated variants were found in all introns except for intron 5, without a clear pattern (Figure S3D). We note that intronic variants are rarely investigated at the functional level and that only the exon flanking regions are sequenced in standard diagnostic analysis.

However, mapping of disease-associated substitution (missense) variants to functional GAA protein domains (Figure 4a), showed a strong enrichment (up to seven-fold) in the catalytic sites relative to the N-terminal signal peptide and the C-terminal distal β-sheet (Figure 4b). Variants associated with the classic infantile phenotype showed a similar enrichment in the catalytic sites. When *GAA* variants in ExAC and dbSNP databases (which contains both disease-associated and non disease-associated variants) were plotted, no such enrichment in the catalytic domain was observed (Figure 4c). These results are in line with the critical function of the catalytic core for GAA enzyme activity.

3.5 | Geographic distribution of the most common disease-associated *GAA* variants

Table 1 shows the most common disease-associated variants per severity (when combined with a null allele), their geographical distributions, and their linked phenotypes. We note that two CRIM negative alleles are required to give rise to a CRIM negative phenotype.

Variants associated with the classic infantile phenotype include the very common variants c.525del (Caucasian, CRIM negative), c.1935C>A (Asian, CRIM positive), and c.2481_102_2446+31del
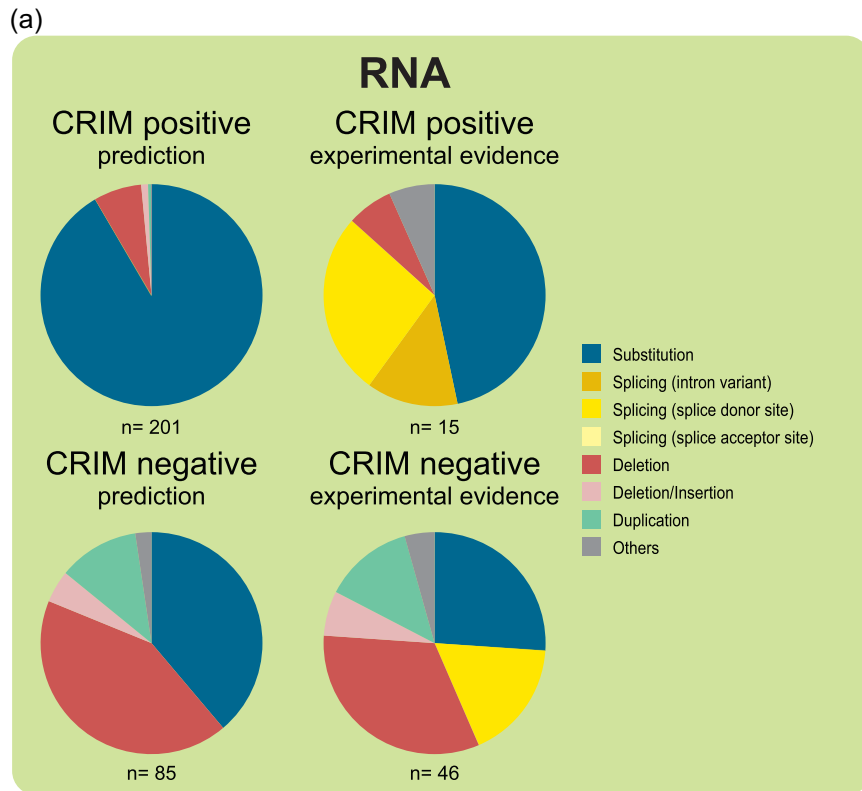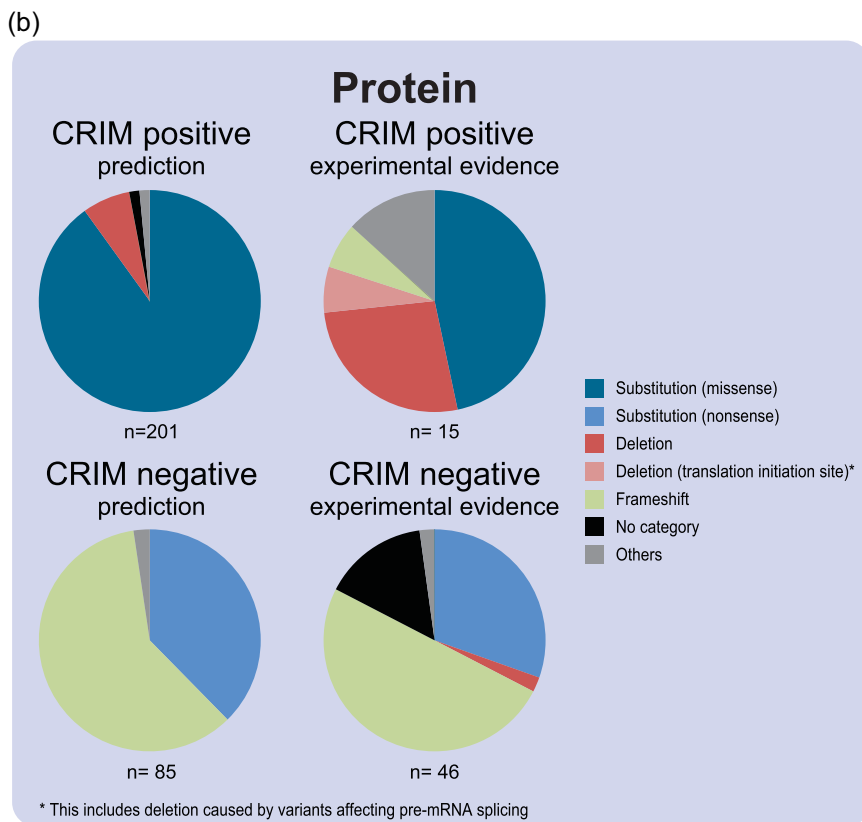
(a)

FIGURE 3 Distribution of CRIM positive and CRIM negative variants per variant type at the RNA level (a) and protein level (b). Left panels in (a) and (b): predicted variants; right panels: experimentally tested variants. CRIM, cross reactive immunologic material

(b)

(delex18; Caucasian, CRIM positive). Other common variants associated with the classic infantile phenotype include the CRIM negative variants c.2560C>T (Latin American and African/African American), c.1411_1414del (Asian), and c.2237G>A (Caucasian), and

the CRIM positive variants c.925G>A, c.1933G>A, c.784G>A, and c.1655T>C (all mostly Caucasian).

Three common variants that are classified as either associated with the classic infantile or childhood phenotypes include c.1064T>C
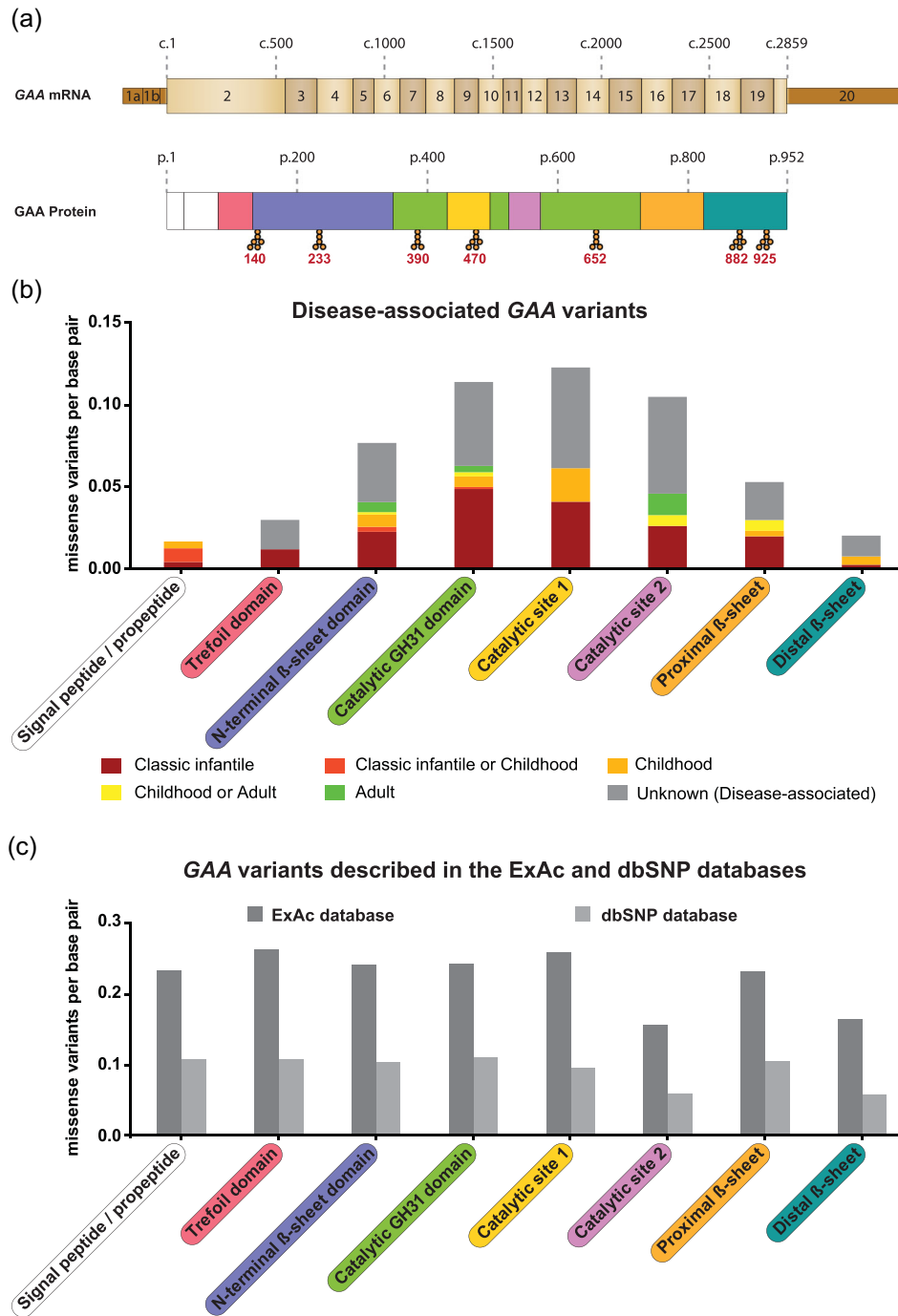
FIGURE 4 Distribution of missense variants along the GAA protein. (a) Cartoon of the *GAA* gene indicating the locations of exons. Dark brown regions indicate the 5′ and 3′ untranslated regions. The translation start codon is indicated as c.1 in exon 2. The second cartoon indicates the GAA protein domains and the sites modified by M6P residues. (b) The distribution of disease-associated variants in GAA protein domains according to frequency and clinical severity rating. Numbers are corrected for the length of each domain. (c) as (b), but now for all *GAA* variants, including both disease-associated and non-disease-associated missense variants, listed in the ExAc database

(Caucasian), c.670C>T (Caucasian and Asian), and c.1561G>A (Asian). Variants associated with the childhood phenotype include the common variants c.1857C>G, c.796C>T (both Asian), c.-32-3C>A (Caucasian and Latin American), c.875A>G (Caucasian), and c.1082C>T (Asian).

Variants that are associated with the childhood or adult phenotypes are strongly dominated by the c.-32-13T>G (IVS1)

variant (Caucasian and Latin American). This variant is present in 86% (253/294) of patients in this category. Other common variants associated with the childhood or adult phenotype include c.2238G>C (Asian), c.2014C>T and 2173C>T (both Caucasian), and c.1634C>T (Caucasian and Asian). Variants associated with the adult phenotype include the common variants c.2647-7G>A (Caucasian) and c.1585_1586delinsGT (Asian). This analysis demonstrates that

TABLE 1 Frequency and geographical distribution of most common GAA variants

Phenotype with a null allele	DNA nomenclature	Caucasian (n)	Asian (n)	African/African American (n)	Latin-American (n)	Undetermined (n)	Total (n)
Classic infantile (CRIM negative)	c.525del	29		1			30
	c.2560C>T	1	1	3	9	1	15
	c.1411_1414del		11				11
	c.2237G>A	5	1				6
	Total	35	13	4	9	1	62
Classic infantile (CRIM positive)	c.1935C>A		68				68
	c.2481+102_2646+31del	20			3	1	24
	c.925G>A	7			1		8
	c.1933G>A	4	2		1		7
	c.784G>A	4	2		1		7
	c.1655T>C*	5			1		6
	Total	40	72		7	1	120
Classic infantile or Childhood	c.1064T>C	9	3		2		14
	c.670C>T	3	3				6
	c.1561G>A	1	3				4
	Total	13	9		2		24
Childhood	c.1857C>G		6				6
	c.796C>T		5				5
	c.-32-3C>A	1			3		4
	c.875A>G	3	1				4
	c.1082C>T	1	3				4
	Total	5	15		3		23
Childhood or Adult	c.-32-13T>G	224	3		18	8	253
	c.2238G>C		21				21
	c.2014C>T	7	1				8
	c.1634C>T	3	2	1			6
	c.2173C>T	5			1		6
	Total	239	27	1	19	8	294
Adult	c.2647−7G>A	9					9
	c.1585_1586delinsGT		4				4
	Total	9	4				13

*The c.1655T>C variant has been classified to be associated with the classic infantile phenotype, although we note that three patients have been reported that carry this variant and showed a childhood onset phenotype.

common disease-associated variants can be found for each phenotype and geographical region.

4 | DISCUSSION

This study describes the extension of the Pompe mutation database www.pompevariantdatabase.nl, which now includes a number of new features to improve its utility. First, by linking variants to the patient information reported in the literature, we have extended severity ratings with the clinical phenotype. Second, we have added information on the MAF, geographical distribution, onset and types of clinical symptoms; and have performed predictions for a) effects on splicing, b) severity of missense variants, and c) CRIM status. Third, we have provided separate lists of null alleles, variants with an 'unknown' clinical severity rating, and variants that can lower the activity of GAA without being capable of causing Pompe disease. An important asset of the database—which is freely accessible online—is that it provides comprehensive information on the genotype-phenotype relationship. In the previous version of the database, the

severity of the majority of GAA variants was predicted in silico. For a subset of variants, expression studies were available. The extended database now considers the clinical consequences of a GAA variant, and it enables a better judgment of the potential severity of variants. This is important for several aspects related to the diagnosis and treatment of Pompe disease as outlined below.

4.1 | Novel aspects of the database

4.1.1 | Clinical information

Pompe disease has a broad spectrum of disease onset and symptoms. It is important to be able to distinguish between classic infantile and late onset phenotypes, because the classic infantile phenotype is so severe that immediate treatment with ERT is required. This is reflected by severe muscle weakness and hypertrophic cardiomyopathy, which results in death within the first year of life if left untreated due tot cardiorespiratory insufficiency. It has also become evident that classic infantile patients slowly develop abnormalities in the central nervous system as detected using nuclear magnetic resonance, which can result in diminished intellectual performance (Ebbink et al., 2018). When

patients have a late onset form of Pompe disease, at present it cannot be accurately predicted when symptoms will arise and how severe these will be. It is therefore important to collect the clinical information on specific disease-associated variants to better understand the genotype–phenotype relationships. These aspects are relevant for newborn screening programs and genetic counseling.

4.1.2 | In silico predictions

Whereas clinical information is crucial for judging the severity of *GAA* variants, in silico predictions can be helpful as they can provide part of the evidence that leads to determination of the severity of variants. In addition, in silico predictions can provide evidence for the underlying mechanism involved in the disruption of GAA enzyme activity. For example, splicing predictions can point to possible effects on splicing of variants that would normally not be suspected of such effects. In particular, missense variants are often not evaluated for their effect on splicing, whereas several reports have suggested that missense variants can often affect splicing (Lim, Ferraris, Filloux, Raphael, & Fairbrother, 2011; Soukarieh et al., 2016). Also in Pompe disease, we recently described a missense variant (c.1075G>A; p.G359R) that appeared to be disease-associated mainly because it affects splicing rather than changing the amino acids sequence. The effect of this variant was predicted in silico, and this was experimentally confirmed (Bergsma et al., 2015). On the other hand, predictions can also fail, such as is the case for the IVS1 variant, which has an experimentally confirmed effect on splicing that cannot be predicted in silico (Boerkoel et al., 1995; Dardis et al., 2014; Huie et al., 1994; van der Wal, Bergsma, Pijnenburg et al., 2017; van der Wal, Bergsma, van Gestel et al., 2017). In addition, comparison of predictions of the severity of amino acid changes with expression studies revealed failure of at least two out of three prediction programs to predict the experimentally found deleterious effect on GAA enzyme activity. This was the case for the following *GAA* missense variants: c.307C>T, c.307C>G, c.380G>T, 701C>G, c.947A>T, c.1040C>G, c.1381G>A, c.1456G>C, c.1834C>T, c.1905C>A, c.2105G>T, and c.2210C>A. Conversely, there were also variants for which in silico programs predicted a deleterious effect, while expression studies showed no effect on enzyme activity. These variants included c.664G>A, c.2132C>G. Finally, it is known that the strength of unnatural transcription termination signals depends on the adjacent sequences and that such signal can be 'leaky', reinforcing the need for experimental confirmation (Dabrowski, Bukowy-Bieryllo, & Zietkiewicz, 2015). In all these cases, it is important to know whether a variant has some residual enzyme activity, whether the GAA protein is still expressed, or whether it is completely deleterious. This is crucial to make a distinction between the classic infantile and late onset forms of Pompe disease, and within the classic infantile population, to distinguish between CRIM positive and CRIM negative patients.

4.1.3 | CRIM status

CRIM negative classic infantile patients generally have a poorer prognosis compared to CRIM positive classic infantile patients when treated with ERT. One of the explanations is the tendency of CRIM negative patients to form high antibody titers during ERT (Kishnani et al., 2010; van Gelder et al., 2016). This can be explained by the lack of any endogenous GAA protein in CRIM negative patients, which results in the recognition of rhGAA by the immune system and the generation of rhGAA-specific antibodies. However, most CRIM positive patients with Pompe disease also develop anti-rhGAA antibodies, likely due to posttranslational differences between the endogenous GAA protein and rhGAA, although on average the antibody titers are lower in CRIM positive patients compared to CRIM negative patients (Kishnani et al., 2010; Poelman et al., 2018). Antibodies have the potential to interfere with ERT by binding to rhGAA and thereby neutralizing its activity and/or uptake by muscle cells. This has led to the exploration of immune modulating strategies before the start of ERT in classic infantile patients (Elder et al., 2013; Mendelsohn, Messinger, Rosenberg, & Kishnani, 2009; Messinger et al., 2012), in particular in CRIM negative patients. At least 32% of disease-associated variants listed in the database have been shown or predicted to be CRIM negative. Whereas the majority of missense variants is predicted to be CRIM positive, missense variants that affect splicing may be CRIM negative if they fully abrogate canonical splicing and change the reading frame to introduce a premature stop codon. For experimental validation of variants CRIM status, future efforts should focus on performing standardized assays such as immunoblot analysis combined with sensitive detection. As the generation of leaky wild type splicing can be detected by RT-PCR analysis and is indicative of a CRIM positive status, such validation should also include splicing analysis (Bergsma, van der Wal, Broeders, van der Ploeg, & Pim Pijnappel, 2018), such as the splicing assay for *GAA* that has been reported by us previously (Bergsma et al., 2015).

4.1.4 | MAF

The extended database now also includes information on the MAF of variants. In general, a MAF of <1% is required for a variant to be considered disease-associated, because otherwise there would be many more patients then found in reality. Relatively high MAFs can be seen in the case of certain disease-associated *GAA* variants that are more frequent. The IVS1 variant, associated with late onset Pompe disease, is truly disease-associated but is clearly enriched in the Caucasian population with a MAF of up to 0.8%. Similarly c.1935C>A, a disease-associated variant associated with classic infantile Pompe disease is enriched in the Asian population with a MAF of 0.17%. Novel variants for which the severity is unknown should first pass the MAF <1% rule, and this is a useful fast criterium to determine whether the variant could be considered disease-associated or not. This rule also applies to variants that can lower GAA activity but not sufficiently to be truly disease-associated (meaning they can cause Pompe disease) on their own. These include the GAA2 (c.271G>A) (MAF up to 4.9%) variant in the Caucasian population and c.1726G>A; c.2065G>A allele (MAF up to 14%) in the Asian population, both of which are variants that lower GAA enzyme activity to some extent

(M. A. Kroos et al., 2008). Considering their MAF and modest effect on GAA activity, they cannot be considered disease-associated variants with sufficient deleterious effect to cause Pompe disease. As screening and diagnostic procedures involve 4-MU as substrate, these variants can cause false-positive results that can be excluded using second tier DNA analysis.

4.1.5 | Geographical information

We have analyzed the geographical distribution of disease-associated variants and this indicated distinct sets of variants in Caucasian and Asian populations. For instance, the IVS1 variant is frequent in the Caucasian population but absent in people from Asian descent, whereas the opposite is true for c.1935C>A. In countries with mixed populations, it is difficult to attribute variants to a certain descent. For example, in Latin America, indigenous populations are mixed with Caucasians and people from African and Asian descent. Many populations including those from Africa, Russia, China, and more areas are currently not covered by the database as it mostly includes variants found in patients from Europe, North America, Australia, Taiwan, and Japan. For future diagnostic purposes in other countries, it will be important to extend the database beyond the current geographical regions.

4.1.6 | Identification of disease-associated variants

There are a number of patients in which only one GAA variant has been identified. It is important to stress that this does not imply that Pompe disease can be caused by only a single disease-associated variant. This is evident from the lack of symptoms in carriers, such as the parents of Pompe patients. Most likely, lack of identification of a second disease-associated GAA variant is caused by variants that are not detected by standard DNA diagnostics. Currently, standard DNA diagnostic analysis involves Sanger sequencing of exonic regions. This means that intronic regions, 5′ and 3′ untranslated regions, and the GAA promoter are not examined, all of which could in principle harbor disease-associated variants. A first clue to the presence of such variant can be provided by analysis at the mRNA level, which can reveal splicing events or a lack of mRNA expression. We have shown that such analysis is useful for detection of deep intronic variants and for elucidating splicing events in fibroblasts (Bergsma et al., 2015), but in principle such analysis may also be performed in blood. Small deletions are analyzed using multiplex ligation-dependent probe amplification or DNA-qPCR at diagnostic departments, but other chromosomal aberrations such as deletions/duplications or uni-parental disomy are currently not systematically analyzed. SNP arrays have recently been shown to be useful for the detection of such cases, which also occur in Pompe disease and may be useful to include in standard DNA diagnostics (Labrijn-Marks et al., 2019).

4.1.7 | Utilization of the database for future personalized medicine

Like ERT with rhGAA, next-generation therapies are now under development. Clinical trials have started for modified versions of rhGAA, such as avaglucosidase alpha (former neoGAA; made by Sanofi Genzyme); and ATB200/AT2221 (made by Amicus), which combines rhGAA with a chaperone to improve the stability of ERT. When used as single treatment without ERT, chaperones could be used to treat a subpopulation of Pompe patients with certain missense variants by preventing degradation of endogenous GAA by the endoplasmatic reticulum-associated protein degradation pathway. The structure of rhGAA, which was solved recently (Roig-Zamboni et al., 2017), should aid the design of next generation chaperones for personalized treatment, a process that will be greatly supported by information in the Pompe mutation database. When disease-associated missense variants were mapped on the GAA structure, we found enrichment of deleterious variants in the catalytic domain, which is important for GAA enzyme activity. The signal peptide/propeptide is involved in protein targeting and activation (Choo, Tan, & Ranganathan, 2005), while the trefoil domain is found in a number of extracellular proteins (http://pfam.xfam.org/family/PF00088) and forms a secondary substrate binding site (Roig-Zamboni et al., 2017). Given their (potential) importance, it is surprising that a relatively low percentage of disease-associated missense variants was found in the signal peptide/propeptide and trefoil domains. Nevertheless, these domains also contained deleterious variants, which may be relevant for the idea that chaperones could be designed for trefoil domain variants that would have enhanced specificity compared to catalytic domain chaperones, because the catalytic domain is conserved in neutral glycosidases that would mediate undesired side effects when targeted with a chaperone (Roig-Zamboni et al., 2017). If approved, other potential therapies currently under preclinical development may, depending on the predicted phenotype, be the most suitable for certain types of Pompe patients. These include AAV-mediated gene therapy directed toward the diaphragm (Corti et al., 2017) or the liver (Puzzo et al., 2017), hematopoietic stem cell-mediated lentiviral gene therapy (van Til et al., 2010), and splice switching antisense oligonucleotides for the common c.-32-13T>G (IVS1) variant and for other more rare childhood/adult variants (van der Wal, Bergsma, Pijnenburg et al., 2017; van der Wal, Bergsma, van Gestel et al., 2017). It will be important to continue updating and improving the database as it forms an important reference point for research on genotype-phenotype relationships, diagnostics, treatment regimen, and clinical trial design.

CONFLICT OF INTEREST

AvdP has provided consulting services for various industries in the field of Pompe disease under an agreement between these industries and Erasmus MC, Rotterdam, The Netherlands.

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REFERENCES

Bergsma, A. J., Kroos, M., Hoogeveen-Westerveld, M., Halley, D., van der Ploeg, A. T., & Pijnappel, W. W. (2015). Identification and characterization of aberrant GAA pre-mRNA splicing in pompe disease using a generic approach. *Human Mutation*, 36(1), 57–68. https://doi.org/10.1002/humu.22705

Bergsma, A. J., van der Wal, E., Broeders, M., van der Ploeg, A. T., & Pim Pijnappel, W. W. M. (2018). Alternative splicing in genetic diseases: Improved diagnosis and novel treatment options. *International Review of Cell and Molecular Biology*, 335, 85–141. https://doi.org/S1937-6448(17)30083-7

Boerkoel, C. F., Exelbert, R., Nicastri, C., Nichols, R. C., Miller, F. W., Plotz, P. H., & Raben, N. (1995). Leaky splicing mutation in the acid maltase gene is associated with delayed onset of glycogenosis type II. *American Journal of Human Genetics*, 56(4), 887–897.

Broomfield, A., Fletcher, J., Davison, J., Finnegan, N., Fenton, M., Chikermane, A., & Vellodi, A. (2016). Response of 33 UK patients with infantile-onset Pompe disease to enzyme replacement therapy. *Journal of Inherited Metabolic Disease (Dordrecht)*, 39(2), 261–271. https://doi.org/10.1007/s10545-015-9898-5. [pii] 10.1007/s10545-015-9898-5.

van Capelle, C. I., van der Beek, N. A., Hagemans, M. L., Arts, W. F., Hop, W. C., Lee, P., & van der Ploeg, A. T. (2010). Effect of enzyme therapy in juvenile patients with Pompe disease: A three-year open-label study. *Neuromuscular Disorders: NMD*, 20(12), 775–782. https://doi.org/S0960-8966(10)00572-9

Chakrapani, A., Vellodi, A., Robinson, P., Jones, S., & Wraith, J. E. (2010). Treatment of infantile Pompe disease with alglucosidase alpha: The UK experience. *Journal of Inherited Metabolic Disease (Dordrecht)*, 33(6), 747–750. https://doi.org/10.1007/s10545-010-9206-3

Choo, K. H., Tan, T. W., & Ranganathan, S. (2005). SPdb--a signal peptide database. *BMC Bioinformatics*, 6, 249. https://doi.org/1471-2105-6-249. [pii] 10.1186/1471-2105-6-249

Corti, M., Liberati, C., Smith, B. K., Lawson, L. A., Tuna, I. S., Conlon, T. J., & Byrne, B. J. (2017). Safety of intradiaphragmatic delivery of adeno-associated virus-mediated alpha-glucosidase (rAAV1-CMV-hGAA) gene therapy in children affected by pompe disease. *Human Gene Therapy. Clinical Development*, 28(4), 208–218. https://doi.org/10.1089/humc.2017.146

Dabrowski, M., Bukowy-Bieryllo, Z., & Zietkiewicz, E. (2015). Translational readthrough potential of natural termination codons in eucaryotes--The impact of RNA sequence. *RNA Biology*, 12(9), 950–958. https://doi.org/10.1080/15476286.2015.1068497

Dardis, A., Zanin, I., Zampieri, S., Stuani, C., Pianta, A., Romanello, M., & Buratti, E. (2014). Functional characterization of the common c.-32–13 T >G mutation of GAA gene: Identification of potential therapeutic agents. *Nucleic Acids Research*, 42(2), 1291–1302. https://doi.org/10.1093/nar/gkt987

van Diggelen, O. P., Oemardien, L. F., van der Beek, N. A., Kroos, M. A., Wind, H. K., Voznyi, Y. V., & Reuser, A. J. (2009). Enzyme analysis for Pompe disease in leukocytes; superior results with natural substrate compared with artificial substrates. *Journal of Inherited Metabolic Disease (Dordrecht)*, 32(3), 416–423. https://doi.org/10.1007/s10545-009-1082-3

den Dunnen, J. T., Dalgleish, R., Maglott, D. R., Hart, R. K., Greenblatt, M. S., McGowan-Jordan, J., & Taschner, P. E. (2016). HGVS recommendations for the description of sequence variants: 2016 Update. *Human Mutation*, 37(6), 564–569. https://doi.org/10.1002/humu.22981

Ebbink, B. J., Poelman, E., Aarsen, F. K., Plug, I., Régal, L., Muentjes, C. … van den Hout, J. M. P. (2018). Classic infantile Pompe patients approaching adulthood: a cohort study on consequences for the brain. *Developmental Medicine & Child Neurology*, 60(6), 579–586. https://doi.org/10.1111/dmcn.13740. PMID: 29573408.

Elder, M. E., Nayak, S., Collins, S. W., Lawson, L. A., Kelley, J. S., Herzog, R. W., … Byrne, B. J. (2013). B-cell depletion and immunomodulation before initiation of enzyme replacement therapy blocks the immune response to acid alpha-glucosidase in infantile-onset Pompe disease. *Jornal de Pediatria*, 163(3), 847–854. https://doi.org/10.1016/j.jpeds.2013.03.002

van Gelder, C. M., Poelman, E., Plug, I., Hoogeveen-Westerveld, M., van der Beek, N., Reuser, A. J. J., & van der Ploeg, A. T. (2016). Effects of a higher dose of alglucosidase alfa on ventilator-free survival and motor outcome in classic infantile Pompe disease: An open-label single-center study. *Journal of Inherited Metabolic Disease (Dordrecht)*, 39(3), 383–390. https://doi.org/10.1007/s10545-015-9912-y

Gungor, D., Kruijshaar, M. E., Plug, I., Rizopoulos, D., Kanters, T. A., Wens, S. C., & van der Ploeg, A. T. (2016). Quality of life and participation in daily life of adults with Pompe disease receiving enzyme replacement therapy: 10 years of international follow-up. *Journal of Inherited Metabolic Disease (Dordrecht)*, 39(2), 253–260. https://doi.org/10.1007/s10545-015-9889-6. [pii] 10.1007/s10545-015-9889-6.

Gungor, D., & Reuser, A. J. (2013). How to describe the clinical spectrum in Pompe disease? *American journal of medical genetics. Part A*, 161A(2), 399–400. https://doi.org/10.1002/ajmg.a.35662

Gungor, D., de Vries, J. M., Brusse, E., Kruijshaar, M. E., Hop, W. C., Murawska, M., & van der Ploeg, A. T. (2013). Enzyme replacement therapy and fatigue in adults with Pompe disease. *Molecular Genetics and Metabolism*, 109(2), 174–178. https://doi.org/S1096-7192(13)00105-4. [pii] 10.1016/j.ymgme.2013.03.016

Hahn, S. H., Kronn, D., Leslie, N. D., Pena, L. D. M., Tanpaiboon, P., Gambello, M. J., & Kishnani, P. S. (2018). Efficacy, safety profile, and immunogenicity of alglucosidase alfa produced at the 4,000-liter scale in US children and adolescents with Pompe disease: ADVANCE, a phase IV, open-label, prospective study. *Genetics in Medicine*, 20, 1284–1294. https://doi.org/gim20182. [pii] 10.1038/gim.2018.2

Reuser, A. J. J., Hirschhorn, R., & Kroos, M. A. (2018). Pompe disease: Glycogen storage disease type II, acid α-glucosidase (acid maltase) deficiency. In Beaudet, A. L., Vogelstein, B., Kinzler, K. W.,

Antonarakis, S. E., Ballabio, A., Gibson, K. M., & Mitchell, G. (Eds.), *The Online Metabolic and Molecular Bases of Inherited Disease.* Lysosomal Storage Disorders New York, NY: The McGraw-Hill Companies, Inc.

Van den Hout, H., Reuser, A. J., Vulto, A. G., Loonen, M. C., Cromme-Dijkhuis, A., & Van der Ploeg, A. T. (2000). Recombinant human alpha-glucosidase from rabbit milk in Pompe patients. *Lancet, 356*(9227), 397–398. https://doi.org/S0140673600025332

Huie, M. L., Chen, A. S., Brooks, S. S., Grix, A., & Hirschhorn, R. (1994). A de novo 13 nt deletion, a newly identified C647W missense mutation and a deletion of exon 18 in infantile onset glycogen storage disease type II (GSDII). *Human Molecular Genetics, 3*(7), 1081–1087.

Kishnani, P. S., Corzo, D., Leslie, N. D., Gruskin, D., Van der Ploeg, A., Clancy, J. P., & Mandel, H. (2009). Early treatment with alglucosidase alpha prolongs long-term survival of infants with Pompe disease. *Pediatric Research, 66*(3), 329–335. https://doi.org/10.1203/PDR.0b013e3181b24e94

Kishnani, P. S., Goldenberg, P. C., DeArmey, S. L., Heller, J., Benjamin, D., Young, S., & Chen, Y. T. (2010). Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Molecular Genetics and Metabolism, 99*(1), 26–33. https://doi.org/S1096-7192(09)00244-3. [pii] 10.1016/j.ymgme.2009.08.003

Kroos, M., Hoogeveen-Westerveld, M., Michelakakis, H., Pomponio, R., Van der Ploeg, A., Halley, D., & Consortium, G. A. A. D. (2012). Update of the pompe disease mutation database with 60 novel GAA sequence variants and additional studies on the functional effect of 34 previously reported variants. *Human Mutation, 33*(8), 1161–1165. https://doi.org/10.1002/humu.22108

Kroos, M., Pomponio, R. J., van Vliet, L., Palmer, R. E., Phipps, M., & Van der Helm, R. GA Database Consortium (2008). Update of the Pompe disease mutation database with 107 sequence variants and a format for severity rating. *Human Mutation, 29*(6), E13–E26. https://doi.org/10.1002/humu.20745

Kroos, M. A., Mullaart, R. A., Van Vliet, L., Pomponio, R. J., Amartino, H., Kolodny, E. H., & Reuser, A. J. (2008). p.[G576S; E689K]: Pathogenic combination or polymorphism in Pompe disease? *European Journal of Human Genetics, 16*(8), 875–879. https://doi.org/10.1038/ejhg.2008.34

Kuperus, E., Kruijshaar, M. E., Wens, S. C. A., de Vries, J. M., Favejee, M. M., van der Meijden, J. C., & van der Beek, N. (2017). Long-term benefit of enzyme replacement therapy in Pompe disease: A 5-year prospective study. *Neurology, 89*(23), 2365–2373. https://doi.org/WNL.0000000000004711. [pii] 10.1212/WNL.0000000000004711

Labrijn-Marks, I., Somers-Bolman, G. M., In 't Groen, S. L. M., Hoogeveen-Westerveld, M., Kroos, M. A., Ala-Mello, S., & Halley, D. J. (2019). Segmental and total uniparental isodisomy (UPiD) as a disease mechanism in autosomal recessive lysosomal disorders: Evidence from SNP arrays. *European Journal of Human Genetics, 27*, 919–927. https://doi.org/10.1038/s41431-019-0348-y

Lim, K. H., Ferraris, L., Filloux, M. E., Raphael, B. J., & Fairbrother, W. G. (2011). Using positional distribution to identify splicing elements and predict pre-mRNA processing defects in human genes. *Proceedings of the National Academy of Sciences of the United States of America, 108*(27), 11093–11098. https://doi.org/10.1073/pnas.1101135108

Mendelsohn, N. J., Messinger, Y. H., Rosenberg, A. S., & Kishnani, P. S. (2009). Elimination of antibodies to recombinant enzyme in Pompe's disease. *New England Journal of Medicine, 360*(2), 194–195. https://doi.org/360/2/194. [pii] 10.1056/NEJMc0806809

Messinger, Y. H., Mendelsohn, N. J., Rhead, W., Dimmock, D., Hershkovitz, E., Champion, M., & Kishnani, P. S. (2012). Successful immune tolerance induction to enzyme replacement therapy in CRIM negative infantile Pompe disease. *Genetics in Medicine, 14*(1), 135–142. https://doi.org/gim20114. [pii] 10.1038/gim.2011.4

Papadopoulos, C., Orlikowski, D., Prigent, H., Lacour, A., Tard, C., Furby, A., & French Pompe Study, G. (2017). Effect of enzyme replacement therapy with alglucosidase alfa (Myozyme(R)) in 12 patients with advanced late-onset Pompe disease. *Molecular Genetics and Metabolism, 122*(1-2), 80–85. https://doi.org/S1096-7192(17)30336-0. [pii] 10.1016/j.ymgme.2017.06.007

van der Ploeg, A. T., Clemens, P. R., Corzo, D., Escolar, D. M., Florence, J., Groeneveld, G. J., & Zivkovic, S. A. (2010). A randomized study of alglucosidase alfa in late-onset Pompe's disease. *New England Journal of Medicine, 362*(15), 1396–1406. https://doi.org/362/15/1396

Poelman, E., Hoogeveen-Westerveld, M., Kroos-de Haan, M. A., van den Hout, J. M. P., Bronsema, K. J., van de Merbel, N. C., & Pijnappel, W. (2018). High sustained antibody titers in patients with classic infantile Pompe disease following immunomodulation at start of enzyme replacement therapy. *Jornal de Pediatria, 195*, 236–243. https://doi.org/10.1016/j.jpeds.2017.11.046

Puzzo, F., Colella, P., Biferi, M. G., Bali, D., Paulk, N. K., Vidal, P., & Mingozzi, F. (2017). Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Science Translational Medicine, 9*(418), eaam6375. https://doi.org/9/418/eaam6375

Regnery, C., Kornblum, C., Hanisch, F., Vielhaber, S., Strigl-Pill, N., Grunert, B., & Schoser, B. (2012). 36 Months observational clinical study of 38 adult Pompe disease patients under alglucosidase alfa enzyme replacement therapy. *Journal of Inherited Metabolic Disease (Dordrecht), 35*(5), 837–845. https://doi.org/10.1007/s10545-012-9451-8

Roig-Zamboni, V., Cobucci-Ponzano, B., Iacono, R., Ferrara, M. C., Germany, S., Bourne, Y. … Sulzenbacher, G. (2017). Structure of human lysosomal acid α-glucosidase-a guide for the treatment of Pompe disease. *Nature Communications, 8*(1), 1111. https://doi.org/10.1038/s41467-017-01263-3.

Schoser, B., Laforet, P., Kruijshaar, M. E., Toscano, A., van Doorn, P. A., van der Ploeg, A. T., & European Pompe, C. (2015). 208th ENMC International Workshop: Formation of a European Network to develop a European data sharing model and treatment guidelines for Pompe disease Naarden, The Netherlands, 26-28 September 2014. *Neuromuscular Disorders: NMD, 25*(8), 674–678. https://doi.org/S0960-8966(15)00131-5

Shigeto, S., Katafuchi, T., Okada, Y., Nakamura, K., Endo, F., Okuyama, T., & Okumiya, T. (2011). Improved assay for differential diagnosis between Pompe disease and acid alpha-glucosidase pseudodeficiency on dried blood spots. *Molecular Genetics and Metabolism, 103*(1), 12–17. https://doi.org/S1096-7192(11)00031-X

Soukarieh, O., Gaildrat, P., Hamieh, M., Drouet, A., Baert-Desurmont, S., Frebourg, T., & Martins, A. (2016). Exonic splicing mutations are more prevalent than currently estimated and can be predicted by using in silico tools. *PLOS Genetics, 12*(1), e1005756. https://doi.org/10.1371/journal.pgen.1005756

Stepien, K. M., Whitby, J., Roberts, M., & Sharma, R. (2015). A 4-year observational clinical study of 24 adult pompe disease patients under alglucosidase alfa enzyme replacement therapy. *Journal of Neuromuscular Diseases, S69*(s1), 69.

Strothotte, S., Strigl-Pill, N., Grunert, B., Kornblum, C., Eger, K., Wessig, C., & Schoser, B. (2010). Enzyme replacement therapy with alglucosidase alfa in 44 patients with late-onset glycogen storage disease type 2: 12-Month results of an observational clinical trial. *Journal of Neurology, 257*(1), 91–97. https://doi.org/10.1007/s00415-009-5275-3

Swallow, D. M., Kroos, M., Van der Ploeg, A. T., Griffiths, B., Islam, I., Marenah, C. B., & Reuser, A. J. (1989). An investigation of the properties and possible clinical significance of the lysosomal alpha-glucosidase GAA*2 allele. *Annals of Human Genetics, 53*(2), 177–184.

van Til, N. P., Stok, M., Aerts Kaya, F. S., de Waard, M. C., Farahbakhshian, E., Visser, T. P., & Wagemaker, G. (2010). Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood*, *115*(26), 5329–5337. https://doi.org/blood-2009-11-252874

de Vries, J. M., van der Beek, N. A., Hop, W. C., Karstens, F. P., Wokke, J. H., de Visser, M., & van der Ploeg, A. T. (2012). Effect of enzyme therapy and prognostic factors in 69 adults with Pompe disease: An open-label single-center study. *Orphanet Journal of Rare Diseases*, *7*(1), 73. https://doi.org/1750-1172-7-73. [pii] 10.1186/1750-1172-7-73

van der Wal, E., Bergsma, A. J., van Gestel, T. J. M., In 't Groen, S. L. M., Zaehres, H., Arauzo-Bravo, M. J., & Pijnappel, W. (2017). GAA Deficiency in Pompe disease is alleviated by exon inclusion in iPSC-derived skeletal muscle cells. *Molecular Therapy. Nucleic Acids*, *7*, 101–115. https://doi.org/S2162-2531(17)30140-3

van der Wal, E., Bergsma, A. J., Pijnenburg, J. M., van der Ploeg, A. T., & Pijnappel, W. (2017). Antisense oligonucleotides promote exon inclusion and correct the common c.-32–13 T > G GAA splicing variant in Pompe disease. *Molecular Therapy. Nucleic Acids*, *7*, 90–100. https://doi.org/S2162-2531(17)30139-7

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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