Pseudo deficiency of acid α-glucosidase: a challenge in the newborn screening for Pompe diseases

Diana Rojas Málaga[1,2], Ana C. Brusius-Facchin[2], Kristiane Michelin-Tirelli[2], Têmis M. Félix[2], Jaqueline Schulte[3], Jamile Pereira[3], Eurico Camargo Neto[3], Claudio Sampaio Filho[4], Roberto Giugliani[1,2,5].

[1]Postgraduate Program in Genetics and Molecular Biology, UFRGS, Porto Alegre, Brazil

[2]Medical Genetics Service, Hospital de Clinicas de Porto Alegre, Brazil

[3]Newborn Screening Center, Porto Alegre, Brazil

[4]Intercientifica, São José dos Campos, Brazil

[5]Department of Genetics, UFRGS, Porto Alegre, Brazil

Corresponding author: Roberto Giugliani

E-mail: rgiugliani@hcpa.edu.br

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ABSTRACT. When a low activity of acid α-glucosidase (GAA) is found, particularly in newborn screening programs, to differentiate α-glucosidase pseudo deficiency from true Pompe disease is important and urgent, as the result generates parental stress and also because this differentiation drives decisions related to the management of the case. Here, we report a case of GAA pseudo deficiency detected in a newborn screening performed by a private laboratory in Brazil. The confirmatory laboratory investigation performed at our service showed reduction of GAA activity on the dried blood spot, with inconclusive results when GAA activity was assayed in leukocytes. Genotyping of the GAA gene with next-generation sequencing revealed the common pathogenic mutation c.-32-13T>G and the "pseudo deficiency allele" p.[Gly576Ser; Glu689Lys], each one in heterozygous state and in trans. This report illustrates the need of newborn screening programs to have the adequate support to perform a comprehensive investigation whenever an abnormality is found in the initial screening test.

KEY WORDS: alfa-glucosidase, pseudo deficiency,

Pompe disease, newborn screening, lysosomal diseases

INTRODUCTION

Pompe disease (PD, OMIM 232300) is an autosomal recessive lysosomal disorder (LD) caused by mutations in the *GAA* gene (17q) that encodes the lysosomal enzyme α-glucosidase (GAA, EC 3.2.1.20). GAA is responsible for glycogen degradation within lysosomes and its deficiency leads to accumulation of lysosomal glycogen especially in cardiac and skeletal muscle (Hirschhorn and Reuser, 2001). To date, over 550 distinct variations in *GAA* have been identified, although not all are considered pathogenic (www.pompecenter.nl). A specific enzyme replacement therapy was developed for Pompe disease, and is already approved in many countries (Chien and Hwu, 2007).

There is a worldwide interest in newborn screening (NBS) for LDs that, like PD, are amenable by enzyme replacement therapy as increasing evidence shows that early treatment intervention results in better outcomes (Chien and Hwu, 2007, 2013). However, some factors affect the detection of PD, including the presence of carriers and pseudo deficiency. The so-called pseudo deficiency allele p.[Gly576Ser; Glu869Lys], or c.[1726A; 2065A], causes, in homozygous state, reduction of GAA activity, which could be as low as the observed in patients affected by PD, but does not lead to the development of the disease (Tajima *et al*., 2002, Labrousse *et al*., 2010). We report here a case that illustrates the challenge of adequately classifying the patient with GAA deficiency as Pompe disease or pseudo deficiency, as this has a very important impact in the decision about the therapeutic measures to be taken.

PATIENT AND METHODS

Case report

The male patient is the first child of a young and non-consanguineous couple. His father has diagnosis of type I Diabetes Mellitus. He was born by C-section after an uneventful pregnancy. His birth weight was 3175g, length 50 cm, OFC 33 cm and Apgar score 9 at first minute. At 4 days old, a routine newborn screening test was performed at a private laboratory and showed GAA activity below the lower reference range (patient: 4.4 µmol/L/hour; reference value: >5.9). The patient was referred to the Medical Genetics Service of HCPA for further investigation as described below. He evolved with normal motor development, held his head at 4 months old and walked unsupported at 11 months old.

Biochemical studies

GAA activity in the DBS was performed using a digital microfluidics platform for multiplexing enzyme assays (Neto et al, 2017). GAA activity in leukocytes was assayed with the substrate 4-methylumbelliferyl-α-D-glucoside, as previously reported (Li *et al*., 2004). GAA activity of parents' patient was assayed as well. For the assays in leukocytes, β-galactosidase was assayed as reference enzyme (Suzuki, 1977).

Molecular Genetic studies

Peripheral blood sample was collected in tubes containing EDTA. DNA was obtained from blood samples by standard procedures (Millet et al., 1988). All coding exons (exons 2 through 20) and as well as the flanking intron/exon junctions (20pb) of the *GAA* gene were sequenced using next-generation sequencing method on Ion Torrent PGM platform employing a prior validated NGS panel that includes the *GAA* gene. The results were visualized in the Integrative Genomics Viewer (IGV) v2.3 (Broad Institute) and Ion Reporter Software v5.0 (Thermo Fisher Scientific). Parental segregation of the mutations was also determined by Sanger sequencing, using primers developed by Oba-Shinjo et al. (2009).

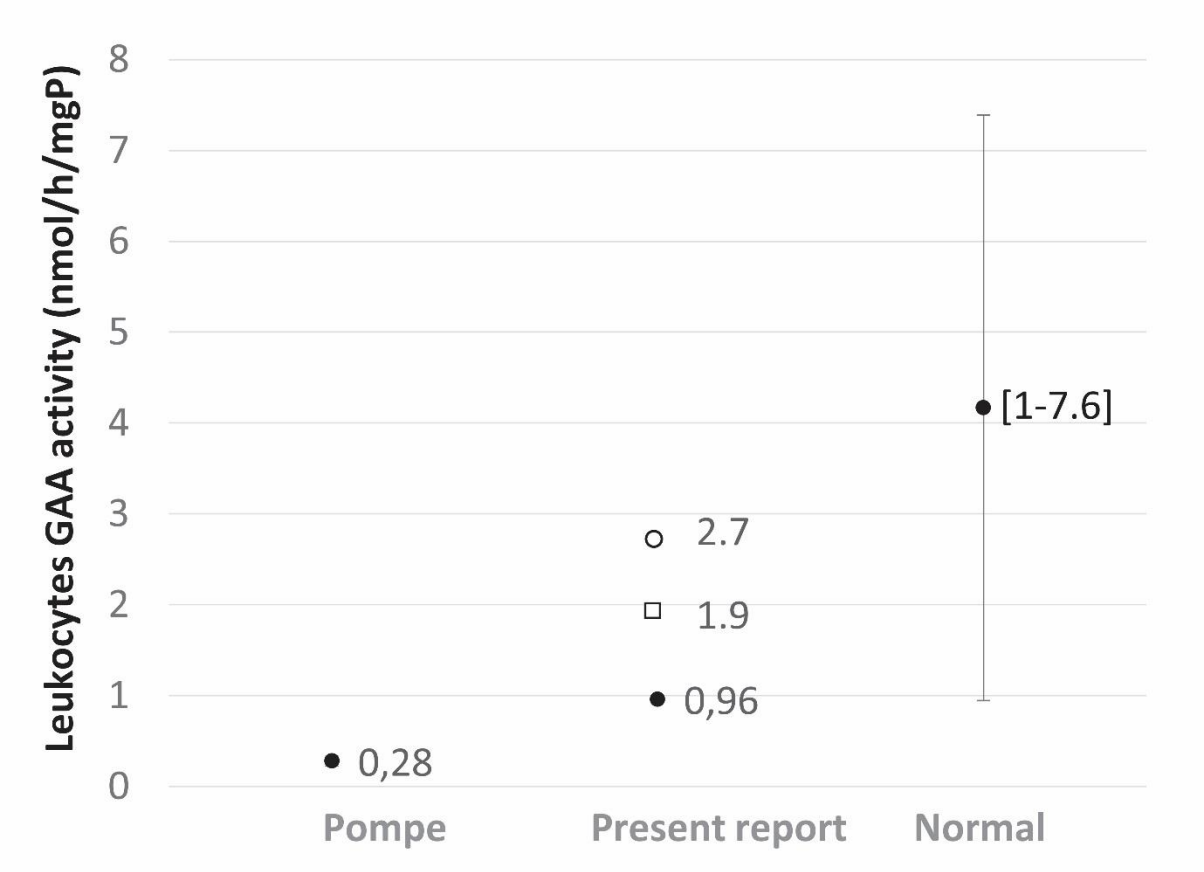
RESULTS

Enzyme analysis

GAA activity in the patient's leukocytes was 0.96 ± 0.03 nmol/h/mg protein, very close to the lower reference limit [reference range: 1.0-7.6] (Figure 1). GAA activities of the parents' leukocytes were within normal range

(mother: 2.7; father: 1.9). Activity of β-galactosidase in leukocytes, assayed as a reference enzyme, was within normal range in all cases, confirming that the samples were adequate.

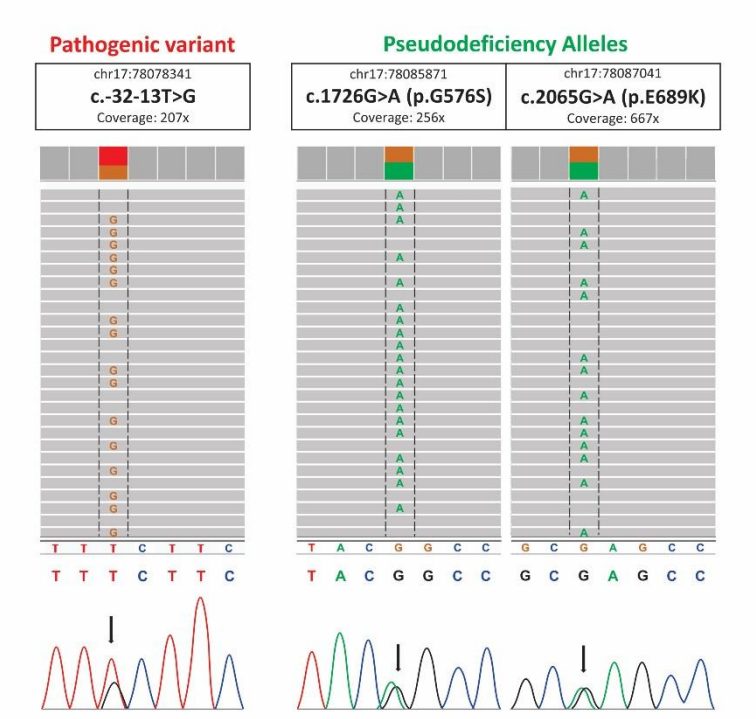
Figure 1. GAA activity in leukocytes. Pompe: results in Pompe patients previously diagnosed; Present report: ○, mother; □ father; ● patient; Normal: Normal reference range.



Molecular genetic analysis

After sequencing the entire coding sequence and splice sites of the *GAA* gene, we identified 3 heterozygous variants when compared with reference sequence NM_001079804.1: the mutation c.-32-13T>G (rs386834236) in the intron 1 and the missense mutations p.Gly576Ser (c.1726G>A) (rs1800307) and p.Glu689Lys (c.2065G>A) (rs1800309) in exon 12 e 15, respectively (Figure 2). These mutations were also confirmed by Sanger sequencing. Parental analysis of the mutations determined that variant c.-32-13T>G was inherited from the father and c. [1726A; 2065A] (or p.Gly576Ser; Glu689Lys]) from the mother, both being heterozygous for the respective mutations.

Figure 2. Identification of mutations by NGS panel, as visualized in Integrative Genomic Viewer (top panel) and confirmed by Sanger sequencing (bottom panel). A, c.-32-13T>G. B, c.1726G>A (p.G576S). C, c.2065G>A (p.E689K) in *GAA*.



These three variants are well-described in the literature as pathogenic or causative of pseudo deficiency. Nonetheless, we present in Table 1 the *in-silico* analyses results using SIFT, Polyphen-2, CADD, Mutation Taster, and Human Splicing Finder v.3 as pathogenicity prediction tools (Kumar *et al*., 2009, Adzhubei *et al*., 2010, Schwarz *et al.*, 2014, Desmet *et al*., 2009).

Table 1. *In silico* analysis of *GAA* three variants found in our patient, using different

Variant	Clinvar	*In silico* Pathogenicity Prediction Tools				
		SIFT	Polyphen-2	CADD	Mutation Taster	HSF
c.-32-13T>G	Pathogenic	N.A	N.A	N.A	Disease causing	Modifier
p.Gly576Ser	Bening/ Likely bening	79%	81%	82%	Polymorphism	N.A
p.Glu689Lys	Bening/ Likely Bening/Pathogenic	82%	73%	65%	Polymorphism	N.A

Note: N.A: not applicable, HSF: Human Splicing Finder.

DISCUSSION

Differentiation between Pompe disease and GAA pseudo deficiency is crucial for reaching a correct diagnosis and taking a decision about the introduction of enzyme replacement therapy, available in many countries, including Brazil. Although it is present in less than 1% of babies in the United States, frequency of pseudo deficiency allele can by quite high in some populations (3.9% in Japan and 3.3% in Taiwan) (Kemper, 2013, Labrousse et al., 2010, Kumamoto et al., 2009). Data on pseudo deficiency frequency for the Brazilian population are not available. Positive results in NBS require second-tier confirmation to address false-positive results. Biochemical assay of the enzyme activity in leukocytes provides additional data, but molecular analysis of the *GAA* gene was needed to discriminate between PD and pseudo deficiency.

We used a prior validated NGS panel (analytical sensitivity of 100%) that includes *GAA* and other genes associated with different lysosomal disorders (LDs). NGS panels proved to be a valid method to genotype samples of suspected patients, in addition to being rapid, accurate and cost effective. The *GAA* intronic variant detected in our patient (c.-32-13T>G) is the most common variant causing late-onset PD (~40-70% of the alleles), and it is

well-described as pathogenic (Huie *et al*., 1994a, Boerkoel *et al*., 1995). This variant causes three aberrant spliced transcripts: the partial or complete exon 2 skipping and a leaky wild-type splicing, which allows a low level of GAA activity, preventing the most severe classic infantile form of PD and leading to a childhood/adult phenotype (Boerkoel *et al*., 1995, Dardis et al., 2014, Bergsma *et al*., 2015). Even though variants p.Gly576Ser and p.Glu689Lys, when analyzed independently, presented inconsistent results after *in silico* analysis for predicting pathogenicity as well as conflicting interpretation of pathogenicity at Clinvar, it has been reported that p.Gly576Ser reduces the GAA activity by about ~80%, while p.Glu689Lys results in 50% of normal GAA activity. The combined effect of both variants, p.[Gly576Ser, Glu689Lys] is like the effect of p.Gly576Ser alone (Kroos *et al*., 2008).

Our results were consistent with previous findings in NBS programs, indicating that newborn with mutation heterozygosity, pseudo deficiency homozygosity without *GAA* mutations and pseudo deficiency heterozygosity with and without a *GAA* mutation, present GAA activity significant lower than in controls, but could be distinguished from patients with Pompe disease, with few exceptions (Labrousse et al., 2010, Scott et al., 2013). Heterozygotes for the pseudo deficiency allele (like patient´s mother) are not at risk for developing PD. However, it has been hypothesized that in some cases the pseudo deficiency allele could modify the effect of another mutation (i.e. p.D645E, p.W746C, p.W746X) (Labrousse et al., 2010, Kroos et al., 2008). It has also been suggested that compound heterozygotes with one p.[Gly576Ser; Glu689Lys] allele and one pathogenic allele may develop a “Pompe-like” disease symptoms late in life due to the very low GAA activity, being a medical follow-up recommended (Kroos *et al*., 2008).

Our finding, and the fact that an individual homozygous for the pseudo deficiency allele was already reported in Brazil (Turaça et al., 2015) suggests that this allele is present in our population. This report of GAA pseudo deficiency detected by newborn screening illustrates and reinforces the need that such programs have a comprehensive protocol including further biochemical and genetic analysis, to provide a final diagnosis to the cases who had a positive result in the initial screening test. This process should be fast and efficient, as it was in the present case, in order to avoid parental stress in the false-positive cases and to enable the prompt start of therapy in confirmed cases.

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