UNIVERSIDADE FEDERAL DE RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

TRIAGEM NEONATAL PARA DOENÇAS LISOSSÔMICAS: AVALIAÇÃO DE PROTOCOLOS PARA INVESTIGAÇÃO DOS CASOS COM ALTERAÇÕES NOS TESTES INICIAIS E ESTIMATIVA DO CUSTO DO PROGRAMA A PARTIR DE UM ESTUDO PILOTO NO BRASIL

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Orientador: Prof. Dr. Roberto Giugliani

Porto Alegre

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Heydy Varinia Bravo Villalta

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LISTA DE ABREVIATURAS

ANVISA: Agência Nacional de Vigilância Sanitária

ARSB: arilsulfatase B/ N-acetil-galactosamina 4-sulfatase

CFOH: Do inglês, *Chinese Foundation of Health*

DLD: Doenças Lisossômicas de Depósito

DMF: Do inglês, *Digital microfluidics*

EIM: Erros Inatos do Metabolismo

EUA: Estados Unidos da América

FDA: Do inglês, *Food and Drug Administration*

GAA: alfa-glicosidase ácida (*α-glucosidase acid*)

GAG: glicosaminoglicanos

GBA: glicocerebrosidase/ *acid β-glucosidase*

GLA: alfa-galactosidase A (α-galactosidase A)

HCPA: Hospital de Clínicas de Porto Alegre

IDS: iduronato 2-sulfatase/ *iduronate 2-sulfatase*

IDUA: alpha-iduronidase /*α-L-iduronidase*

MeSH: Do inglês, *Medical Subject Heading*

MPS I: Mucopolissacaridose tipo I

MPS II: Mucopolissacaridose tipo II

MPS III A: Mucopolissacaridose tipo IIIA

MPS IVA: Mucopolissacaridose tipo IVA

MPS VI: Mucopolissacaridose tipo VI

MPS: Mucopolissacaridose

MS/MS: Espectrometria de massa em tandem

NCBI: Do inglês, *National Center for Biotechnology Information*

NPC: Niemann-Pick tipo C

NTUH: Do inglês, *National Taiwan University Hospital*

OMS: Organização Mundial da Saúde

PCDT: Protocolo Clínico e Diretrizes Terapêuticas

PKU: Fenilcetonúria

PNTN: Programa Nacional de Triagem Neonatal

rhASB: N-acetilgalactosamina-4-sulfatase humana recombinante

RUSP: Do inglês, *Recommended Uniform Screening Panel*

SACHDNC: Do inglês, *Secretary's Advisory Committee on Heritable Disorders in Newborns and Children*

SBTN: Sociedade Brasileira de Triagem Neonatal

SGM: Serviço de Genética Médica

SNC: Sistema nervoso central

SRTN: Serviços de Referência em Triagem Neonatal

SUS: Sistema Único de Saúde

TCTH: Transplante de células-tronco hematopoiéticas

TN: Triagem neonatal

TRE: Terapia de Reposição Enzimática

TRS: Terapia de redução de substrato

WHO: Do inglês, *World Health Organization*

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RESUMO

A triagem neonatal (TN) tornou-se uma importante intervenção de saúde pública para a detecção precoce e prevenção de doenças, ajudando a reduzir a morbidade e a mortalidade em crianças. As doenças lisossômicas de depósito (DLDs) são distúrbios genéticos, clinicamente heterogêneos, causados principalmente por defeitos em genes que codificam enzimas lisossômicas que degradam macromoléculas. As opções terapêuticas para essas doenças evoluíram bastante nos últimos anos e muitas das DLDs têm atualmente tratamento disponível que pode melhorar a evolução clínica, especialmente se forem introduzidos precocemente. Esse fato mostra claramente a necessidade de diagnóstico precoce e oportuno, de preferência durante a fase assintomática da doença, o que apoia o desenvolvimento de TN para alguns desses distúrbios. Embora vários estudos já tenham estabelecido métodos de triagem adequados para uso em programas de TN, pouco foi discutido sobre outros procedimentos, posteriores à triagem. Portanto, nesse estudo, avaliamos protocolos para o diagnóstico confirmatório de casos que resultarão alterados nos programas de TN para DLDs tratáveis, considerando sua potencial implementação no Brasil. Inicialmente, revisamos, coletamos e resumimos informações sobre protocolos e estratégias para confirmação diagnóstica de casos triados positivos para a doença de Fabry, doença de Gaucher, doença de Pompe e Mucopolissacaridose (MPS) tipo I, II e VI. Duas estratégias foram identificadas para o diagnóstico de quase todas essas doenças. Um baseado apenas na análise molecular e outro baseado principalmente na análise enzimática e na análise molecular. Depois, avaliamos as estratégias de diagnóstico confirmatório, previamente reportados, para investigar quatro casos que foram encaminhados a partir de um estudo de TN para DLDs realizado em mais de 10.000 recém-nascidos no Brasil. Dois casos de pseudodeficiência foram identificados, um para a doença de Pompe e um para MPS I. Além disso, dois portadores (heterozigotos) também foram identificados, um para MPS I e um para a doença de Gaucher. Esse estudo discute também as limitações das análises enzimáticas e moleculares na confirmação do diagnóstico, os desafios que podem ser encontrados durante a interpretação dos resultados e a necessidade de um protocolo abrangente para a investigação precisa de casos em suspeita, identificados por estudos de TN para DLDs. Por conseguinte, um protocolo foi desenvolvido com recomendações para o diagnóstico confirmatório de casos que resultarão alterados após o teste inicial para MPS I, proposto como modelo para a futura elaboração de protocolos para as demais doenças.

Finalmente, o custo global de um programa de TN para DLDs foi estimado, na base do estudo piloto realizado no Brasil, considerando a fase da triagem e a estrutura do protocolo de confirmação diagnóstica. Esse estudo representa uma base para avaliações mais abrangentes destes programas, em vista de uma possível implementação em grande escala.

ABSTRACT

Newborn screening (NBS) has become an important public health intervention for early detection and prevention of diseases, helping to reduce the morbidity and mortality in children. Lysosomal storage diseases (LSD) are genetic disorders, clinically heterogeneous, caused mainly by defects in genes encoding lysosomal enzymes that degrade macromolecules. Therapeutic options for these disorders have substantially evolved in the last years and many of the LSDs currently have available treatment that may improve clinical outcomes, especially if early introduced. This fact clearly raises the need for early and timely diagnosis, preferably during the asymptomatic stage of the disease, which supports the development of NBS for some of these disorders. While several studies have already established screening methods suitable for use in NBS programs, little has been discussed on further procedures. In this study, we evaluated protocols for the confirmatory diagnosis of cases that will result altered in NBS programs for treatable LSDs, considering its potential implementation in Brazil. Initially, we reviewed, collected and summarized information on protocols and strategies for diagnosis confirmation of cases screened positive for Fabry disease, Gaucher disease, Pompe disease and Mucopolysaccharidosis (MPS) type I, II and VI. Two strategies were identified for the diagnosis of almost all these diseases. One based only in the molecular analysis and the other based mainly in both the enzyme analysis and molecular analysis. Then, we evaluated the previously reported confirmatory diagnostic strategies to investigate four cases that were referred from a NBS study for LSDs performed in over 10,000 newborns in Brazil. Two cases of pseudodeficiency were identified, one for Pompe disease and one for MPS I. In addition, two carriers (heterozygotes) were also identified, one for MPS I and one for Gaucher disease. This report discusses the limitations of enzymatic and molecular analyses in diagnosis confirmation, the challenges that may be found during result interpretation and the need for a comprehensive protocol for precise investigation of cases in suspicion identified by NBS studies for LSDs. Therefore, a protocol was developed that comprises recommendations for the confirmatory diagnosis of cases that will result abnormal after the initial testing for MPS I, proposed as a template for the future development of similar protocols for the other diseases. Finally, the overall cost of a NBS program for LSDs was estimated, based on the pilot study conducted in Brazil, considering the screening phase and the structure of the diagnosis confirmation protocol. This study

provides a basis for more comprehensive assessments of these programs in view of potential large-scale implementation.

CAPÍTULO 1. INTRODUÇÃO

1.1 TRIAGEM NEONATAL

A Triagem Neonatal (TN) tornou-se uma importante intervenção em saúde pública para a detecção precoce e prevenção de doenças, ajudando para reduzir a morbidade e mortalidade em crianças, evitando, dessa maneira, diferentes tipos de deficiência que afetariam o indivíduo e sua família e, por consequência, a sociedade.

Triagem, de maneira geral, refere-se à identificação presumida de uma doença ou defeito através de exames ou outros procedimentos, pelos quais os indivíduos assintomáticos são separados em aqueles que provavelmente têm a doença, e os que não têm (Wilson and Jungner, 1968). O resultado positivo de um exame de triagem não é diagnóstico, porém representa a suspeita inicial para continuar com procedimentos adicionais até o diagnóstico confirmatório (Wilson and Jungner, 1968).

A triagem neonatal visa a detectar doenças tratáveis em recém-nascidos antes de se tornarem sintomáticos, e por isso, são vários os procedimentos já estabelecidos para TN. Eles incluem: 1) o exame clínico; 2) a triagem para doenças hematológicas, metabólicas e hormonais; 3) a triagem para surdez; a triagem para cardiopatias e outros defeitos congênitos (WHO, Fact Sheet N° 370 Congenital Anomalies).

1.2 TRIAGEM NEONATAL PARA DOENÇAS GENÉTICAS E METABÓLICAS

Nos anos 60 iniciou-se no mundo a TN para doenças genéticas e metabólicas, depois que Horst Bickel estabeleceu uma terapia dietética eficaz para Fenilcetonúria (PKU) e Robert Guthrie desenvolveu o primeiro método para dosagem de fenilalanina em amostras de sangue seco em papel-filtro (Hoffman *et al*., 2010).

Desde então, a TN vem se expandindo em inúmeros países e já faz parte dos programas de saúde pública em muitos deles. Para as doenças serem inclusas na TN, os programas seguem critérios ainda baseados nos princípios de triagem propostos por J. Wilson e G. Jungner em um relatório da Organização Mundial da Saúde (OMS) em 1968 (Tabela 1A). Contudo, trabalhos envolvendo novos critérios para triagem têm também surgido (Tabela 1B), incorporando o progresso tecnológico das últimas décadas, sem desconsiderar os princípios de Wilson e Jungner (Anderman *et al.,* 2008).

Tabela 1. Critérios para a Triagem Neonatal

A. Princípios de triagem segundo Wilson e Jungner (1968)
1. A condição deve ser um importante problema de saúde. 2. Deve haver um tratamento aceito para os pacientes com uma doença reconhecida. 3. Devem estar disponíveis instalações para o diagnóstico e tratamento. 4. Deve haver um estado reconhecível latente ou sintomático precoce. 5. Deve haver um exame ou teste apropriado. 6. O teste deve ser aceitável para a população. 7. A história natural da doença, incluindo o desenvolvimento do estado latente para o estabelecimento final da doença, deve ser entendida de forma adequada. 8. Deve haver uma política consensuada sobre quem tratar como pacientes. 9. O custo de caso detectado (incluindo diagnóstico e tratamento dos pacientes diagnosticados) deve ser economicamente equilibrado em relação com as possíveis despesas na assistência médica como um todo. 10. A detecção de casos deve ser um processo contínuo.
B. Critérios emergentes segundo Anderman *et al*. (2008)
1. O programa de triagem deve responder a uma necessidade reconhecida. 2. As metas de triagem devem ser definidas desde o início. 3. Deve haver uma população-alvo definida. 4. Deve haver evidências da eficácia de programas de triagem. 5. O programa deve integrar educação, exames, serviços clínicos e programas de atendimento. 6. Deve haver garantia de qualidade, com mecanismos para minimizar os riscos potenciais da triagem. 7. O programa deve garantir uma escolha informada, confidencialidade e respeito à autonomia. 8. O programa deve promover a equidade e o acesso à triagem de toda a população. 9. A avaliação do programa deve ser planejada desde o início. 10. Os benefícios da triagem devem compensar os danos.

1.3 TRIAGEM NEONATAL NO BRASIL

No Brasil, a TN iniciou-se em 1976, com um projeto de triagem neonatal para fenilcetonúria e posteriormente para o hipotireoidismo congênito. No entanto, foi só em 1992 que sua incorporação ao Sistema Único de Saúde (SUS) foi proposta (Portaria GM/MS n.º 22).

Em 2001, o Ministério da Saúde instituiu a Triagem Neonatal no SUS, criando o Programa Nacional de Triagem Neonatal (PNTN) (Portaria GM/MS n.º 822, de 6 de junho de 2001) (Ministério de Saúde, 2002). A partir de então, os estados passaram a participar do PNTN, através de Serviços de Referência em Triagem Neonatal (SRTN) credenciados. Todos possuem uma estrutura de diagnóstico, busca ativa, tratamento e acompanhamento das doenças triadas, remunerados com recursos do SUS destinados para esse fim (Sociedade Brasileira de Triagem Neonatal - SBTN, História).

Desde 2014, o PNTN inclui a triagem de Hipotireoidismo Congênito, Fenilcetonúria, Hemoglobinopatias, Fibrose Cística, Hiperplasia Adrenal Congênita e Deficiência da

Biotinidase. As duas últimas doenças foram as mais recentes a serem incorporadas no PNTN, pela Portaria GM/MS № 2829, de 14 de dezembro de 2012. É importante ressaltar que os exames de triagem são realizados em cada Estado conforme a habilitação e as fases de implantação estabelecidas pelo Ministério da Saúde (Ministério de Saúde, 2013; SBTN, 2014).

1.4 DOENÇAS LISOSSÔMICAS DE DEPÓSITO (DLDs)

1.4.1 Definição, etiologia, manifestações clínicas

O conceito de doenças lisossômicas foi desenvolvido pelo pesquisador H. G. Hers a partir da descoberta dos lisossomos por Christian de Duve na década de 1950 (Ballabio and Gieselmann, 2009; Platt and Lachmann, 2009).

As doenças lisossômicas de depósito (DLDs) são um grupo de distúrbios genéticos, considerados raros quando avaliados individualmente, no entanto em conjunto apresentaram uma prevalência de 1: 7.700 indivíduos (Meikle *et al.*, 1999).

As DLDs são causadas principalmente por defeitos monogênicos, em genes que codificam enzimas lisossômicas que degradam macromoléculas como glicolipídeos, glicoproteínas e mucopolissacarídeos. Podem ser causadas também por defeitos genéticos em receptores lisossômicos, proteínas ativadoras e de membrana, ou em transportadores lisossômicos. Esses defeitos produzem um acúmulo lisossômico anormal e progressivo de substratos específicos, causando alterações estruturais e deterioração da função celular, que levam à manifestação clínica da doença (Platt and Lachmann, 2009; Hoffman *et al.*,2010; Wang *et al.*, 2011).

A tabela 2 mostra uma lista representativa destas doenças e algumas características, tais como: a enzima deficiente ou o defeito lisossômico, o substrato acumulado, o gene respectivo e o padrão de herança.

Tabela 2. Doenças lisossômicas de depósito*

Doença	Enzima deficiente ou defeito	Produto de depósito	Gene	Padrão de herança
Esfingolipidoses				
Farber	Ceramidase ácida	Ceramida	*ASAH*	AR
Fabry	α-Galactosidase A	Gb3	*GLA*	Ligado ao X
Gaucher	β-Glucocerebrosidase	Glicosilceramida	*GBA*	AR
GM1-gangliosidose	β-Galactosidase	GM1	*GLB1*	AR
GM2-gangliosidose B	β-hexosaminidase subunidade-α	GM2	*HEXA*	AR
GM2-gangliosidose O	β-Hexosaminidase subunidade -β	GM2	*HEXB*	AR
Krabbe	β-galactocerebrosidase	Galactosilceramida	*GALC*	AR
Leucodistrofia metacromática	Arilsulfatase A	Sulfatideos	*ARSA*	AR
Niemann-Pick tipos A e B	Esfingomielinase ácida	Esfingomielina	*SPMPD1*	AR
Wolman	Lipase ácida	Ésteres de colesterol, triglicerídeos	*LIPA*	AR
Doenças de depósito de glicogéneo				
Pompe	α-glucosidase ácida	glicogêneo	*GAA*	AR
Mucopolissacaridoses				
MPS I	α-L-iduronidase	Dermatan sulfato, Heparan sulfato	*IDUA*	AR
MPS II	Iduronato-2-sulfatase	Dermatan sulfato, Heparan sulfato	*IDS*	Ligado ao X
MPS IIIA	Heparan N-sulfatase	Heparan sulfato	*SGS*	AR
MPS IIIB	N-acetyl-a-glucosaminidase	Heparan sulfato	*NAGLU*	AR
MPS IIIC	α-Glucosaminida-acetil-CoA transferase	Heparan sulfato	*TMEM76, HGSNAT*	AR
MPS IIID	N-acetylglucosamina-6-sulfatase	Heparan sulfato	*GNS*	AR
MPS IV-A	N-acetilgalactosamina-6-sulfatase	Keratan sulfato Sulfato de condroitina	*GALNS*	AR
MPS IVB	β-galactosidase ácida	Keratan sulfato	*GLB1*	AR
MPS VI	Arilsulfatase B	Dermatan sulfato	*ARSB*	AR
Glucoproteinoses				
α-manosidose	α-manosidase	oligossacarídeos	*MAN2B1*	AR
Sialidose	Sialidase, α-neurominidase-1	oligossacarídeos	*NEU1*	AR
Multiple enzyme deficiencies				
Mucolipidose tipo II	N-acetil 1-glucosamina fosfotransferase, subunidades α/β	Lipideos e oligossacarídeos	*GNPTAB*	AR
Deficiência múltipla de sulfatases	Enzima geradora de formil-glicina	Sulfatídeos, mucopolissacarídeos	*SUMF1*	AR

Continua

Tabela 2. Doenças lisossômicas de depósito* (*Continua*)

Doença	Enzima deficiente ou defeito	Produto de depósito	Gene	Padrão de herança
Defeitos de transporte lisossômico				
Cistinose	Cistinosina	cistina	*CTNS*	AR
Salla	Sialina	Ácido siálico	*SLC17A5*	AR
Defeitos de tráfico lisossômico				
Niemann-Pick tipo C1	NPC1	Colesterol, fosfolipídeos, glicoesfingolipídios	*NPC1*	AR
Niemann-Pick tipo C2	NPC2	Colesterol, fosfolipidios, glicoesfingolipídios	*NPC2*	AR
Mucolipidose tipo IV	Mucolipina-1	Fosfolideos, glicoesfingolipídios, mucopolissacarídeos	*MCOLN1*	AR
Danon	LAMP2	Glicogênio	*LAMP2*	Ligado ao X
Lipofuscinoses ceróide neuronal (CLN)				
CLN1	PPT1, proteina de palmitoil tioesterase	SAPs	*CLN1* ou *PPT1*	AR
CLN2	TPP1, tripeptidil peptidase I	SCMAS	*CLN2* ou *TPP1*	AR
CLN3	CLN3	SCMAS	*CLN3*	AR
CLN4	CLN4	SCMAS	*DNAJC5*	Dominante
CLN5	CLN5	SCMAS	*CLN5*	AR
CLN6	CLN6	SCMAS	*CLN6*	AR
CLN8	CLN8	SCMAS	*CLN8*	AR
CLN10	Catepsina D	SAPs	*CTSD*	AR

*Trata-se de uma lista parcial de doenças lisossômicas de depósito. AR: Autossómica recessiva. Gb3: globotriaosilceramida, GM1 e GM2: gangliosídeos. LAMP2: Proteína 2 de Membrana Associada ao Lisossoma. MPS: mucopolissacaridose. NPC: proteína Niemann-Pick tipo C. SAPs: *sphinolip activator porteins*/ proteínas ativadoras esfingolipídicas; SCMAS: *subunit c mitochondrial ATP synthase*/subunidad c mitocondrial ATP sintetase. CLN: *neuronal ceroid lipofuscinosis*/proteína lipofuscinose ceróide neural.

Clinicamente, as DLDs são doenças heterogêneas e progressivas, caracterizadas por um comprometimento geralmente multissistêmico, que pode se manifestar na forma de dismorfias, organomegalias, alterações cardíacas, respiratórias e neurológicas (Hoffman *et al.*, 2010).

1.4.2 Diagnóstico das DLDs

Em pacientes sintomáticos, o diagnóstico das DLDs exige testes bioquímicos e/ou genéticos em sangue (usualmente), realizados em laboratórios especializados. Na maioria dos casos, o diagnóstico é feito após se demonstrar a deficiência da atividade da enzima

lisossômica específica para a doença em estudo. Nos casos de DLDs causadas por defeitos não enzimáticos, o diagnóstico necessita de um teste funcional específico para essa doença (Winchester, 2014). Outros procedimentos, dependendo da doença, podem incluir: o exame radiológico do esqueleto para casos de disostose multiplex (graves anormalidades no desenvolvimento da cartilagem e do osso), análise de depósitos em leucócitos e outras células, e avaliação de órgãos parenquimatosos. A urina pode também ser investigada para identificar, por exemplo, a presença de glicosaminoglicanos (GAGs) e oligossacarídeos anormais (Hoffman *et al.*, 2010). A análise genética é utilizada geralmente como exame confirmatório, e consiste na identificação de mutações causadoras da doença (Winchester, 2014).

1.4.3 Tratamento das DLDs

Embora não exista ainda cura para as doenças lisossômicas, diferentes opções terapêuticas têm surgido nestes últimos anos e muitas das DLDs têm, na atualidade, a disponibilidade de um ou mais tratamentos. O transplante de células-tronco hematopoiéticas (TCTH) e a terapia de reposição enzimática (TRE) foram aprovados para várias DLDs e encontram-se atualmente em uso clínico. Outras terapias surgiram nas últimas décadas e algumas já estão em uso comercial, enquanto outras se encontram em diferentes fases de ensaios clínicos. Dentre elas, a terapia com pequenas moléculas (terapia de redução do substrato – TRS, e as chaperonas farmacológicas) e a terapia gênica (Heese, 2008; Platt and Lachmann, 2009; Hoffman *et al.*, 2010; Pastores, 2010; Parenti *et al.*, 2013, 2015; Giugliani *et al.*, 2016; Gonzalez e Baldo, 2017) (Tabela 3).

Esses tratamentos mostraram beneficio em várias condições, melhorando as manifestações clínicas e a qualidade de vida.

1.4.4 Impacto da introdução precoce do tratamento para DLDs

Estudos têm demonstrado que o início precoce do tratamento melhora as manifestações clínicas das DLDs, seja retardando ou prevenindo importantes alterações patológicas (van Capelle *et al.*, 2008; McGill *et al.*, 2010; Anderson *et al.*, 2014; Aldenhoven *et al.*, 2015; Gabrielli *et al.*, 2016).

Tabela 3. Opções de tratamento para as DLDs

Tipo de tratamento	Doenças Em uso: Nome da doença (nome do tratamento disponível) Pesquisa: nome da doença	Efeito /Objetivo do tratamento
TCTH	Em uso: MPS I (Hurler). Proposto: MPS VI, MPS VII, Krabbe, Leucodistrofia Metacromática, Alfa-fucosidose, alfa-manosidose,Gaucher, Niemann-Pick tipo B.	Usado em condições em que há comprometimento do sistema nervoso central.
TRE Uso de enzimas recombinantes que substituem a enzima deficiente. Aprovada pela primeira vez para uso clínico em 1991 pela FDA (EUA).	Em uso: Gaucher tipo 1(Imiglucerase).	Reduz a hepatoesplenomegalia e as crises de dor. Melhora a anemia e trombocitopenia.
	Em uso: Fabry (beta-agalsidase)	Melhora os sintomas gastrointestinais e a dor, diminui a hipertrofia cardíaca e estabiliza a função renal.
	Em uso: Pompe infantil (Alfa-alglicosidase)	Melhora função muscular e reduz cardiomegalia.
	Em uso: Deficiência de Lipase Ácida (Sebelipase alfa)	Normaliza os níveis de colesterol e previne as complicações da doença, como a cirrose hepática.
	Em uso: MPS I (Laronidase)	Reduz a hepatoesplenomegalia; melhora a mobilidade articular e a doença respiratória. Diminui a excreção urinária de GAGs.
	Em uso: MPS II (Idursulfase)	Melhora a hepatoesplenomegalia, a doença cardiopulmonar e reduz a excreção urinária de GAGs.
	Em uso: MPS VI (Galsulfase)	Melhora a hepatoesplenomegalia, o movimento articular, a função cardiopulmonar e a dor. Reduz a excreção urinária de GAGs.
	Em uso: MPS IVA (Elosulfase alfa)	Melhora a caminhada de 6 minutos, os GAGs urinários.
	Em uso: MPS VII (Vestronidase alfa)	Melhora a caminhada de 6 minutos, os GAGs urinários.
Terapia com pequenas moléculas: TRS	Em uso: Gaucher, Niemann Pick tipo C (miglustat). Em uso: Gaucher (Eliglustat) Pesquisa: Fabry, GM2-gangliosidose de início tardio, Sandhoff, MPSs (genisteína)	Reduzir produção de material de depósito anormal. Tratamento oral.
Chaperonas	Pesquisa: Fabry, Gaucher tipo 1, Niemann-Pick tipo C, Pompe, GM1-gangliosidose GM2-gangliosidose, MPS IIIC, Batten.	Melhorar a função da enzima existente. Tratamento oral.
Terapia gênica	Pesquisa: MPS IIIA, MPS I, Leucodistrofia Metacromática, Sandhoff, Tay-Sachs	Inserir uma seqüência funcional do gene mutado para substituir a enzima defeituosa.

TCTH: transplante de células-tronco hematopoiéticas. TRE: terapia de reposição enzimática. TRS: terapia de redução do substrato. MPS: mucopolissacaridose. FDA: Food and Drug Administration. EUA: Estados Unidos da América. GAGs: glicosaminoglicanos.

Um desses estudos comparou a eficácia e os benefícios da TRE para MPS VI, N-acetilgalactosamina-4-sulfatase humana recombinante (rhASB), em dois irmãos (McGill *et al*., 2010). O irmão mais velho iniciou sintomas aos 14 meses de idade, apresentando macrocefalia, hepatoesplenomegalia e manifestações cardíacas. Ele iniciou o tratamento aos 3,6 anos de idade. Após 182 semanas de tratamento, observou-se melhora da mobilidade articular e diminuição/estabilização da patologia cardíaca e da escoliose. O irmão mais novo iniciou o tratamento com 8 semanas de idade, e após 182 semanas de tratamento, não desenvolveu escoliose e apresentava preservação do movimento das articulações, válvulas cardíacas e morfologia facial. No entanto, o efeito do tratamento foi nulo para a opacificação da córnea e algumas das alterações esqueléticas.

Outro estudo comparou os resultados da TRE, depois de 12 anos de seguimento, entre dois irmãos diagnosticados com a forma atenuada de MPS I (Gabrielli *et al*., 2016). O irmão mais novo, de 12 anos, tratado a partir dos 5 meses de idade, apresentou a aparência facial, a taxa de crescimento linear e o volume do fígado e do baço normais, e um grau mínimo de envolvimento da doença articular, vertebral e valvular (cardíaca) em comparação com sua irmã afetada, de 17 anos, que iniciou a terapia aos 5 anos de idade.

Assim, o tratamento pode prevenir alterações patológicas irreversíveis, ou minimizar significativamente as manifestações da doença, especialmente quando introduzidas precocemente; fato que ratifica a necessidade de um diagnóstico precoce, de preferência durante a fase assintomática da doença.

1.5 TRIAGEM NEONATAL DE DOENÇAS LISOSSÔMICAS

1.5.1 Tecnologia e métodos na TN de DLDs

Ao longo da última década, vários métodos e tecnologias foram desenvolvidos para a triagem neonatal de doenças lisossômicas (Tabela 4). A maioria são ensaios de alto rendimento, com capacidade de analisar simultaneamente (multiplex) a atividade enzimática relacionadas com 4 a 6 doenças, a partir de uma única amostra de sangue seco impregnado em papel filtro.

Tabela 4. Triagem Neonatal para DLDs no mundo

Pais	Doença triada	Técnica para a Triagem	Total de RN Triados	Total confirmados[a]	Frequência observada	Referência
Itália	Fabry	Fluorometria	37.134 (males)	12	1: 3.094	Spada *et al.*, 2006
	Pompe	Fluorometria	3.403	0		Paciotti *et al.*, 2012
	Gaucher	Fluorometria	3.403	1	1: 3.403	
	Fabry	Fluorometria	3.403	0		
	MPS I	Fluorometria	3.403	0		
Taiwan	Pompe	Fluorometria	132.538	4	1: 33.134	Chien *et al.*, 2008
	MPS I	Fluorometria	35.285	2	1: 17.642	Lin *et al.*,2013
	Pompe	Fluorometria	47.738	27	1: 17.546	Chiang *et al.*,2012
	Fabry	MS/MS	191.767	64	1: 2.996	Liao *et al.*, 2014
	Pompe	MS/MS	191.786	16	1: 11.987	
	Gaucher	MS/MS	103.134	3	1: 34.378	
	MPS I	MS/MS	60,473	0		
Áustria	Gaucher	ESI-MS[b]	34.736	2	1: 17.368	Mechtler *et al.*, 2012
	Pompe	ESI-MS[b]	34.736	4	1: 8.684	
	Fabry	ESI-MS[b]	34.736	9	1: 3.859	
	NP-A/B	ESI-MS[b]	34.736	0		
Hungria	Fabry	MS/MS[b]	40.024	3	1: 13.341	Wittmann *et al.*, 2012
	Gaucher	MS/MS[b]	40.024	3	1: 13.341	
	Pompe	MS/MS[b]	40.024	9	1: 4.447	
	NP-A/B	MS/MS[b]	40.024	2	1: 20.012	
EUA	Fabry	MS/MS[b]	108.905	7	1: 15.558[c]	Scott *et al.*, 2013
	Pompe	MS/MS[b]	111.544	4	1: 27.886	
	MPS I	MS/MS[b]	106.526	3	1: 35.509	
	Pompe	DMF	43.701	8	1: 5.463	Hopkins *et al.*, 2015
	Fabry	DMF	43.701	15	1: 2.913	
	Gaucher	DMF	43.701	1	1: 43.701	
	MPS I	DMF	43.701	3	1: 14567	
	Fabry	MS/MS[b]	42.391	3	1: 14.130	Elliott *et al.*, 2016
	Gaucher	MS/MS[b]	44.485	1	1: 44.485	
	Krabbe	MS/MS[b]	44.441	0		
	MPS I	MS/MS[b]	43.530	4	1: 10.882	
	NP-A/B	MS/MS[b]	44.432	1	1: 44.432	
	Pompe	MS/MS[b]	44.074	1	1: 44.074	

DMF: *digital microfluidics*. ESI-MS: *electrospray ionisation tandem mass spectrometry*. EUA: Estados Unidos da América. MS/MS: espectrometria de massas em tandem. MPS I: mucopolissacaridose tipo 1. NP-A/B: Niemann-Pick tipo A e B. RN: Recém-nascido. [a] Inclui só casos relatados no estudo como comfirmado. [b] Multiplex assay. [c] Informado como 1: 7.800 (em 54.800 RN meninos) em Scott *et al.*, 2013.

Uma das primeiras abordagens foi o ensaio fluorimétrico desenvolvido por Chamoles *et al.* (2001a), que utilizou substratos fluorogênicos (4-metilumbeliferona) para medir a atividade de várias enzimas lisossômicas. Essa abordagem permite a detecção de várias DLDs incluindo Fabry (alfa-galactosidase A), MPS I (alfa-L-iduronidase), Gaucher (glucocerebrosidase), Niemann-Pick (esfingomielinase ácida), Pompe (alfa-glicosidase ácida), MPS II (iduronato-2-sulfatase) e a MPS VI (arilsulfatase B) entre outras doenças (Chamoles *et al.*, 2001a, 2001b, 2001c, 2002, 2004).

Outra abordagem é a espectrometria de massa em tandem (MS/MS) que detecta vários produtos enzimáticos em uma análise multiplex. Foi usado para detectar as doenças de Fabry, Gaucher, Krabbe, MPS I, MPS II, MPS IVA, MPS VI, Niemann-Pick-A / B e Pompe (Li *et al.*, 2004a, 2004b, Blanchard *et al.*, 2008, Liao *et al.*, 2014, Kumar *et al.*, 2015).

Finalmente, a plataforma digital de microfluidos (DMF do inglês, *digital microfluidics*) é um sistema automático baseado no método fluorimétrico; porém que manipula líquidos como microgotículas separadas sob o controle de um software. Requer pequenas quantidades de amostra, que são analisadas em um chip localizado em cartucho descartável (Millington *et al.*, 2010). Essa abordagem foi utilizada para detectar simultâneamente as doenças de Pompe, Fabry, MPS I (Hurler), Gaucher e MPS II (Sista *et al.*, 2013; Hopkins *et al.*, 2015).

Todas essas abordagens foram direcionadas para a análise inicial (*first-tier*) da triagem e mostraram adequação para seu uso em programas de triagem neonatal em grande escala.

1.5.2 Estudos de Triagem Neonatal para DLDs No Mundo

O fato do que o tratamento específico foi desenvolvido para algumas doenças lisossômicas, e que a sua introdução precoce poderia ser vantajosa para melhorar os resultados no longo prazo, ao prevenir alterações patológicas irreversíveis, ou ao minimizar significativamente as manifestações da doença, fez que alguns países incluíssem a triagem neonatal de algumas doenças lisossômicas nos seus programas de triagem, seja como programas estabelecidos ou programas piloto. Na tabela 4 encontra-se uma lista de países onde a triagem neonatal para DLDs foi desenvolvida. A seguir, alguns exemplos da experiência nesses países.

Taiwan

O centro de triagem neonatal do Hospital Universitário Nacional de Taiwan (NTUH, do inglês, *National Taiwan University Hospital*) realizou um programa piloto de triagem

neonatal em grande escala entre outubro de 2005 e março de 2007 e confirmou quatro casos de doença de Pompe após a triagem de 132.538 recém-nascidos (Chien *et al.*, 2008). Esse e outro relatório indicam o estabelecimento de um programa de triagem neonatal para a doença de Pompe desde 2005.

Outro centro, a Fundação Chinesa da Saúde (CFOH, do inglês, *Chinese Foundation of Health*), responsável por um terço do programa de triagem nacional (60.000-70.000 casos), iniciou um programa de triagem neonatal em larga escala para a doença de Fabry em 2006, utilizando o método fluorimétrico e reportou a triagem em mais de 300.000 amostras de sangue em papel filtro para doenças de Pompe e Fabry. CFOH foi o primeiro centro de triagem neonatal a usar a tecnologia multiplex MS/MS para triagem em larga escala para doenças lisossômicas na Ásia desde 2010 e tem relatado um estudo piloto de triagem neonatal para doenças de Fabry, Pompe, Gaucher e MPS I (Liao *et al.*, 2014).

Áustria

Um estudo nacional prospectivo anônimo realizou a triagem para a doença de Gaucher, doença de Pompe, doença de Fabry, e doença de Niemann-Pick tipos A e B. As amostras de sangue seco de 34.736 recém-nascidos coletadas de janeiro a julho de 2010 foram analisadas por espectrometria de massa em tandem com ionização por electrospray e análises da mutação genética eram feitas nas amostras com suspeita de deficiência da enzima (Mechtler *et al.*, 2012). Um total de quinze casos foi confirmado. Deles dois foram casos para a doença de Gaucher, quatro casos para a doença de Pompe e nove casos para a doença de Fabry.

Estados Unidos da América (EUA)

Nos EUA, o Comitê Consultivo da Secretaria de Saúde e Serviços Humanos sobre Distúrbios Hereditários em Recém-Nascidos e crianças (SACHDNC, do inglês, *Secretary's Advisory Committee on Heritable Disorders in Newborns and Children*) é responsável para revisar evidências e realizar as recomendações sobre as condições propostas para serem adicionadas ao seu Painel de triagem uniforme recomendado (RUSP, do inglês, *Recommended Uniform Screening Panel*). No entanto, cada estado determina se seguirá ou não as recomendações. A doença de Pompe, MPS I, doença de Krabbe,

doenças de Niemann-Pick A e B e a doença de Fabry foram condições oficialmente propostas de serem incluídas nesse painel (Matern *et al.*, 2015,), sendo que, Pompe e MPS I já tiveram a recomendação aprovada (*Advisory Committee on Heritable Disorders in Newborns and Children*).

O estado de Nova Iorque faz a triagem para a doença de Krabbe desde 2006 (Heese, 2008). O estado de Missouri está realizando testes para cinco doenças lisossômicas (Fabry, Gaucher, Hurler, Krabbe e Pompe) desde 2013 (Missouri Department of Health &Senior Services; Hopkins *et al.*, 2015). Assim, a triagem está em andamento em alguns estados e em fase de planejamento ou implementação em vários outros.

1.5.3 TN de DLDs na América Latina

A maioria dos países da América Latina inclui a triagem neonatal em seus programas de saúde pública. No entanto, existem diferenças importantes entre eles, como o número de doenças triadas e a cobertura.

Em relação às doenças lisossômicas, não são conhecidos programas que tenham sido implementados a nível nacional. O Brasil é um dos poucos da região que desenvolveu e implementou um programa piloto de triagem neonatal, inicialmente para uma comunidade com alto risco para MPS VI, o que vem permitindo o atendimento precoce dos indivíduos afetados e o aconselhamento genético para o paciente e as famílias (Giugliani, 2012; Acosta *et al*., 2013).

1.4.5 DLDs candidatas para TN no Brasil

Breve descrição de DLDs com potencial para serem inclusas em programas de triagem neonatal.

Doença de Fabry

A doença de Fabry é um distúrbio do grupo das esfingolipidoses causado pela deficiência da enzima alfa-galactosidase A (GLA; EC 3.2.1.22), que acarreta o depósito de globotriaosilceramida (Futerman and van Meer, 2004). A doença é ligada ao cromossomo

X, sendo os homens os principais afetados, mas mulheres também podem ter manifestações clínicas.

Os sintomas podem aparecer na infância ou adolescência e incluem dor em queimação nas mãos e pés, hipoidrose, náuseas, dor abdominal, diarreia pós-prandial, e dificuldades escolares. Os sintomas progridem e usualmente desenvolve-se proteinúria, mais frequente nos homens afetados. Eventualmente, pode haver o desenvolvimento de insuficiência renal. Lesões em outros órgãos afetados também progridem, levando a manifestações cardíacas e cerebrovasculares (Mehta and Hughes, 2002), com redução da expectativa de vida.

A prevalência estimada da doença varia de 0,12 – 0,85 casos por 100.000 nascidos vivos (Kingma *et al*., 2015) até 2,5 por 100.000 (Zarate and Hopkin, 2008).

No Brasil, o tratamento disponível inclui a TRE endovenosa com betagalsidase e alfagalsidase, aprovadas pela ANVISA em 2006 e 2009, respectivamente (Sartori Junior *et al*., 2012).

Doença de Gaucher

A doença de Gaucher é uma esfingolipidose causada por deficiência de glucocerebrosidase também conhecida como beta-glucosidase ácida (GBA; EC 3.2.1.45), levando ao acúmulo de glicosilceramida (Futerman and van Meer, 2004). O padrão de herança é autossômico recessivo e a prevalência foi estimada em 0,25-1,75 casos por 100.000 nascidos vivos (Kingma *et al*.,2015).

Essa doença apresenta um espectro clínico que vai com grave e início precoce até formas mais leves e início tardio. A ausência de comprometimento neurológico define o tipo I, que é caracterizado por doença óssea, hepatoesplenomegalia, anemia e trombocitopenia, além de doenças pulmonares (Gene Reviews, Gaucher disease; Grabowski 2008). Já a presença de características neuropáticas define os tipos II (mais grave) e III (mais atenuado).

Os tratamentos disponíveis no Brasil e aprovados pela ANVISA incluem a TRE e TRS. A TRE endovenosa, com imiglucerase e alfavela glicerase, indicado para o tipo I e III da doença, e a TRS com miglustate, também indicado para o tipo I e III (Ministério da saúde, PCDT-Gaucher, 2014).

Doença de Pompe

A doença de Pompe é uma doença de depósito lisossômico rara, progressiva, com um padrão de herança autossômico recessivo. A prevalência estimada da doença de Pompe é de 0,17-2,66 casos por 100.000 nascidos vivos (Kingma *et al*., 2015). A deficiência parcial ou total da enzima ácido α-glucosidase (GAA; EC 3.2.1.20) ocasiona o armazenamento intralisossômico de glicogênio, o que conduz a dano celular, que é mais pronunciado nos músculos.

O espectro clínico varia de um fenótipo clássico infantil, rapidamente progressivo, para formas mais leves que se apresentam em crianças e adultos. Os primeiros sintomas em pacientes com a forma clássica infantil aparecem nos primeiros meses de vida e a hipertrofia cardíaca é uma característica fundamental. Pacientes não tratados morrem antes de um ano de idade. Pacientes com fenótipos não clássicos têm formas mais leves da doença, que progride mais lentamente. Eles comumente sofrem de fraqueza muscular de cinturas e/ou problemas respiratórios causados pela debilidade dos músculos respiratórios, incluindo o diafragma.

Durante muito tempo, não existia nenhum meio para deter a progressão da doença, mas a aprovação da terapia de reposição enzimática mudou substancialmente as perspectivas para os pacientes (van der Ploeg and Reuser, 2008; van der Meijden *et al.*, 2015). O tratamento disponível no Brasil inclui a TRE endovenosa com alfa-alglicosidase, aprovada pela ANVISA (2007).

Mucopolissacaridoses

As mucopolissacaridoses (MPS) são doenças de curso crônico e progressivo, causadas pela deficiência da atividade de enzimas lisossômicas necessárias para degradar

os glicosaminoglicanos (GAGs). O acúmulo lisossômico dos GAGs ocasiona disfunção celular e tissular em vários órgãos dos indivíduos afetados, o que leva ao comprometimento de múltiplos sistemas (Giugliani *et al*., 2010).

MPS I

A Mucopolissacaridose tipo I (MPS I) é uma doença multissistêmica progressiva causada pela deficiência de alfa-L-iduronidase (IDUA; EC 3.2.1.76), que ocasiona o depósito de dermatan e heparan sulfato (Futerman and van Meer, 2004). O padrão de herança é autossômico recessivo. A prevalência estimada para essa doença vai de 0,25 – 1,19 casos por 100.000 nascidos vivos (Kingma *et al*.,2015).

Os indivíduos afetados são classificados em um de três fenótipos: síndrome de Hurler, síndrome de Hurler-Scheie e síndrome de Scheie, mas não existem diferenças bioquímicas identificadas e os achados clínicos se sobrepõem. Os indivíduos afetados podem ser descritos como tendo MPS I grave ou atenuada, uma distinção que influencia as opções terapêuticas. As manifestações clínicas incluem: face grosseira, opacidade da córnea, hérnias inguinal e umbilical, hepatoesplenomegalia, disostose óssea múltipla, alterações cardíacas, respiratórias, articulares e neurológicas (Giugliani *et al*., 2010, Beck *et al*., 2014). O comprometimento cognitivo é observado nos três fenótipos, porém na forma grave (síndrome de Hurler) é mais profundo.

O tratamento disponível no Brasil inclui o TCTH e a TRE intravenosa com laronidase. Essa última aprovada pela ANVISA em 2005 (Giugliani *et al*., 2010).

MPS II

Mucopolissacaridose tipo II (MPS II), ou síndrome de Hunter, é causada pela deficiência da enzima iduronato-2-sulfatase (IDS, EC 3.1.6.13), o que leva ao depósito de dermatan sulfato e heparan sulfato (Futerman and van Meer, 2004). A doença está ligada ao cromossomo X, sendo a imensa maioria dos afetados do sexo masculino, sendo muito raro que mulheres apresentem alguma manifestação da doença. Estima-se uma prevalência de 0,10 para 1,09 casos por 100.000 nascidos vivos (Kingma *et al*.,2015).

A doença é multissistêmica e de início, gravidade e progressão variável. Os casos graves apresentam deterioração cognitiva progressiva, disfunção progressiva cardíaca e das vías aéreas, podendo levar à morte nas primeiras duas décadas da vida. Nos casos atenuados, o sistema nervoso central (SNC) está pouco ou quase não afetado e tem se observado casos de adultos com inteligência normal, mas o efeito nos outros sistemas pode ser como na forma grave. Outras manifestações são: baixa estatura; macrocefalia com ou sem hidrocefalia comunicante; macroglossia; voz áspera; perda auditiva condutiva e neurossensorial; hepatomegalia e/ou esplenomegalia; disostose múltipla e contraturas articulares incluindo anquilose da articulação temporomandibular; estenose do canal medular e síndrome do túnel do carpo (Giugliani *et al*., 2010; Scarpa *et al*., 2011).

O tratamento disponível no Brasil para MPS II é a TRE intravenosa com idursulfase, aprovada pela ANVISA em 2008 (Giugliani *et al*., 2010).

MPS VI

Mucopolissacaridose tipo VI, ou síndrome de Maroteaux–Lamy, é uma doença metabólica progressiva e rara, que tem um padrão de herança autossômico recessivo. A incidência estimada é de 0,05-2,51 casos por 100.000 nascidos vivos (Kingma *et al*.,2015). Porém, de maneira particular, em uma pequena cidade do Estado de Bahia, no Brasil, a MPS VI apresenta uma prevalência muito mais alta, estimada em 25 casos por 100.000 nascidos vivos (Vairo *et al*., 2015).

Essa doença é causada pela deficiência da enzima lisossômica N-acetil-galactosamina 4-sulfatase ou arilsulfatase B (ARSB, EC 3.1.6.12), que leva ao depósito de dermatan sulfato. Apresenta-se como um espectro clínico com diferentes progressões da doença e da gravidade da mesma, manifestando características faciais grosseiras, hepatoesplenomegalia, função pulmonar restritiva, anormalidades cardíacas e das articulações.

O tratamento disponível no Brasil é a TRE endovenosa com galsulfase, aprovada pela ANVISA em 2009.

1.6 DOENÇAS LISOSSÔMICAS DE DEPÓSITO (DLDs) NO BRASIL E O PAPEL DO SERVIÇO DE GENÉTICA DO HOSPITAL DE CLINICAS DE PORTO ALEGRE

Embora, na atualidade, dados sobre incidência e/ou prevalência das DLDs no Brasil sejam inexistentes, um estudo mostrou que as DLDs eram as doenças mais frequentes (59,8%) entre várias doenças metabólicas diagnosticadas (Coelho *et al.*, 1997). O estudo foi baseado em dados do Laboratório de Erros Inatos do Metabolismo (LEIM), do Serviço de Genética Médica (SGM) do Hospital de Clínicas de Porto Alegre (HCPA). Nesse estudo, pacientes com sinais e sintomas sugestivos para doenças do grupo de erros inatos do metabolismo (EIM), provenientes de diferentes regiões do Brasil, foram investigados entre janeiro de 1982 até abril de 1995. De 9.901 pacientes diagnosticados para doenças de EIM, 387 corresponderam a DLDs. Outro dado mais recente do mesmo laboratório sobre pacientes de alto risco investigados para EIM, entre os anos 1982 e 2014, indicou que 71% dos casos diagnosticados corresponderam a DLDs. (M Burin, comunicação pessoal).

O Serviço de Genética Médica (SGM) do Hospital de Clínicas de Porto Alegre (HCPA) é um centro de referência para o Brasil e para a América Latina para a atenção e acompanhamento dos pacientes com doenças genético-metabólicas, incluindo DLDs.

Desde 2004 é reconhecido como Centro Colaborador da OMS para o Desenvolvimento de Serviços de Genética Médica na América Latina e desde 2016 está habilitado como Serviço de Referência em Doenças Raras. Sua função é assistencial, educacional e de pesquisa. O serviço tem experiência particular na atenção, acompanhamento, monitorização, adequação do tratamento e aconselhamento genético para pacientes com Doenças Lisossômicas, com suspeita ou diagnóstico estabelecido. Dispõe também de diferentes laboratórios incluindo os de genética bioquímica para a investigação das doenças através de técnicas modernas que incluem ensaios enzimáticos específicos, espectrometria de massa em tandem e o laboratório de Genética Molecular (Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre).

Além disso, foram estabelecidas várias redes de informação e diagnóstico de diversos tipos de doenças genético-metabólicas, incluindo a “Rede EIM Brasil”, a “Rede MPS Brasil”, a ”Rede DLD Brasil” e a “Rede NPC Brasil”, que estão relacionadas com as doenças lisossômicas.

A Rede EIM Brasil, por exemplo, visa a produzir um mapeamento dos Erros Inatos do Metabolismo no Brasil, identificando as regiões de maior prevalência de situações específicas, informação essa que poderia ser utilizada para planejar ações dentro do Programa Nacional de Triagem Neonatal (Rede EIM Brasil).

1.7 PROTOCOLOS PARA A CONFIRMAÇÃO DIAGNÓSTICA DE DLDs EM CASOS POSITIVOS DE TRIAGEM NEONATAL

1.7.1 Definição de protocolo

Protocolos e algoritmos são utilizados para orientar os processos de decisão. Protocolo é definido no dicionário médico Dorland, como “plano detalhado e explícito de um experimento, procedimento ou teste”. E algoritmo refere-se a “um método gradativo de resolução de problemas ou tomada de decisões como a elaboração de um diagnóstico” (Dorland, 2004).

Na prática médica, os protocolos são também conhecidos como diretrizes clínicas (*clinical guidelines*). Protocolo clínico é definido no *Medical Subject Heading* (MeSH) do *National Center for Biotechnology Information* (NCBI) dos EUA como “os planos precisos e detalhados para o estudo de um problema médico ou biomédico e/ou planos para um regime de terapia”.

1.7.2 Protocolos para diagnóstico de Doenças Lisossômicas

A maior parte dos protocolos e algoritmos produzidos é para o diagnóstico a partir de suspeita clínica em casos sintomáticos. Os relatórios de protocolos ou algoritmos a partir de estudos de triagem neonatal, ou seja, a partir de casos assintomáticos são

escassos. Em relação a esses últimos, um estudo analisou os resultados de um programa de triagem neonatal em grande escala para a doença de Pompe, e estabeleceu um protocolo de diagnóstico para a forma mais grave da doença de Pompe, o tipo infantil, para promover um tratamento mais precoce e melhores resultados (Yang *et al.*, 2014). Outro estudo desenvolveu um algoritmo de triagem neonatal para a doença de Krabbe a partir da medição da atividade enzimática por espectrometria de massa em tandem e a definição de pontos de corte, com uma análise posterior de DNA (Orsini *et al.*, 2009).

Outro estudo mais abrangente desenvolveu orientações para a confirmação do diagnóstico e manejo clínico de indivíduos pré-sintomáticos com suspeita de um grupo de doenças lisossômicas. O estudo foi baseado na revisão de literatura em língua inglesa e discussões em um painel de consenso de um grupo internacional de especialistas em diagnóstico clínico e laboratorial, tratamento e manejo de triagem neonatal, e nos aspectos genéticos das doenças estudadas. Segundo esse relato a identificação de indivíduos pré-sintomáticos poderia ser feita por triagem neonatal, ou por teste baseado na família após a identificação do probando, ou por teste do portador em populações de risco. O relato incluiu as doenças de Fabry, Gaucher, Pompe, Krabbe e Niemann-Pick A/B, leucodistrofia metacromática, e mucopolissacaridose tipo I, II e VI (Wang *et al.*, 2011).

1.8 ESTUDOS DE CUSTO DE TRIAGEM NEONATAL PARA DOENÇAS LISOSSÔMICAS

Atualmente, estudos de custo de triagem neonatal para doenças lisossômicas são quase inexistentes. Na pesquisa da literatura foi encontrado um único estudo que avaliou o programa de triagem neonatal para a doença de Krabbe no Estado de Nova York (EUA). Essa avaliação informou que o custo anual total do programa a partir da perspectiva do Estado era de 750 652 dólares e o custo estimado da adição da triagem para a doença de Krabbe ao painel de triagem neonatal era de 2,5 dólares por criança triada (Salvenson, 2011).

O fato de haver carência de dados em relação ao custo da triagem neonatal para doenças lisossômicas foi também ressaltado num recente artigo de revisão sobre a triagem neonatal para doenças lisossômicas (Matern *et al.*, 2015). Esse relato também comentou

sobre a probabilidade de semelhança nos custos dos métodos utilizados na triagem primária com os custos de outras triagens neonatais já estabelecidas (Matern *et al.*, 2015).

Vale ressaltar que os estudos de custo fazem parte das avaliações de atividades de saúde, que incluem avaliações econômicas, que direcionam a tomada de decisão em relação a uma ou mais alternativas. Uma avaliação econômica define-se como a análise comparativa dos cursos alternativos de ação em relação a seus custos e consequências. Existem avaliações consideradas como parciais e completas. As avaliações econômicas parciais incluem: descrição de custos, análise de custos, descrição de custo-resultado, enquanto as avaliações econômicas completas incluem a análise de minimização de custo, a análise de custo-efetividade, a análise de custo-utilidade e a análise de custo-benefício (Drummond *et al.*, 1987).

CAPÍTULO 2. JUSTIFICATIVA

A Organização Mundial de Saúde (OMS) recomendou prioridades para a comunidade internacional para ajudar no estabelecimento e fortalecimento de programas nacionais para a prevenção e os cuidados de anomalias congênitas, onde também estão incluídas as doenças genéticas e metabólicas (WHO. Fact sheet N°370. Congenital Anomalies, 2016).

Os programas de triagem neonatal provaram ser intervenções importantes em saúde pública para a detecção precoce e a prevenção destas doenças. Sabe-se que, nesses casos, a introdução precoce de medidas terapêuticas pode evitar a progressão de diferentes tipos de deficiência e, assim, também a prevenir a morbidade e mortalidade, especialmente infantil.

Um grupo de doenças lisossômicas (MPS I, MPS II, MPS VI, Pompe, Fabry e Gaucher) são candidatas a ser incluídas nos programas de triagem neonatal porque, na atualidade, dispõe-se de alternativas terapêuticas aprovadas pelas autoridades de saúde e que têm trazido melhora clínica significativa. Contudo, para isso, é importante também o diagnóstico e a intervenção precoces.

No Brasil, o Programa Nacional de Triagem Neonatal visa à identificação de doenças genéticas e/ou congênitas em fase pré-sintomática, permitindo o tratamento precoce e o acompanhamento multidisciplinar dos pacientes. Além disso, desde 2014, foi instituída a Política Nacional de Atenção Integral às Pessoas com Doenças Raras no âmbito do Sistema Único de Saúde (SUS) (Portaria Nº 199, de 30 de janeiro de 2014).

Por outra parte, o Serviço de Genética Médica (SGM) do Hospital Clínicas de Porto Alegre (HCPA) é um dos centros de referência do país para o atendimento, manejo e acompanhamento de pacientes com doenças raras, que incluem as doenças lisossômicas de depósito.

Um estudo piloto de um programa de triagem neonatal está sendo desenvolvido no SGM do HCPA para doenças lisossômicas de depósito tratáveis. Os grupos de pesquisa já estão trabalhando para aperfeiçoar as técnicas bioquímicas e moleculares a serem utilizados neste estudo.

Junto com esse trabalho existem outros aspectos que fazem parte da possível implementação de um programa de triagem neonatal que precisarão também ser considerados. Isso significa, for exemplo, que será necessário determinar qual conduta deve ser tomada até chegar-se a uma confirmação diagnóstica da doença em casos que apresentem uma triagem inicial alterada.

A esse respeito, existem alguns poucos relatórios com orientações sobre os procedimentos a seguir após resultados iniciais anormais de uma triagem neonatal e, portanto, alguns algoritmos têm sido propostos para algumas doenças lisossômicas tratáveis. Essas propostas se baseiam em práticas realizadas em outros países, que podem não estar adequadas à realidade brasileira, tornando-se recomendável o estabelecimento de protocolos e algoritmos passíveis de serem usados neste país. Os resultados deste estudo serão importantes para orientar a possível implementação futura da triagem para DLDs no PNTN do Brasil.

CAPÍTULO 3. OBJETIVOS

3.1 Objetivo Geral

Desenvolver e avaliar protocolos e algoritmos para a avaliação com vistas ao diagnóstico confirmatório em casos que resultem alterados em um programa piloto de triagem neonatal para doenças lisossômicas tratáveis no Brasil e estimar o custo global de um programa de triagem neonatal para as doenças pesquisadas neste estudo, considerando sua possível implementação em larga escala.

3.2 Objetivos Específicos

- Coletar informações sobre protocolos para o diagnóstico confirmatório de casos que resultarão anormais na triagem neonatal para as doenças lisossômicas: Mucopolissacaridose I (MPS I), Mucopolissacaridose II (MPS II), Mucopolissacaridose VI (MPS VI), doença de Fabry, doença de Gaucher e doença de Pompe.

- Aplicar os protocolos de diagnóstico confirmatório na investigação de casos que apresentarão resultados iniciais anormais em um estudo de triagem neonatal para doenças lisossômicas a ser realizado no Brasil.

- Desenvolver um protocolo para o diagnóstico confirmatório de casos que resultarão anormais nos programas de triagem neonatal para Mucopolissacaridose I (MPS I), considerando seu uso potencial no Brasil e em outros países.

- Estimar os custos financeiros globais da triagem neonatal para as doenças lisossômicas pesquisadas, considerando o custo do programa de triagem e a estrutura do protocolo do diagnóstico confirmatório.

CAPÍTULO 4

Newborn Screening for Lysosomal Storage Diseases: Protocols for confirmation of diagnosis in cases screened positives

Artigo a ser submetido para publicação

Newborn Screening for Lysosomal Storage Diseases: Protocols for confirmation of diagnosis in cases screened positives

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ABSTRACT

Lysosomal storage diseases (LSDs) are genetic disorders that cause progressive and multisystemic manifestations. Since treatment is available for some of these disorders and its early introduction seems to be more beneficial to improve clinical outcomes, inclusion of these diseases in newborn screening (NBS) programs is increasingly being considered in various countries. For that, different aspects will also need consideration; as the follow-up process of cases screened positive in NBS studies for LSDs. Therefore, in this study, we report strategies for the confirmatory diagnosis of those cases that screened positive in NBS for six treatable diseases, which are Fabry disease, Gaucher disease, Pompe Disease, Mucopolysaccharidosis (MPS) I, MPS II and MPS VI.

Keywords: Lysosomal storage diseases, newborn screening, diagnosis confirmation.

INTRODUCTION

Lysosomal storage diseases (LSD) are metabolic disorders caused mainly by monogenic defects in genes encoding lysosomal enzymes that degrade macromolecules such as glycolipids, glycoproteins and mucopolysaccharides. These defects produce abnormal and progressive lysosomal accumulation of specific substrates, leading to structural changes and deterioration of the cellular function. Clinically, LSDs are heterogeneous disorders, characterized by progressive manifestations that may include neurological, skeletal, organomegaly and dysmorphic features.[1-4] In spite of that, their therapeutic options have increasingly evolved these last years and many of the LSDs currently have treatment available that consist of enzyme replacement, transplantation of hematopoietic cells and substrate synthesis inhibitors.[1,2] Treatment may prevent irreversible pathological changes, or significantly minimize disease manifestations, especially when early introduced. This fact clearly raises the need for early diagnosis, preferably during the asymptomatic stage of the disease, which supports the development of newborn screening (NBS) for some of these disorders and the establishment of mass screening programs.

In fact, studies on NBS for some LSDs have been already developed in different countries. For instance, Taiwan established a NBS program for Pompe disease since 2005[5] and in the United States of America (USA), Pompe disease, Mucopolysaccharidosis I (MPS I), Krabbe disease, Niemann-Pick disease types A and B and Fabry disease were officially proposed for inclusion to the recommended uniform screening panel (RUSP) [6]. Pompe disease and MPS I achieved approval (Advisory Committee on Heritable Disorders in Newborns and Children).

Once the NBS for LSDs is developed and the screening methods established, the next step should be to confirm or not the diagnosis in cases identified by these NBS programs. Then, some questions will arise on how the abnormal results after initial screening testing should be managed and which further procedures for confirmatory diagnosis would be necessary. Therefore, this study aimed to collect information on protocols and/or procedures performed for the confirmatory diagnosis of cases that will result altered in newborn screening for six treatable LSDs, such are the Mucopolyssacharidosis I (MPS I), Mucopolysaccharidosis II (MPS II), Mucopolysaccharidosis VI (MPS VI), Fabry disease, Gaucher disease and Pompe disease.

METHODS

Literature searching was conducted initially to identify guidelines or protocols addressing diagnosis confirmation procedures for cases screened positive at NBS for LSDs. Search was performed at electronic databases including Cochrane Database of Systematic Reviews (CDSR), Database of Abstract of Reviews of Effectiveness (DARE), Central Register of Controlled Trials (CENTRAL), PubMed Clinical Queries for Systematic Reviews. Terms used for searching included: #1 Lysosomal Storage Diseases", #2 newborn screening or "neonatal screening, #3 guidelines, #4 algorithms". Where data were lacking, systematic searches were conducted at Medline/PubMed database to identify population studies on NBS for LSDs to collect and assess the procedures they carried out for confirming the diagnosis. MeSH terms and text terms were used and included: #1 newborn screening, #2 mucopolysaccharidosis I, #3 mucopolysaccharidosis II, #4 mucopolysaccharidosis VI, #5 Fabry disease, #6 Gaucher disease, #7 Pompe disease, #8 Diagnosis, #9 infant,newborn, #10 asymptomatic disease, #11 guideline and #12 protocol. Last date of search: July, 2016.

RESULTS

Few studies about protocols for confirmatory diagnosis in asymptomatic newborns with abnormal results after NBS for Fabry diasease, Gaucher diasease, Pompe disease, MPS I, MPS II and MPS VI were found. One of these studies was a guideline addressing the confirmatory diagnosis for these LDSs in presymptomatic newborns[3]. Other studies were pilot studies of NBS for most of these LSDs.

Based on the above data, we summarized the process for confirmatory diagnosis reported in these studies, for cases screened positive in NBS for Fabry disease, Gaucher disease, Pompe disease and MPS I (Table 1-4, figures 1-4). We did not find NBS studies for MPS II, and MPS VI, and then the process for confirmatory diagnosis, showed in figures 5 and 6, was based mainly on the guideline of Wang *et al.*, (2011). Below, we briefly describe the diagnosis confirmation process for each disease targeted in this study.

Newborns in suspicion of Fabry disease, identified through a NBS, could be further investigated in two ways, depending on the NBS platform (Table 1, figure 1). When a fluorometric platform is used for NBS, the confirmatory diagnosis is performed by the

measurement of the α-galactosidase A (GLA) activity in leukocytes and plasma, analyzed also by a fluorometric assay. If deficient activity for GLA is confirmed, then *GLA* gene sequencing is performed from whole blood to identify the disease-causing variants. When mass spectrometry in tandem (MS/MS) platform is used for NBS, then *GLA* gene sequencing in DBS is directly performed in dried blood spot (DBS) or whole blood samples.

For cases in suspicion of Gaucher disease identified by NBS, the investigation could be done by the *GBA* (acid β-glucosidase) gene sequencing in DBS or whole blood, when the initial screening testing was a MS/MS. When a fluorometric screening is performed, the confirmatory diagnosis includes the determination of the GBA enzyme activity in leukocytes by a flourometric analysis (Table 2, figure 2). If deficient activity for GBA is confirmed, *GBA* gene sequencing is performed to identify pathogenic variants. The measurement of chitotriosidase activity in plasma by fluorometry should be also added to the analysis. It is expected a higher activity for chitotriosidase in comparison to its normal range to complement diagnosis confirmation.

Cases identified through NBS for Pompe disease, can be further investigated for diagnosis confirmation following two strategies (Table 3, figure 3). One is to perform directly *GAA* (acid α-glucosidase) gene sequencing in DBS or whole blood to identify disease-causing variants. The other strategy is to measure the GAA activity by a fluorometric enzyme assay in leukocytes. If GAA activity is deficient, then, direct sequencing of the *GAA* gene should be performed.

For cases in suspicion of MPS I, identified by NBS using a fluorometric platform, the process of confirmatory diagnosis start by the urinary GAGs analysis, and the α-L-iduronidase (IDUA) activity measurement by fluorometry in leukocytes. If IDUA activity is deficient, the *IDUA* gene sequencing is analyzed from blood samples, to identify disease-causing variants. Abnormal results of the urinary GAGs analysis support the diagnosis of an IDUA deficiency. Same as for the other diseases, when a MS/MS platform is used for screening, then confirmatory diagnosis is usually performed directly by *IDUA* gene sequencing. (Table 4, figure 4)

Regarding the confirmatory diagnosis of positive cases in NBS for MPS II, as above mentioned, no reports on NBS for MPS II were found including follow-up information.

Thus, according with the Wang *et al*. (2011) guidelines, the confirmatory diagnosis of these cases can be performed by measuring simultaneously the iduronate 2-sulfatase (IDS) activity in plasma, and both the arylsulfatase A (ARSA) and arylsulfatase B (ARSB) activities in leukocytes. If only deficient IDS activity is confirmed, *IDS* gene sequencing should be performed to determine MPS II-causing variants and finally confirm the disease (Figure 5). Urinary GAGs analysis can be also performed initially in the investigation of suspected cases for MPS II, since any MPSs may present abnormal excretion of GAGs and/or electrophoretic pattern for dermatan and heparan sulfates [15]. ARSA and ARSB activities are determined to exclude the possibility of a multiple sulfatase deficiency (MSD) that will be considered when both enzymes are deficient.

For cases in suspicion of MPS VI, identified by NBS, the confirmatory diagnosis is based in the enzyme activity of ARSA and ARSB measured simultaneously. A normal ARSA and a deficient ARSB, confirm the diagnosis for MPS VI that will need the *ARSB* gene sequencing to identify the disease-causing variants for further diagnosis confirmation (Figure 6). Same as for other cases in suspicion for MPS, urinary GAGs analysis should be performed to initiate the investigation of these cases[15].

DISCUSSION

NBS for LSDs is receiving an increased interest for inclusion in regional or national NBS programs around the world. This will demand from the programs to consider not only the screening phase but also the follow-up for cases with abnormal results. In this study, the process of confirmatory diagnosis for cases identified by NBS for six treatable LSDs was collected and revised from available reports. Such studies showed that the strategies for diagnosis confirmation of Fabry disease, Gaucher disease, Pompe disease and MPS I may depend on the platform used for the initial NBS testing. That is, if a fluorometry-based platform for NBS is used, then an enzymatic assay by fluorometry in leukocytes is performed, as well as sequencing the respective enzyme-coding gene. If a MS/MS platform is used, direct gene sequencing is usually performed in DBS, without a previous enzymatic analysis for diagnosis confirmation. For newborns that will need confirmation diagnosis for MPS II and MPS VI, even though no reports on this diagnostic process from NBS

studies exist, it is suggested to perform the enzymatic assay of at least two sulfatases and the molecular analysis.

Although this is a simplified manner to show the diagnosis confirmation of these cases identified by NBS, the decision on which strategy for diagnosis confirmation will be determined by each NBS program. For that, it is worthy to remark that this process for confirmatory diagnosis should be performed in a specialized center with expertise in metabolic and genetic disorders, since results interpretation may have some challenges in some cases, because all diagnostic procedures to date have some limitations. For instance, abnormal enzyme activity caused by pseudodeficiency[16] of alleles and molecular analysis of gene that codifies the defective lysosomal enzyme may present variants with unknown significance (VOUS) that will require further evaluations.

Details on the technical procedures and reference values for the analyses suggested for diagnosis confirmation of cases identified by NBS were not included in this report, since most of them are methods used for confirmatory diagnosis of clinically suspected cases, whose laboratorial procedure may vary in each specialized center.

Conclusion

We reported the procedures for diagnosis confirmation of cases identified by NBS for six LSDs that included Fabry disease, Gaucher disease, Pompe disease and three types of Mucopolysaccharidosis (MPS I, MPS II and MS VI). The procedures for confirmatory diagnosis were collected from reported studies and consist mainly of enzyme activity determination and gene analysis of the respective defective gene product.

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Table 1. Strategies for diagnosis confirmation after the positive results of NBS for Fabry disease.

References[7-13]	Spada et al., 2006	Paciotti et al., 2012	Mechtler et al., 2012	Wittmann et al., 2012	Scott et al., 2013	Liao et al., 2014	Hopkins et al., 2015
SCREENING							
First DBS, method	Fluorometry	Fluorometry	ESI-MS	MS/MS	MS/MS	MS/MS	DMF
Enzyme activity, cut off	≤20% of normal mean. SD (7.7 ±3.1 U/ml whole blood)	< 25% of median (median 33.1 nmol/h/ml)	2.8 µmol/L/h	2.5 µmol/L/h	≤1.9 µmol//L blood (≤19% daily mean)	1,5 µmol/L/h	5.5 µmol/L/h
Retest at same DBS	No	No	Yes	Yes	No	No	Yes
Enzyme activity, cut off	n.a	n.a	2.8 µmol/L/h		n.a.	n.a.	Not reported
Recall for second sample	Yes	Yes	Not Reported	Not Reported	No	379 (0.20%)	No
Enzyme activity, cut off	≤1.5 U/ml	< 25% of median (median 33.1 nmol/h/ml)			n.a.	1 µmol/L/h	
CONFIRMATION							
Enzyme assay	Plasma GLA activity ≤ 2.5 U/ml.Mean,SD=14.3 ±3.2 U/ml.	GLA in leukocytes	Not performed	Not performed	Not performed	not performed	Performed
Sample	Whole blood	Whole blood	n.a.	n.a.	n.a.	n.a.	Not reported
Technique	Fluorometry	Fluorometry	n.a.	n.a.	n.a.	n.a.	Not reported
Molecular analysis	*GLA* gene sequencing[a]	No needed.	*GLA* gene sequencing	*GLA* gene sequencing	*GLA* gene sequencing	*GLA* gene sequencing	Performed
Sample	Whole blood		DBS	DBS	DBS	whole blood	Not reported

DBS: dried blood spot. DMF: Digital microfluidics. ESI-MS: electrospray ionisation tandem mass spectrometry. GLA: α-Galactosidase A. MS/MS: tandem mass spectrometry. n.a.: not apply. SD: standard deviation. [a]Gene sequencing: 7 exons, intron/exon boundaries. No needed: enzyme assay result normal.

Table 2. Strategies for diagnosis confirmation after the positive results of NBS for Gaucher disease.

References[8-10, 12,13]	Paciotti et al., 2012	Mechtler et al., 2012	Wittmann et al., 2012	Liao et al., 2014	Hopkins et al., 2015
SCREENING					
First DBS, method	Fluorometry	ESI-MS	MS/MS	MS/MS	DMF
Enzyme activity, cut off	< 25% of median (Median=24.5nmol/h/ml)	4.0 μmol/L/h	3.5 μmol/L/h	7,5 (μmol/L/h)	4.5 μmol/L/h
Retest at same DBS	no	yes	yes	no	yes
Enzyme activity, cut off	n.a.	4.0 μmol/L/h		n.a.	Not reported
Recall for second DBS	yes			Yes	No
Enzyme activity, cut off	< 25% of median (24.5 nmol/h/ml)			7,5 μmol/L/h	n.a.
CONFIRMATION					
Enzyme assay	GBA: 2.81 nmol/h/mg protein−normal mean: 14.1±5 nmol/h/mg protein	no	no	no	Performed
Sample	Leukocytes (Blood)				Not reported
Technique	Fluorometry				Not reported
Molecular analysis	*GBA* gene sequencing	*GBA* gene sequencing	*GBA* gene sequencing	*GBA* gene sequencing	Performed
Sample	Leukocytes (blood)	DBS	DBS	whole blood	Not reported
Other	Chitotriosidase (Plasma, Fluorometry)80 nmol/h/ml (Reference range:15–1251 nmol/h/ml)				

DBS: dried blood spot. DMF: Digital microfluidics. ESI-MS: electrospray ionisation tandem mass spectrometry. GBA: acid β-glucosidase. MS/MS: tandem mass spectrometry. n.a.:not apply.

Table 3. Strategies for diagnosis confirmation after the positive results of NBS for Pompe disease.

References[5, 8-13]	Chien et al., 2008	Paciotti et al., 2012	Mechtler et al., 2012	Wittmann et al., 2012	Scott et al., 2013	Liao et al., 2014	Hopkins et al., 2015
SCREENING							
First DBS	Fluorometry	Fluorometry	ESI-MS	MS/MS	MS/MS	MS/MS	DMF
Enzyme activity, cut off	GAA activity < 55% of mean	GAA activity, cut off < 35% of median (median=25 nmol/h/ml blood).	GAA activity cut off=2.0 µmol/L/h	GAA activity, cut off=3.0 µmol/L/h	Cut off=≤15% daily mean or ≤2.6 µmol//L blood.	Cutoff = 1.6 µmol/L/h	Cut off:8.0 µmol/L/h
Retest in same DBS	Yes[a] Fluorometry	No	Yes[a]	No	No	Yes[a]	Yes
Enzyme activity, cut off	GAA and NAG. If GAA activity < 25% of mean and NAG/GAA > 25	n.a	2.0 µmol/L/h	n.a.	n.a.		
Recall for 2nd DBS	Yes	Yes	no	no	no	Yes	no
Enzyme activity, cut off	GAA, tGAA, and NAG. If GAA activity < 8% of mean and % tGAA inhibition > 80% and NAG/GAA > 60	GAA activity, cut off < 35% of median (median=25 nmol/h/ml blood).	n.a.	n.a.	n.a.	GAA activity cutoff= 1µmol/L/h	n.a.
CONFIRMATION							
Enzyme assay	GAA activity. <5% of normal mean.	GAA activity	No	No	No	No	Performed
Sample	mononuclear blood cell (purified lymphocytes).	leukocytes	n.a.	n.a.	n.a.	n.a.	Not reported
Technique	Fluorometry	Fluorometry					Not reported
Molecular analysis	Not performed	Not performed (no cases confirmed at enzyme assay)	*GAA* gene sequencing	*GAA* gene sequencing	*GAA* gene sequencing	*GAA* gene sequencing	Performed
Sample			DBS	DBS	DBS	Whole blood	Not reported
Other	Physical examination, cardiologic evaluations[b], blood CK, CK myocardial band.					In confirmation center.	

CK: creatine kinase. DBS: dried blood spot. DMF: Digital microfluidics. ESI-MS: electrospray ionisation tandem mass spectrometry. GAA: acid α-glucosidase. MS/MS: tandem mass spectrometry. NAG: total neutral glucosidase activity. tGAA: total GAA. n.a.: not apply. [a]Duplicates. [b]Electrocardiograms, chest radiography.

Table 4. Strategies for diagnosis confirmation after the positive results of NBS for MPS I

References[8,14,11,13]	Paciotti et al., 2012	Lin et al., 2013	Scott et al., 2013	Hopkins et al., 2015
SCREENING				
First DBS, method	Fluorometry	Fluorometry	MS/MS	DMF
Enzyme activity, cut off	< 25% of median (8.2 nmol/h/ml)	≤19.82 µmol/L Blood/20h;	≤1.15 µmol//L blood; ≤32% daily mean	4.0 µmol/L/h
Retest at same DBS	no	yes	not performed	yes
Enzyme activity, cut off	n.a.	≤9.03 µmol/L Blood/20h	n.a.	Not reported
Recall for second sample	yes	yes	not performed	No
Enzyme activity, cut off	< 25% of median (8.2 nmol/h/ml)	≤9.03 µmol/L Blood/20h	n.a.	n.a.
CONFIRMATION				
Enzyme assay	IDUA activity	IDUA activity: <5% of normal range	Not performed	Performed.
Sample	Leukocytes (Blood)	Whole blood	n.a.	Not reported
Technique	Fluorometry	Fluorometry	n.a.	Not reported
Molecular analysis	No needed.	*IDUA* gene sequencing	*IDUA* gene sequencing	Performed
Sample		Whole blood	DBS	Not reported
Other		Urinary GAGs quantification: normal DMB/CRE ratio (44.6 ± 23.7 mg/mmol creatinine). 2D-electrophoresis: DS pattern, slight HS pattern.		

DBS: dried blood spot. DMF: Digital microfluidics. DMB/CRE: dimethylmethylene blue/creatinine. DS: dermatan sulfate. ESI-MS: electrospray ionisation tandem mass spectrometry. GAGs: glycosaminoglycans. HS: heparan sulfate. IDUA: α-L-iduronidase. MS/MS: tandem mass spectrometry. 2D: two-dimensional. n.a.: not apply. No needed: enzyme assay result normal.

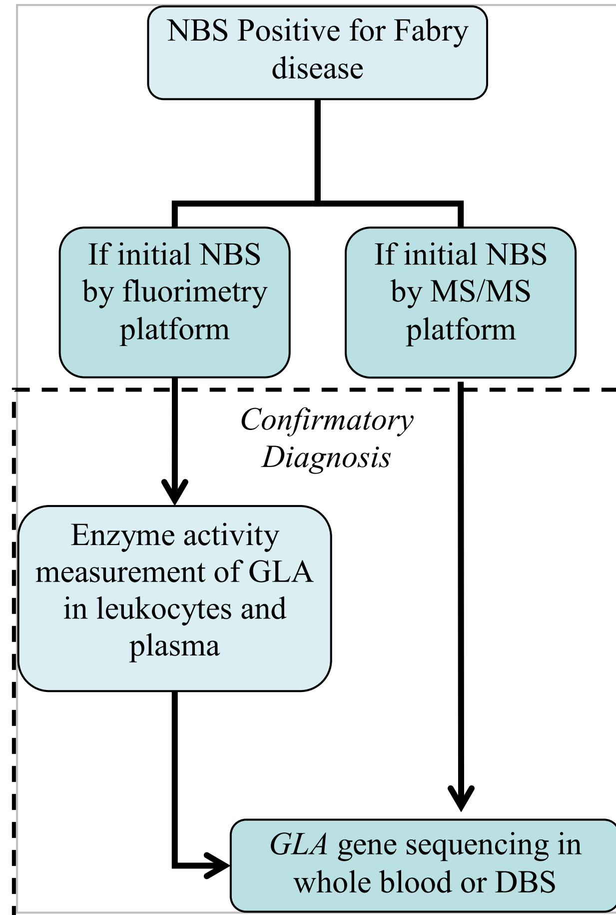

Figure 1.

Algorithm showing the strategies for diagnosis confirmation of Fabry disease after an abnormal result at the initial NBS for Fabry disease. The procedures for diagnosis are shown into the square with dashed lines. DBS: dried blood spots. GLA: α-galactosidase A. MS/MS: tandem mass spectrometry. NBS: newborn screening.

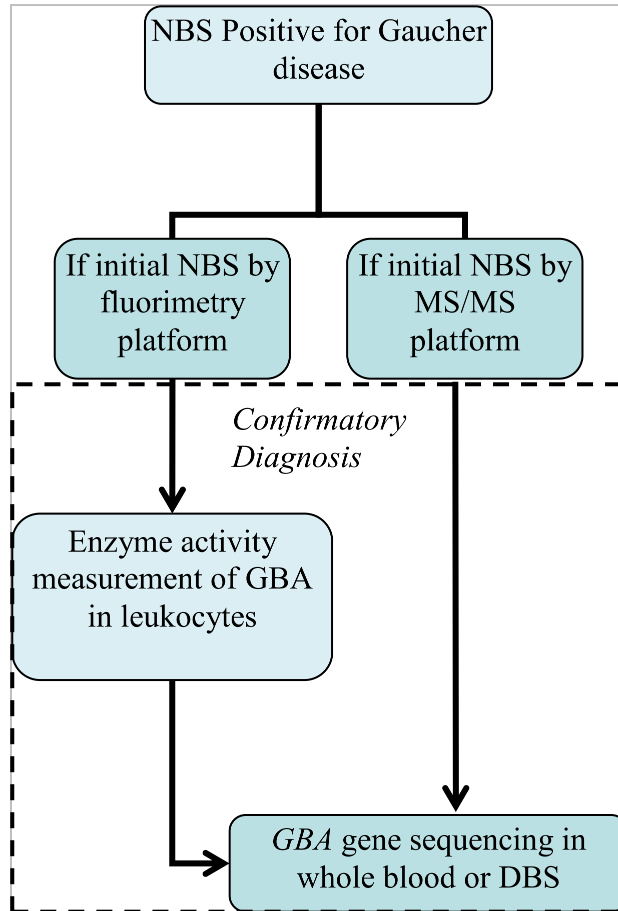

Figure 2.

Algorithm showing the strategies for diagnosis confirmation of Gaucher disease after an abnormal result at the initial NBS for Gaucher disease. The procedures for diagnosis are shown into the square with dashed lines. DBS: dried blood spots. GBA: acid β–glucosidase or glucocerebrosidase. MS/MS: tandem mass spectrometry. NBS: newborn screening.

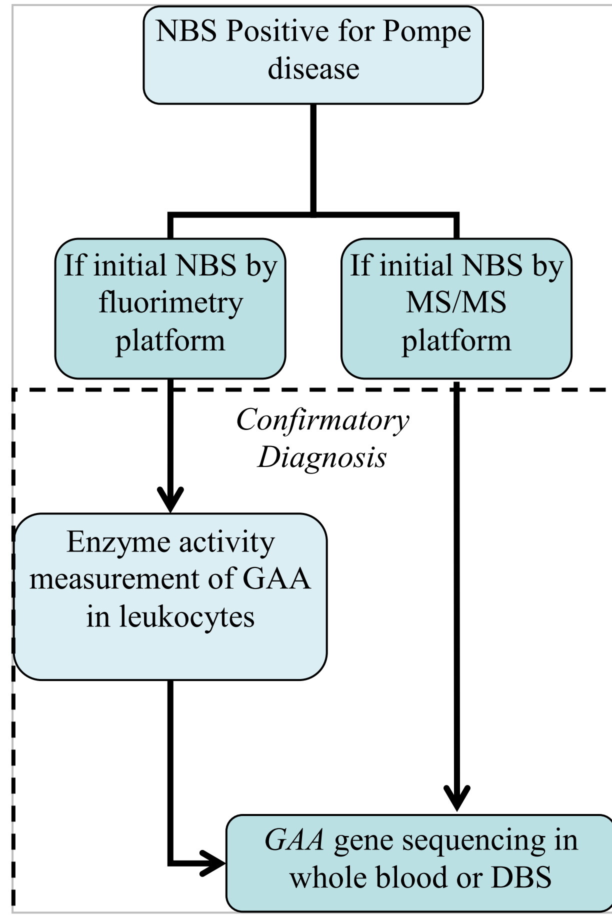

Figure 3.

Algorithm showing the strategies for diagnosis confirmation of Pompe disease after an abnormal result at the initial NBS for Pompe disease. The procedures for diagnosis are shown into the square with dashed lines. DBS: dried blood spots. GAA: acid α–glucosidase. MS/MS: tandem mass spectrometry. NBS: newborn screening.

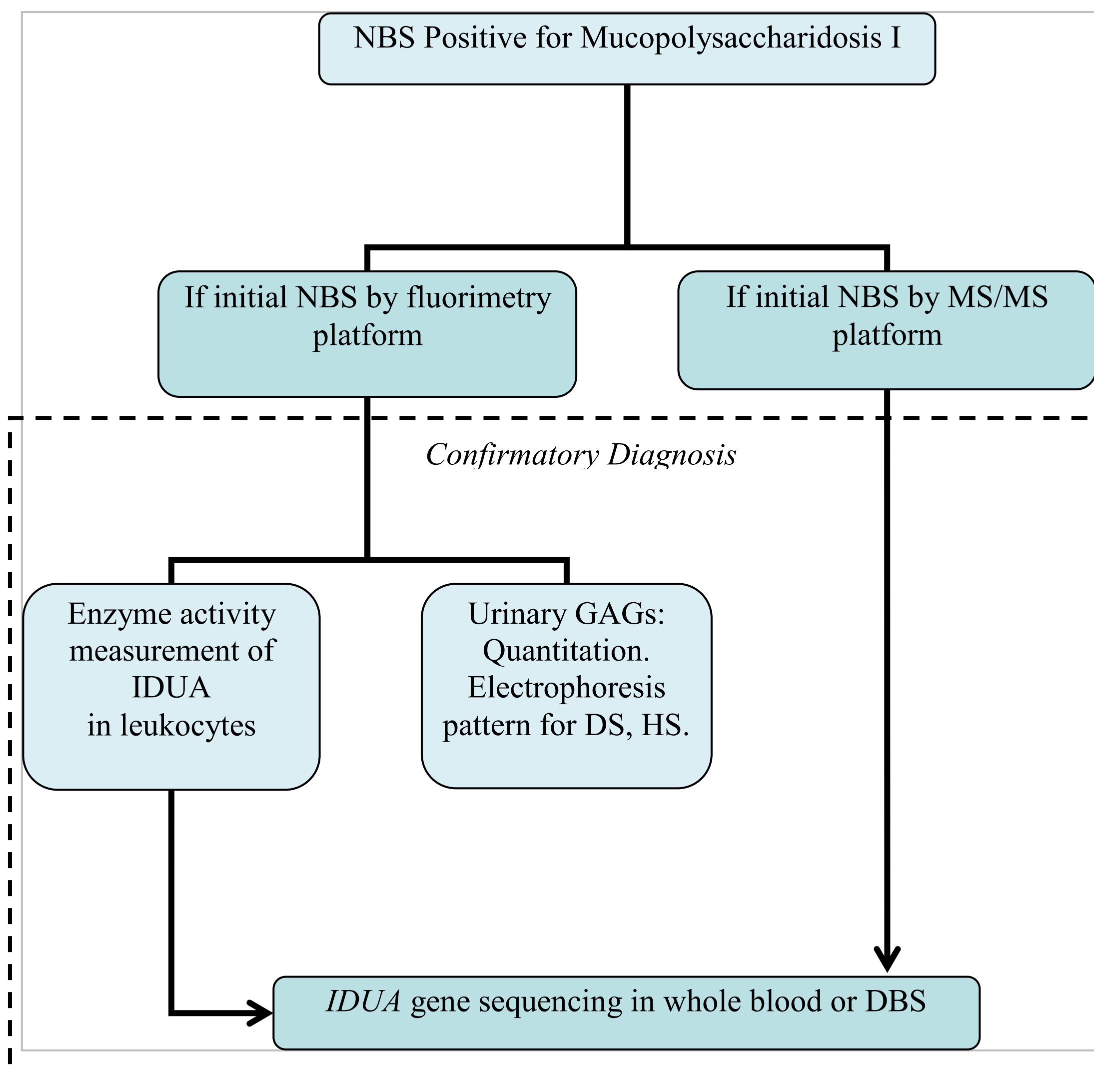

Figure 4.
Algorithm showing the strategies for diagnosis confirmation of Mucopolysaccharidosis I (MPS I) after an abnormal result at the initial NBS for MPS I. The procedures for diagnosis are shown into the square with dashed lines. DBS: dried blood spots. DS: dermatan sulfate. GAGs: glycosaminoglycans. HS: heparan sulfate. IDUA: α–L-iduronidase. MS/MS: tandem mass spectrometry. NBS: newborn screening.

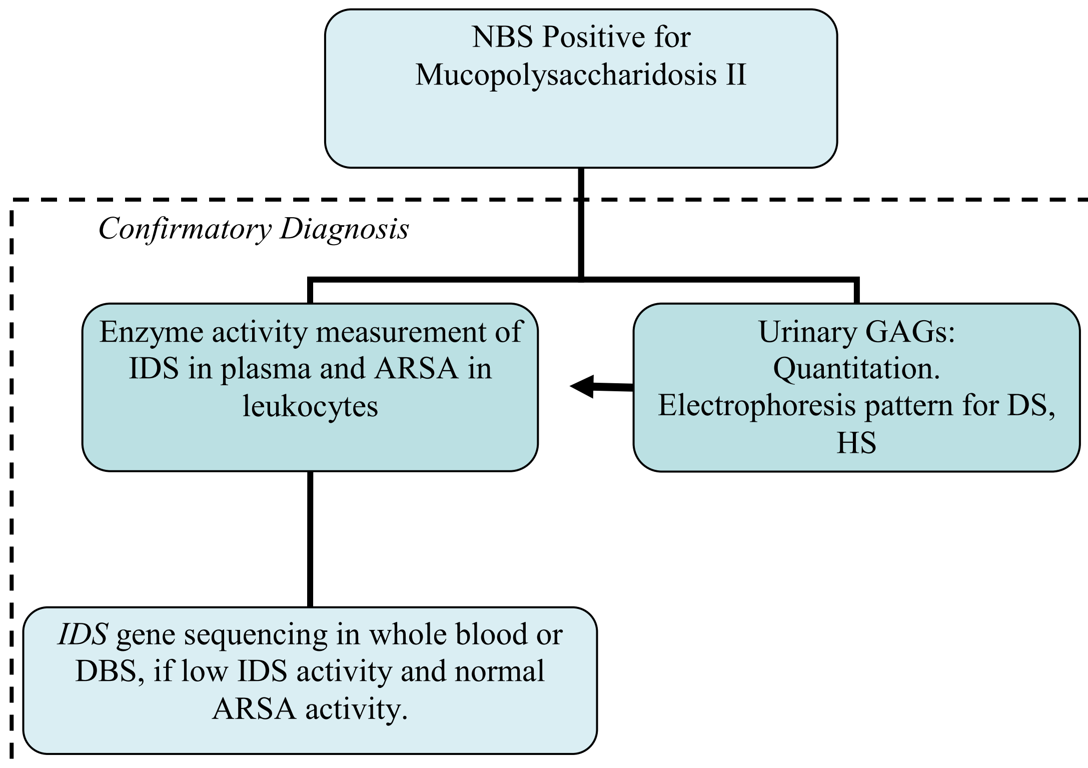

Figure 5.
Algorithm showing the strategies for diagnosis confirmation of Mucopolysaccharidosis II (MPS II) after an abnormal result at the initial NBS for MPS II. The procedures for diagnosis are shown into the square with dashed lines. ARSA: arylsulfatase A. DBS: dried blood spots. DS: dermatan sulfate. GAGs: glycosaminoglycans. HS: heparan sulfate. IDS: iduronate 2-sulfatase. NBS: newborn screening.

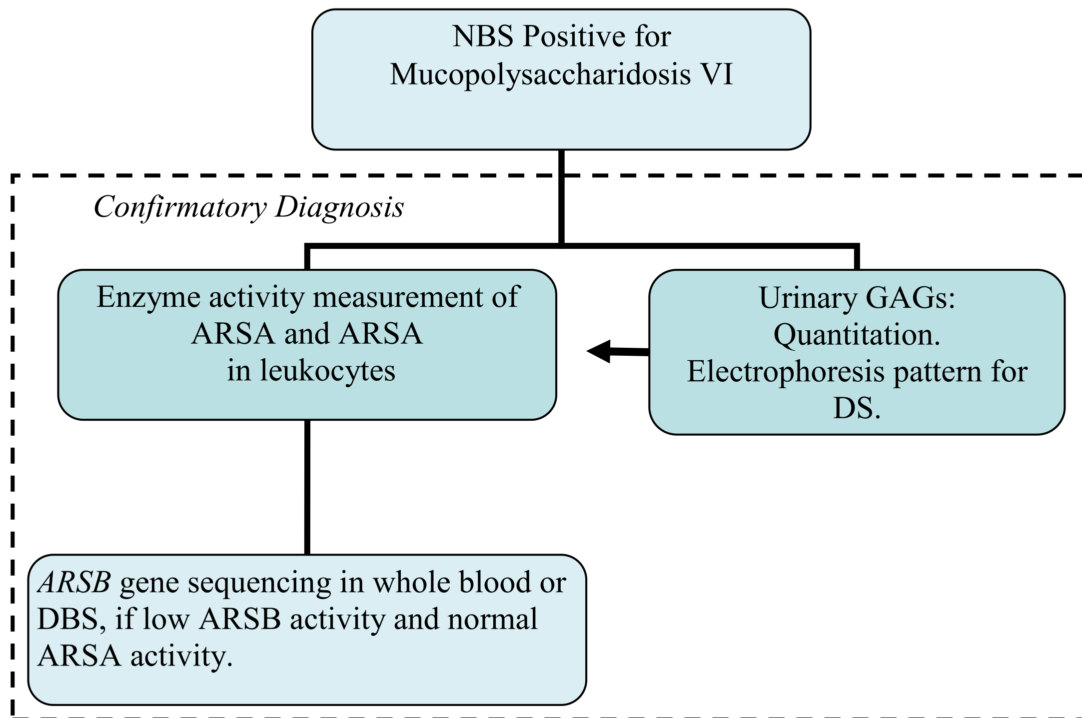

Figure 6.
Algorithm showing the strategies for diagnosis confirmation of Mucopolysaccharidosis VI (MPS VI) after an abnormal result at the initial NBS for MPS VI. The procedures for diagnosis are shown into the square with dashed lines. ARSA: arylsulfatase A. ARSB: arylsulfatase B. DBS: dried blood spots. DS: dermatan sulfate. GAGs: glycosaminoglycans. NBS: newborn screening.

CAPÍTULO 5

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

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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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A B S T R A C T

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 Mother: p.[Gly576Ser; Glu689Lys]		Father: c.1205G > A 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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CAPÍTULO 6

Development and Evaluation of a Protocol for Diagnosis Confirmation of Cases with Abnormal Newborn Screening Results for Mucopolysaccharidosis I

Artigo a ser submetido para publicação

Development and Evaluation of a Protocol for Diagnosis Confirmation of Cases with Abnormal Newborn Screening Results for Mucopolysaccharidosis I

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ABSTRACT

Introduction: Mucopolysaccharidosis I (MPS I) is a genetic disorder caused by deficient activity of the lysosomal enzyme alpha-L-iduronidase (IDUA) that leads to intracellular accumulation of the glycosaminoglycans (GAGs), namely dermatan and heparan sulfates. MPS I has been proposed for inclusion in early detection programs, such as newborn screening (NBS), because of treatment availability that reduces a number of the disease symptoms, especially when introduced early. While several studies have established techniques suitable for NBS of Lysosomal storage diseases (LSDs), including MPS I, and mass screening studies are underway in some NBS programs, little has been discussed on which further procedures should be performed. Therefore, we aimed to develop a protocol for the confirmatory diagnosis of MPS I in those cases that resulted positive at the initial testing in NBS programs for MPS I. **Methods:** We developed a protocol based on the WHO Handbook for Guideline Development and The Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach. Procedures included systematic search of literature at various electronic databases. Relevant evidence was selected, synthesized and evaluated, to then formulate the recommendations. **Results:** Based on evidence, confirmatory diagnosis of cases with abnormal results in NBS programs for MPS I should be based on IDUA activity, urinary GAGs and IDUA gene analyses. GRADE evidence profiles were created based on these analyses that guided the formulation of recommendations. Overall, evidence was evaluated as of moderate quality. Lack of randomized trials and systematic reviews were observed as limitations of evidence in the study. **Conclusion**: We provided an evidence-based protocol intended to facilitate decision-making during the confirmatory diagnosis process in newborns likely to be asymptomatic, who will result screened-positive in NBS programs for MPS-I.

Keywords: Newborn screening; Mucopolysaccharidosis I; Diagnosis confirmation; Protocol

INTRODUCTION

Mucopolysaccharidosis type I (MPS I) is an inherited metabolic disorder, belonging to the group of lysosomal storage diseases (LSDs). It has an autosomal recessive pattern of inheritance and its prevalence ranges from 0.25 to 1.33 cases per 100,000 live births (Kingma *et al.*, 2015). MPS I is caused by the alpha-L-iduronidase (IDUA) deficiency, mainly due to defects in the *IDUA* gene that lead to intracellular accumulation of the glycosaminoglycans (GAGs) dermatan and heparan sulfate (Futerman and van Meer, 2004). This progressive and multisystemic disorder includes among its clinical manifestations coarse facial features, corneal clouding, hepatosplenomegaly, hernias, skeletal and articular abnormalities, cardiac valve abnormalities and cognitive impairment (Beck *et al.*, 2014, Clarke 2002). The age of onset and degree of severity of these manifestations is variable among the three MPS I phenotypes: Hurler, Hurler-Scheie and Scheie syndromes, also known as the severe, intermediate and attenuated forms, respectively. This phenotypic distinction influences the management and selection of the therapeutic strategy, because hematopoietic stem cell transplantation (HSCT) will be preferred as the standard of care for patients with the severe form and early disease onset; and the enzyme-replacement therapy (ERT) with laronidase (human recombinant α-L-iduronidase, Aldurazyme) mainly for patients with the attenuated forms (Muenzer *et al.*, 2009, Giugliani *et al.*, 2010). Evaluations of these treatments have shown that clinical improvements were greater when treatment was early introduced (Aldenhoven *et al.*, 2015; Al-Sannaa *et al.*, 2015; Gabrielli *et al.*, 2016). This fact has led to consider the need for a timely and early diagnosis, and consequently the proposal of MPS I for inclusion in early detection programs, such as newborn screening (NBS).

Currently, several studies have established techniques suitable for NBS of MPS I. These are mainly multiplexed approaches for the screening of selected LSDs that uses fluorometry and tandem mass spectrometry platforms (Chamoles *et al*., 2001; Blanchard *et* al., 2008; Metz *et al*., 2011; Sista *et al*., 2013; Gelb *et al.*,2015).

While several reports show established screening approaches, suitable for NBS programs for LSDs, and mass screening studies are ongoing in various countries (Paciotti *et al.*, 2012; Lin SP *et al*., 2013; Scott *et al*., 2013; Liao *et al*., 2014; Hopkins *et al*., 2015; Elliott *et al.*, 2016), little has been discussed on further procedures, such as the process for

confirmatory diagnosis in newborns with an abnormal result at the initial testing of NBS for MPS I and what should be taken in consideration.

In clinical settings, systematically developed statements included in protocols or guidelines, help to assist practitioner in decisions about appropriate health care for specific clinical circumstances (Eccles *et al*., 2012). Therefore, in this study, we aimed to develop a protocol for the confirmatory diagnosis of cases that will result altered at the NBS programs for MPS I, considering its potential future use in Brazil and other countries.

METHODS

Protocol development was based on the WHO Handbook for Guideline Development (World Health Organization, 2012). The steps carried out are described as follow.

1) Planning, scoping, needs assessment

The scope of this protocol was defined as a protocol intended for NBS programs and related health care centers. Its target population will include asymptomatic newborns with an initial abnormal newborn screening result for MPS I that needs further diagnosis evaluation for confirmation or not. For this protocol surrogates outcomes were selected instead of patient important outcomes (e.g. morbidity and mortality) because of lack of data. Then, considering that these are diagnostic tests, accuracy testing measures (true positive/true negative rates) were used and thus, sensitivity and specificity were calculated when data were available.

For the protocol scope, three main priority topics were selected after conducting a preliminary search of literature at Cochrane Database of Systematic Reviews (CDSR), Database of Abstract of Reviews of Effectiveness (DARE), Central Register of Controlled Trials (CENTRAL), PubMed Clinical Queries for Systematic Reviews and other existing reports.

2) Key question formulation (PICO approach)

Questions were formulated based on the priority topics using the PICO approach (PICO: Population, Intervention, Comparison, Outcome). They were used for the search strategy

and guided the retrieval of studies and further steps until the recommendations formulations.

3) Evidence retrieval and synthesis process

Literature was first searched at CDSR and PubMed Clinical Queries to identify relevant systematic reviews for each priority topic. Where data were lacking, systematic searches were conducted at Medline/PubMed, EMBASE, LILACS, Global Health Library and WHOLIS. The key terms used for the search strategy are shown in Table 1. No restriction was applied for the study design of retrieved reports. Language was not limited either, but English, Spanish, Portuguese and French were mainly considered. Last date of search was July 11, 2017.

Next, studies were selected based on the approaches suggested in Cochrane Reviews for selecting studies (Higgins and Green, 2011). EndNote was used to manage the references retrieved from the systematic search. After removing duplicates, 141, 142 and 115 titles and abstracts were examined for topic 1, 2 and 3, respectively. From them, 48, 33 and 52 studies were selected for full-text examination based on eligibility criteria (Table 1). At this step, studies that met at least one criterion for that topic were included.

Then, full-texts were examined and data were collected using a checklist form that contained items of interest for each topic (Table S1). Final decision on inclusion of studies for data collection and synthesis was based on more specific eligibility criteria showed in Table 1. Where no reports were found, expert opinion was consulted. Then, relevant evidence was selected and synthesized.

4) Evidence quality assessment by GRADE

Collected evidence was evaluated by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach. Evidence profiles for each question related to the main topics were generated by using the online tool GRADEpro GDT. For each question, data on test accuracy, either sensitivity or specificity, was appraised as outcomes. Quality of evidence was graded as very low, low, moderate and high, based on the study design, and five factors that were: risk of bias, indirectness (with respect to the target population), inconsistency (of the results across the available studies), imprecision

and publication bias. Quality was defined as the extent to which one could be confident that an estimate of evidence is correct.

5) Development of recommendations.

Recommendations were formulated based on the questions for each topic. The strength of the recommendation - strong, conditional and “not recommended” - was determined by considering the quality of evidence, the balance between benefits and harm, and variability of values and preferences.

RESULTS

A protocol for the confirmatory diagnosis of cases with abnormal results at the initial testing in NBS for MPS I was developed and below presented. Its content includes recommendations derived of three topics identified as priority for the diagnosis confirmation process of MPS I. These are: the IDUA activity analysis, the urinary GAGs analysis and the *IDUA* gene analysis. These analyses were mainly assessed to determine its accuracy as diagnostic testing in consideration to its potential use for diagnosis confirmation procedure in newborns screened positive at NBS programs for MPS I. Questions that guided the identification and formulation of these recommendations are shown in table 2, together with the GRADE evidence profiles.

Target Audience

These recommendations are intended primarily for use by healthcare providers in newborn screening programs and children health programs.

DIAGNOSIS CONFIRMATION FOR MPS I: RECOMMENDATIONS

Topic 1: IDUA activity analysis

The enzymatic analysis of α-L-iduronidase activity by a fluorometric assay, in peripheral blood leukocytes, using 4-methylumbelliferyl (4-MU) α-L-iduronide as substrate, is considered the gold standard procedure for diagnosis confirmation of MPS I in clinically symptomatic, suspected cases (Hopwood *et al*., 1979). Regarding asymptomatic newborns,

a pilot study on NBS for MPS I reported to confirm the diagnosis of those abnormal screening results by using this analysis (Lin *et al*., 2013).

Usually, detection of no enzyme activity is linked with Hurler phenotype, and residual activity is associated with the intermediate and attenuated phenotypes. However, this technique does not differentiate among the three phenotypes and the carrier status (Wang et al., 2011).

Recommendation 1*:* IDUA activity measured by fluorometry in leukocytes should be used to confirm the diagnosis of MPS I in newborns that screened positive in the NBS program for MPS I. (Quality of evidence: moderate. Strong recommendation)

Evidence and summary of findings

In order to determine the diagnosis accuracy of the measurement of IDUA activity by a fluorometric assay and to establish mainly its suitability for newborns, we searched and identified initially 8 studies from 141 that met these criteria. All were then excluded since enzymatic assays were for screening purpose and not diagnostic (Wang *et al.*, 2005; Campos *et al*., 2013; Sista *et al.*, 2013a, 2013b; Hopkins *et al*., 2015; Johnson *et al.*, 2015; Matern *et al.*, 2015; Millington *et al.*, 2017).
Since studies on both newborns and this specific assay seem to be scarce, then, studies on diagnosis accuracy of the fluorometric assay, regardless of age or symptomatic status of the studied population, were evaluated. Of 48 studies selected for data collection, only two showed data for assessing the procedure accuracy. These two studies (Hopwood *et al*. 1979; Manami *et al*. 1980) were case-control studies that compared α-L-iduronidase activities assayed with two different artificial substrates: 4-MU α-L-iduronide and Phenyl-α-L-iduronide. Both assays were performed in leukocytes whole cell homogenates from affected patients and family members (parents, siblings) with Hurler and Scheie syndrome. The assay using 4-MU α-L-iduronide showed a higher sensitivity, lower incubation time and a simpler assay system. Then, this was considered suitable and useful for diagnosis of IDUA deficiency in MPS I suspected cases. It was also observed that cases with Hurler phenotype showed undetected enzyme activity, and Scheie phenotype some residual activity. However, this is not uniformly observed and this technique does not differentiate among the three phenotypes.

Regarding the reference range values, they differed among studies. Each study has its own range and mean value determined for its population. Therefore, this should be also taken in consideration.

Recommendation 2. IDUA activity measured by fluorometry should not be used to determine heterozygote status, due to overlapping of IDUA activity values with control subjects. (Quality of evidence: moderate. Strong recommendation)

Evidence and summary of findings

IDUA activity values of MPS I's obligate heterozygotes and control subjects overlap. Observational studies that determined the IDUA activity in parents (obligate heterozygotes) of affected MPS I individuals showed that some of them had values into the range observed in healthy control subjects. The overlapping was evident either for obligated heterozygotes of affected patient with Hurler syndrome or Scheie syndrome. No overlapping was observed between affected cases and heterozygotes. The studies measured the enzyme activity in blood leukocytes samples by fluorometry and used 4-MU as substrate (Hopwood *et al*., 1979; Manami *et al*., 1980, Tsvetkova *et al*., 1991; Mandelli *et al*., 2001).

Recommendation 3. IDUA activity measured by fluorometry in DBS samples should be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I, when leukocytes or fibroblast samples are unavailable. (Quality of evidence: moderate. Conditional recommendation)

Evidence and summary of findings

Seven observational studies were identified in which the IDUA activity was analyzed in DBS samples by a fluorometric method for the diagnosis of MPS I (Civallero *et al*., 2006; Muller *et al*., 2010, 2011; Campos *et al*., 2013, 2014; Cobos *et al*., 2014; Abdi *et al*., 2015). Five of them showed that this approach discriminated the enzyme activities from affected and normal cases, with a sensitivity and specificity of 100% (Civallero *et al*., 2006; Muller *et al*., 2011; Campos *et al*., 2013; Cobos *et al*, 2014; Abdi *et al*., 2015). These studies measured the IDUA activity from patients that had the diagnosis of MPS I previously confirmed by a fluorometric approach in leukocytes and compared them to the

enzyme activities of controls mainly adults. Two studies also evaluated the IDUA activity from unaffected neonates, one showed a statistical difference with the activities of adult controls, although a variable overlapping of activities was also observed in both studies (Civallero *et al*, 2006; Campos *et al*., 2013). The fluorometric method used for most of these studies was a modification from the study of Chamoles *et al*. (2001), and the substrate was the 4-MU-iduronide.

About heterozygote status, it was observed that the values of IDUA activity measured in obligate heterozygotes of MPS I cases overlap into the range of healthy controls (Civallero *et al*., 2006; Campos *et al*., 2013, 2014). About other concerns for the assay, it was reported that the enzyme IDUA might be less stable in DBS than other enzymes tested (Cobos *et al*., 2014). Likewise, a correction factor related to creatinine or protein concentration is suggested to be incorporated to this approach to improve the quantitative analysis (Civallero *et al*., 2006).

Topic 2: Urinary GAGs analysis

The determination of urinary GAGs concentration by quantitative method and the identification of the urinary GAG's type by electrophoresis are both procedures performed as preliminary step in the diagnosis of clinically suspected MPS I cases (Winchester, 2014). An elevated concentration of GAGs in urine is indicative of any MPSs, but its normality does not exclude the diagnosis. The electrophoresis is a qualitative method that helps to identify the presence of dermatan sulfate and heparan sulfate; excreted in MPS I affected cases. One study of NBS for MPS I reported to use these approaches as part of a set of tests that also included enzymatic and molecular assays to confirm the diagnosis of newborns with positive screening result (Lin *et al*., 2013).

Recommendation 4. Total urinary GAGs quantitation should be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I. (Quality of evidence: moderate. Strong recommendation)

Evidence and summary of findings

Quantitation of urinary GAGs was performed as one of the first steps in the process of diagnosis of MPSs, which included the MPS I. The only study that we identified among

several studies on urinary GAGs quantitation allows us to evaluate its test accuracy on diagnosis. Piraud *et al*. (1993) measured the urinary GAGs in 2,000 clinically suspected patients for MPSs and 746 non-affected individuals. Of the 43 MPS I patients, 42 showed an increased excretion of total GAGs that gives a sensitivity of 97% for this approach. The remaining patient had a normal excretion. The report also remarked that urinary GAGs excretion decrease with age in healthy subjects, for that reason comparison of results should be age-related. The approach used the 1,9-dimethylmethylene blue (DMB) solution.

Recommendation 5. Urinary GAGs electrophoresis should be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I. (Quality of evidence: moderate. Strong recommendation)

Evidence and summary of findings

One study, the same above reported, identified the expected electrophoretic patterns for 41 of 43 patients with MPS I. The 43 patients were after confirmed with the diagnosis of MPS I by enzymatic assays in leukocytes. Then, two MPS I cases do not showed the expected electrophoretic patterns. One showed a normal GAGs excretion and the other could not be confirmed by enzyme assay. The approach to identify the GAGs consisted of a monodimensional electrophoresis and it was estimated to have a sensitivity of 95%. (Piraud *et al*., 1993)

Topic 3: *IDUA* gene analysis

Molecular analysis of the *IDUA* gene is the preferred strategy for additional confirmatory diagnosis of MPS I, usually performed after a biochemical diagnosis, in both symptomatic and asymptomatic individuals. The analysis is mainly performed by direct sequencing from DNA extracted of peripheral blood leukocytes. It includes the 14 exons, exon-intron boundaries and the 5’ untranslated region (UTR) of the *IDUA* gene. Diagnosis of MPS I is usually confirmed when 2 known pathogenic variants are identified, in *trans* conformation.

Recommendation 6. *IDUA* gene analysis by direct sequencing should be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I. (Quality of evidence: high. Strong recommendation).

Evidence and summary of findings

Several studies, including NBS studies, showed that direct sequencing was used as main analysis for molecular diagnosis of MPS I cases. We identified thirteen studies from which we evaluated the accuracy of this diagnostic test. The studies were mainly on mutational analysis in patients diagnosed clinically and biochemically as MPS I. Different populations from around the world were analyzed. Considering all the studies, a total of 292 MPS I patients, including the phenotypes Hurler, Hurler/Scheie and Scheie syndrome, were analyzed. Of them, 259 patients had their diagnosis also confirmed by this molecular approach and the remaining 32 cases were genotyped as heterozygotes (one disease-causing variant), or reported as no variant identified. From these results, we estimated a sensitivity of 89%. All these studies performed the sequencing in automatic sequencers and the analysis included the14 exons and respective intron/exon boundaries of the *IDUA* gene. The type of variants identified included: missense mutations, nonsense mutations, deletions of variable length, insertions, splice site mutations. The p.W402X and p.Q70X were the most frequent variants identified in all populations, but heterogeneity was also the most common feature observed in all the studies.(Beesley *et al*., 2001; Venturi *et al*., 2002; Matte *et al*., 2003; Lee *et al*., 2004; Laradi *et al*., 2005; Vazna *et al*., 2009; Bertola *et al*., 2011; Chkioua *et al*., 2011; Pollard *et al*., 2013; Pineda *et al*., 2014; Sánchez-Suárez *et al*., 2014; Atçeken *et al*., 2016; Kwak *et al*., 2016).

Recommendation 7. Whole blood sample should be used for *IDUA* gene analysis to diagnose MPS I in newborn screened positive in a NBS for MPS I. (Quality of evidence: moderate. Strong recommendation).

Evidence and summary of findings

Peripheral whole blood has been the most used sample for the molecular analysis of *IDUA* gene. We identified twelve studies that reported the use of whole blood samples either for direct sequencing or PCR-RFLP method (Alif *et al*., 1999; Matte *et al*., 2003; Laradi *et al*., 2005; Vazna *et al*., 2009; Chkioua *et al*., 2011; Prommajan *et al*., 2011; Viana *et al*., 2011; Pollard *et al*., 2013; Pineda *et al*., 2014; Sánchez Suárez *et al*., 2014; Atçeken *et al*., 2016; Kwak *et al*., 2016). We estimated a sensitivity of 90% for the molecular approaches when

used whole blood samples after considering that 123 out of 137 previously confirmed MPS I cases had its diagnosis also confirmed by this molecular analysis.

Recommendation 8. DBS sample should be used for *IDUA* gene analysis to diagnose MPS I in newborn screened positive in a NBS for MPS I (Quality of evidence: Low. Conditional recommendation)

Evidence and summary of findings

DBS samples have been also considered for the molecular analysis of *IDUA* gene. Two studies were identified using this type of sample (Scott *et al.*, 2013; Elliott *et al.*, 2016). These were NBS studies for MPS I, from which, cases with low enzyme activity at the screening phase were sent for genotyping. Of 15 cases, seven had it diagnosis confirmed as affected, and the remaining cases were genotyped as heterozygotes or wild type.

All the above recommendations were based on best evidence available, but the lack of systematic reviews and randomized control trials, and the few studies in newborns were common limitations to collect evidence data.

Nevertheless, as observed in studies on NBS for MPS I (Paciotti *et al.*, 2012; Lin *et al.*, 2013; Scott *et al.*, 2013), diagnosis confirmation of cases screened positive usually include the IDUA activity, urinary GAGs analysis and *IDUA* gene analysis. The strategies may only differ according to the initial screening approach (Fig.1). Studies using a mass spectrometry (MS/MS) approach for screening have directly refer the abnormal screening results for *IDUA* gene analysis. Studies using a fluorometric-based approach for screening have opted for the enzyme assay in leukocytes first, to after, sequencing the *IDUA* gene.

DISCUSSION

We developed a protocol for the confirmatory diagnosis of MPS I in newborns that will have an abnormal screening result at NBS programs. The included recommendations are related to three main procedures, which are analyses of the IDUA activity, urinary GAGs and the *IDUA* gene, identified through a systematically search of evidence.

These are well-known procedures for the evaluation of clinically suspected cases of MPS I (Giugliani *et al*, 2010; Winchester, 2014), usually diagnosed at older ages than the newborn period (Beck et al., 2014), but their suitability and accuracy for diagnosis in asymptomatic newborns is still little explored, as we evidenced by the few studies retrieved during the development of this protocol.

In spite of that, recommendations were based on available evidence that allow us to evaluate the accuracy of each diagnostic procedure, in order to determine its inclusion to the protocol. Most of the procedures showed a high sensitivity to distinguish affected cases of control subjects. For instance, the enzymatic assay for IDUA activity by fluorometric assay in leukocytes showed 100% sensitivity and the *IDUA* gene analysis by sequencing 89% (Table 2). Therefore, based on these data, we strongly recommend measuring IDUA activity in leukocytes by fluorometry, together with or after the quantitative and qualitative analysis of urinary GAGs, to confirm or not the diagnosis of MPS I in cases that resulted in suspicion through NBS. Likewise, it is strongly recommended to analyze the *IDUA* gene by sequencing from a whole blood sample, after the previous biochemical analysis, to reassure or finally confirm the diagnosis.

Two reports have also suggested similar process of diagnosis confirmation for cases identified through NBS programs. Wang *et al*. (2011), for instance, developed guidelines for diagnosis confirmation and management of presymptomatic cases of selected LSDs, including MPS I. They suggested as first testing the leukocyte IDUA enzyme activity and if this were low, referring the case to a metabolic center for further evaluations that comprise the *IDUA* gene sequencing for diagnosis confirmation and multidisciplinary evaluations for management of confirmed cases. Likewise, Clarke *et al*. (2017) developed an algorithm for follow-up testing of these cases. They also proposed to perform the IDUA enzyme analysis from a blood sample for diagnosis confirmation and emphasized the assessment by a genetic or metabolic disease specialist after confirming a deficient enzyme activity for prompt evaluation and delineation of the disease severity with further biochemical and molecular analyses, which include the *IDUA* gene sequencing as critical component for decision-making.

In our study, besides these main diagnosis procedures, we also considered some of the assay features; such is the type of sample to analyze, that might influence the procedure

accuracy. Whole blood and DBS samples are preferred samples for analysis. As above mentioned, whole blood is the preferred sample for diagnosis confirmation by the enzymatic and molecular analyses. Its use has showed a high sensitivity for those diagnostic procedures. DBS samples have been also used in some studies due to its easy transportation and shipment. They have shown a high sensitivity to distinguish affected cases from controls, when using a fluorometric method, as we evaluated in some studies. However, DBS also have shown limitations for diagnosis use possibly due to its small volume that might cause false positive results after using a fluorometric assay, as other study has also remarked (Clarke *et al*., 2017). In addition, the IDUA enzyme seems to be less stable in DBS than other enzymes (Cobos *et al*., 2014). Therefore, we only recommend using DBS samples for diagnosis confirmation of MPS I when whole blood sample or other more reliable tissue sample is unavailable for analysis. In relation to the use of DBS samples for diagnosis confirmation by *IDUA* gene analysis, evidence is still insufficient to evaluate its diagnosis accuracy.

It is also recommended not to use the fluorometric approach, either in whole blood or DBS, to establish a heterozygous status, since overlapping of activity values are observed, mainly with control subjects. Although this has been observed in studies of clinically suspected cases, some NBS studies for LSDs, that included MPS I, reported that some of the cases classified as false positive could be carriers/heterozygotes since enzyme activity results for diagnosis confirmation were in the low-normal range (Hopkins *et al*., 2015).

One of the main objectives of NBS is to detect early cases, which can benefit of specific therapy. Treatment selection for cases identified by NBS programs and confirmed for MPS I will depend on the expected phenotype: the attenuated forms (Hurler-Scheie and Scheie syndromes) or the severe form (Hurler syndrome). In our study we have not examined the accuracy of the approaches included in the recommendations for distinguishing the MPS I phenotypes, but from the studies assessed during the development of this protocol, we did not find any study demonstrating that one of these approaches could precisely distinguish the three phenotypes or the severe and attenuated forms. Regarding this matter, Clarke *et al*. (2017) addressed the challenges and limitations of these analyses to predict the severity

of MPS I, in cases that were identified by NBS. As we also discussed, enzyme activity assays have limitations distinguishing the MPS I phenotypes and the *IDUA* gene sequencing may be challenging at the moment to interpret the resulting genotypes, mainly when variants that have not been previously described as pathogenic are identified and whose significance is therefore unknown. Nonetheless, the proposed guideline by Clarke *et al*. (2017) to delineate the severity of the confirmed cases is based in the *IDUA* gene sequencing. That approach has been also proposed by Giugliani *et al*. (2016) to differentiate the MPS I phenotypes, considering that patients with the severe phenotype have shown mainly gene deletions/rearrangements, stop-codon and frameshift mutations, while the attenuated phenotypes were more associated to missense mutations, but not exclusively.

Our protocol intended to be based on high quality evidence, however we found some limitations. The evidence was collected from observational studies due to lack of randomized clinical trials, which is expected for rare disease studies. Moreover, we found very few studies on both asymptomatic newborns and the diagnostic accuracy of the included procedures for confirmation of MPS I in this population. Then, we developed and evaluated each recommendations based on studies performed on older subjects, clinically symptomatic.

Despite that, we can observe that pilot studies of NBS for MPS I have used same diagnosis strategy as for symptomatic cases (Paciotti *et al.*, 2012; Lin *et al.*, 2013; Scott *et al.*, 2013), with the difference that those studies using a fluorometric NBS platform (Paciotti *et al.*, 2012; Lin *et al.*, 2013), performed both the enzymatic and molecular diagnosis procedures, and those studies that used the MS/MS platform performed directly the *IDUA* gene sequencing (Scott *et al.*, 2013).

It will depend on each center to decide on the strategy to be used during the diagnostic confirmation process, however we consider and the evidence shows that the combination of the three procedures should be used, given their complementarity among them.

Nevertheless, more studies will be necessary to validate the accuracy of these diagnostic procedures in newborns, and other issues will also need to be evaluated such as the pseudodeficiency.

CONCLUSIONS

We presented an evidence-based protocol that comprises recommendations for the confirmatory diagnosis of cases that resulted altered after the initial testing for MPS I in a newborn screening. The collected evidence was thoroughly structured following relevant sources of guideline development and based on these data, it was suggested that the confirmatory diagnosis in these cases should be performed by enzyme assays to determine the deficiency of α–L-iduronidase in peripheral blood leukocytes, and this biochemical confirmation should be followed by the detection of the causative mutations by a DNA sequencing analysis of *IDUA* gene. We consider that recommendations included in this protocol may be used to facilitate decision-making during the process of confirmatory diagnosis after an abnormal result of newborn screening for MPS I.

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ONLINE SOURCES

Central Register of Controlled Trials (CENTRAL), http://onlinelibrary.wiley.com/cochranelibrary/search?searchRow.searchOptions.searchProducts=clinicalTrialsDoi (April, 2016)

Cochrane Database of Systematic Reviews (CDSR), http://onlinelibrary.wiley.com/cochranelibrary/search/ (April, 2016)

Database of Abstract of Reviews of Effectiveness (DARE), http://www.crd.york.ac.uk/CRDWeb/ResultsPage.asp (April, 2016)

EMBASE, https://www.embase.com/login

EndNote https://access.clarivate.com/#/login?app=endnote

Global Health Library, http://www.globalhealthlibrary.net/php/index.php

GRADEpro GDT: GRADEpro Guideline Development Tool [Software]. McMaster University, 2015 (developed by Evidence Prime, Inc.). https://gradepro.org/

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PubMed Clinical Queries for Systematic Reviews, https://www.ncbi.nlm.nih.gov/pubmed/clinical (April, 2016)

Searching the WHO Library Database (WHOLIS), http://dosei.who.int/uhtbin/webcat

Table 1. Evidence retrieval tasks for MPS I protocol development

Task	Topic 1	Topic 2	Topic 3
Keywords for search strategy. Used as MeSH terms and text words.	#1newborn #2 NOT newborn screening #3 alpha-l-iduronidase #4 fluorometry #5 fluorometric #6 fluorometric method #7,diagnosis #8 mucopolysaccharidosis type 1 #9 mucopolysaccharidosis I #10 diagnostic accuracy #11 enzyme activity #12 enzyme activity assay #13 sensitivity #14 specificity	#1newborn infant #2 glycosaminoglycans #3 quantitation method #4 mucopolysaccharidosis type 1 #5 Mucopolysaccharidosis I #6 diagnosis #7 diagnostic accuracy #8 dermatan sulfate #9 heparan sulfate	#1 newborn OR Newborns #2 “Infant, newborn” #3 Neonate OR neonatal #4 IDUA gene #5 IDUA, gene analysis #6 IDUA protein, human” #7 alpha iduronidase #8Molecular DiagnosticTechniques #9 DNA Mutational Analysis #10 Sequence Analysis, DNA #11 Iduronidase #12 diagnosis,diagnostic accuracy”, #13 hurler syndrome
After duplicate removal	141	142	115
Selection studies for full-text examination: title and abstract appraisal. Inclusion criteria	Studies on: Newborns Enzyme assay Diagnosis Fluorometry Diagnosis accuracy Sensitivity Specificity.	Studies on: Diagnosis by GAGs quantitation, for newborns or symptomatic patients. Diagnosis accuracy of the quantitation method. Diagnosis of GAGs by the qualitative method, either for newborns or symptomatic cases. Diagnosis accuracy of the qualitative technique.	Studies on: Gene or mutational analysis either by Sanger sequencing or NGS, in newborns or affected or suspected cases with MPS I. Diagnosis accuracy of gene sequencing method.
Exclusion criteria	Studies on: Neonatal screening techniques Treatment Clinical manifestations reports. Reasons for exclusion, studies were on: animal model, prenatal diagnosis, molecular analysis, treatment, other disease and review articles.	Studies on quantitative method when qualitative method was examined and vice versa. Reason for exclusion, studies on: Other disease: MPS VII, MPS II, mucolipidosis, osteogenesis. Treatment. Other sample: skin culture. Animal model study. No abstract/other topic/Review articles.	Other techniques: enzyme assays, MS/MS. Prenatal diagnosis Animal model Gene therapy One novel mutation report Analysis in relatives Other disease: MPS II, MPS IVA, MPS VI, mucolipidosis. No abstract/other topic.

Continued

Table 1. Evidence retrieval tasks for MPS I protocol development (*Continued*)

Task	Topic 1	Topic 2	Topic 3
Total selected	48	33	52
Data collection. Inclusion criteria	Studies on: Newborns and enzyme activity measured by fluorometry. Diagnosis accuracy and fluorometry in leukocytes, in newborns or other.	Studies on: Quantitative and qualitative methods were examined separately.	Studies on: IDUA gene analysis by direct sequencing with automatized, both newborns and/or patients.
Reason for exclusion	Other substrate: phenyl-α-L-iduronide. Other samples: urine, skin fibroblast, DBS. Other analyses: fluorometry for NBS, MS/MS, biochemical/kinetic parameter determination.	Other sample: Serum, dried urine sample in filter paper. One case report. Other technique: MS/MS No data for diagnosis accuracy assessment.	Targeted polymorphism analysis. No automatized sequencer. Evaluation of one or two cases. New method evaluation. Bioinformatic study.

Notes: DBS: dried blood spot. GAG: glycosaminoglycan. IDUA: alpha-L-iduronidase. MeSH: medical subject heading. MS/MS: Tandem mass spectrometry. MPS: mucopolysaccharidosis. NGS: next-generation sequencing.PGD: pre-implantation genetic diagnosis.

Table 2. GRADE evidence profiles of test accuracy for IDUA activity, Urinary GAGs and *IDUA* gene analyses.

Outcome	№ of studies (№ of patients)	Study design	Factors that may decrease quality of evidence					Test accuracy Quality of Evidence
			Risk of bias	Indirectness	Inconsistency	Imprecision	Publication bias	
Question: Should IDUA activity measured by fluorometry in leukocytes be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I?								
True positives Sensitivity 100% Specificity 100%	2 studies (4 patients)	case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should IDUA activity measured by fluorometry be used to diagnose heterozygous status of MPS I in newborns that screened positive in a NBS for MPS I?								
True positives (patients Heterozygotes of MPS I)	4 studies (11 patients)	case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should IDUA activity measured by fluorometry in DBS be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I?								
True positive Sensitivity 100% Specificity 100%	7 studies (56 patients) 7 studies (417 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should total urinary GAGs quantitation be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I?								
True positives Sensitivity 97 %	1 studies (42 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should urinary GAGs electrophoresis be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I?								
True positives Sensitivity 95 %	1 studies (41 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should *IDUA* gene analysis by direct sequencing be used to diagnose MPS I in newborns that screened positive in a NBS for MPS I?								
True positives Sensitivity 89 %	13 studies (259 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕⊕ HIGH
Question: Should whole blood sample be used for *IDUA* gene analysis to diagnose MPS I in newborns screened positive in a NBS for MPS I?								
True positives Sensitivity 90%	12 studies (123 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕⊕◯ MODERATE
Question: Should DBS sample be used for *IDUA* gene analysis to diagnose MPS I in newborns screened positive in a NBS for MPS I?								
True positives	2 studies (7 patients)	Case-control	not serious	serious [a]	not serious	not serious	none	⊕⊕◯◯ LOW
Explanations: [a] no newborn case.								

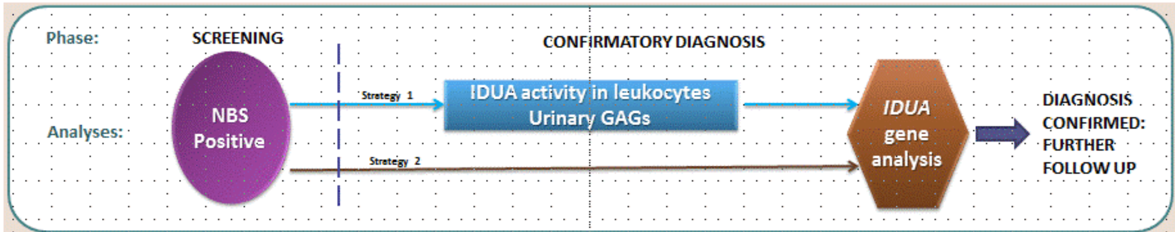

Figure 1. Strategies for diagnosis confirmation of MPS I in newborns screened positive in NBS for MPS I, as reported across NBS studies. Strategy 1: Initial screening testing is fluorometric. Strategy 2: Initial screening testing is tandem mass spectrometry. IDUA: α-L-iduronidase. GAGs: glycosaminoglycans.

Table S1. Check list form for data collection

Form for Topic 1 and 2	Form for Topic 3
SOURCE Study ID (created by review author). Review author ID (created by review author). Citation and contact details. Type of publication Study main purpose ELIGIBILITY Confirm eligibility for review. Study on Diagnosis accuracy and diagnose MPS 1 (here) Mention if explicit Sensitivity for the studied method Specificity for the studied method Other measure of accuracy (Indicate which) Reason for exclusion. METHODS PARTICIPANTS Total number. Type of Sample Setting. Diagnostic criteria. Age. Sex. Country. INTERVENTIONS Specific intervention. Intervention details (sufficient for replication, if feasible). RESULTS/ OUTCOMES Neonates Age (if specific: e.g days, weeks) IDUA activity /GAG value (Indicated Mean) SD 95% CI (confidence Interval).Range Heterozygotes Number of participants(n) Age IDUA activity/GAG value (Indicated Mean) SD, 95% CI (confidence Interval), Range Cases/Target population/Patients Number of participants(n) Age IDUA activity/GAG value (Indicated Mean) SD, 95% CI (confidence Interval), Range (or min-max) Healthy individuals no newborns Number of participants(n) Age IDUA activity/GAG value (Indicated Mean) SD 95% CI (confidence Interval), Range (or min-max) Difference between groups (p value)Test Name OTHER RESULTS/more results MISCELLANEOUS Key conclusions of the study authors. Miscellaneous comments from the study authors. References to other relevant studies. Correspondence required. Miscellaneous comments by the review authors.	SOURCE Study ID (created by review author). Review author ID (created by review author). Citation and contact details. Type of publication Study main purpose ELIGIBILITY Confirm eligibility for review. Study on Diagnosis accuracy and diagnose MPS 1 (here) Mention if explicit Sensitivity for the studied method Specificity for the studied method Other measure of accuracy (Indicate which) Reason for exclusion. METHODS Study design Total study duration. Sequence generation*. Allocation sequence concealment*. Blinding* Other concerns about bias*. PARTICIPANTS Total number. Age Sex. Previous Diagnosis (Criteria used in the study in revision to add as participant): Clinical presentation, IDUA activity, GAGs, other. Type of Sample for gene/molecular analysis (if other no gene analysis) Gene analysis method Type of sequencer Size analyzed regions analyzed Country. Ethnicity. INTERVENTIONS Total number of intervention groups. (For each intervention and comparison group of interest:) Specific intervention. RESULTS (after gene analysis) Number of patient studied Number of patient with established molecular diagnosis (Homozygous, heterozygous compound) Number of patient without a established molecular diagnosis Reason(VUS, no variant identified, other) Some additional data (frequency of allele identified) Number of patients with genetic diagnosis) Neonates Cases/Target population/patients (Hetero) Cases/Target population/patients Healthy individuals no newborns OTHER RESULTS/more results MISCELLANEOUS Key conclusions of the study authors. Miscellaneous comments from the study authors. References to other relevant studies. Correspondence required. Miscellaneous comments by the review authors.

CAPÍTULO 7

Newborn Screening for Lysosomal Storage Diseases: program cost estimation from a pilot study in Brazil

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Artigo a ser submetido para publicação

Short Report

Newborn Screening for Lysosomal Storage Diseases: program cost estimation from a pilot study in Brazil

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ABSTRACT

Newborn screening (NBS) for lysosomal storage diseases (LSDs) has gain interest the last years because of treatment availability for an increasing number of these diseases and the evidence of improved clinical outcome when this is introduced early. Besides, the NBS technology and studies on suitability of them in mass-screening programs, other issues are also necessary to consider. Here, we report the estimate of the overall cost of a newborn screening program for treatable LSDs, based mainly on a pilot study in Brazil, considering a potential large-scale implementation.

Keywords: Lysosomal storage diseases, newborn screening, cost analysis

INTRODUCTION

Newborn screening (NBS) for lysosomal storage diseases (LSDs) has become target of interest these last years because of the availability of treatment for an increasing number of these diseases and the evidence of a better clinical outcome when treatment is introduced early (McGill *et al*., 2010; Aldenhoven *et al*., 2015; Gabrielli *et al*., 2016; Yang *et al*., 2016).

LSDs are caused mainly by monogenic defects in genes encoding lysosomal enzymes that degrade macromolecules such as glycolipids, glycoproteins and mucopolysaccharides. That leads to the abnormal and progressive lysosomal accumulation of specific substrates that consequently produce structural changes and deterioration of the cellular function (Futerman and van Meer, 2004). LSDs present heterogeneus and progressive clinical manifestations, usually multisystemic, including organomegalies, dysmorphic features, and in various cases neurological and skeletal involvement (Heese 2008; Platt and Lachmann 2009; Hoffman *et al*., 2010; Wang *et al*., 2011). Treatment that comprises enzyme replacement, transplantation of hematopoietic stem cells and substrate synthesis inhibitors (Platt and Lachmann, 2009, Hoffman *et al.*, 2010) reduces disease manifestations and may prevent irreversible pathological changes, when early introduced (McGill *et al*., 2010; Aldenhoven *et al*., 2015; Gabrielli *et al*., 2016; Yang *et al*., 2016). Therefore, early diagnosis should be performed, preferably during the asymptomatic stage of the disease, especially for the severe form cases.

The latter supported the development of newborn screening for some of these disorders and therefore, several pilot studies of NBS for LSDs have been already performed in different countries, showing mainly the suitability of NBS approaches for various LSDs, using either a fluorometric or mass spectrometry platform for screening, performed in a multiplex manner or not (Spada *et al*., 2006; Chien *et al*., 2008; Mechtler *et al*., 2012; Paciotti *et al*., 2012; Wittmann *et al*., 2012; Lin SP *et al*., 2013; Scott *et al*., 2013; Liao *et al*., 2014; Hopkins *et al*., 2015; Elliott *et al*., 2016). In spite of that there are still issues that remain unsolved, especially when implementation of such a program is considered. Cost is one of these still unmet needs that will require being further assessed. To date, reports on cost evaluation of NBS for LSDs programs are scarce and mainly focus in cost description of the screening phase only (Mechtler *et al*., 2012, supplemental material; Gelb *et al*., 2014, supplemental material). Therefore, this study aimed to estimate the overall cost of a

newborn screening program for treatable lysosomal diseases, based on a pilot study in Brazil, and considering both the cost of the NBS program and the structure of the diagnosis confirmatory protocol, in view of a potential large-scale implementation.

METHODS

A list of the procedures performed for the confirmatory diagnosis of the four cases that were investigated at the Medical Genetic Service (SGM) of the 'Hospital de Clinicas de Porto Alegre' (HCPA), Brazil, was collected from a previous report (Bravo *et al*., 2017). The cases were those referred from the NBS study for LSDs (Fabry disease, Gaucher disease, Mucopolysaccharidosis type 1- MPS I and Pompe disease) performed at the newborn screening laboratory, the CTN (Centro de Triagem Neonatal), based in Porto Alegre, Brazil (Camargo-Neto *et al*., in press). Information on cost for each procedure of the list was then collected from informative cost tables of the SGM-HCPA and annotated in Brazilian currency, Brazilian real (BRL). Total cost per sample was calculated in BRL and converted to US dollar (USD), according with the exchange rate of the day of data collection. The cost for the screening phase was not available, and then literature reports were used for estimation.

RESULTS AND DISCUSSION

First, cost of the diagnosis confirmation process for the cases screened positive at the NBS of MPS I (two cases), Pompe disease and Gaucher disease were estimated. Table 1 shows the cost of each procedure performed in each case.

Based on these data, the cost for the confirmatory diagnosis of the case in suspicion of MPS I was estimated as 1,984.00 BRL or its equivalent, 604.88 USD. For the case in suspicion of Pompe disease, the cost was 1,719.98 BRL, or its equivalent, 579.26 USD. For the case in suspicion of Gaucher disease the total cost for the confirmatory diagnosis process was estimated as 2,334.44 BRL or 711.72 USD.

Cost for the screening process was not possible to be calculated from the pilot study performed in Brazil; however cost estimation was taken from other reported studies. These studies estimated that cost per newborn per LSD might be below or close to one USD, using either a fluorometric platform or a tandem mass spectrometry platform (Mechtler *et al*., 2012, supplemental material; Gelb *et al*., 2014, supplemental material). It is also

believed that primary screening methods will not be more expensive that other already used in NBS (Matern *et al*., 2015).

Therefore, based on the above data, the overall cost of a newborn screening program for four treatable lysosomal diseases including MPS I, Fabry disease, Pompe disease and Gaucher disease would be 44,608.74 USD, considering the screening of 10,527 newborns and the investigation for confirmatory diagnosis of four cases derived from the same screening.

It should be noted that this report comprises the cost description of assay procedures only and not include cost for equipment, work-time of personnel and other indirect cost. Even though, it gives an estimation of cost that includes not only the screening cost assay but also the cost of the procedures performed to confirm or not the diagnosis, which are not usually included in cost calculation data reported in literature. Therefore, the cost estimation of our study can be used as template to further analyze the overall cost of a NBS program for LSDs, considering a potential implementation.

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Table 1. Cost per case for the confirmatory diagnosis in a NBS for LSDs

For MPS I cases		**For Pompe Disease case**		**For Gaucher Disease case**	
List of Procedures	**Cost (R$)**	**List of Procedures**	**Cost (R$)**	**List of Procedures**	**Cost (R$)**
Urinary GAGs (DMB)	120.00				
Urinary GAGs (Electrophoresis)	180.00				
ENZYME Analysis					
α-L-iduronidase in DBS (Fluorometry)	144.00	Protein β-Galactosidase in Leukocytes	180.00	Acid β-glucosidase in DBS	300.00
Prot. β-Galactosidase in leukocytes	180.00	acid α-glucosidase in leukocytes	240.00	β-Galactosidase in DBS	144.00
α-L-iduronidase in plasma	180.00			Chitotriosidase	144.00
α-L-iduronidase in leukocytes	180.00			Chitotriosidase in DBS	144.00
				Acid β-glucosidase in leukocytes	300.00
GENE Analysis					
DNA extraction (Blood)+ *IDUA* Sequencing	1000.00	DNA extraction (Blood)	96.00	DNA extraction (Blood)	96.00
		NGS (Panel, per sample, 500rxns)	3.98	NGS (Panel, per sample, 500rxns)	6.44
		GAA Gene Sequencing	1200.00	*GBA* Gene Sequencing	1200.00
TOTAL Brazilian Real (R$)	1984.00		1719.98		2334.44
Total Cost in Dollars (Exchange rate 8 December 2017: 3,28R$/1 USD)	604.88		579.26		711.72

MPS I: Mucopolysaccharidosis type 1. GAGs: Glycosaminoglycans. DMB: dimethylmethylene blue. DBS: dried blood spot. IDUA: α-L-iduronidase. GAA: acid α-glucosidase. GBA: acid β-glucosidase. Prot: protein. NGS: Next-generation sequencing

CAPÍTULO 8. DISCUSSÃO

A triagem neonatal constitui uma intervenção importante em saúde pública que visa à redução da morbidade e mortalidade em crianças por meio da detecção precoce de doenças. Nesse sentido, a triagem neonatal para doenças lisossômicas tem ganhado uma crescente atenção nos últimos anos principalmente pela disponibilidade de tratamento para um grupo dessas doenças e a evidência de melhora clínica quando as medidas terapêuticas são introduzidas precocemente.

Uma possível implementação e inclusão dessas doenças em programas de triagem neonatal estabelecidos requer avaliação prévia de vários aspectos relacionados à sua estrutura e organização. Isso abrange os procedimentos para a triagem, para o diagnóstico confirmatório e para o manejo e acompanhamento dos casos confirmados.

Neste estudo, foram avaliados os procedimentos necessários para a investigação com vistas ao diagnóstico confirmatório de casos que resultem alterados nos testes iniciais da triagem neonatal para as doenças de Fabry, Gaucher, Pompe e as Mucopolissacaridoses tipo I, II e VI (MPS I, MPS II e MPS VI), doenças lisossômicas consideradas candidatas para inclusão em programas de triagem neonatal, por serem tratáveis.

No primeiro artigo, mostramos os procedimentos de diagnóstico que poderiam ser utilizados na determinação do diagnóstico dos casos cujo resultado de triagem neonatal foi positivo. A informação foi coletada principalmente das estratégias utilizadas para o diagnóstico em estudos prévios de triagem neonatal de DLDs relatados na literatura, dada a falta de relatórios sobre protocolos, específicos para a confirmação diagnóstica desses casos.

Os procedimentos diagnósticos incluem principalmente análise enzimática e análise molecular. A determinação da atividade enzimática é realizada pelo método fluorimétrico em leucócitos, um método considerado padrão-ouro para o diagnóstico dessas doenças em casos sintomáticos. A análise molecular é realizada pelo método de seqüenciamento do gene que codifica a enzima que estaria deficiente e causaria a doença. O diagnóstico é estabelecido quando se determina uma atividade enzimática deficiente e se identifica

variantes patogênicas presentes em genótipos homozigotos ou heterozigotos compostos em conformação *trans*.

No segundo artigo, avaliamos as estratégias de diagnóstico confirmatório, reportadas previamente, para investigar quatro casos que derivaram de um estudo de triagem neonatal de DLDs, o primeiro estudo em grande escala realizado no Brasil, que utilizou uma nova plataforma fluorométrica para a triagem. Os casos que eram positivos para triagem neonatal das doenças de Gaucher, Pompe e MPS I (dois casos) foram avaliados no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, centro especializado em doenças genéticas e metabólicas. Nenhum dos quatro casos foi diagnosticado como afetado. No entanto, foi evidenciada a presença de pseudodeficiência em dois casos suspeitos de doença de Pompe e MPS I; e dois casos de portadores/heterozigotos para doenças de Gaucher e MPS I. Outros estudos identificaram também esse tipo de casos e os consideraram como falsos positivos (Hopkins *et al*., 2015).

Nesse estudo também foram demonstradas as limitações das análises enzimáticas e moleculares no diagnóstico, relatado também em outros estudos de triagem neonatal para DLDs, que classificaram esses casos como indeterminados ou pendentes (Hopkins *et al*., 2015, Burton *et al*., 2017). Foi observado que os casos de pseudodeficiência e de heterozigotos apresentam valores baixos para a atividade enzimática, mas que não correspondem aos observados nos casos afetados. Do mesmo modo, a análise molecular, que acabou confirmando o diagnóstico, também apresentou desafios para a interpretação dos resultados, devido à presença de variantes de significado desconhecido.

No terceiro artigo, foi desenvolvido um protocolo para a confirmação diagnóstica de casos que resultarão positivos após uma triagem neonatal para MPS I. O protocolo tem a particularidade de ter sido elaborado seguindo uma diretriz para o desenvolvimento de protocolos relatado pela OMS, que na elaboração sugere também a avaliação estruturada da qualidade da evidência usando a abordagem GRADE.

No protocolo desenvolvido, foram sugeridas recomendações relacionadas aos três principais processos diagnósticos utilizados para a confirmação diagnóstica desses casos e

incluem análise da atividade enzimática da IDUA, determinação quantitativa e qualitativa da excreção urinária de GAGs e análise molecular do gene *IDUA*.

Na elaboração deste protocolo, comprovamos a falta de estudos para a validação da acurácia diagnóstica desses procedimentos e seu uso em recém-nascidos. Embora tenha sido demonstrado que os procedimentos de confirmação diagnóstica utilizados pelos estudos de triagem neonatal seriam os mesmos usados no diagnóstico de casos sintomáticos, é questionável se seu uso é apropriado para casos de recém-nascidos assintomáticos. Sabe-se que valores de referência dos testes laboratoriais em recém-nascidos são geralmente diferentes dos infantes e dos indivíduos de maior idade. Portanto, seria aconselhável realizar estudos de validação para esta faixa etária. Principalmente em relação ao uso do método fluorimétrico para a determinação da atividade enzimática em leucócitos, que não possui estudos de validação em recém-nascidos.

A decisão sobre qual estratégia de confirmação de diagnóstico será utilizada irá depender de cada programa de triagem neonatal. No entanto, é importante observar que o processo de diagnóstico confirmatório deve ser realizado em um centro especializado com experiência em doenças metabólicas e genéticas, uma vez que a interpretação de resultados pode ter alguns desafios em alguns os casos, devido às limitações que todos os procedimentos de diagnóstico, atualmente utilizados, têm.

Finalmente, no quarto artigo, realizamos uma estimativa do custo de um programa de triagem neonatal para doenças lisossômicas tratáveis. Essa estimativa considerou especificamente o custo da fase de triagem e do processo de confirmação diagnóstica, na base do estudo piloto realizado em Porto Alegre, Brasil e apresentado no segundo artigo deste projeto.

Os relatórios sobre avaliação de custo para programas de triagem neonatal para DLDs são ainda escassos e se concentram apenas na descrição do custo da fase de triagem (Mechtler *et al*., 2012, material suplementar; Gelb *et al*., 2014, material suplementar). Portanto, a estimativa de custo do nosso estudo pode ser usada como base para uma análise mais abrangente do custo global de um programa triagem neonatal para DLDs.

Consideramos que a informação coletada em este estudo, pode ser usada também pelos os responsáveis pela implementação e organização dos programas de triagem neonatal, como fonte primária para avaliação adicional e desenvolvimento de diretrizes.

Entre as perpectivas deste trabalho, a metodologia no desenvolvimento de protocolos pretende ser usada para desenvolver e avaliar protocolos para outras doenças lisossômicas.

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APÊNDICES

APÊNDICE 1. Trabalho aceito para publicação e relacionado com o projeto

Newborn screening for four lysosomal storage diseases with a digital microfluidics platform: initial results in Brazil

Journal:	*Genetics and Molecular Biology*
Manuscript ID	GMB-2017-0227.R1
Manuscript Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	Camargo Neto, Eurico; CTN Diagnósticos Schulte, Jaqueline; CTN Diagnósticos Pereira, Jamile; CTN Diagnósticos Bravo, Heydy; Universidade Federal do Rio Grande do Sul Instituto de Biociencias, Programa de Pós-Graduação em Genética e Biologia Molecular - PPGBM; Instituto Nacional de Ciência e Tecnologia de Genética Médica Populacional - INAGEMP Sampaio Filho, Cláudio; Intercientífica Giugliani, Roberto; Universidade Federal do Rio Grande do Sul, Departamento de Genética; Hospital de Clinicas de Porto Alegre, Serviço de Genética Médica
Keyword:	Newborn Screening, Digital Microfluidics, Brazil, Lysosomal Storage Diseases

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TITLE

Neonatal screening for four lysosomal storage diseases with a digital microfluidics platform: initial results in Brazil

AUTHORS

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ABSTRACT

We describe the initial results of neonatal screening program for four lysosomal storage diseases (MPS I, Pompe, Gaucher and Fabry) using digital microfluidics methodology. The method successfully identified patients previously diagnosed with these diseases and was used to test dried blood spot samples obtained from 10,527 newborns aged 2 to 14 days. Digital microfluidic technology shows potential for a simple, rapid and high-throughput screening for these four diseases in a standard neonatal screening laboratory.

KEYWORDS: Lysossomal storage diseases, neonatal screening, digital microfluidics, Brazil

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Neonatal screening for lysosomal storage diseases (LSDs) has been gaining considerable interest as a result of the new policies to promote early diagnosis of rare diseases, the development of new screening methods, and the availability of enzyme replacement and other specific therapies for several LSDs. Also, LSDs are not considered too rare anymore, with a combined incidence for the around 50 LSDs estimated to be around 1/7,000 [Meikle et al, 1999; Poorthuis et al, 1999].

LSDs have a wide spectrum of clinical signs and symptoms, with overlapping findings across different diseases and with most disorders presenting large variability [Gieselmann, 2005]. This makes the diagnosis particularly difficult, especially as it needs to be confirmed with sophisticated biochemical and genetic tools [Wang et al, 2011]. For these reasons there is often a delay in the diagnosis of LSDs [Vieira et al, 2008]. This delay may influence the outcome of treatment, already available for several of these conditions [Giugliani et al, 2016].

The development of high-throughput protocols with multiplexing capabilities for use with dried blood spot (DBS) samples has facilitated the establishment of NBS programs for LSDs around the world [Spada et al, 2006; Hwu et al, 2009; Hopkins et al, 2015].

We present here the initial results obtained with a fluorimetric digital microfluidics (DMF) platform used for the neonatal screening of 4 LSDs (Mucopolysaccharidosis I (MPS I), Gaucher, Fabry, and Pompe diseases).

A DMF platform was used to assay the activities of α-L- iduronidase (IDUA), to screen for MPS I; acid α-glucosidase (GAA), to screen for Pompe; acid β-glucosidase (GBA), to screen for Gaucher and acid α-galactosidase (GLA), to screen for Fabry in the newborn's samples. We used one workstation with 4 digital microfluidic instruments to run a multiplexed fluorometric enzymatic assay platform as described previously [Sista et al, 2011; Sista et al, 2013]. All necessary hardware and reagents for GAA, GBA, GLA, and IDUA were supplied by Baebies Inc (Durham, NC, USA).

DBS samples from patients previously diagnosed with MPS I, Gaucher, Pompe or Fabry diseases were initially tested to check the capability of the method in identifying the respective enzyme deficiencies (Table 1).

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DBS samples were then collected from 10,527 newborns aged 2 to 14 days of life, randomly selected for testing among the cards routinely received by the Neonatal Screening Center, based in Porto Alegre, Brazil. Samples from newborns were spotted in the filter paper and shipped at room temperature to the reference laboratory, arriving in 24 to 72 hours .Tests were performed no more than 48 hours after sample arrival to the lab.

The DMF assay protocol to determine the enzyme activity in a multiplex format took less than 4 hours, allowing 3 runs per day (eventually, one overnight) and yielded 152 results in each run (total, 456 per day) when using 1 working station with 4 fluorimeters in parallel.

Overall coefficient of variation (CV) values between cartridges, days, instruments, and operators ranged from 4 to 22%. Linearity correlation coefficients were ≥ 0.98 for all assays.

Cutoff values of 5.1 µmol/L/hour for IDUA, 5.9 µmol/L/hour for GAA, 3.9 µmol/L/hour for GBA, and 5.7 µmol/L/hour for GLA were established during a preliminary phase using 1,000 samples from the cards routinely collected and DBS specimens from patients with confirmed LSDs previously diagnosed. These cut-offs values were not very different from the ones established by Hopkins et al [8], using the same method (4.0, 8.0, 4.5, and 5.5 µmol/L/hour, respectively).

The method demonstrated to correctly discriminate 9 samples of affected patients (3 from MPS I, 2 from Pompe, 2 from Gaucher and 2 from Fabry cases) previously diagnosed, from samples of normal subjects. Further information on cutoffs and results in known patients are displayed in Table 1.

Four samples out of the 10,527 tested showed activity below the cutoff and were further investigated until a final conclusion was established. These cases were studied by another group and eventually as pseudodeficiency (low activity of enzyme without generation of storage and with no clinical consequences, usually found in patients who present mutations already known as related to pseudodeficiency) of α-L-iduronidase (2 cases), pseudodeficiency of α-glucosidase (1 case) and carriership for Gaucher disease (1

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4 case) (Bravo H et al, 2017).
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6 The availability of effective therapies for several LSDs [Aronivich et al, 2015] and the
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8 evidence that early introduction of therapy may bring a better outcome [Gabrielli et al, 2016]
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10 make neonatal screening for these conditions an option to be considered. This is especially
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12 true as several high-throughput platforms became available to perform tests in a large
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14 number of samples at a low cost per assay [Matern et al, 2015].
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16 In the present report we demonstrated that DMF was efficient in identifying samples
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18 from patients previously diagnosed with MPS I, Gaucher, Fabry and Pompe diseases.
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20 Although we cannot discard that false negatives could occur, this possibility is considered
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22 very small as affected patients usually have extremely low enzyme activities, and all affected
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24 patients tested were identified by the method in the validation process (data not shown).
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26 The usefulness of this platform was already reported by Hopkins et al [2015], who detected
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28 1/1,618 newborns affected by one of these four diseases in 43,686 samples evaluated. We
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30 identified of 4 samples among the 10,527 DBS from newborns tested with a low enzyme
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32 activity (1/2,631). Although subsequent testing informed that these were not true LSDs, all
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34 these cases had true low enzyme activity, which was detected by the screening program
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36 using the DMF method.
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38 This is the first report of neonatal screening for multiple LSDs conducted in Brazil. It
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40 is important to mention that this program was performed in a standard clinical biochemistry
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42 laboratory, indicating that, in addition to be efficient and robust, the platform is suitable to
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44 less sophisticated labs, as are most of the labs in Latin America. The successful use of this
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46 strategy to simultaneously measure four enzyme activities indicates that this method could
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48 be an option for Brazil and other developing countries.
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Authors' Contributions

EC Neto supervised the testing program and wrote the first draft. J Schulte and J Pereira performed the analyses. C Sampaio-Filho provided expert advice and designed the table. . H Bravo, C Sampaio-Filho and R Giugliani provided comments to the first draft. All authors contributed to the final version and granted approval.

Conflicts of Interest

EC Neto, J Schulte and J Pereira are employees of Centro de Triagem Neonatal. H Bravo has nothing to disclose. C Sampaio-Filho is employee of Intercientifica. R Giugliani received investigator fees, and or speaker honoraria and/or travel grants from Actelion, Amicus, BioMarin, Lysogene, Sanofi-Genzyme, Shire and Ultragenyx.

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Table 1. Averages for each enzyme activity in normal subjects, cut-off values and results in confirmed cases previously diagnosed

MPS-1	Results (µmol/L/h)
Samples (n=9864)	
IDUA average	17.1
calculated IDUA cut-off (30 %)	5.1
Positive samples (n=3)	
Patient 1	3.4
Patient 2	4.9
Patient 3	3.7

FABRY	Results (µmol/L/h)
Samples (n=9988)	
GLA average	18.9
calculated GLA cut-off (30 %)	5.7
Positive samples (n=2)	
Patient 1	4.2
Patient 2	4.7
Patient 3	3.6

POMPE	Results (µmol/L/h)
Samples (n=9560)	
GAA average	19.8
calculated GAA cut-off (30 %)	5.9
Positive samples (n=2)	
Patient 1	3.4
Patient 2	3.1

GAUCHER	Results (µmol/L/h)
Samples (n=9878)	
GBA average	13
calculated GBA cut-off (30 %)	3.9
Positive samples (n-2)	
Patient 1	3.3
Patient 2	3.5

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