Novel GAA Variants and Mosaicism in Pompe Disease Identified by Extended Analyses of Patients with an Incomplete DNA Diagnosis

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Pompe disease is a metabolic disorder caused by a deficiency of the glycogen-hydrolyzing lysosomal enzyme acid α-glucosidase (GAA), which leads to progressive muscle wasting. This autosomal-recessive disorder is the result of disease-associated variants located in the GAA gene. In the present study, we performed extended molecular diagnostic analysis to identify novel disease-associated variants in six suspected Pompe patients from four different families for which conventional diagnostic assays were insufficient. Additional assays, such as a generic-splicing assay, minigene analysis, SNP array analysis, and targeted Sanger sequencing, allowed the identification of an exonic deletion, a promoter deletion, and a novel splicing variant located in the 5′ UTR. Furthermore, we describe the diagnostic process for an infantile patient with an atypical phenotype, consisting of left ventricular hypertrophy but no signs of muscle weakness or motor problems. This led to the identification of a genetic mosaicism for a very severe GAA variant caused by a segmental uniparental isodisomy (UPD). With this study, we aim to emphasize the need for additional analyses to detect new disease-associated GAA variants and non-Mendelian genotypes in Pompe disease where conventional DNA diagnostic assays are insufficient.

INTRODUCTION

Pompe disease (glycogen storage disease type II, OMIM 232300) is a rare metabolic disorder that leads to progressive muscle wasting caused by acid α-glucosidase (GAA) enzyme deficiency. This autosomal-recessive disorder is caused by disease-associated variants located in the GAA gene.1 Classic infantile Pompe patients have a complete deficiency of GAA enzymatic activity and present hypertrophic cardiomyopathy and severe muscle weakness shortly after birth. Patients with residual GAA activity can be classified as childhood- or adult-onset Pompe patients, based on the age at symptom onset, and generally do not show cardiac involvement.

Enzyme replacement therapy (ERT) is currently the only available treatment. Without treatment, classic infantile Pompe patients do not survive the first year of life due to cardiorespiratory failure.2,3 In order to become eligible for ERT, a patient needs to present a clinical phenotype related to Pompe disease and have a GAA deficiency, and certain countries require the identification of two disease-associated variants in the GAA gene.4 GAA activity measurements are performed using dried blood with spot sampling or with primary fibroblasts or leukocytes using either glycogen or 4-methylumbelliferone-α-d-glucopyranoside (4MU) as a substrate.5 False-positive results are known to occur using blood-based assays, for example, those caused by known pseudodeficiency alleles; therefore, DNA analysis is recommended in order to identify disease-associated variants.6,7 Routine diagnostic DNA analysis usually focuses only on the coding regions of GAA using PCR reactions and subsequent Sanger sequencing.6,8 This method detects variants in the coding regions and in close proximity to splice sites but not in the promoter, UTRs, and most of the intronic regions.4,9 Reported variants are listed in the “Pompe disease GAA variant database” (http://www.pompevariantdatabase.nl), which contains over 400 disease-associated variants in GAA and has recently been extended to include clinical phenotypes.10 When the disease-associated variants of both alleles are identified using DNA sequencing, a general prediction of the patient phenotype is possible using the information in this database. Recent findings indicate that analysis of the modifier c.510C>T variant, which is silent but modulates splicing in patients carrying the common c.-32-13T>G (IVS1) variants, is also important.11

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Previously, we described an exon-flanking RT-PCR that can be used to detect novel disease-associated variants that affect pre-mRNA splicing, irrespective of their location.10,12 This assay is important for diagnosis but also to allow the design of antisense oligonucleotides that can restore splicing.12,13 Other work has shown single-nucleotide polymorphism (SNP) array analysis to be capable of explaining phenotypes by detecting large deletions or genomic copy number variations (CNVs).16,17 In addition, SNP array analysis can be used to elucidate homozygote variants incompatible with Mendelian inheritance by detecting uniparental isodisomies (UPDs) and regions of homozygosity (RoHs).16,17

Here, we utilized the additional diagnostic assays described above to identify the genetic cause of Pompe disease in six patients from four families with incomplete DNA sequencing results. This resulted in the identification of three novel disease-associated variants located in non-coding regions of GAA. Additionally, we describe the genetic analysis performed for a patient with an atypical Pompe phenotype, first described in Labrijn-Marks et al.17 We provide the clinical symptoms and the series of experiments that were performed in order to make this diagnosis and to strengthen the conclusion that this is a mosaic patient.

RESULTS
The six patients (including three siblings) described here had symptoms that could be attributed to a GAA deficiency. Enzymatic activity of GAA was found deficient in primary fibroblasts and indicated that Pompe disease is a probable cause of symptoms. Genetic screenings of the coding regions of GAA were insufficient to provide a genotype that corresponds with the observed phenotype (Table 1).

Patient 1
The first indication of Pompe disease was observed during prenatal ultrasound, which found evidence of cardiac hypertrophy. Muscle weakness presented shortly after birth, and cultured primary fibroblasts showed no GAA enzyme activity (Table 1). Sanger sequencing of the coding regions identified the heterozygous c.2331+2T>A variant (Table 1; Figure 1A). The intronic c.2331+2T>A variant has been classified as “very severe” and is associated with the classic infantile phenotype when combined with a null allele.10,17 This variant is located close to the exon 16 splice donor site and affects splicing of intron 16, ultimately resulting in a frameshift, the generation of an early stop codon, and mRNA degradation. The second disease-associated variant was not identified by standard DNA sequencing.

Exon-flanking RT-PCR for all GAA exons was performed as previously described9 to test for the presence of a non-coding intronic variant and revealed an aberrant product of exons 8, 9, and 10 (Figure 1B; product 2). Sanger sequence analysis of this product showed the exclusion of exon 9 from the transcripts, but it failed to identify a candidate splicing variant around the splice junctions (Figure 1C). To test the possibility of a deletion that could have been obscured by the presence of the other allele, we performed flanking exon PCR analysis of exon 9 (ranging from exon 8 to exon 10) using genomic DNA as a template (Figure 1D). This showed the presence of an additional band in the patient DNA that was not found in the healthy control, one that corresponded with the expected product ranging from exon 8 to 10 and one that contained a 343-bp deletion that spanned exon 9 and parts of the neighboring introns (band A in Figure 1E).

The resulting c.1327-61_1437+171del variant explains the exclusion of exon 9 at the RNA level and why the initial DNA sequencing analysis was unable to detect this variant, as the annealing sites for PCR amplification were included in the deleted region. The deletion of exon 9 has been shown to result in a total disruption of GAA enzyme activity in previous work.9 The combination of c.1327-61_1437+171del and c.2331+2T>A explains the lack of enzyme activity and the classic infantile phenotype of the patient (Figure 1F).

Patient 2
The second patient presented mild muscle weakness at 15 years of age. GAA activity in fibroblasts was found to be reduced to 13.7 nmol/h/mg (patient range: 0–20 nmol/h/mg). Sanger sequencing analysis performed on the patient’s DNA identified the c.-32-13T>G

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(IVS1) variant in a heterozygous state but failed to identify a second disease-associated variant (Table 1; Figure 2A). Exon-flanking RT-PCR of cDNA, derived from the patient’s fibroblasts, showed no aberrant splicing products, except for those caused by the IVS1 variant around exon 2 (Figure 2B).13,20,21

Next, we performed SNP array analysis to test for the presence of large genomic aberrations. This analysis revealed a deletion on chromosome 17 (GRCh37/hg19 [Chr17:78.059.821–78.076.592]) (Figure 2C). Further evaluation of this 17kb deletion indicated that it starts upstream of GAA in the CCDC40 gene and included the promoter, transcription start site (TSS), and non-coding exons 1A and 1B of GAA.

Formally proving the pathogenicity of this deletion was complicated, due to the residual GAA expression and activity originating...
from the IVS1 allele. To address this, the sequence of exon 3 in the patient’s DNA and mRNA was analyzed for allele-specific SNPs (Figure 2D). We identified the c.596A>G SNP (rs1042393, minor allele frequency [MAF] in Dutch population: 75%) in a heterozygous state in the patient’s DNA. With the use of an IVS1-specific PCR (Figure S1), we found the c.596A>G SNP not to be present on the allele containing the IVS1 variant. Sequence analysis of the cDNA failed to detect transcripts that contained the c.596A>G SNP, indicating that the allele containing the deletion is not expressed.
Patients 3, 4 & 5:

Allele 1

Allele 2

GAA expression after transfection

% of wild-type

Wild-type Mutant Mock

Patients 3, 4 & 5:
In conclusion, the 17kb deletion, including the promoter and TSS, completely abrogates GAA expression, and the combination with the late-onset IVS1 variant on the other allele (Figure 2E) explains the late-onset phenotype presented by the patient.

**Patients 3, 4, and 5**

Patients 3, 4, and 5 are siblings, all of whom showed progressive muscle weakness later in life. Primary fibroblasts were only available for patient 3. GAA activity in lymphocytes was deficient in all three siblings; this was confirmed in the primary fibroblasts of patient 3 (Table 1). Sanger sequence analysis revealed the 2065G>A variant in a homozygous state for all three siblings but was unable to identify any disease-associated variants (Figure 3A). Previous work has shown that the c.2065G>A variant reduces GAA activity to 54% in a GAA cDNA expression construct. This reduction is not sufficient for a patient to present symptoms associated with Pompe disease but is known to lead to a pseudodeficiency of GAA.

Exon-flanking RT-PCR of cDNA derived from fibroblasts of patient 3 did not detect any aberrant splicing events but showed an overall low expression of GAA (Figure 3B). Quantitative real-time PCR (qRT-PCR) analysis confirmed this, showing a reduction of GAA expression to 8% of healthy control values (Figure 3C). SNP array analysis showed several large RoHs in all siblings that were likely derived from regions that were homologous in both parents, suggesting consanguinity between the parents. Interestingly, all three siblings were found to have a RoH ranging from 17q25.3 to 17qter (including GAA) but this was not present in the parental DNA (Figure 3D; Figure S2). The consanguinity of the parents explains the presence of the multiple RoHs in the three siblings via a combination of several identical blocks. However, this finding does not explain the decreased expression of GAA mRNA, and we hypothesized the presence of a variant in the 5' UTR or the promoter. To this end, Sanger sequencing was performed for the non-coding first exons and the GAA promoter (Figure 3E). Sanger sequence analysis of this region revealed the presence of the homozygous variant c.-113+2T>A in patient 3. This variant is located close to the splice acceptor of exon 1A, which is, according to recent annotations, the 5' part of exon 1. Our unpublished RNA sequencing (RNA-seq) data indicate that in agreement with the recent annotations, exon 1 consists of two small exons (exon 1A and exon 1B) that are spliced in cells from healthy individuals, as well as patients with Pompe disease. The c.-113+2T>A variant was also found in a homozygous state in the two siblings (patients 4 and 5) and in a heterozygous state in parental DNA. This variant has not been described previously. DNA from the mother was unavailable.

To investigate the pathogenic nature of c.-113+2T>A, a previously published minigene model was modified to include the genomic region of exon 1A. Two new constructs were generated, one wild-type and one carrying the c.-113+2T>A variant (Figure 3F). qRT-PCR of cDNA obtained from transfected HEK293T cells using primers located in exon 2 showed that c.-113+2T>A reduced GAA expression to 35% of the wild-type minigene (Figure 3G). Exon-flanking PCR for exons 1 to 3 showed that c.-113+2T>A lowered expression of mRNA containing exon 1B-exon 3 (Figure 3H, left panel). An additional exon-flanking RT-PCR was performed to detect aberrant splicing events between exons 1A and 1B (Figure 3H). The wild-type construct spliced both exons correctly, whereas the mutant completely abolished expression of the mRNA transcript containing the exon 1A-exon 1B splice junction (Figure 3H). We note that we were unable to test these splicing events in patient fibroblasts due to the low expression of GAA mentioned previously.

In summary, the c.-113+2T>A variant decreases expression of GAA. We note that the reduction in the minigene was less strong (65%) compared to the reduction in fibroblasts (92%). This difference might be due to promoter-dependent effects of the c.-113+2T>A variant, as in the patient’s fibroblasts, mRNA expression is regulated by the endogenous GAA promoter, whereas expression from the minigene model is regulated by the cytomegalovirus (CMV) promoter. Additionally, all three patients carry both the c.-113+2T>A and c.2065G>A variants at a homozygous state (Figure 3I). The c.2065G>A missense variant is known to decrease activity to ~50%. The combined action of both variants likely reduces endogenous GAA activity to pathogenic levels.

**Patient 6**

Indications for a superior vena cava syndrome were found during a routine prenatal ultrasound. Further cardiac screening was performed at the age of 2.5 months and revealed a left ventricular hypertrophy (LVH) with cardiac ultrasound, whereas electrocardiography showed a shortened PQ interval time without signs of pre-excitation (Table 2). At this time, the patient was normotonic with good psychomotor development and showed no signs of muscle weakness. Exome screening of cardiac genes was negative, and a metabolic examination of plasma and urine showed mildly elevated creatine kinase (CK) and transaminase levels. GAA activity was found to be only slightly reduced to 17% in lymphocytes from patient 6. An accompanying cartoon for the RT-PCRs, the location of the primers are indicated using arrows. (l) Cartoon of the disease-associated variant c.-113+2T>A and the pseudodeficiency variant c.2065G>A, both at a homozygous state in patient 3. Error bars in C and G indicate standard deviation (S.D.)
Table 2. Clinical and Molecular Information Accompanying Patient 6

| Patient Information | |
|---------------------|------------------|
| Age at analysis     | 2.5 months to 4 years |
| Gender              | Male |

| GAA Activity        | Patient Range |
|---------------------|---------------|
| Fibroblasts         | 8.1 nmol/h/mg | 0–20 nmol/h/mg |
| Leukocytes          | 26 nmol/h/mg  | 0–10 nmol/h/mg |

Clinical Description

- Left ventricular hypertrophy detected
- No signs of respiratory insufficiency
- No muscle weakness

Cardiac Muscle Measurements

- Left ventricular mass: 122.1 g (Z score +3.7)
- Shortening fraction: 37.1% (Z score +0.14)
- CK levels: 243 U/L (ref < 230 U/L)

Disease-Associated Variants

| Allele 1             | c.925G>A |
|----------------------|----------|
| Allele 2             | no variant found |

Parental Disease-Associated Variants

| Father (Asymptomatic) | |
|-----------------------|------------------|
| Allele 1              | c.925G>A |
| Allele 2              | no variant found |

| Mother (Asymptomatic) | |
|-----------------------|------------------|
| Allele 1              | no variant found |
| Allele 2              | no variant found |

SNP Array Result

- SNP Array Result

Segmental abnormality range: Chr17q41.5-17qter

decreased in leukocytes, whereas in primary fibroblasts, enzymatic activity was within the range of late-onset Pompe disease (Table 2). Histochemical analysis of a muscle biopsy showed normal morphology, but some enlarged lysosomes were visible using acid phosphatase staining (Figure S3). In addition, periodic acid-Schiff (PAS) staining showed minor glycogen accumulation (Figure S3). These findings suggested that the patient might have late-onset Pompe disease, but there were two inconsistencies: the lack of GAA deficiency in lymphocytes and the LVH, a characteristic of the classic infantile phenotype but not late-onset Pompe disease. Sanger sequencing revealed only the disease-associated variant c.925G>A at a heterozygous state (Figure 4A). This variant is associated with the classic infantile phenotype when combined with a null allele. Sanger sequencing analysis of the parents (both showed no signs of Pompe disease) found the c.925G>A only in a heterozygous state in paternal DNA. No other disease-associated variants were identified in the patient nor in his parents (Table 2). Exon-flanking RT-PCR of cDNA from primary fibroblasts of the patient did not detect any splicing aberrations (Figure 4B). Sequence analysis of the GAA mRNA transcripts showed that the majority of transcripts contained the c.925G>A variant (Figure 4C). This indicated that the second GAA allele was present but was expressed at reduced levels. SNP array analysis identified a 39.5-Mb allelic imbalance, ranging from 17q21.31 to the 17qter (Figure 4D). The imbalance showed no change in CNV but a change in chromosomal equilibrium (i.e., a change in the B allele frequency of heterozygous SNPs) for SNPs located in the affected segment. Within the imbalanced segment, GAA was present, whereas all other genes present within this region were unlikely to be the cause of the presented phenotype (Table S1).

When analyzing the SNP array data, it became clear that the allelic imbalance was more profound in fibroblasts compared to leukocytes. We estimated a deviation in B allele frequency (ΔBAF) of ±25% in fibroblasts compared to ±17% in leukocytes (Figure 4D). This was consistent with the GAA activity, which showed that activity in fibroblasts was lower compared to leukocytes (Table 2).

Additional Sanger sequencing analysis of the patient’s tissues showed a skewed contribution in favor of the c.925A variant in primary fibroblasts, leukocytes, and also in skeletal muscle tissue (Figure 4E). Microsatellite analysis of chromosome 17 showed a skewed composition in favor of the paternal allele in the affected segment, whereas markers located in the unaffected segment were distributed evenly (Figure S4). Fluorescence in situ hybridization (FISH), using probes located on 17p12 and 17q22 to detect both ends of chromosome 17, showed a normal karyotype in the patient’s fibroblasts (Figure 4F). Both ends of the chromosome were present at equal numbers, confirming that this imbalance is not the result of a change in genomic copy number.

The results are in line with a mosaic segmental UPD, in which affected cells contain two copies of the paternal GAA that harbor the c.925G>A variant. The patient’s tissue is comprised of two distinct cell populations: one with the c.925G>A variant in a homozygous state, resulting in a classic infantile Pompe genotype, and one with...
the c.925G>A variant in a heterozygous state, which results in a healthy cell population. To confirm this further, two protein-based assays were performed to examine the biochemical properties of the patient’s cells. Figure 4G shows the results of the glycogen accumulation and GAA activity assays, which were performed on the same cellular extract. Patient fibroblasts accumulated intracellular glycogen, a characteristic that normally only occurs in vitro in fibroblasts derived from classic infantile Pompe patients but not in late-onset patients. However, GAA enzymatic activity (Figure 4G) was present and in the range of late-onset Pompe disease. This is in agreement with a genetic mosaicism with the sample consisting of two cell populations: one that accumulates glycogen in vitro and one that still possesses enzymatic GAA activity and thereby does not accumulate glycogen.

In summary, the atypical phenotype for Pompe disease presented by patient 6 can be explained by a genetic mosaicism for a segment of chromosome 17 that includes GAA. It is likely that in cardiac cells, the contribution of homozygous cells to heart tissue is relatively large, causing a LVH. In skeletal muscle cells, the mixture of homozygous and heterozygous nuclei in muscle fibers likely causes only mild pathology.

**DISCUSSION**

Conventional diagnostic procedures are able to identify and diagnose most individuals with Pompe disease; however, a number of cases remain unexplained. Here, we emphasize the need to introduce new methods in the diagnostic validation of Pompe disease in cases for which conventional procedures prove insufficient. With the use of extended diagnostic analyses, we identified three new variants in the GAA gene. With the use of minor adjustments to already-existing assays, we proved the pathogenicity of the variants and completed the diagnosis for six cases of Pompe disease derived from four families (Table 3).

In previous work, the exon-flanking RT-PCR analysis proved useful to identify novel variants located outside the coding regions, for example, as shown by the identification of c.2190-343A>G. Here, we showed this assay to be capable of detecting novel deletions and revealing differential gene expression as a result of GAA variants. However, the 17kb deletion in patient 2 and thereby other deletions spanning more than one exon remained undetected, and additional assays were required. To detect the deletion of exon 18, which is a common variant in Caucasian patients with Pompe disease, our diagnostic center performs a separate PCR reaction. However, other large deletions are not routinely analyzed and will therefore not be identified. This is supported by the under-representation of gross deletions and other types of variants, such as promoter variants in GAA.

Table 3. Novel Variants Identified with Proof of Pathogenicity

| Disease-Associated Variant on Allele 1 | Disease-Associated Variant/Event on Allele 2 | Proof of Pathogenicity for Novel Variant |
|---------------------------------------|---------------------------------------------|-----------------------------------------|
| Patient 1  c.2331+2T>A                | c.1327-61_1437+171del  | resulting deletion in the transcript is known to cause a deficiency of GAA |
| Patient 2  c.-32:13T>G                | 17 kb deletion, including promoter and transcription start site of GAA  | no RNA expression originating from the allele containing the deletion |
| Patients 3, 4, and 5 c.-113+2T:A       | c.-113+2T:A                           | expression and splicing analysis of variant in minigene model |
| Patient 6  c.925G>A                   | mosaicism c.925G>A                      | SNP arrays of different tissues, Sanger sequencing, glycogen accumulation assay |

*Novel variants.*

*Pseudodeficiency variant.*

Patients with an atypical phenotype often represent a challenge when diagnosing genetic disorders. Patient 6 presented a unique pathological condition: LVH, a characteristic usually observed only in the classic infantile phenotype, while lacking any sign of muscle weakness after 4 years of age. This phenotype was corroborated by the differences in...
GAA activity in the patient’s tissues. With the use of the SNP array analysis, we detected a chromosomal imbalance on chromosome 17, which was crucial to identify the genetic mosaicism of Pompe disease. Genetic mosaicisms are often associated with a milder symptomatology due to the presence of a healthy or less affected cell population.7 32

The mosaicism in patient 6 appears to be the result of a segmental UPD. UPDs have been associated with many inherited disorders and appropriately explain a subset of patients carrying homozygous disease-associated variants.33 Whole-chromosome UPDs are reported to occur in 1/3,500 births,33 whereas segmental UPDs occur less frequently and are the result of somatic recombination.34–36 The mosaic nature of the segmental UPD and the high percentage of affected cells in several of the patient tissues suggest that the recombination event occurred early in the postzygotic stage of embryonal development.34–36 Tissues of this patient were therefore comprised of two cell populations, with the c.925G>A disease-associated variant being present at either a heterozygous (healthy) or homozygous (classic infantile) state.

The presence of a population of healthy cells in the patient did not prevent the development of LVH, which normally only occurs in classic infantile patients. However, at 4 years of age, the patient has not yet manifested any other symptoms related to Pompe disease. The lack of muscle weakness in this patient was reflected by mild pathology in his muscle biopsies. This is likely due to the multinucleation of muscle fibers, whereby the genetic mosaicism in this patient is offset by the presence of both affected and unaffected nuclei in individual muscle fibers, which could be sufficient to prevent muscle pathology. A comprehensive follow-up of this patient, including CNS magnetic resonance imaging (MRI) and neuropsychological investigations, will be required to rule out additional symptoms, such as white matter changes in the brain, which can manifest in classic infantile Pompe patients.37,28

This study shows the need for the implementation of additional diagnostic assays and research as a follow-up when conventional procedures prove insufficient or when a patient’s symptoms or biochemical characteristics do not match to a disorder. For monogenic disorders, such as Pompe disease, more specific assays can be implemented for an improved analysis of a singular gene. The implementation of this follow-up will likely result in the identification of novel variants and provide a complete DNA diagnosis for patients carrying non-coding variants.

MATERIALS AND METHODS

Patients
The patients described here were selected for their clinical and/or biochemical diagnosis that could be attributed to a deficiency of GAA. Informed consents were obtained to perform extended diagnostic assays to identify GAA variants and explain the presented phenotypes. Analysis of the patients analyzed in this study was approved by the medical ethics committee of the Erasmus MC. Primary fibroblasts were not available for patient 4 and 5, assays were performed for patient 3, and findings were thereafter confirmed in DNA from patients 4 and 5.

DNA Analysis
GenBank: NM_001079803.2 and NM_0001079803.2 were used as reference sequences for GAA DNA and mRNA, respectively, where c.1 represents the first nucleotide of the translation start codon ATG. NP000143.2 was used as a reference for GAA protein. We note that in previous annotations, exon 1 comprised 334 nt, whereas in current annotations, this region is divided into two exons (exon 1A and 1B) and a 185-nt intron. All SNP arrays analyses were performed using the Illumina Infinium CytoSNP-850K BeadChip platform. Analysis of genes related to genetic disorders located within specified genomic regions was performed using Genomic Oligoarray and SNP Array Evaluation Tool v3.0.

Histology and Imaging
Acid phosphatase staining was performed on cryosections, as described.39 PAS staining was performed on sections of glycolmethacrylate (GMA)-processed tissue.40 Hematoxylin and eosin (H&E) stainings were performed on both GMA fixed and cryosections. A Hamamatsu NanoZoomer 2.0 (Hamamatsu Photonics) was used for imaging, and images were analyzed with NDP.view software.

RNA Isolation and cDNA Synthesis
Total RNA was isolated from cultured fibroblasts using the RNeasy minikit (QIAGEN), including a DNaseI (QIAGEN) treatment. RTPCR was performed using the iScript cDNA synthesis kit (Bio-Rad) in reactions with 300–800 ng RNA input.

PCR and Sanger Sequencing
RT-PCR and qRT-PCR were performed as described.9 Sanger sequencing of PCR products was performed using a ABI3730XL DNA analyzer (Thermo Fisher Scientific) and analyzed using ApE software.

Generation of the Minigene Constructs and Transfection
The minigene model described in Bergsma et al.12 was modified as follows: with the use of a primer with a XhoI overhang and a SBF1 restriction site, the exon 1a region was amplified and cloned in the construct. We used the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) to introduce the c.-113+2T>A variant. Transfection of the minigene constructs was performed in HEK293T cells using Lipofectamine 2000 (Invitrogen). RNA was harvested 48 h after transfection.

Enzyme Activity and Glycogen Assay
GAA activity was measured in leukocytes or fibroblasts using 4MU, as described.9 All 4MU assays were performed in diagnostic settings, with the exception of Figure 4G. For this experiment, fibroblasts had been confluent for 3 weeks and starved in glucose-free medium, 24 h before harvest, after which, GAA activity was measured using the
4MU. Intracellular glycogen was measured with a two-step protocol using amylase and glucose-oxidase. 

Fragment Analysis of Microsatellite Markers
15 probes were selected for their location on chromosome 17. Analysis of the VIC- or FAM-labeled products was performed using an ABI3730XL DNA analyzer (Thermo Fisher Scientific).

Fluorescence In Situ Hybridization Analysis
FISH was performed on cultured fibroblasts at a low passage number. Two probes located on 17p12 and 17q22 were used to visualize both ends of the chromosome. The cells were not confluent when fixed and classified as having a normal karyotype when two or four copies of each probe were visible.

Statistics
Data were analyzed using IBM SPSS statistics, version 26. For all experiments, normal distribution of data was determined based on calculated residuals. Significance between normally distributed data from two groups was tested using an unpaired two-tailed t test. For experiments with three or more groups, a one-way ANOVA of independent samples with Tukey honestly significant difference (HSD) or Games-Howell post hoc multiple correction (depending on homogeneity of the variance) was performed. Non-normal distributed data were statistically tested using the Kruskal-Wallis method of independent samples with Bonferroni multiple comparison for three or more groups. A p value of less than 0.05 was considered significant for all tests.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conceptualization, S.L.M.i.G., A.T.v.d.P., A.J.B., and W.W.M.P.P.; Methodology, S.L.M.i.G., D.O.S.d.F., and A.J.B.; Validation Verification, M.-A.B.R., and P.W.; Formal Analysis, S.L.M.i.G., A.I., and J.J.S.; Investigation, S.L.M.i.G., D.O.S.d.F., H.D., T.D., and G.M.S.-B.; Resources, T.D., P.W., and M.-A.B.R.; Data Curation, H.D., T.D., and G.M.S.-B.; Writing – Original Draft, S.L.M.i.G. and A.I.; Writing – Review & Editing, all authors; Supervision Project Administration, A.J.B., J.M.P.v.d.H., L.H.H., and W.W.M.P.P.; Funding Acquisition, A.T.v.d.P. and W.W.M.P.P.

CONFLICTS OF INTEREST
A.T.v.d.P. 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. All other authors declare no competing interests.

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