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Investigation of newborns with abnormal results in a newborn screening program for four lysosomal storage diseases in Brazil

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ABSTRACT

Lysosomal storage diseases (LSDs) are genetic disorders, clinically heterogeneous, mainly caused by defects in genes encoding lysosomal enzymes that degrade macromolecules. Several LSDs already have specific therapies that may improve clinical outcomes, especially if introduced early in life. With this aim, screening methods have been established and newborn screening (NBS) for some LSDs has been developed. Such programs should include additional procedures for the confirmation (or not) of the cases that had an abnormal result in the initial screening. We present here the methods and results of the additional investigation performed in four babies with positive initial screening results in a program of NBS for LSDs performed by a private laboratory in over 10,000 newborns in Brazil. The suspicion in these cases was of Mucopolysaccharidosis I - MPS I (in two babies), Pompe disease and Gaucher disease (one baby each). One case of pseudodeficiency for MPS I, 1 carrier for MPS I, 1 case of pseudodeficiency for Pompe disease and 1 carrier for Gaucher disease were identified. This report illustrates the challenges that may be encountered by NBS programs for LSDs, and the need of a comprehensive protocol for the rapid and precise investigation of the babies who have an abnormal screening result.

1. Introduction

Lysosomal storage diseases (LSDs) are genetic disorders with an estimated overall prevalence of 1 in 7,700 live births [1]. They are mainly caused by monogenic defects in genes encoding lysosomal enzymes that degrade macromolecules such as glycolipids, glycoproteins and mucopolysaccharides. These defects produce an abnormal and progressive lysosomal accumulation of specific substrates, leading to structural changes and deterioration of the cellular function. LSDs are clinically heterogeneous, being usually undetectable at birth, and characterized by progressive manifestations that may include different organs and systems in the body [2]. Treatment for LSDs, already available for several of them, consists of enzyme replacement, transplantation of hematopoietic stem cells, substrate synthesis inhibition, pharmacological chaperones and some other strategies [2,3]. The specific treatment, when introduced early, may prevent irreversible pathological changes or significantly minimize disease manifestations [4,5].

These facts have motivated the development of screening methods to be used in large scale, enabling strategies such as newborn screening (NBS). Once NBS programs for LSDs are established, additional procedures for confirmatory diagnosis should be available as a mandatory part of these programs, to rule out false positives and to enable the prompt start of therapy whenever indicated in true positive cases.

Recently, NBS for LSDs was introduced by a newborn screening laboratory, the CTN (*Centro de Triagem Neonatal*), based in Porto Alegre, Brazil. The program was a pilot project to evaluate the use of a digital microfluidic (DMF) platform to measure simultaneously the activities of

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α-L-iduronidase (IDUA), acid α-glucosidase (GAA), acid β-glucosidase (GBA) and α-galactosidase (GLA) to screen for MPS I, Pompe disease, Gaucher disease and Fabry disease, respectively [Neto EC, personal communication]. The procedures for the first-tier screening were performed as described previously by Sista et al. [6,7], and are already being used in newborn screening programs for LSDs [8]. Cut off values were estimated as the activity 30% below the mean enzyme activity obtained with the analysis of DBS samples from 1,000 unaffected babies samples. These cutoffs were validated with the blind analysis of samples obtained from previously confirmed cases of MPS I, Gaucher, Fabry and Pompe diseases [Neto EC, personal communication].

Here, we present the results of the additional investigation performed in the cases that presented initial abnormal results in the above screening program. This investigation was based on biochemical and molecular genetics approaches. We also discuss the challenges encountered in the interpretation of these results.

2. Materials and methods

2.1. Samples

The cases with initial abnormal results in the program of NBS for LSDs were referred from the NBS laboratory (CTN) to the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (HCPA). Both institutions are located in Porto Alegre, Rio Grande do Sul State, Brazil.

Dried blood spots (DBS), whole blood and urine samples were collected from the cases that had abnormal results in the initial screening for one of the four LSDs tested, for further investigation at the reference center. Blood samples were also collected from the parents in three of the cases for related analyses.

The biochemical and genetic investigations were performed at the Laboratory of Inborn Errors of Metabolism and at the Laboratory of Molecular Genetics, respectively, of the Medical Genetics Service (SGM) of HCPA. SGM/HCPA is a reference center for rare diseases in Brazil, and a WHO Collaborating Center for the Development of Medical Genetic Services in Latin America since 2004 [9].

2.2. Enzyme activity analyses

Enzyme activities of α-L-iduronidase (IDUA; EC 3.2.1.76), acid α-glucosidase (GAA; EC 3.2.1.20) and acid β-glucosidase (GBA; EC 3.2.1.45) were measured in leukocytes by fluorometric assays following procedures previously described [10–12]. Likewise, enzyme activities in DBS and plasma were measured by fluorometric assays in accordance with previous reports [10,13].

Chitotriosidase was measured in plasma by a fluorometric assay as reported previously [14].

2.3. Urinary glycosaminoglycans (GAGs) analysis

Urinary GAGs were analyzed by standard quantitative and qualitative methods, the dimethylmethylene blue (DBM) colorimetric assay and the monodimensional electrophoresis, respectively [15–17].

2.4. Gene analysis

2.4.1. Analysis of IDUA gene (OMIM *252800) for MPS I

Genomic DNA was isolated from peripheral blood sample in EDTA for case 1 and from blood impregnated in filter paper for case 4. The 14 exons and flanking regions of the *IDUA* gene were amplified by PCR and subsequently sequenced [18]. Identified variants were interpreted based on information found in the Human Gene Mutation Database (HGMD), dbSNP, ClinVar, ExAc and literature review [19–22]. New variants were analyzed *in silico* to predict pathogenicity using softwares such as Poly-Phen2 and SIFT [23,24].

2.4.2. Analysis of GAA gene (OMIM *606800) for Pompe disease

Genomic DNA was isolated from peripheral blood cells samples and used for sequencing in the Ion Torrent Personal Genome Machine (Thermo Scientific™), using a customized panel (Ion AmpliSeq™ Thermo Scientific™) that included the *GAA* gene. Analysis of data used the platform of bioinformatics from Ion Torrent Suite and Ion Reporter (Thermo Scientific™) version 5.0. All procedures were performed in accordance of the manufacturer's recommendations.

Sanger sequencing using ABI 3500 Genetic Analyzer (Applied Biosystems) was also used for the analysis of intron 1, exon 12 and 15 of *GAA* gene of proband's parents, as previously described [25]. Identified variants were interpreted using different databases such as Human Gene Mutation Database (HGMD), dbSNP, ClinVar, ExAc, Pompe Disease Mutation Database (Erasmus MC: Pompe Center), and literature review [19–22,26].

2.4.3. Analysis of GBA gene (OMIM *606463) for Gaucher disease

Genomic DNA was isolated from peripheral blood samples and then sequenced in the Ion Torrent Personal Genome Machine (Thermo Scientific™), using a customized panel (Ion AmpliSeq™ Thermo Scientific™) that included the *GBA* gene. Then, data were analyzed at the platform of bioinformatics from Ion Torrent Suite and Ion Reporter (Thermo Scientific™) version 5.0. All the above procedures followed the manufacturer's recommendations. Analysis was complemented by Sanger sequencing of exon 10 of the *GBA* gene to evaluate the presence of a pseudodeficiency allele [27]. Identified variants were interpreted using different databases such as Human Gene Mutation Database (HGMD), dbSNP, ClinVar, ExAc and literature review [19–22].

3. Results

Four cases, that screened positive among the first 10,567 babies tested in the program of NBS for LSDs, were further investigated. Data of the analyses performed for diagnostic confirmation and the results observed for each case are shown in Table 1. Description of each case is presented below.

3.1. Case 1: suspicion of MPS I

A female baby was referred for further investigation, after resulting positive for a NBS for MPS I, which revealed a low IDUA activity (0.8 μmol/L/h; cut off: > 5.0) measured on DBS.

Urinary GAGs were analyzed and showed a normal GAGs quantitation for the age and a normal GAGs pattern at the qualitative analysis. IDUA activity was measured in DBS, plasma and leukocytes samples. IDUA activity was reported as undetectable in DBS. Measurement in plasma showed a normal enzyme activity and the analysis in leukocytes revealed an IDUA activity below the normal range (11 nmol/h/mg protein, with normal reference range from 27 to 171).

After considering all the biochemical results, it was not possible to reach a conclusion about the MPS I diagnosis. Therefore, molecular analysis of the *IDUA* gene was performed, with the identification of the variant c.251G > C [p.(Gly84Ala)] and the variant NM_000203.4(IDUA):c.246C > G (p.His82Gln). The variant p.(Gly84Ala) was a recently reported variant, predicted as possibly pathogenic by *in silico* analysis and located at the same codon where two pathogenic variants were already described [18]. The variant p.His82Gln was previously described as benign and possibly leading to pseudodeficiency, resulting to low *in vitro* enzyme activity in normal subjects [28–30].

Thus, putting together the results of normal urinary GAGs, low IDUA activity in leukocytes (but higher than that usually observed in affected cases for MPS I) and a genotype with a possibly pathogenic variant and a variant associated with pseudodeficiency, the conclusion was that the baby presented pseudodeficiency for MPS I.

Table 1
Confirmatory investigation of cases screened positive in a program of NBS for LSDs in Brazil.

	Case 1	Case 2	Case 3	Case 4
	MPS I?	Pompe?	Gaucher?	MPS I?
Enzyme analysis	IDUA	GAA	GBA	IDUA
DBS-fluorometry	Undetectable	NP	2.8 nmol/h/mL (2.2–17)	NP
Plasma-fluorometry	11 nmol/h/mL (6.6–34)	NP	NP	NP
Leukocytes-fluorometry	11 nmol/h/mg protein (27–171)	1.00 nmol/h/mg protein (1.00–7.60) Father: 1.9 Mother: 2.70	5.6 nmol/h/mg protein (10–45) Father: 8.1 Mother: 22.0	27 nmol/h/mg protein (27–171)
Urinary GAGs				
Quantitation (DMB - colorimetry)	197 μg/mg creatinine (133–460)	NP	NP	272 μg/mg creatinine (133–460)
Electrophoresis (qualitative)	Normal GAG pattern	NP	NP	Normal GAG pattern
Gene analysis	*IDUA*	*GAA*	*GBA*	*IDUA*
Mutation 1	c.251G > C	c.-32-13T > G	c.1226A > G	c.1205G > A
Effect	p.(Gly84Ala)	Splice site variant	p.Asn409Ser (N370S)	p.Trp402Ter
Significance	Predicted pathogenic	Pathogenic variant	Pathogenic variant	Pathogenic variant
Mutation 2	c.246C > G	c.[1726G > A; 2065G > A]	No pathogenic variant identified	No pathogenic variant identified
Effect	p.His82Gln	p.[Gly576Ser; Glu689Lys]		
Significance	Pseudodeficiency allele	Pseudodeficiency allele		
		Father: c.-32-13T > G		Father: c.1205G > A
		Mother: p.[Gly576Ser; Glu689Lys]		Mother: No pathogenic variant

Numbers in parenthesis, in enzyme analysis and urinary GAGs, are reference values. IDUA: α-L iduronidase; GAA: acid α-glucosidase; GBA: acid β-glucosidase; MPS I: mucopolysaccharidosis type 1. DBS: dried blood spot; GAGs: glycosaminoglycans. NP: not performed.

3.2. Case 2: suspicion of Pompe disease

A male baby, clinically normal, was referred for further investigation after presenting a low GAA activity (4.3 μmol/L/h; cut off: > 10) in a NBS for Pompe disease.

For confirmatory diagnosis, GAA activity was measured in leukocytes and resulted in slightly low (0.94 nmol/h/mg protein, with normal reference range from 1.00 to 7.60) in an initial measurement and at the lower limit of the reference range (1.0 nmol/h/mg protein) when the analysis was repeated.

Given the slightly low enzyme activity (although higher than that usually observed in patients with Pompe disease), a conclusion about the tentative Pompe diagnosis was not possible. Then, *GAA* gene sequencing was performed to elucidate the case. It was detected a known pathogenic variant in heterozygosis, the NM_000152.4(GAA):c.-32-13T > G in one chromosome, and in the other chromosome a previously reported pseudodeficiency allele [31,32] that consists of two variants, the NM_000152.4(GAA):c.1726G > A (p.Gly576Ser) and the NM_000152.3(GAA):c.2065G > A (p.Glu689Lys). Variants found by NGS were confirmed using Sanger sequencing.

Additionally, the parents of the infant were also evaluated by enzymatic and molecular analyses. The enzyme assays revealed a normal GAA activity in leukocytes for both parents. The molecular analysis showed that the father was carrier of the variant c.-32-13T > G and the mother was carrier for the two variants, c.1726G > A (p.Gly576Ser) and c.2065G > A (p.Glu689Lys).

Hence, based on all the above results in the infant and the information provided for the analysis in the parents, the case was defined as pseudodeficiency for Pompe disease.

3.3. Case 3: suspicion of Gaucher disease

A male newborn, referred for further investigation after a result in the NBS for Gaucher disease that showed a low GBA activity (6.1 μmol/L/h; cut off: > 7) in a DBS sample.

In the additional investigation, GBA activity in DBS exhibited a normal activity. The enzyme assay performed in leukocytes resulted in a low GBA activity (5.6 nmol/h/mg protein, with normal reference range from 10 to 45). Chitotriosidase was not helpful, as it was evaluated in DBS (activity undetectable, with reference range from 0 to 44 nmol/h/mL) and in plasma (activity 0.1 nmol/h/ml, with normal reference values ranging from 8.8 to 132). As biochemical results were not conclusive, *GBA* gene sequencing was performed, and the variant NM_001005741.2(GBA):c.1226A > G (p.Asn409Ser) was identified in heterozygosis. This is a well-known pathogenic variant also described as p.N370S. Additionally, it was discarded the possibility of pseudodeficiency after identifying a normal sequence for exon 10 of *GBA* gene that is the usual location of complex recombination between the *GBA* gene and the pseudogene.

The parents were also evaluated. Analysis of GBA activity in leukocytes resulted in a low activity for the father only, being normal for the mother. This sample was unsuitable for molecular analysis, which was not performed in the parents as they did not return for blood collection.

Then, gathering all the above information, the conclusion was that this baby was as a carrier for Gaucher disease.

3.4. Case 4: suspicion of MPS I

A female newborn was referred for further investigation after being screened positive for a NBS for MPS I. The screening resulted in a low IDUA activity (2.4 μmol/L/h; cut off: > 5.0).

Evaluation of this case started with the urinary GAGs analysis that resulted normal in the quantitative and qualitative analyses. Then, enzyme activity was measured in leukocytes and revealed an IDUA activity at the lower limit of the reference range (27 nmol/h/mg protein, with reference range from 27 to 171). Given this borderline result of the enzyme activity and the normal urinary excretion of GAGs,

biochemical results were considered inconclusive.

Molecular analysis with sequencing of the *IDUA* gene was then performed in the baby, with the identification of a known pathogenic variant in heterozygosis, the NM_000203.4(IDUA):c.1205G > A (p.Trp402Ter). Targeted gene analysis was also performed in both parents, by sequencing of the affected exon. It demonstrated the presence of this variant in heterozygosis at the father's DNA and absent in the mother's sample.

Based on the enzymatic assay and the gene analysis results, together to normal excretion of GAGs in urine, the conclusion was that the baby is a carrier for MPS I.

4. Discussion

We report the investigation performed in the four presumptive cases for LSDs identified in a pilot study of NBS for 4 LSDs (MPS I, Fabry, Gaucher, and Pompe diseases) carried out in a NBS laboratory in Brazil. Two of the cases had suspicion of MPS I, one had suspicion of Gaucher disease and one had suspicion of Pompe disease. The investigation included biochemical and molecular analyses performed in the babies and in their parents. No affected subject for any of the diseases was diagnosed. However, we did not classify these cases as false positives, as they were identified as having pseudodeficiency (one case of suspected MPS I and one case of suspected Pompe disease) or as carriers (one case of suspected MPS I and one case of suspected Gaucher disease).

The first baby had a suspicion of MPS I. MPS I, caused by IDUA deficiency that fail to degrade the glycosaminoglycans heparan and dermatan sulfate, is diagnosed by measuring mainly a reduced IDUA activity in leukocytes or in other nucleated cell and by either one or both increased excretion of GAGs in urine and a pattern of heparan and dermatan sulfate excretion at the electrophoresis [33]. Biochemical investigation showed normal GAG excretion, suggesting an absence of functional impact of an apparent IDUA deficiency on GAGs degradation. Normal GAG excretion with low IDUA activity suggests the possibility of pseudodeficiency, and molecular analysis is recommended to elucidate the diagnosis. Despite the presence of a possibly pathogenic variant p.(Gly84Ala), the presence of a pseudodeficiency allele p.His82Gln allowed normal degradation of GAGs. Pseudodeficiency condition was found in other NBS programs for MPS I, with an estimated frequency of 0.01% to 0.02% of the total screened samples in each study [8,34]. These NBS programs, carried out mainly in U.S.A. (Missouri, Illinois and New York), reported pseudodeficiency cases among the screened positive samples for MPS I and the number of confirmed pseudodeficiency cases was higher than the true affected cases. Although NBS programs of other countries such as Taiwan and Italy did not report pseudodeficiency cases for MPS I [35,36], the possibility to find this condition in the evaluation of suspected MPS I should be clearly taken in consideration. Therefore, this case was identified as pseudodeficiency for MPS I, without pathogenic consequences, allowing the prediction of a normal child.

Pseudodeficiency has been already described as a possible confounder in the interpretation of enzymatic assay results for some LSDs [37], including Pompe disease. Diagnosis of Pompe disease is established by a decreased GAA activity in leukocytes or fibroblast and a genotype demonstrating pathogenic variants of the *GAA* gene in homozygosis or in compound heterozygosis [38]. Because enzyme assay has limitations to discriminate pseudodeficiency and carrier status of affected or normal cases, gene analysis is required to establish the diagnosis. The genotyping of the baby with suspected Pompe disease allowed the identification of a combination of a previously reported pseudodeficiency allele with a known pathogenic mutation, both in heterozygosis, which explain the slight reduction of the GAA activity. Previous *in vitro* studies have shown that the two variants of the pseudodeficiency allele, when combined, reduce the GAA activity by approximately 80% in comparison to the expression of wild-type cDNA [31] and are highly frequent in Asian populations [32]. Likewise, the c.-32-13T > G, a splice site variant of intron 1, has been reported as the most frequent pathogenic variant in adult onset Caucasian patients [39] and may reduce the GAA activity to a range of 3% to 20% of the normal when presented in compound heterozygous state, combined with other deleterious *GAA* gene variants [40,41]. Since this variant was observed mostly in juvenile and adult form of Pompe disease, it is considered of mild effect. Combination of a pseudodeficiency allele and a pathogenic variant may exhibit different levels of reduction of the GAA activity as observed in the case investigated in this study and contrasted by other study where the described case showed an important decrease of GAA activity, which may be accounted for the effect of a nonsense mutation considered more deleterious p.[Gly576Ser; Glu689Lys]/p.Trp746Ter [31]. Other newborn screening studies for Pompe disease have also reported similar cases of carriers with an additional pseudodeficiency allele that were part of the false-positive cases found in that screening program [32,42,43]. Thus, caution has been already recommended in the interpretation of enzyme activity results in cases when pseudodeficiency alleles are present. The diagnosis of this case was established as pseudodeficiency for Pompe disease, allowing the prediction of a normal clinical course for the proband.

One baby had a suspicion of Gaucher disease, which is caused by a deficient GBA activity, leading to glucocerebroside accumulation in cells of monocyte or macrophage lineage. Its diagnosis is usually established after demonstrating enzyme deficiency in leukocytes or fibroblasts [44]. The case showed a low enzyme activity in leukocytes but not so reduced as observed in affected cases [45]. When enzyme activity results show an overlap of the values found in carriers and in non-carriers, *GBA* gene analysis should be performed [44]. Chitotriosidase activity could provide important information if elevated, which would suggest Gaucher disease. When it is very low, as in the present case, results are not as informative as it could be caused by a common mutation that affects its activity [46,47]. To elucidate the case, molecular analysis of the *GBA* gene was performed, being identified the most common disease-causing variant (N370S), that has been associated to Gaucher disease type 1 [48]. Carriers for Gaucher disease were identified in other NBS programs, such as those performed in Washington, Illinois and New York in the U.S.A., Hungary and Taiwan, with a frequency estimated in the range of 0.002% to 0.02% of total screened samples [49–51]. Genotypes included different variants, but the p.Asn409Ser (p.N370S) was observed in all these NBS studies and reported as the most common allele among the identified alleles [34]. Therefore, in our study, as the pathogenic variant was found in a heterozygous state, the baby was only a carrier and consequently there should be no risk to developing clinical disease.

Our last case was, again, one with a suspicion of MPS I. The measurement of IDUA activity in leukocytes was inconclusive, with an enzyme activity in the lower limit of the reference range. The molecular analysis of the *IDUA* gene elucidated the diagnosis demonstrating a common pathogenic variant (p.Trp402Ter) in heterozygous state. This variant in homozygous state has been associated with the severe phenotype of MPS I [52]. A Brazilian study showed that this variant accounted for 38% of the alleles in patients with MPS I [53]. Other NBS programs also found carriers for MPS I with an estimated frequency of 0.001% to 0.005% of the total screened samples, including all cases reported as confirmed carriers [8,34,35,43,50]. Although, not all these studies reported the genotype identified, the reported variants were different to the one found in our study. Being a carrier for MPS I, this baby is not at risk of developing clinical disease.

The investigation performed in these cases illustrates the possible strategies for confirmatory diagnosis in asymptomatic subjects from NBS programs for LSDs and the challenges that may be faced during its interpretation. Previous studies on NBS for LSDs discuss briefly on the additional procedures used for the investigation of suspected cases, with variable strategies according to the laboratory. Some perform enzymatic and molecular analyses simultaneously, while others use only the molecular analysis. Among the challenges during

interpretation, the presence of pseudodeficiencies or carrier status represents situations difficult to diagnose by biochemical methods, which, however, are important to identify the functional status of the patient.

Molecular analysis seems to be critical for the understanding of each case, but may also show some difficulties in the interpretation when new gene variants of unknown significance are identified, that will require further prediction exercises and functional studies to elucidate its effect and validate its significance.

Therefore, all these aspects should be considered in the process of diagnostic confirmation, especially when the cases are identified in mass screening programs of clinically normal subjects, as it is the case of NBS.

Finally, it is worthy to mention the absolute need of having comprehensive diagnostic protocols in place when a NBS for LSDs is performed. In the investigation of babies screened positive, the integration of the different pieces of the screening team, (screening lab, biochemical diagnosis lab, molecular genetics lab and clinical group) is very important to establish the correct diagnosis of each case.

5. Conclusions

Biochemical and molecular procedures for confirmatory investigation of newborns who had abnormal results in the initial test in NBS programs for LSDs should be an essential part of the program, and should be performed, whenever possible, in reference centers with high expertise in the diagnosis of these diseases. This allows a rapid and precise investigation of the babies who have an abnormal screening result, reducing parental anxiety in false-positives and allowing prompt initiation of therapy in the cases with confirmed disease.

Author contributions

HB and RG conceived the investigation for confirmatory diagnosis, wrote the first draft and analyzed the data; ECN supervised the NBS for LSDs program; JS and JP performed the NBS analyses; CSF provided expert advice on NBS; FB and FS performed the enzyme analysis for confirmatory diagnosis; RRG performed the urine GAGs analysis; KM-T supervised the enzyme and GAGs analyses; ACB-F, GP, DRM and FBT performed the molecular analyses for confirmatory diagnosis; RG supervised the whole procedures of the investigation for confirmatory diagnosis; all authors revised and approved the final version of this manuscript.

Conflicts of interest

The authors declare no conflict of interest to report in relation to this manuscript.

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