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

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INTRODUCTION

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.

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. 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.7 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.3,5,7 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. This assay is important for diagnosis but also to allow the design of antisense oligonucleotides that can restore splicing. 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). 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).

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. 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.

<p>| Table 1. Basic Information for All Patients at Start of Analysis |
|-----------------|-----------------|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Age at Symptom Onset</th>
<th>GAA Activity in Fibroblasts (Patient Range: 0–20 nmol/h/mg)</th>
<th>Disease-Associated Variant 1</th>
<th>Disease-Associated Variant 2</th>
<th>Initial Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>&lt;1 month</td>
<td>0 nmol/h/mg</td>
<td>c.2331+2T&gt;A</td>
<td>?</td>
</tr>
<tr>
<td>Patient 2</td>
<td>15 years</td>
<td>13.7 nmol/h/mg</td>
<td>c.32-13T&gt;G</td>
<td>?</td>
</tr>
<tr>
<td>Patient 3 (sibling 1)</td>
<td>24 years</td>
<td>7.5 nmol/h/mg</td>
<td>? + c.2065G&gt;A</td>
<td>? + p.(Glu689Lys)</td>
</tr>
<tr>
<td>Patient 4 (sibling 2)</td>
<td>41 years</td>
<td>deficient in lymphocytes</td>
<td>? + c.2065G&gt;A</td>
<td>? + p.(Glu689Lys)</td>
</tr>
<tr>
<td>Patient 5 (sibling 3)</td>
<td>21 years</td>
<td>deficient in lymphocytes</td>
<td>? + c.2065G&gt;A</td>
<td>? + p.(Glu689Lys)</td>
</tr>
<tr>
<td>Patient 6</td>
<td>&lt;1 month</td>
<td>8.1 nmol/h/mg</td>
<td>c.925G&gt;A</td>
<td>?</td>
</tr>
</tbody>
</table>

The resulting c.1327-61_1437+171del variant explains 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 second disease-associated variant was not identified by standard DNA sequencing. 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.

Exon-flanking RT-PCR for all GAA exons was performed as previously described 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).
(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
- c.2065G>A

### Allele 2
- c.2065G>A

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**Patients 3, 4 & 5:**

### Allele 1
- c.2065G>A

### Allele 2
- c.2065G>A

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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 paternal 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 paternal 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
Patient 6: c.925G>A

Allele 1

Allele 2

ΔBAF 25%

ΔBAF 17%

GAA activity (% of control)

Glycogen accumulation (μg/mg protein)

(legend on next page)
Table 2. Clinical and Molecular Information Accompanying Patient 6

<table>
<thead>
<tr>
<th>Patient Information</th>
<th>GAA Activity</th>
<th>Patient Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at analysis</td>
<td>2.5 months to 4 years</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>male</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>8.1 nmol/h/mg</td>
<td>0–20 nmol/h/mg</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>26 nmol/h/mg</td>
<td>0–10 nmol/h/mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease-Associated Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 1</td>
</tr>
<tr>
<td>Allele 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parental Disease-Associated Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father (Asymptomatic)</td>
</tr>
<tr>
<td>Allele 1</td>
</tr>
<tr>
<td>Allele 2</td>
</tr>
<tr>
<td>Mother (Asymptomatic)</td>
</tr>
<tr>
<td>Allele 1</td>
</tr>
<tr>
<td>Allele 2</td>
</tr>
<tr>
<td>SNP Array Result</td>
</tr>
</tbody>
</table>

| Segmental abnormality range: Chr17:41,681,527–Chr17qter | 25.2 Mb |

| Left ventricular hypertrophy detected |  |
| No signs of respiratory insufficiency |  |
| 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 |

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.  

Additional Sanger sequence 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 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.
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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease-Associated Variant on Allele 1</th>
<th>Disease-Associated Variant/Event on Allele 2</th>
<th>Proof of Pathogenicity for Novel Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>c.2331+2T&gt;A</td>
<td>c.1327-61_1437+171del</td>
<td>resulting deletion in the transcript is known to cause a deficiency of GAA</td>
</tr>
<tr>
<td>Patient 2</td>
<td>c.-32-13T&gt;G</td>
<td>17 kb deletion, including promoter and transcription start site of GAA</td>
<td>no RNA expression originating from the allele containing the deletion</td>
</tr>
<tr>
<td>Patients 3, 4, and 5</td>
<td>c.-113+2T&gt;A  + c.2065G&gt;A</td>
<td>c.-113+2T&gt;A    + c.2065G&gt;A</td>
<td>expression and splicing analysis of variant in minigene model</td>
</tr>
<tr>
<td>Patient 6</td>
<td>c.925G&gt;A</td>
<td>mosaicism c.925G&gt;A</td>
<td>SNP arrays of different tissues, Sanger sequencing, glycogen accumulation assay</td>
</tr>
</tbody>
</table>

*Novel variants.*

*Pseudodeficiency variant.*

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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 mosaicsisms are often associated with a milder symptomatology due to the presence of a healthy or less affected cell population.32-34

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,35 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 thereafter 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.37,38

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. RT-PCR 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 independence of the variance. This work was funded through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brazil), the Sophia Foundation for Medical Research (SSWO; project number s17-32), and Metakids (project number 2016-063). This project has received funding from the Ministry of Economic Affairs under TKI allowance under the TKI-Programme Life Sciences & Health. The collaboration project is co-funded by the PPP Allowance made available by Health ~ Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships (project numbers LSHM16008 and LSHM19015). The collaboration project is co-initiated by the Prinses Beatrix Spierfonds.

**REFERENCES**


**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.12.016.

**AUTHOR CONTRIBUTIONS**


**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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