CLINICS

RAPID COMMUNICATION

Diagnosing lysosomal storage diseases in a Brazilian non-newborn population by tandem mass spectrometry

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OBJECTIVES: High-throughput mass spectrometry methods have been developed to screen newborns for lysosomal storage disorders, allowing the implementation of newborn screening pilot studies in North America and Europe. It is currently feasible to diagnose Pompe, Fabry, Gaucher, Krabbe, and Niemann-Pick A/B diseases, as well as mucopolysaccharidosis I, by tandem mass spectrometry in dried blood spots, which offers considerable technical advantages compared with standard methodologies. We aimed to investigate whether the mass spectrometry methodology for lysosomal storage disease screening, originally developed for newborns, can also discriminate between affected patients and controls of various ages.

METHODS: A total of 205 control individuals were grouped according to age and subjected to mass spectrometry quantification of lysosomal α-glucosidase, β-glucocerebrosidase, α-galactosidase, acid sphingomyelinase, galactocerebrosidase, and α−L-iduronidase activities. Additionally, 13 affected patients were analyzed.

RESULTS: The median activities for each enzyme and each age group were determined. Enzyme activities were significantly lower in individuals aged older than 18 years compared with those in newborns. Affected patients presented enzymatic activities corresponding to less than 20% of the age-matched controls.

CONCLUSIONS: Our data indicate that the mass spectrometry methodology can be used for the screening of lysosomal storage diseases in non-newborn patients. However, for some diseases, such as Fabry and mucopolysaccharidosis I, a combination of biochemical and clinical data may be necessary to achieve accurate diagnoses.

KEYWORDS: Dried Blood Spot Analysis; Pompe Disease; Fabry Disease; Gaucher Disease; Krabbe Disease.

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INTRODUCTION

Lysosomal storage diseases (LSDs) consist of approximately 50 genetic disorders, which have a combined prevalence of 1:7,000~8,000 live births (1). These conditions are caused mainly by mutations in genes encoding enzymes or other essential proteins involved in the lysosomal degradation of intermediate metabolites (2). The progressive accumulation of these molecules leads to cellular destruction, which may cause tissue and organ dysfunction (2,3).

Diagnosing this group of diseases is, in many cases, challenging. The age of onset of clinical manifestations and rate of substrate accumulation are variable, even among patients with identical genotypes (4). Nevertheless, these conditions should be diagnosed as soon as possible, for early therapeutic interventions have been considered beneficial to patients. Newborn screening (NBS) for LSDs by tandem mass spectrometry (MS/MS) has been implemented in North America and European countries as pilot projects (5-7). Early detection of LSDs in newborns, especially in Latin America, will take many years before a considerable fraction of the population is covered (8). Up until then, inexpensive and high throughput biochemical assays that can reliably diagnose infants, children and adults would be desirable.

The introduction of MS/MS assays for the measurement of lysosomal enzyme activities in dried blood spots (DBSs) combined sensitivity and specificity with high throughput

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(9). The current study addresses the implementation of the detection of LSDs by MS/MS in the Sarah Network of Rehabilitation Hospitals in a non-newborn population in Brazil. Patients were screened for Pompe (GAA), Gaucher (ABG), Fabry (GLA), Niemann-Pick A/B (ASM), and Krabbe (GALC) diseases, as well as MPS I (IDUA). An adapted version of the NBS methodology was employed and validated using DBSs of the CDC Quality Assurance Program (9,10). The median activities of these enzymes in control individuals were determined, and patients presenting with Pompe, Krabbe, Fabry, Gaucher, and MPS I diseases were also evaluated. To the authors' knowledge, this is the first report concerning the screening of LSDs by MS/MS in Brazil.

MATERIALS AND METHODS

Reagents

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme substrates (S) and internal standards (IS) were kindly donated by the Newborn Screening Translation Research Initiative (NSTRI) at the North American Center for Disease Control (CDC, Atlanta, USA).

Subjects

A total of 205 control patients were divided according to age into 5 groups: 50 newborn patients, 32 patients aged 1 month to 5 years, 35 patients aged 5 to 10 years, 23 patients aged 10 to 18 years, and 65 patients aged 18 to 77 years. Thirteen affected patients diagnosed at our institution by classic fluorimetric methods, as well as DBSs from three other genetics services in Brazil, were used as positive controls. Informed consent was obtained from all the patients. This research was approved by the ethics committee of the Sarah Network of Hospitals.

Enzyme assays

An adapted version of the original methodology described in the literature was used (9,11). Assay cocktails were prepared by resuspending vials containing substrates and internal standards for GAA, GLA, ABG, ASM, IDUA, and GALC according to the supplied instructions. DBS extraction and incubation with enzyme substrates were performed as instructed, with the following modifications. We used Eppendorf tubes instead of 96-well plates, and solid phase extraction was performed using Strata SI-1 (Phenomenex, Torrance, CA, USA) and a Visiprep 24-port vacuum manifold (Sigma). Electrospray ionization mass spectrometry (ESI-MS/MS) analyses were performed using an API 3200 triple quadrupole instrument (AB Sciex, Framingham, MA, USA) in the positive-ion, multiple-reaction monitoring mode. Blank DBSs, as well as quality control materials, were assayed (10). Enzyme activities were expressed as μmol/h/L.

Statistical analyses were performed using SPSS ver. 21.

RESULTS

Assay validation

Quality control (QC) materials provided by the CDC were assayed to evaluate the entire methodological procedure. These materials correspond to a pool of inactivated cord blood (BP) supplemented with 5% (low), 50% (medium), and 100% (high) unprocessed cord blood (10). Enzyme activities were comparable to those reported by the CDC, and all the assayed enzymes showed a linear dependency between the enzyme activity and percentage of unprocessed blood in the DBSs with $R^2>0.99$ (data not shown).

Table 1 - Lysosomal storage disorder enzyme activities of individuals according to age. Data are expressed as the median ± median absolute deviation. The percent residual enzyme activities in affected patients according to their age groups are shown in parentheses.

		Enzyme (μmol/h/L)					
Controls/Patients	Age	GAA	GLA	GALC	ABG	ASM	IDUA
Group 1	0 to 28 days	13.01±3.34	8.77±3.08	2.15±0.91	17.83±5.03	5.60±1.33	9.31±1.71
Group 2	29 days to 5 years	9.59±2.61	3.69±0.84	2.31±0.96	9.72±2.37	4.44±1.17	8.29±2.19
Group 3	5 to 10 years	8.65±0.94	3.44±0.77	1.86±0.77	10.17±3.27	4.22±1.44	8.23±2.94
Group 4	10 to 18 years	6.40±1.79	3.50±0.85	1.45±0.32	9.13±1.63	3.81±0.89	7.14±2.24
Group 5	>18 years	8.11±1.63	3.32±1.14	1.36±0.42	7.71±2.52	3.75±0.82	4.64±2.74
Pompe [a]	45 years	0.24 (3%)					
Pompe [b]	53 years	0.18 (2%)					
Pompe [c]	30 years	1.19 * (15%)					
Pompe [d]	51 years	0.32 (4%)					
Pompe	10 months	0.18 (2%)					
Pompe	4 months	0.11 (1%)					
Fabry	44 years		0.36 (11%)				
Krabbe	17 years			0.19 # (13%)			
Gaucher	N/A				0.72		
Gaucher	26 years				0.78 (10%)		
MPS I	N/A						0.54
MPS I [e]	13 years						1.33 (19%)
MPS I [f]	1 year						1.09 (13%)

GAA = α-glucosidase, ABG = β-glucocerebrosidase, GLA = α-galactosidase, ASM = acid sphingomyelinase, GALC = galactocerebrosidase, and IDUA = α−L-iduronidase.

*Patient undergoing enzyme replacement therapy (blood collected immediately before enzyme infusion). #Diagnosis pending confirmation by gene sequencing. N/A = Not available.

Confirmatory enzyme activities using the 4-MU substrate (% of the normal control activity): GAA: a = 2 (4%), b = 2 (3%), c = 0 (0%), and d = 6 (15%) (reference range = 20 to 60 nmol/h/mg of protein). IDUA: e = 0 (0%) and f = 0 (0%) (reference range = 3 to 19 nmol/h/mg of protein).

Enzyme activities in newborn and non-newborn individuals

Enzyme activities in control patients were not normally distributed for the five age groups and six evaluated enzymes. Therefore, non-parametric descriptive statistics and tests were used. As reported in the literature, only minor discrepancies were found between the population mean and median (12). The median enzyme activities of the control and affected patients are listed in Table 1 and shown in Figure 1. The median activities of the enzymes GAA, GLA, ABG, and ASM were significantly higher in newborns (Group 1) than in all the other groups (Mann-Whitney U-test, $p<0.05$), findings that were not observed for IDUA and GALC. For all the evaluated enzymes, patients older than 18 years presented, on average, only ~50% of the median enzyme activity of newborns. GLA was the enzyme most affected by age; specifically, patients older than 18 years had only ~38% of the median activity of newborns.

Diagnosing LSDs in non-newborn patients

In the present study, there were no overlaps between the enzime activities of the affected and healthy patients of any age group for the enzymes GAA, GLA, and ABG (Figure 1). Indeed, all Pompe, Fabry, Gaucher, and Krabbe disease patients considered in this cohort had enzyme activities lower than 15% of the median activities of their respective

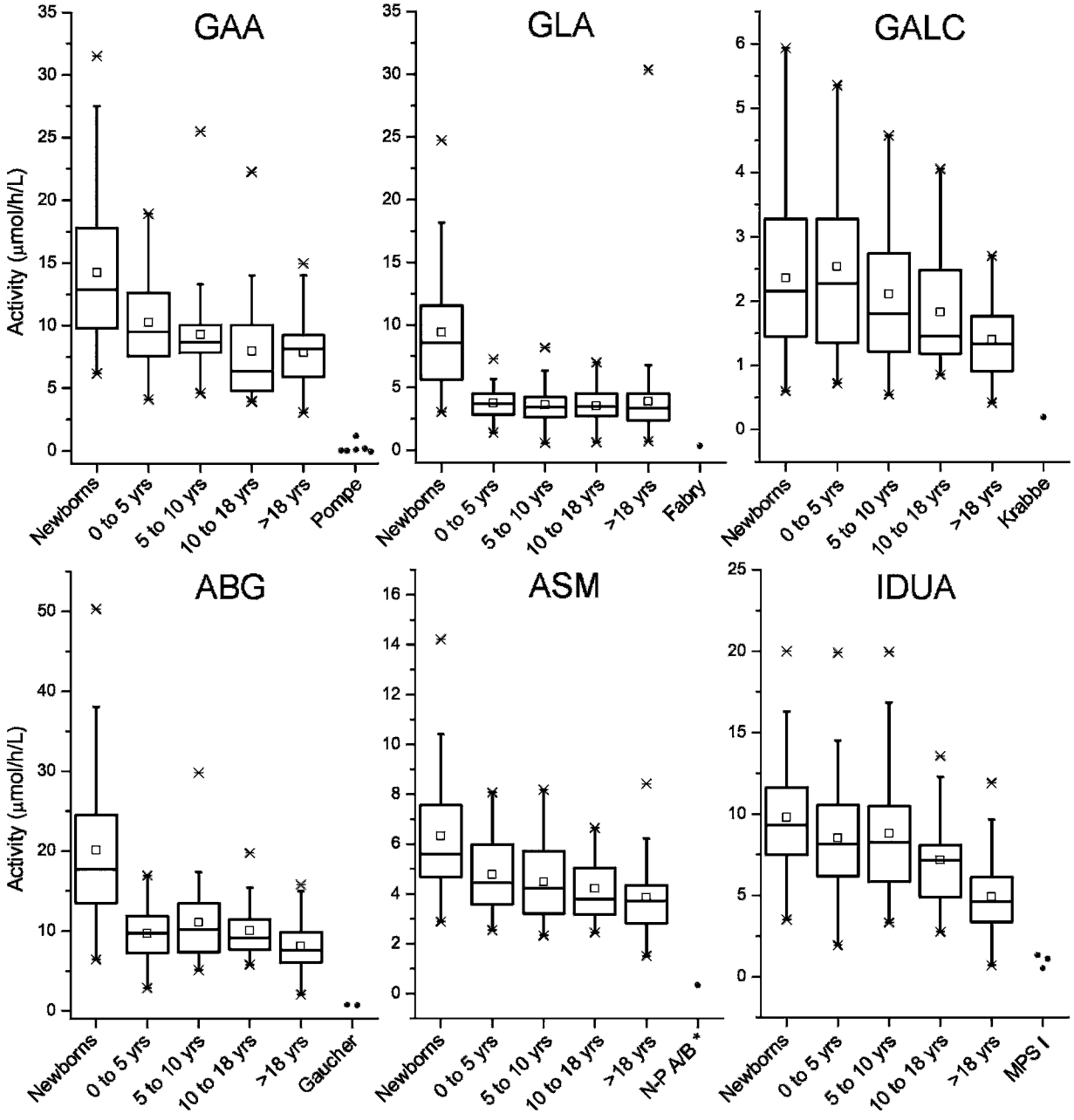

Figure 1 - Comparison of the enzyme activities (µmol/h/L) between DBS samples from newborns and non-affected adults grouped according to age and DBS samples from patients with LSDs. *No patients with Niemann-Pick A/B disease were available. This sample corresponds to a DBS from a healthy patient subjected to protein denaturing conditions (heat inactivation).

groups (Table 1, in parentheses). A 17-year-old individual presenting classical clinical symptoms of Krabbe disease was identified by our genetics service and tested. GALC activity in this patient was 13% of the median activity of Group 4 (Table 1), and confirmation by gene sequencing is still pending. No DBSs from Niemann-Pick A/B disease patients could be obtained. MPS I patients presented 13% (patient aged 1 year) and 19% (patient aged 13 years) of the median IDUA activity of age-matched controls (Table 1). There was an overlap between the activity of IDUA in subjects affected by MPS I and control individuals older than 18 years (Figure 1).

DISCUSSION

Screening of LSDs by tandem mass spectrometry was originally developed to provide a suitable methodology for NBS programs, which require fast and parallel assays (9). However, there is a demand, particularly in Latin America, for reliable, inexpensive, and accessible tests to identify not only asymptomatic newborns but also undiagnosed children and adults carrying late-onset alleles (8). Alleles presenting late-onset clinical manifestations are indeed common, as demonstrated by pilot studies in Austria, Italy, and Taiwan (7,13,14).

In the present cohort, the median GAA, GLA, GALC, ABG, ASM, and IDUA activities in the DBSs of newborns were comparable to those reported in the literature (5,7,15,16). It was possible to discriminate between the enzyme activity in non-affected infants, children, and adults of any age and the enzyme activity in patients affected with Pompe, Fabry, Gaucher, and Krabbe diseases (the latter is still pending confirmation by gene sequencing) as shown in Table 1 and Figure 1. This finding indicates that, for these particular diseases, the biochemical assays alone can provide reliable diagnostics. The overlap between MPS I patients and non-affected adults aged older than 18 years (Figure 1) indicates that the identification of patients in these groups may require other confirmatory biochemical assays. However, at this age, MPS I clinical symptoms are usually evident. As the number of screened individuals increases, group overlaps might occur for other enzyme measurements. Indeed, heterozygous carriers have approximately one-half the enzyme activity of normal adults, a finding that is particularly relevant in screening female Fabry patients (9). Discerning between adult heterozygotes who have low enzyme activities and affected patients based on the enzymes GLA, GALC, and IDUA may eventually be challenging. Nevertheless, in these cases, a combination of standard biochemical assays and clinical data should provide sufficient information to enable accurate diagnoses.

The MS/MS methodology evaluated in this study introduces further benefits compared with standard fluorimetric assays as follows: control DBSs provide an external reference; the methodology allows a more comprehensive screening of diseases that may have overlapping clinical manifestations; and several enzyme quantifications can be performed using a single and robust methodology to optimize the laboratory routine. Laboratories capable of performing lysosomal enzyme determinations are scarce in Latin America. This simple, robust, and inexpensive methodology will allow more laboratories to perform lysosomal enzyme determinations. Dried blood spots may be sent by regular mail in a plain envelope with minimal financial costs, granting access to specialized genetic tests to populations located in remote areas of the continent. Diagnoses will lead to earlier treatment, reducing the morbidity of the diseases and improving patient survival and quality of life.

The present study discusses the use of tandem mass spectrometry assays to screen for Pompe, Fabry, Gaucher, Krabbe, and Niemann-Pick A/B diseases, as well as MPS I, in infants, children, and adults. The data presented herein indicate that this methodology is adequate; however, in some cases, further clinical and laboratory evaluation may be necessary. This methodology may become standard in the near future as the spectra of tested disorders expand to include other types of LSDs.

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AUTHOR CONTRIBUTIONS

Brand GD, Matos HC, Cruz GC, and Fontes NC conducted the experiments. Brand GD wrote the manuscript. Buzzi M, and Brum JM designed the experiments and revised the manuscript.

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