UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

MORTE SÚBITA E INESPERADA NA INFÂNCIA ASSOCIADA A ERROS INATOS DO METABOLISMO NO BRASIL: UMA ABORDAGEM EPIDEMIOLÓGICA E GENÉTICA

FERNANDA HENDGES DE BITENCOURT

PORTO ALEGRE

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de **Doutora em Genética e Biologia Molecular.**

PORTO ALEGRE

2018

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Albert Pine

RESUMO

Introdução: Morte súbita inesperada e inexplicada na infância ou morte súbita infantil inesperada (SUDI, do inglês *Sudden unexpected death in infancy)* é uma das mais frequentes causas de óbito no primeiro ano de vida. De 3 a 6% das crianças com SUDI podem ter algum erro inato do metabolismo (EIM – doenças genéticas geralmente autossômicas recessivas), sendo que SUDI pode ser a única manifestação dessas doenças. A deficiência de acil-CoA desidrogenase de cadeia media (MCADD), um defeito de beta-oxidação de ácidos graxos, parece ser o principal EIM associado à SUDI. Até o momento, não há na literatura qualquer dado sobre a frequência de SUDI associada a EIM no território brasileiro. Além disso, não existe na literatura dados sobre a frequência de MCADD e suas variantes no Rio Grande do Sul (RS). Como muitos dos EIM são tratáveis, o seu diagnóstico e tratamento precoces poderiam prevenir a ocorrência de SUDI.

Objetivos gerais: 1) Estimar e caracterizar por região do Brasil, os óbitos relacionados a EIM associados a SUDI em neonatos e lactentes <1ano de idade no período de 2002 a 2014 2) Avaliar a fração atribuível dos EIM nas situações de SUDI em neonatos e lactentes <1ano de idade na população do RS no período de 2002 a 2014.

Metodologia: *Etapa 1 (Análise Epidemiológica):* estudo transversal de base populacional utilizando as bases de dados do Sistema de Informações sobre Mortalidade (SIM) correspondentes ao período 2002-2014. A partir do SIM, foram identificados os óbitos de crianças <1ano no Brasil e no RS cuja causa básica corresponda a alguns dos EIM associados a SUDI (CID10-E70 – distúrbios do metabolismo de aminoácidos aromáticos, E71 – distúrbios do metabolismo de aminoácidos de cadeia ramificada e do metabolismo de ácidos graxos, E72 – outros distúrbios do metabolismo de aminoácidos, E74 – outros distúrbios do metabolismo de carboidratos) e à SUDI (CID10-R95 – Síndrome de morte súbita na infância, R96 – outras mortes súbitas de causa desconhecida, e R99 – outras causas mal definidas e não especificadas de mortalidade). Para os dados do RS, foram solicitadas à Secretaria Municipal de Saúde de Porto Alegre as declarações de óbito (DOs) dessas crianças, as quais foram analisadas em relação às variáveis de interesse. Para os óbitos ocorridos no RS, foi realizado o georreferenciamento dos casos a fim de avaliar a presença de *cluster* de SUDI e de EIM associados a SUDI. *Etapa 2 (Análise Genética):* Estudo observacional e transversal de detecção de heterozigotos para as variantes patogênicas mais frequentes nos genes *ACADM* (MCADD; c.985A>G e c.199T>C). Os participantes foram doadores voluntários de sangue que comparecerem ao Banco de Sangue do Hospital de Clínicas de Porto Alegre.

Resultados: *Etapa 1:* de 2002 a 2014 foram registrados 199 óbitos de crianças <1ano no Brasil devido a EIM associados à SUDI, sendo a prevalência de óbitos calculada de 0,67:10.000 nascidos vivos (IC95% 0,58-0,77). Destes 199 óbitos, 18 (9,0%) ocorreram na região Norte do país, 43 (21,6%) na região Nordeste, 80 (40,2%) na região Sudeste, 46 (23,1%) na região Sul e 12 (6,0%) na região Centro-Oeste. Em todo o território nacional, CID10-E74 foi o predominante. Entre 2002 e 2014, 21 crianças <1ano faleceram no RS devido aos EIM pesquisados. No mesmo período, 650 faleceram devido à SUDI, sendo que a taxa calculada foi de 0,53:10.000 nascidos vivos (IC95% 0,33-0,81) e de 16,0:10.000 nascidos vivos

(IC95% 15-18), respectivamente. A análise das DOs mostrou que há um alto grau de incomplitude, principalmente no que diz respeito a afecções iniciais, sequenciais e terminais (que levam a identificação da causa básica de óbito) nos casos de SUDI, não permitindo a associação entre SUDI e EIM. O georreferenciamento dos óbitos de criança <1ano por CID10-R95 mostrou que o município de Pejuçara é um isolado geográfico com uma alta taxa de óbitos infantis. Com relação ao CID10-R99, os municípios de Nova Bréscia e Paraí também são isolados geográficos com altas taxas de óbitos infantis por esse CID. Nova Bréscia e Paraí seguem como isolados geográficos quando todos os óbitos por SUDI foram considerados (CID10-R95, R96 e R99). O georreferenciamento de óbitos por CID10-R96 e EIM associados a SUDI não foi realizado devido ao pequenos número de registros de óbitos por esses CIDs no período avaliado (13 e 21 casos, respectivamente). *Etapa 2:* Até o momento, foram analisados 300 indivíduos para a variante c.199T<C. Não foram encontrados alelos para essa variante no gene *ACADM* na população estudada.

Discussão/Conclusões: Este é o primeiro estudo a avaliar o número de óbitos de crianças <1ano atribuído a SUDI e a EIM associados à SUDI no território nacional e, em especial, no RS num período de treze anos. O baixo número de registros de óbitos por EIM associados à SUDI no Brasil pode ser resultado não da raridade dos distúrbios, mas sim, da subnotificação e/ou subdiagnóstico. Apesar de MCADD ser o principal EIM associado à SUDI, o CID10-E71 (no qual estão incluídos os defeitos de beta-oxidação de ácidos graxos), foi o menos prevalente. No RS, por sua vez, o número de óbitos por EIM associados à SUDI reflete o panorama nacional, com apenas 21 casos registrados no período avaliado. Por outro lado, os óbitos por SUDI não permitem a associação destes com EIM devido à escassez de informações nas DOs, principalmente no que diz respeito às afecções iniciais, sequenciais ou terminais que conduziram ao estabelecimento da causa básica de óbito. A presença de isolados geográficos com altas taxas de óbitos de crianças <1ano nos municípios de Paraí, Nova Bréscia e Pejuçara pode ser um indicador da presença de EIM na região. Essa hipótese é apoiada por fatores tais como a dificuldade de diagnóstico de doenças tão complexas como os EIM; a ausência de necropsia metabólica no Brasil; a ancestralidade europeia dessas regiões, a qual pode estar associada a uma maior incidência de MCADD, principal EIM relacionado a SUDI. Com relação à análise genética do gene *ACADM,* os resultados ainda são parciais, não permitindo o estabelecimento da frequência de heterozigotos para as mutações c.199T>C e c.985A>G na população saudável do RS, e nem a determinação da incidência de MCADD na região.

Palavras-chave: morte súbita, erros inatos do metabolismo, SUDI, mortalidade infantil

ABSTRACT

Introduction: Sudden unexpected death in infancy (SUDI) is one of the most frequent causes of death during the first year of life. From 3% to 6% of children with SUDI may have some inborn error of metabolism (IEM – genetic diseases usually with autosomal recessive inheritance), and SUDI may be the main manifestation of these diseases. Medium-chain acyl-CoA dehydrogenase deficiency (MCADD), a fatty acid oxidation disorder, appears to be the major IEM associated with SUDI. To date, there is no data on the frequency of IEM associated with SUDI associated in Brazil. In addition, there is no literature on the frequency of MCADD and its pathogenic variants in Rio Grande do Sul (RS). Since many of these IEM are treatable, early diagnosis and treatment could prevent the occurrence of SUDI.

Principal objectives: 1) To estimate and characterize, by region of Brazil, deaths related to IEM associated with SUDI in neonates and infants <1year old from 2002 to 2014; 2) To evaluate the attributable fraction of IEM in SUDI situations in neonates and infants <1year old in RS population from 2002 to 2014.

Methodology: Phase 1 (Epidemiological Analysis): a cross-sectional population-based study using the Mortality Information System (SIM) database for the period of 2002 to 2014. From SIM data, the deaths of children <1year old in Brazil and RS, whose underlying cause of death corresponds to some of the IEM associated with SUDI (ICD10-E70 – disorders of aromatic amino-acid metabolism, E71 - disorders of branched-chain amino-acid metabolism and fatty-acid metabolism, E72 - Other disorders of amino-acid metabolism, and E74 - **o**ther disorders of carbohydrate metabolism) and SUDI (ICD10-R95 - Sudden infant death syndrome, R96 - Other sudden death, cause unknown, and R99 - Other ill-defined and unspecified causes of mortality) were identified. For RS data, Death Certificates (DCs) of these children were obtained from the Municipal Health Department of Porto Alegre, which were analyzed in relation to the variables of interest. For the deaths occurred in RS region, georeferencing of the cases was carried out in order to evaluate the presence of a cluster of SUDI and EIM associated with SUDI. Phase 2 (Genetic Analysis): Observational and transversal study for the detection of heterozygotes for the most frequent pathogenic variants in the *ACADM* genes (MCADD; c.985A>G and c.199T>C). Participants were volunteer blood donors who attended the Blood Bank of the Hospital de Clínicas of Porto Alegre.

Results: Phase 1: 199 deaths of children <1year old in Brazil were recorded from 2002 to 2014 due to IEM associated with SUDI, with a estimated mortality rate of deaths of 0.67: 10,000 live births (CI95% 0.58-0.77). Of these 199 deaths, 18 (9.0%) occurred in the North, 43 (21.6%) in the Northeast, 80 (40.2%) in the Southeast, 46 (23.1% and 12 (6.0%) in the Central-West region of Brazil. In all regions, ICD10-E74 was the most frequent. From 2002 to 2014, 21 children <1year old died in RS due to the IEM selected. In the same period, 650 children <1year old died due to SUDI, and estimated mortality rate was 1.5:10,000 live births (CI95% 0.33-0.81) and 45.0:10,000 live births (CI95% 15-18), respectively. The analysis of the DC showed that there is lack of information, especially regarding to initial, sequential and terminal conditions (leading to identification of the underlying cause of death) in the cases of SUDI, not allowing the association between SUDI and IEM.
In addition to that, there was no concomitance of ICD related to EIM and ICD related to SUDI in the analyzed DCs. The georeferencing of death of children <1year old by

ICD10-R95 showed that Pejuçara city is a geographical isolate with a high rate of infant deaths. With regard to ICD10-R99, Nova Bréscia city and Paraí city are both geographical isolates with high rates of infant deaths. Nova Bréscia and Paraí are also geographical isolates when all deaths by SUDI were considered (ICD10-R95, R96 and R99). The georeferencing of deaths by ICD10-R96 and EIM associated with SUDI was not performed due to the small number of death registries by these codes in the period evaluated (13 and 21 cases, respectively). Phase 2: To date, 300 individuals included in the study were analyzed for the pathogenic variant c.199T> C. Partial results show that there is no heterozygote for the pathogenic variant c.199T>C in *ACADM* gene.

Discussion/Conclusions: This is the first study to evaluate the number of deaths of children <1year attributed to SUDI and IEM associated with SUDI in Brazil, and especially in RS within a period of thirteen years. The low number of death records for IEM associated with SUDI in Brazil may result not from the rarity of the disorders, but from underreporting and/or underdiagnosis of these condition. Although MCADD is the major IEM associated with SUDI, ICD10-E71 (which includes fatty acid oxidation disorders), was the least frequent. In RS the number of deaths due to IEM associated with SUDI reflects the national panorama, with only 21 cases registered in the period evaluated. On the other hand, due to the lack of information in the DCs it was not possible to associated cases of SUDI to IEM, especially with regard to the initial, sequential or terminal conditions that led to the establishment of the underlying cause of death. The presence geographical isolates with high rates of death of infants <1year old in Paraí, Nova Brescia and Pejuçara may be an indicator of the presence of IEM in those regions. This hypothesis is supported by some facts such as the difficulty of diagnosing of IEM which are complex diseases; the absence of metabolic necropsy in Brazil; the European ancestry of these regions, which may be associated with a higher incidence of MCADD, the most common IEM associated with SUDI. Regarding the genetic analysis of the *ACADM* gene, the results are still partial, not allowing the establishment of the frequency of heterozygotes for the c.199T> C and c.985A> G mutations in the healthy population of RS and nor the determination of the incidence of MCADD in the region.

Keywords: sudden death, inborn errors of metabolism, SUDI, infant mortality.

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ABREVIATURAS

CID: Classificação Internacional de Doenças

DO: declaração de óbito

EIM: erros inatos do metabolismo

EUA: Estados Unidos da América

LCAD: acil-CoA desidrogenase de cadeia longa

LCEH: 2,3-enoil-CoA hidratase de cadeia longa

LCHAD: 3-hidroxi-acil-CoA de cadeia longa

LKAT: 3-cetoacil-CoA tiolase de cadeia longa

MCAD: acil-CoA desidrogenase de cadeia media

MCADD: deficiência de acil-Coa desidrogenase de cadeia média

MKAT: 3-cetoacil-CoA tiolase de cadeia media

MS: Ministério da Saúde

MTP: complexo da proteína trifuncional mitocondrial

ODM: Objetivos do Desenvolvimento do Milênio

OMS: Organização Mundial de Saúde

PIDCER: Plano Integral de Defeitos Congênitos e Doenças Raras – Uruguai

PNPNL: Programa Nacional de Triagem Neonatal – Uruguai

PNTN: Programa Nacional de Triagem Neonatal

RS: Rio Grande do Sul

SCAD: acil-CoA desidrogenase de cadeia curta

SCHAD: 3-hidroxi-acil-CoA de cadeia curta

SCHE: 2,3-enoil-CoA hidratase de cadeia curta

SIDS: *sudden infant death syndrome* (síndrome de morte súbita infantil)

SIM: Sistema de Informações sobre Mortalidade

SKAT: 3-cetoacil-CoA tiolase de cadeia curta

SMSL: síndrome de morte súbita do lactente

SNC: sistema nervoso central

SUDI: *sudden unexpected death in infancy*

SUID: *sudden unexplained infant death*

SUS: Sistema Único de Saúde

UVE: Unidade de Vigilância Epidemiológica

VLCAD: acil-CoA desidrogenase de cadeia muito longa

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INTRODUÇÃO

1 INTRODUÇÃO

1.1 Erros inatos do metabolismo

Doenças metabólicas hereditárias ou erros inatos do metabolismo (EIM) – nome sugerido por Sir Archibald Garrod, são enfermidades relativamente raras individualmente, mas frequentes como grupo. Apesar de ser um único grupo de doenças, os EIM representam uma vasta, diversa e heterogênea coleção de condições genéticas que são uma causa significante de morbidade e mortalidade, principalmente na infância (PAMPOLS, 2010; SAUDUBRAY-CAZORLA, 2018). A maioria dos EIM é causada por deficiências enzimáticas ou defeitos em proteínas de transporte (SAUDUBRAY-CAZORLA, 2018). Quando há diminuição da atividade enzimática, ocorre um bloqueio total ou parcial de uma rota metabólica, que tem como consequência o acúmulo do substrato e a falta do produto final. Os indivíduos com EIM podem apresentar sintomatologia variada, e a gravidade do quadro clínico de cada paciente depende da rota metabólica afetada, bem como do metabólito acumulado ou deficiente. São, na sua grande maioria, doenças graves e que podem levar o paciente a óbito quando não tratadas corretamente (CHUANG e SHIH, 2001; GOMES; SANTOS; VILANOVA, 2005).

Os EIM apresentam, geralmente, herança autossômica recessiva, com risco de recorrência de 25% para cada gestação de pais heterozigotos. Atualmente são conhecidos aproximadamente 1000 EIM, com uma incidência total estimada em 1:800 (CHUANG; SHIH, 2001; OLIVEIRA et al., 2001; SANDERSON et al., 2006; SAUDUBRAY e GARCIA-CAZORLA, 2018).

Entre as doenças genéticas, os EIM destacam-se por serem complexas em termos de diagnóstico e, por muitas vezes serem, ao contrário da maior parte das demais doenças genéticas, possíveis de serem tratadas (GIUGLIANI, 1998).

1.1.1 Classificação dos erros inatos do metabolismo

Segundo Saudubray e Garcia-Cazorla, os EIM podem ser classificados de em duas grandes categorias. A categoria 1 inclui doenças que envolvem apenas um único sistema funcional (como sistema endócrino, sistema imune, ou fatores de coagulação) ou que afetam apenas um órgão ou sistema anatômico (como intestino, túbulos renais, eritrócitos ou tecido conjuntivo). Os sintomas são uniformes e o diagnóstico é geralmente fácil de ser estabelecido. A categoria 2, por sua vez, inclui doenças nas quais o defeito bioquímico base afeta uma via metabólica comum a um grande de número de células ou órgãos (por exemplo, doenças lisossômicas) ou é restrita a um órgão, mas que desencadeia consequências sistêmicas ou humorais (por exemplo, defeitos do ciclo da ureia, glicogenose hepática). As doenças dessa categoria, por sua vez, podem ser divididas em três grupos (Quadro 1) (SAUDUBRAY GARCIA-CAZORLA, 2018).

Quadro 1. Classificação clínica dos erros inatos do metabolismo (Adaptado de SAUDUBRAY e GARCIA-CAZORLA, 2018).

Grupo 1	Distúrbios que afetam o metabolismo intermediário de pequenas moléculas
Grupo 2	Distúrbios que envolvem o metabolismo energético
Grupo 3	Distúrbios envolvendo moléculas complexas

O grupo 1 é formado doenças que afetam a rede de reações bioquímicas de degradação (catabolismo), síntese (anabolismo) e reciclagem, a qual permite troca contínua entre células e nutrientes através da alimentação (carboidratos, lipídeos e proteínas) e da respiração (oxigênio). Inclui EIM que causam intoxicação aguda ou progressiva devido ao acúmulo de compostos próximos ao ponto de bloqueio metabólico, como, por exemplo, EIM do catabolismo de aminoácidos (fenilcetonúria, doença da urina do xarope do bordo, etc), a maioria das acidemias orgânicas (metilmalônica, propiônica, isolvalérica, entre outras), distúrbios do ciclo da uréia e doenças relacionadas, defeitos no metabolismo de galactose e frutose e porfirias (SAUDUBRAY e GARCIA-CAZORLA, 2018)

O grupo 2, por sua vez, é formado por EIM com sintomas que, pelo menos parcialmente, levam à deficiência na produção ou uso de energia no fígado, miocárdio, músculo, cérebro e outros tecidos. Defeitos mitocondriais são os mais

graves e geralmente sem tratamento. Os sintomas podem envolver sinais de intoxicação agudos (geralmente acionados por jejum, catabolismo, febre) ou crônicos. Os defeitos citoplasmáticos são geralmente menos graves e incluem distúrbios da glicólise, do metabolismo do glicogênio e gliconeogênese, hiperinsulinismo e defeitos no transportador da glicose, distúrbios no metabolismo da creatina e EIM da vida da fosfato pentose (SAUDUBRAY e GARCIA-CAZORLA, 2018).

O grupo 3 inclui doenças que envolvem a síntese, processamento, controle de qualidade e catabolismo de moléculas complexas. Esses processos complexos ocorrem em organelas (mitocôndrias, lisossomos, peroxissomos, retículo endoplasmático e complexo de Golgi). Os sintomas são permanentes, geralmente progressivos e independentes de eventos intercorrentes ou da ingestão alimentar. O grupo inclui doenças de depósito lisossomal, distúrbios peroxissomais, erros inatos da purina e pirimidina, erros inatos da síntese do colesterol, distúrbios dos triglicerídeos, fosfolipídeos e glicoesfingolipídeos, entre outros (SAUDUBRAY e GARCIA-CAZORLA, 2018)

1.1.2 Sintomatologia

O diagnóstico de EIM é bastante complexo para a maioria dos médicos. O número de EIM conhecidos é provavelmente tão grande quanto o número de sintomas que podem indicar um distúrbio metabólico (Quadro 2). Além disso, os pacientes podem apresentar hipoglicemia e acidose metabólica (SAUDUBRAY e CHARPENTIER, 2001).

Quadro 2. Exemplos de erros inatos do metabolismo e sintomas associados (Adaptado de BEAUDET *et al.,* 2001; APPLEGARTH, TOONE e LOWRY, 2000; MEIKLE *et al.,* 1999)

Sintoma	**Exemplos de doenças**
Diarreia	Deficiência de lactase Doenças mitocondriais
Intolerância a exercícios	Distúrbios da oxidação de ácidos graxos Distúrbios de glicogenólise Distúrbios mitocondriais
Infarto agudo do miocárdio	Doença de Fabry Homocistinúria
Emese recorrente	Galactosemia
Neuropatia periférica	Doenças mitocondriais Leucodistrofia metacromática Doenças congênitas de glicosilação
Sintomas de pancreatite	Doenças mitocondriais Glicogenose tipo I

1.1.2.1 Sintomatologia em neonatos e lactentes

Alguns dias ou semanas após o nascimento, um neonato previamente saudável pode começar a apresentar sinais de uma doença metabólica oculta. Apesar de o quadro clínico poder variar, neonatos e crianças com doenças metabólicas tipicamente apresentam letargia, diminuição do apetite, vômitos, taquipneia e convulsões (Quadro 3). Com o progresso da doença, podem surgir anormalidades em relação ao tônus (hipo ou hipertonia), postura e movimentos, além de apneia do sono (CLARKE, 2002). Elevado nível plasmático de amônia, hipoglicemia e acidose metabólica são sugestivos de EIM (Quadro 4). Além disso, os cuidadores ou médicos podem perceber um odor não usual no lactente, o qual pode estar associado a algumas doenças metabólicas (ex.: doença da urina do xarope do bordo, fenilcetonúria, acidemia isovalérica). Por fim, uma doença similar à Síndrome de Reye (encefalopatia hepática não específica, possivelmente acompanhada de hipoglicemia) pode estar presente em anormalidades da gliconeogênese, da oxidação de ácidos graxos, da cadeia de transporte de elétrons ou de ácidos orgânicos (RAGHUVEER, GARG e GARF, 2006).

Quando uma criança com EIM não diagnosticado vai a óbito, este fato geralmente é só atribuído a sepse (em doenças que predisponham o surgimento de condições infecciosas), resultando em um erro de diagnóstico (LINDOR e KARNES, 1995).

Quadro 3 Manifestações clínicas dos erros inatos do metabolismo em neonatos e lactentes (Adaptado de BURTON, 1987; SEASHMORE; RINALDO, 1993 e SAUDUBRAY-CAZORLA, 2018).

Atraso no desenvolvimento	Vômito e/ou diarreia
Letargia ou coma	Hipo ou hipertonicidade
Convulsões	Hepatomegalia e/ou hepatopatia
Dificuldade respiratória e/ou apneia	Icterícia
Face grotesca	Odores não usuais (urina, suor)
Face dismórfica	Cabelos anormais
Macroglossia	Achados oculares anormais
Atraso no crescimento	Miopatia
Infecções frequentes	Hipoglicemia
Acidose metabólica	Sepse

Quadro 4 Erros inatos do metabolismo e achados laboratoriais associados (Adaptado de BEAUDET *et al.,* 2001; APPLEGARTH, TOONE e LOWRY, 2000; MEIKLE *et al.,* 1999).

Achados laboratoriais	**Exemplos de doenças**
Função hepática anormal	Intolerância à frutose Doenças mitocondriais Galactosemia Doença de Gaucher
Hipoglicemia	Doenças do metabolismo de carboidratos Distúrbios de oxidação de ácidos graxos Doenças de depósito de glicogênio Galactosemia Acidemias orgânicas
Cetose	Aminoacidopatias Acidúrias orgânicas
Acidose metabólica	Aminoacidopatias Acidúrias orgânicas

1.2 Morte súbita inesperada na infância

Morte súbita inesperada e inexplicada na infância ou morte súbita infantil inesperada (do inglês, *Sudden unexpected death in infant* – SUDI ou *Sudden unexplained infant death* - SUID) é uma das mais frequentes causas de morte no primeiro ano de vida após o período neonatal. De acordo com Hube, SUDI é um segmento da mortalidade infantil no qual a morte ocorre de forma súbita e inesperada (HAUCK e TANABE, 2008). SUDI é responsável por cerca de 4600 mortes infantis por ano nos Estados Unidos da América (EUA) (SHAPIRO-MENDOZA, 2007). Os termos SUDI e SUID têm sido atualmente utilizados para óbitos que ocorrem de forma repentina e inesperada, geralmente em crianças aparentemente saudáveis. A categoria SUDI inclui, mas no entanto, não se limita

somente a, mortes relacionadas a sufocamento não intencional ou Síndrome de Morte Súbita Infantil (do inglês, *Sudden infant death syndrome* – SIDS), também denominada Síndrome de Morte Súbita do Lactente (SMSL). SIDS é uma classificação de exclusão, sendo definida como a "morte súbita de uma criança com menos de um ano de idade que permanece inexplicada após uma investigação completa do caso, incluindo a realização de uma autópsia completa, análise do local de morte e revisão da história clínica" (KROUS *et al.,* 2004). As principais causas de SUDI são listadas abaixo:

Síndrome da Morte Súbita Infantil (SIDS): nos EUA, SIDS é a maior causa de morte em crianças de um mês a um ano de idade e a terceira causa de mortalidade infantil, após anomalias congênitas e prematuridade/baixo peso ao nascer. SIDS contribui com 2300 mortes por ano nos EUA. SIDS ocorre mais frequentemente em crianças de dois a quatro meses de idade e raramente após os oito meses. SIDS ocorre inesperadamente e geralmente em período de sono, não sendo causada por sufocamento, aspiração, abuso ou negligência. SIDS ocorre durante uma fase crítica de rápido crescimento e desenvolvimento do cérebro durante os primeiros seis meses de vida. Esse período compreende 90% das mortes relacionadas à SIDS. A causa da SIDS ainda é desconhecida (SHAPIRO-MENDOZA, 2007).

Asfixia ou sufocamento: asfixia ou sufocamento são causados pela inabilidade de respirar. Essa condição conduz a uma falta de oxigenação do corpo, o que pode gerar perda de consciência e morte. A principal causa de asfixia reportada em crianças é o sufocamento acidental e estrangulamento na cama (SHAPIRO-MENDOZA, 2007).

Doença metabólica: os EIM são doenças genéticas raras que podem parar ou evitar que o organismo de transforme alimento em energia. Quando o organismo não pode processar os alimentos, pode ocorrer um acúmulo de substâncias tóxicas ou a deficiência de substâncias necessárias para o funcionamento corporal normal. A deficiência de desidrogenase de acil-CoA de cadeia média (MCADD) é um tipo de doença metabólica associada a um pequeno percentual de SUDI. Outros exemplos são a doença da urina do xarope de bordo, fenilcetonúria, deficiência de glicose-6-fosfato desidrogenasse (G6PD) e galactosemia (SHAPIRO-MENDOZA, 2007).

Lesão ou trauma: lesões podem ser fatais ou não fatais e podem ocorrer de forma intencional ou não intencional. Exemplos de lesões não intencionais incluem o choque da criança em um brinquedo pequeno ou a queda da cama. O abuso infantil, por outro lado, é ume exemplo de lesão intencional (DEAL, 2000).

Causas desconhecidas ou inclassificáveis: o termo desconhecido ou inclassificável é aplicado como causa da morte se a investigação da cena da morte e/ou a autópsia foram incompletas ou não realizadas e a declaração de óbito não possui evidência suficiente para registrar uma causa de morte mais específica (SHAPIRO-MENDOZA, 2007).

Em outras palavras, SUDI é um grupo muito heterogêneo e que inclui todas as categorias de morte súbita e inesperada na infância (BAJANOWSKI *et al.,*2007). Em resumo, após uma investigação completa dos casos, estas mortes podem ser diagnosticadas como sufocamento, asfixia, infecção, doenças metabólicas, arritmias cardíacas, trauma, SIDS, entre outras. Em alguns casos em que a evidência não é clara, ou não exista informação suficiente, a morte é considerada de causa indeterminada (SHAPIRO-MENDOZA, 2007).

Apesar da SUDI em bebês aparentemente saudáveis ser reconhecida desde a antiguidade, esse tipo de óbito só obteve atenção médica no século 20. A queda das nas taxas de mortalidade infantil no mundo do século 20 para os dias atuais mostra que, a cada dia, mais atenção tem sido depositada nos casos de mortalidade onde as causas de óbito são desconhecidas ou apresentam pouca explicação (FLEMING, BLAIR e PEASE, 2015). No Brasil, a taxa de mortalidade infantil caiu de 162,4 a cada 1.000 nascido vivos em 1930 para 13,82 a cada 1.000 nascidos vivos em 2015 (IBGE, 2013).

1.2.1 Morte súbita inesperada na infância e erros inatos do metabolismo

Os EIM constituem potenciais causas de morte súbita, quer pelas crises que ocasionam com intoxicação e comprometimento da sobrevivência do indivíduo, quer por provocarem alterações que aumentam o risco de falência de determinados

órgãos. (EMERY *et al*). De 0,9% a 6% dos casos de SUDI envolvem EIM (Van RIJT *et al.,* 2016).

A primeria referência à associação entre uma doença metabólica (hiperplasia congênita da supra-renal) e a SUDI data de 1962 (CLEVELAND; GREEN). No entanto, só em meados dos anos 80 é que surgiram numerosos estudos dando especial atenção à associação entre EIM e SUDI (BONHAM e DOWNING, 1992; BENNETT, VARIEND, POLLIT, 1986), os quais se focalizaram predominantemente nas alterações da beta-oxidação mitocondrial de ácidos graxos e mais concretamente na MCADD (HOWAT *et al.,* 1984; ROE *et al.,* 1986). Mais casos de MCADD em SUDI foram identificados, com uma notável alta ocorrência de mortes prévias não esclarecidas em irmãos. SUDI é o principal sintoma apresentado por pelo menos um quarto dos casos de MCADD (LOUGHREY, PREECE, GREEN, 2014).

Outras doenças da beta-oxidação de ácidos graxos, e outros EIM, incluindo acidemias orgânicas, aminoacidopatias, e doenças da cadeia respiratória, foram subsequencialmente implicadas em casos de SUDI. Entretanto, a complexidade do diagnóstico dessas doenças, combinada com a falta de expertise e de recursos para proceder com a investigação metabólica de SUDI, resulta em subinvestigação e subdiagnóstico (LOUGHREY, PREECE, GREEN, 2014; MOORE *et al.,* 2000).

Os defeitos de beta-oxidação de ácidos graxos parecem ser responsáveis por cerca de 5% dos casos de morte súbita em neonatos (YAMAMOTO *et al.,* 2015). A maioria das doenças de beta-oxidação de ácidos graxos impedem o uso de gordura ou proteína como uma fonte alternativa durante períodos de jejum e/ou períodos de aumento de demanda metabólica. Essas condições podem levar a quadros de hipoglicemia e crises metabólicas, as quais podem resultar rapidamente em morte súbita (TAYLOR *et al*, 2016). Uma revisão da literatura proposta por van Rijt e colaboradores mostra que, no mínimo, 43 EIM estão associados à morte súbita e/ou a síndrome de Reye sendo que 26 destes já mostram sintomas durante o período neonatal. Ao menos 32 dos EIM relatados são passíveis de tratamento e diversos estão associados exclusivamente a SUDI (quadro 5) (van RIJT *et al.,* 2016).

Quadro 5 Erros inatos do metabolismo associados à morte súbita. Adaptado de van RIJT *et al.,* 2016.

Doenças metabólicas associadas com morte súbita
Metabolismo de aminoácidos e peptídeos Doenças do ciclo da ureia Deficiência de carbamoilfosfato sintetase Deficiência de ornitina transcarbamilase Citrulinemia tipo I Acidúria arginosuccínica Acidemias orgânicas Acidemia glutárica tipo I Acidemia metilmalônica Acidemia isovalérica Acidemia metilglutânica tipo II Acidemia L-2-hidroxiglutárica Metabolismo da biotina Deficiência de biotinidase Doenças do metabolismo da fenilalanina e da tirosina Tirosinemia tipo I Doenças do metabolismo da glicina e da serina Hiperglicinemia não cetótica Doenças do transporte de aminoácidos Intolerância à proteína lisinúrica
Metabolismo de carboidratos Doenças de gliconeogênese Deficiência da fosfoenolpiruvato carboxiquinase Doenças de depósito de glicogênio Glicogenose tipo Ia Glicogenose tipo Ib Glicogenose tipo II
Metabolismo de ácidos graxos e corpos cetônicos Doenças de transporte da carnitina e do ciclo da carnitina Deficiência do transporte da carnitina Deficiência da carnitina palmitoiltransferase I Deficiência da carnitina-acilcarnitina translocase Deficiência da carnitina palmitoiltransferase II Doenças mitocondriais de beta-oxidação de ácidos graxos Deficiência de acil-CoA desidrogenase de cadeia muito longa Deficiência de acil-CoA desidrogenase de cadeia média Múltiplas deficiências de acil-CoA desidrogenases Deficiência da desidrogenase de 3-hidróxi-acilCoA de cadeia longa
Metabolismo energético Distúrbios da cadeia respiratória mitocondrial

Os EIM associados à SUDI serão descritos mais detalhadamente na próxima seção.

1.3 Beta-oxidação de ácidos graxos

Os ácidos graxos representam uma importante fonte de energia em períodos de estresse catabólico relacionado ao aumento da atividade muscular, jejum

prolongado ou doenças febris, onde cerca de 80% da energia destinada ao coração, músculo esquelético e fígado é derivada dos mesmos. Os ácidos graxos têm um papel importante em neonatos uma vez que eles apresentam limitada reserva de glicogênio e alta taxa metabólica (RINALDO, MATERN e BENETT, 2002).

A beta-oxidação de ácidos graxos é a principal fonte de energia para o músculo esquelético e ao coração, enquanto que o fígado oxida ácidos graxos em condições de jejum prolongado, durante episódios de doença e durante período de intensa atividade física. A beta-oxidação de ácidos graxos também desempenha um papel essencial no metabolismo intermediário do fígado (EATON, BARTLETT e POURFARZAM, 1996).

A beta-oxidação mitocondrial dos ácidos graxos é realizada em quatro etapas e por meio de quatro reações enzimática, resultando na remoção sequencial de unidades de dois carbonos na forma de acetil-Coenzima A (Figura 1). Os ácidos graxos plasmáticos são transportados ativamente através da membrana, esterificados para coenzima A, carreados por proteínas de ligação através do citoplasma para a mitocôndria, e translocados para dentro da matriz da mitocondrial por canais de carnitina. Uma vez dentro da matriz, os ácidos graxos são clivados, perdendo dois carbonos, pelas quatro reações da espiral de beta-oxidação. Cada etapa é catalisada por de duas a quatro enzimas distintas, codificadas por diferentes genes, mas que apresentam sobreposição de substratos. A primeira etapa da espiral é a reação da acil-CoA-desidrogease, catalisada pela acil-CoA desidrogenase de cadeia muito longa (VLCAD) e suas enzimas homólogas, acil-CoA desidrogenase de cadeia longa (LCAD), de cadeia média (MCAD) e de cadeia curta (SCAD). A segunda etapa adiciona uma molécula de água e é catalisada tanto pela 2,3-enoil-CoA hidratase de cadeia longa (LCEH) ou de cadeia curta (SCHE). A terceira etapa é catalisada pelas desidrogenases de 3-hidróxi-acilCoA de cadeia longa (LCHAD) ou curta (SCHAD), as quais oxidam a posição 3-hidróxi produzindo 3-cetoacil-CoA. A quarta e última etapa é mediada pela 3-cetoacil-CoA tiolase de cadeia longa (LKAT), média (MKAT) ou curta (SKAT), as quais reduzem o substrato de acil-CoA graxo pela clivagem de um resíduo de acetil-Coa. Para ácidos graxos de cadeia longa, as últimas três etapas são mediadas pelo complexo da proteína trifuncional mitocondrial (MTP) (RECTOR, PAYNE e IBDAH, 2008).

Os defeitos de beta-oxidação de ácidos graxos, descritos na década de 70, são um importante grupo de EIM, apresentando uma grande heterogeneidade clínica e alta morbidade e mortalidade. As manifestações clínicas ocorrem geralmente no primeiro ano de vida, especialmente após eventos catabólicos como febre, vacinação, jejum e exercício prolongado. As manifestações mais comuns durante as crises são vômito, letargia, coma e morte súbita (RECTOR, PAYNE e IBDAH, 2008).

Em 1982, foi descrita MCADD em pacientes que apresentavam descompensação metabólica com sintomas neurológicos durante o jejum (KOLVRAA *et al.,* 1982). Atualmente, já estão descritas pelo menos 23 doenças nesse grupo (BLAU *et al.,* 2014). Dentre os principais defeitos de β-oxidação de ácidos graxos, encontram-se defeitos no transporte de carnitina na membrana plasmática, nas enzimas carnitina palmitoiltransferase I e II, carnitina/acilcarnitina translocase, nas VLCAD, MCAD, SCAD, na 2,4-dienoil-CoA redutase e nas LCHAD e SCHAD, bem como na MTP, sendo que MCADD é o mais comum entre eles (ROE e DING, 2001).

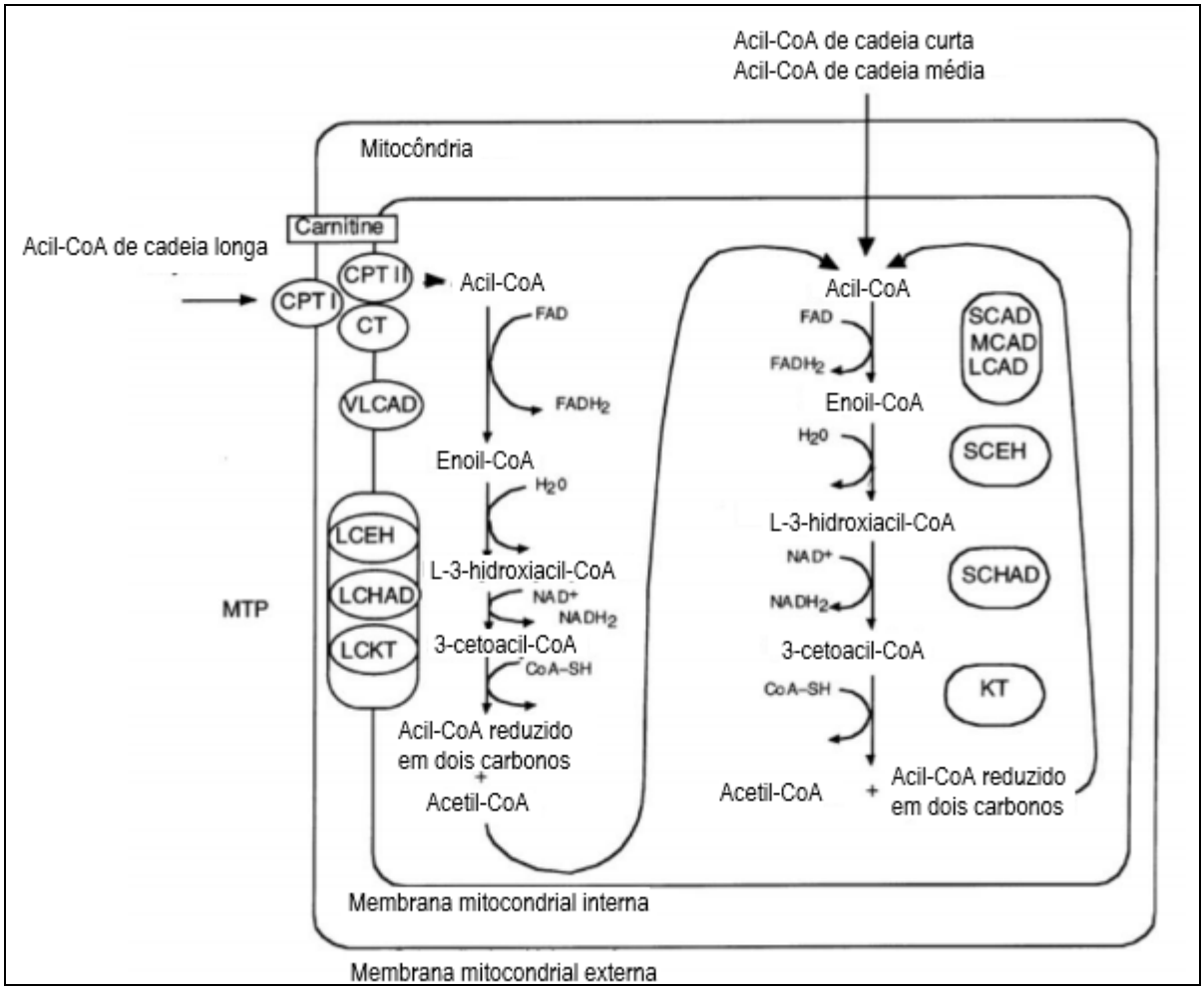


Figura 1 Esquema da beta-oxidação dos ácidos graxos na mitocôndria.

Carnitina palmitoil transferase I (CPTI), carnitina palmitoiltransferase II (CPT2), desidrogenases de acil-CoA de cadeia muito longa (VLCAD), proteína trifuncional mitocondrial (MTP), 3-cetoacil-CoA tiolase de cadeia longa (LCKT), desidrogenase de 3-hidróxi-acilCoA de cadeia longa (LCHAD), desidrogenase Acil-CoA de cadeia média (MCAD), desidrogenases de 3-hidróxi-acilCoA de cadeia curta (SCHAD), enoil-CoA hidratase de cadeia curta (SCEH), ketotiolase (KT), acil-CoA desidrogenase de cadeia curta (SCAD) (Adaptado de Tyni e Pihko, 1999).

1.3.1 Deficiência da desidrogenase de acil-CoA de cadeia média

A MCADD foi descrita pela primeira vez em 1976 é o defeito mais comum de beta-oxidação de ácidos graxos, sendo sua incidência de 1:10.000 a 1:27.000 nascidos vivos (GREGERSEN, LAURITZEN e RASMUSSEN, 1976; MAIER *et al.,* 2005; ROE e DING, 2001; LINDNER, HOFFMANN e MATERN, 2010). Em

caucasianos, MCADD afeta 1:9.000 1:15.000 nascidos vivos (ANDRESEN *et al.,* 2001; GROSSE *et al.,* 2006).

O defeito enzimático resulta num bloqueio da degradação de ácidos graxos de cadeia média, sendo bioquimicamente caracterizado pelo acúmulo de grandes quantidades de octanoato, decanoato, cis-4-decenoato e seus derivados de carnitina, assim como acidose láctica durante episódios de descompensação metabólica (SCHATZ e ENSENAUER, 2010; ROE e DING, 2001).

1.3.1.1 Fisiopatologia

A beta-oxidação de ácidos graxos consiste em quatros reações sequenciais catalisadas por dois conjuntos de enzimas de comprimento específico, as quais produzem, a cada final de ciclo, uma molécula de acetil-CoA e uma molécula de acil-CoA com dois carbonos a menos. A MCADD resulta na oxidação incompleta de ácidos graxos de cadeia média (C6-C10) e a principal manifestação clínica é a hipoglicemia hipocetótica iniciada pelo jejum. O principal distúrbio na MCADD é um inadequado fornecimento de acetil-CoA.

Normalmente, os ácidos graxos são convertidos pela via da beta-oxidação em acetil-CoA que, por sua vez, são oxidados para gerar ATP. O ATP é necessário no fígado para a síntese de glicose a partir de substratos como lactato e alguns aminoácidos (substratos que não carboidratos) pela gliconeogênese. Esse método de produção de glicose é vital para a manutenção plasmática de glicose – o principal combustível para o funcionamento do sistema nervoso central (SNC) (BHAGAVAN, 2002a).

Acetil-CoA também pode ser convertida em corpos cetônicos como o β-hidroxibutirato e o acetoacetato no fígado. Apesar de a glicose ser o combustível de escolha para o funcionamento do SNC, corpos cetônicos podem ser utilizados durante longos períodos de jejum ou fome. Além disso, ATP também é necessário para outras funções metabólicas vitais, incluindo a conversão de amônia em ureia no fígado (BHAGAVAN, 2002a).

A MCADD leva ao acúmulo de acil-CoA de ácidos graxos de cadeia média dentro das mitocôndrias. Eleva-se, então, a razão acil-CoA:CoA, causando a inibição das enzimas piruvato desidrogenase e α-cetoglutarato desidrogenase, que utilizam a coenzima A como substrato. Assim, ocorre a diminuição da conversão de piruvato em acetil-CoA e a diminuição na velocidade do ciclo do ácido cítrico, visto que a síntese de citrato e a conversão de α-cetoglutarato em succinil-CoA também estão reduzidas. Além disso, a succinil-CoA ligase também é inibida pelo ácido octanóico e também por intermediários de acil-CoA. Com a baixa produção de acetil-CoA, há redução na síntese de citrato. O citrato, por sua vez, é precursor do malato, substância necessária para a produção de glicose, via gliconeogênse, e precursor do malonil-CoA, o principal regulador inibitório da carnitina palmitoiltransferase I, enzima responsável pela entrada de ácidos graxos de cadeia longa na mitocôndria. Portanto, a diminuição dos níveis de citrato ocasionada pelo acúmulo de octanoato e outros ácidos graxos na MCADD provoca também uma diminuição da gliconeogênese e um aumento da entrada de ácidos graxos de cadeia longa na mitocôndria, o que deve ser um agravante para a hipoglicemia e deve provocar o acúmulo de derivados de acil-CoA graxos nos pacientes (ROE e DING, 2001).

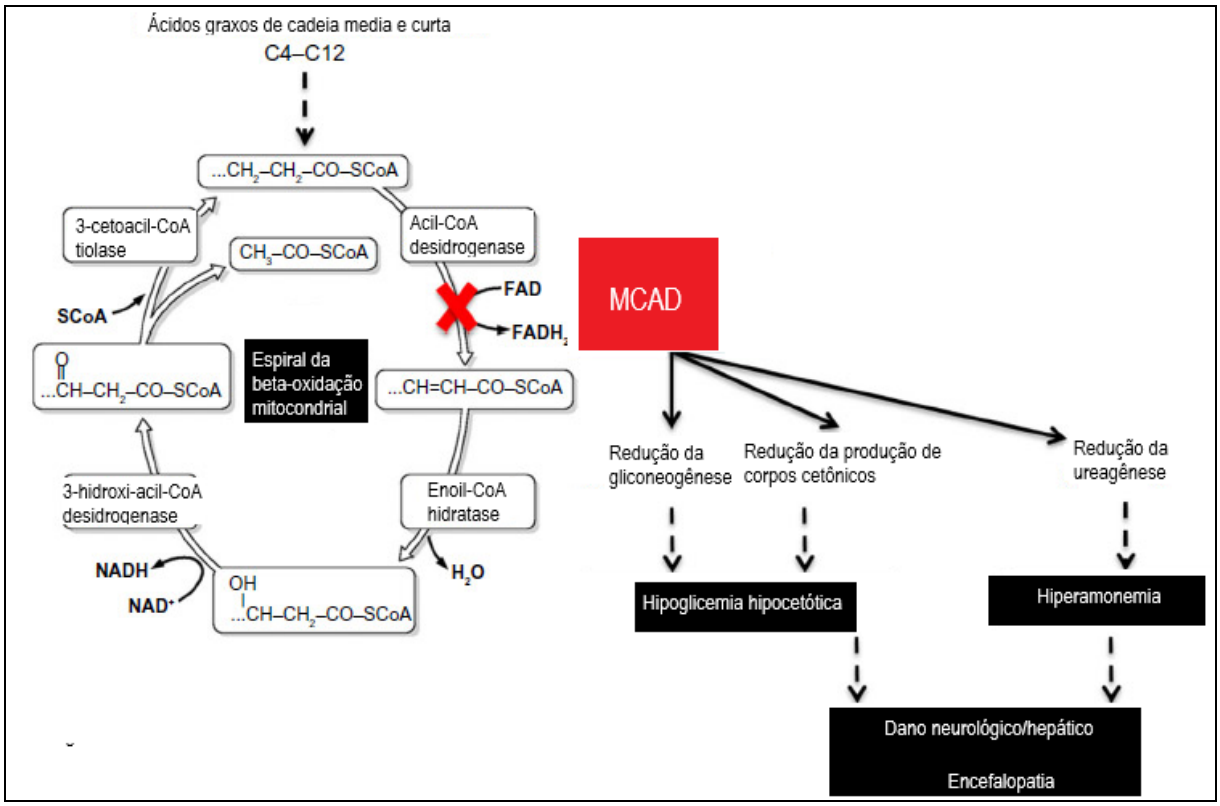
1.3.1.2 Manifestações clínicas

A MCADD pode causar hipoglicemia hipocetótica grave, hiperamonemia, encefalopatia grave, síndrome de Reye ou morte súbita (RAGHUVEER *et al.,* 2006). Durante os primeiros dias ou primeiras semanas de vida, o bebê, antes perfeitamente saudável, começa a apresentar doença metabólica em resposta a períodos longos de jejum e/ou episódios intercorrentes de infecções, o que causa perda de apetite e aumento da necessidade de utilização de energia quando a febre está presente, o que conduz a óbito em 20 a 40% doa casos nos primeiros cinco anos de vida (DERKS *et al.,* 2006).

As manifestações clínicas geralmente ocorrem durante períodos de jejum ou outras situações que envolvem estresse metabólico e são caracterizadas por letargia, convulsões e coma, bem como hipoglicemia hipocetótica. Hepatomegalia e

doença hepática aguda com hiperamonemia também podem aparecer durante as crises (TOUMA e CHARPENTIER, 1992). A baixa disponibilidade de substratos no cérebro (glicose e cetonas) combinado com a hiperamonemia e o potencialmente tóxico acúmulo de ácidos graxos de cadeia média e/ou seus derivados provavelmente são os responsáveis pela interrupção das funções energética cerebrais e pelo desenvolvimento de encefalopatia (BENNET, 2010; Figura 2).

Figura 2 Deficiência de desidrogenase de acil-CoA de cadeia media: bioquímica e consequências fisiopatológicas. Adaptado de FICICIOGLU, SOLER ALFONSO e BENNET, 2016.



Bioquimicamente falando, a doença é caracterizada pelo aumento dos níveis sanguíneos de acilcartininas C6, C8, C10 e C10:1 (RAGHUVEER *et al.,* 2006).

Estudos mostram que uma das manifestações mais frequentes da MCADD é a morte súbita (ROE e DING, 2001; LOVERA *et al.,* 2012; YUSUPOV *et al.,* 2010). Quando os sintomas não são detectados, aproximadamente 20-25% dos bebês irão à óbito ou sofrerão de danos neurológicos permanentes como consequência de primeira descompensação metabólica aguda (ARNOLD *et al.,* 2010). Apesar de o prognóstico ser excelente quando o diagnóstico é rapidamente estabelecido, morte

súbita ainda pode ocorrer ainda na idade adulta como consequência de um estresse metabólico. Os achados na autópsia incluem edema cerebral e infiltração gordurosa hepática, renal e cardíaca (BOLES *et al.,* 1998).

1.3.1.3 Aspectos genéticos

A MCADD é uma doença autossômica recessiva causada por variantes patogênicas no gene *ACADM* (acil-coenzima A desidrogenase, C-4 de cadeia de C-12 em linha reta; NM_000016.4), o qual é localizado no cromossomo 1p31 e apresenta 12 éxons (ZHANG *et al.,* 1992).

Até o momento, mais de 160 variantes patogênicas já foram identificadas nesse gene, sendo a maioria delas mutações *missense (*HGMD Professional Database, HGMD, disponível em http://www.hgmd.org). A variante patogênica mais comum na MCADD é a c.985A>G (p.Lys329Glu) no éxon 11 do gene *ACADM,* o que leva à substituição de uma lisina por um ácido glutâmico na posição 329 do precursor da proteína (posição 304 da proteína madura), sendo referenciada como K329E (ou K304E) (MATSUBARA *et al.,* 1992). Quase 80% dos clinicamente identificados com MCADD são homozigotos para c.985A>G, 18% são heterozigotos para essa variante e 2% apresentam ambos os alelos com variantes gênicas raras associadas à doença (GREGERSEN *et al.,* 1991; ANDRESEN *et al.,* 2001).

Pacientes homozigotos para a variante patogênica c.985A>G não apresentam qualquer atividade enzimática e mostram os sintomas clássicos associados à doença (ROE e DING, 2001). Além da variante patogênica previamente descrita, cerca de 6% dos indivíduos com MCADD apresentam a variante patogênica c.199T>C (p.Tyr67His), sendo heterozigotos compostos, a qual é associada com uma atividade residual da enzima MCAD (ANDRESEN *et al.,* 2001).

Um estudo de meta-regressão realizado por Leal *et al.,* 2014 mostrou que a frequência de heterozigotos para a variante patogênica c.985A>G oscila de acordo com a origem do paciente. Segundo Leal, os países das regiões ocidentais e do norte da Europa apresentam uma frequência menor da variante patogênica

c.985A>G quando comparados com os países do oeste europeu (LEAL *et al.,* 2014). A literatura aponta que a origem da variante patogênica é predominantemente no Norte da Europa (entre 1:40 e 1:100 indivíduos), o que sugere um efeito fundador (LEAL *et al.,* 2014; GREGERSEN *et al.,* 1993; TANAKA *et al.,* 1997). A Tabela 2 mostra a frequência alélica da variante patogênica c.985A>G em diferentes países. Trabalho realizado por Netto, 1997, verificou a presença da variante patogênica c.985A>G numa população do RS Para os 243 indivíduos incluídos no estudo não foi encontrada a variante patogênica pesquisada, o que pode ser resultado do pequeno amanho amostral (NETTO, 1997). Outro estudo que avaliou retrospectivamente 1722 indivíduos, mostrou que no Brasil a frequência de heterozigotos para a variante patogênica c.985A>G é de 1:246, enquanto que a incidência da MCADD é de 1:250.000 indivíduos (FERREIRA *et al.,* 2009*)*. Com relação à incidência da doença, estudos mostram que a península Ibérica apresenta uma incidência de 1:11.945 nascidos vivos (uma das maiories incidências já descritas na população mundial), enquanto que Portugal, 1:8.380 (ROCHA *et al.,* 2014). Na Alemanha, por sua vez, a incidência depende da localização geográfica, variando de 1:4.900 nascidos vivos na região norte para 1:13.400 na região sudoeste do país (SANDER *et al.,* 2001; LINDNER *et al.,* 2011). Na parte central da Itália, a incidência de MCADD é de 1:23.000 nascidos vivos (CATARZI *et al.,* 2013).

A atividade residual da enzima em indivíduos heterozigotos é bastante variável. Dependendo do tipo de variante patogênica e se o indivíduo é heterozigoto composto (c.985A>G e outro alelo mutante), a atividade residual da enzima pode variar entre 0% e 80% da atividade enzimática encontrada nos indivíduos com ambos os alelos selvagens (ROE e DING, 2001).

Tabela 1 Frequência alélica da variante patogênica c.985A>G em diferentes países (adaptado de LEAL *et al.,* 2014; FERREIRA *et al.,* 2009 e NASLAVSKY *et al.,* 2017)

País	Região	Frequência do alelo c.985A>G para cada 1.000 indivíduos (IC 95%)
Australia	Melbourne	7,0 (2,2-14,4)
Canadá	Quebec	7,1 (5,8-8,5)
Dinamarca	Copenhagen	5,0 (2,3-8,6)
	País (como um todo)	4,9 (3,0-7,3)
Alemanha	Bavária	7,2 (5,9-8,5)
Itália	Piemonte	1,5 (0,3-3,6)
	Regiões de Florença, Prato, Pistoia e Toscana	4,2 (2,0-6,8)
Espanha	Catalunha	3,5 (1,4-6,5)
	País (como um todo)	2,5 (1,2-4,2)
	Murcia	6,2 (2,9-10,0)
	Galícia	5,8 (3,7-8,1)
Inglaterra	Trent	7,3 (2,7-14,3)
	West Midlands	3,6 (2,9-4,4)
Estados Unidos	Estado de Nova Iorque	7,1 (0,2-26,3)
	Carolina do Norte	5,9 (4,0-8,2)
	Pensilvania, Ohio, Nova Jersey, Illinois, Florida e Carolina do Norte	5,8 (3,7-8,1)
Brasil	Região sudeste São Paulo	2,0 (----)* Alelo não encontrado

IC95% : intervalo de confiança de 95%; *intervalo de confiança não estabelecido nesse estudo.

1.3.1.4 Diagnóstico

Durante os períodos de descompensação metabólica, pacientes com MCADD apresentam excreção urinária de ácidos dicarboxílicos C6-C10 e conjugados de acilglicina e acilcarnitina (ROE e COATES, 2014). Em particular, a identificação da excreção de acilglicinas por cromatografia gasosa/espectrometria de massas (CG/MS) tem sido utilizada durante os episódios de descompensação metabólica (GREGERSEN *et al.,* 1993).

Como a excreção urinária desses metabólitos é extremamente baixa quando os pacientes não apresentam descompensação metabólica, CG/MS não é a técnica de escolha em casos de triagem neonatal ou diagnóstico de indivíduos assintomáticos. Nesses casos, o diagnóstico da MCADD pode ser realizado através da dosagem de acilcarnitinas no sangue por espectrometria de massas em *tandem* (MS/MS) ou cromatografia líquida (HPLC) (CHACE *et al.,* 1997), princípio básico da triagem neonatal. O perfil de acilcarnitinas de indivíduos com MCADD é

caracterizado pelo acúmulo de espécies de C6 a C10, com proeminente concentração de octanoilcarnitina (C8), com níveis de C8>C6 e com aumento da razão entre C8:C10. Pacientes que são homozigotos para a variante patogênica c.985A>G, apresentam níveis de C8 acima de 10mmol/L durante as primeiras 24h a 72h de vida. Em torno da primeira semana de vida, esses valores baixam para 1 a 6mmol/L. Qualquer recém-nascido com níveis de C8 acima de 1mmol/L apresenta forte suspeita de MCADD. Recém-nascidos com valores de C8 entre 0,3 e 1,0mmol/L podem apresentar reduzida atividade da enzima, possivelmente devido a outras variantes patogênicas que não a c.985A>G ou a uma heterozigosidade composta. Elevados níveis de C8 parecem estar relacionados a uma diminuição da atividade de MCAD (LEHOTAY *et al.,* 2004). No Brasil, a triagem neonatal por MS *tandem* não está coberta pelo Sistema Único de Saúde, sendo apenas disponível na rede privada.

A confirmação do diagnóstico pode ser feita por análise do gene *ACADM,* através da identificação dos alelos patogênicos ou através da determinação do fluxo da beta-oxidação de ácidos graxos em fibroblastos ou através da determinação da atividade enzimática de MCAD em leucócitos, fibroblastos ou outros tecidos (MATERN e RINALDO, 2015).

1.3.1.5 Tratamento

O principal objetivo do tratamento da MCADD é prevenir o desenvolvimento de deficiência intelectual com anormalidades cerebrais e morte súbita (KOMPARE e RIZZO, 2008). O tratamento de indivíduos sintomáticos consiste na reversão do catabolismo e a manutenção do anabolismo mediante a administração de carboidratos simples por via oral ou por via endovenosa, garantindo assim, uma ingestão adequada de calorias (ROE e DING, 2001; SAUDUBRAY *et a.,* 2014). Os períodos de jejum em um bebê com MCADD desde o seu nascimento até os 4 meses de idade não deve ser maior que 4 horas; entre 5 e 12 meses, uma hora adicional pode ser agregada a cada mês. A prática de exercícios intensos deve ser acompanhada de ingesta adequada de carboidratos e intensa hidratação. Fórmulas

contendo triglicerídeos de cadeia media não são apropriadas para crianças com MCADD. Para adultos, 30% das Kcal da dieta devem ser provenientes de gordura, sendo que a mesma deve incluir frutas e verduras, além de carboidratos complexos (FRAZIER, 2008).

Apesar de ainda controversa, a suplementação com L-carnitina associada à suplementação de riboflavina podem ser de valia na MCADD. A L-carnitina parece reduzir o número e a gravidade das crises de descompensação metabólica em alguns pacientes pela correção da deficiência secundária desse composto e provavelmente devido à sua capacidade de ligar-se ao metabólitos tóxicos acumulados, aumentando, assim, sua excreção urinária. A L-carnitina também pode restaurar a razão acil-CoA/CoA a qual é fundamental para as funções mitocondriais (DERKS *et al.,* 2014; LEE *et al.,* 2005). Entretanto, é necessário salientar que a melhora clínica com esse tipo de suplementação ainda não foi provada, ainda que alguns estudos mostrem que a L-carnitina e a riboflavina melhoram o fenótipo bioquímico de pacientes com MCADD (SPIERKERKOETTER *et al.,* 2010).

1.4 Classificação Internacional de Doenças

A Classificação Internacional de Doenças e Problemas Relacionados à Saúde (também conhecida como Classificação Internacional de Doenças – CID) é publicada pela Organização Mundial de Saúde (OMS) e visa padronizar a codificação de doenças e outros problemas relacionados à saúde de forma a ser uma importante ferramenta de diagnóstico padronizada para epidemiologia, gestão em saúde e finalidade clínica. Além disso, a CID é utilizada para monitorar a incidência e prevalência de doenças e outros agravos, promovendo um grande quadro da situação geral de saúde dos países e de suas populações. A última versão da CID, a versão 10 (CID-10) fornece códigos relativos à classificação de doenças e de uma grande variedade de sinais, sintomas, aspectos anormais, queixas, circunstâncias sociais e causas externas para ferimentos ou doenças. A cada estado de saúde é atribuída uma categoria única à qual corresponde um código CID-10 (WHO, 2014. Disponível em: http://www.who.int/classifications).

A CID é uma forma de classificação de eixo variável sendo estruturada de forma a agrupar as doenças da seguinte maneira: doenças epidêmicas, doenças constitucionais ou gerais, doenças localizadas organizados pelo local afetado, doenças do desenvolvimento e lesões. Esse padrão pode ser identificado nos capítulos da CID-10. Cada capítulo é subdividido em blocos homogêneos de categorias formadas por três caracteres. Dentro de cada bloco, algumas das categorias de três caracteres são para uma única condição clínica, enquanto que outras são para um grupo de doenças com algumas características comuns. A maioria das categorias de três caracteres também é subdividida, sendo utilizada, por exemplo, para identificar diferentes locais ou variedades quando a categoria de três caracteres é para um grupo de doenças. Em resumo, a CID básica é uma lista de código únicos compostas por categorias de três caracteres, sendo que cada uma pode ser dividida em até dez subcategorias de quatro caracteres cada. Os códigos variam entre A00.0 a Z99.9 (WHO, 2014. Disponível em: http://www.who.int/classifications).

Os distúrbios metabólicos são descritos nos grupos E70 a E90 (Quadro 7).

Quadro 6 Classificação de distúrbios metabólicos pela Classificação Internacional de Doenças (CID10)

CID10	**Doenças**
E70	Distúrbios do metabolismo de aminoácidos aromáticos
E71	Distúrbios do metabolismo de aminoácidos de cadeia ramificada e do metabolismo dos ácidos graxos
E72	Outros distúrbios do metabolismo de aminoácidos
E73	Intolerância à lactose
E74	Outros distúrbios do metabolismo de carboidratos
E75	Distúrbios do metabolismo de esfingolípides e outros distúrbios de depósito de lípides
E76	Distúrbios do metabolismo do glicosaminoglicano
E77	Distúrbios do metabolismo de glicoproteínas
E78	Distúrbios do metabolismo de lipoproteínas e outras lipidemias
E79	Distúrbios do metabolismo de purina e pirimidina
E80	Distúrbios do metabolismo da porfirina e da bilirrubina
E83	Distúrbios do metabolismo de minerais
E84	Fibrose cística
E85	Amiloidose
E86	Depleção de volume
E87	Outros transtornos do equilíbrio hidroeletrolítico e ácido-básico
E88	Outros distúrbios metabólicos
E89	Transtornos endócrinos e metabólicos pós-procedimentos, não classificados em outra parte
E90	Transtornos nutricionais e metabólicos em doenças classificadas em outra parte

SUDI é um termo amplo que includi todos os tipos de morte súbita e o qual pode ser dicotomizado em mortes de causa explicada ou não explicada, sem código CID. De acordo com a CID10, SIDS é codificada como código R95 – “síndrome de morte súbita na infância” (após diagnóstico de exclusão). O código 96 inclui “outras mortes súbitas de causa desconhecidas,” entre elas, morte instantânea (R96.0) e morte que ocorre em menos de 24 horas após o início dos sintomas e que não pode ser explicada (R96.1). O código R98, por sua vez, inclui os casos de “morte sem assistência”, sendo definida como indivíduo encontrado morto ou morte em circunstâncias nas quais o corpo do falecido foi encontrado e não se pode descobrir causa. Mortes por causas incertas ou duvidosas são codificadas como código R99 – “Outras causas mal definidas e não especificadas de mortalidade” – na qual a causa da morte permanece desconhecida mesmo após investigação. As únicas mortes incluídas nessa categoria são aquelas em que somente esta causa está descrita no certificado de óbito (SHAPIRO-MENDONZA *et al.,* 2006).

O código CID10 final para a causa de óbito depende, portanto, da avaliação de achados no local de óbito e na autopsia. Além de haver grande variabilidade no tipo de avaliação executada após o óbito, uma grande proporção de todos os casos de SUDI não possuem um diagnóstico conclusivo, mesmo após intensa investigação. A variabilidade dos códigos utilizada para designar SUDI tornam as análises bastantes complexas. De um modo geral, o termo SIDS é amplamente utilizado para se referir ao óbito repentino e inesperado de uma criança que não pode ser explicado após investigação. Um estudo realizado em 2015 tentou comparar os códigos de CID 10 utilizados para designar SUDI em oito diferentes países (Australia, Canadá, Alemanha, Japão, Holanda, Nova Zelândia, Inglaterra e Estados Unidos). A heterogeneidade dos códigos é imensa, sendo que qualquer óbito registrado sob os CID10 código R95 e R98 são considerados como SUDI. A proporção de óbitos codificados como R99 varia de 3,6% até 36% e somente o Japão considera o CID10 código R96 como SUDI. Desta forma, nenhum código parece ser usado de forma consistente para designar SUDI, sendo que, talvez o ideal, seja utilizar um conjunto deles para avliar morte súbita, como CID10 R95, R96 e R99 (TAYLOR *et al.,* 2015).

De acordo com o Ministério da Saúde (MS), prioritariamente para óbito de menores de 1 ano de idade, os EIM (capítulo IV) são considerados causas de óbito redutíveis por ações de prevenção, diagnóstico e tratamento precoces (Quadro 6) (BRASIL, 2001). De acordo com a OMS, anomalias congênitas, nas quais estão incluídos os erros inatos do metabolismo, são a segunda causa de óbito infantil no Brasil, nas quais estão incluídos os erros inatos do metabolismo (EIM; OMS, 2017). No RS, as anomalias congênitas desde 2015 são a primeira causa de óbito infantil (FRANÇA *et al.,* 2017).

Quadro 7 Lista de algumas doenças redutíveis por ações de prevenção, diagnóstico e tratamento precoces. Adaptado de BRASIL, 2011.

Capítulo	Grupo de causas	Códigos CID-10
I	Outras doenças bacterianas (exceto tétano do recém-nascido, outros tipos de tétano, difteria, coqueluche e síndrome de Waterhouse-Friderichsen); outras doenças por espiroquetas; outras doenças causadas por clamídias; infecções virais do SNC (exceto poliomielite aguda e raiva); infecções virais caracterizadas por lesões da pele e mucosas (exceto varicela e sarampo); micoses	A30-A32, A34, A38, A39.0, A39.2-A.49, A65-A74, A81, A83-A89, B00, B02-B04, B06-09, B35-B49
III	Doenças do sangue e dos órgãos hematopoéticos e alguns transtornos imunitários (exceto anemia por deficiência de ferro não especificada, anemia por deficiência de folato não especificada, anemia por deficiência de proteínas, anemia escorbútica e alguns transtornos que comprometem o mecanismo imunitário)	D50.0-D50.8, D51.0-D52.8, D53.1, D53.8-D53.9, D55-D77
IV	Doenças endócrinas, nutricionais e metabólicas (exceto desnutrição e outras deficiências nutricionais)	E00-E35, E65-E90
V	Retardo mental; transtornos globais do desenvolvimento	F70-F79, F84

1.5 Sistema de Informações sobre Mortalidade

O Sistema de Informações sobre Mortalidade (SIM), criado pelo Ministério da Saúde do Brasil (MS) em 1975, é produto da unificação de mais de quarenta modelos de instrumentos utilizados, ao longo dos anos, para coletar dados sobre mortalidade no país. Possui variáveis que permitem, a partir da *causa mortis* atestada pelo médico, construir indicadores e processar análises epidemiológicas que contribuam para a eficiência da gestão em saúde. O SIM foi informatizado em 1979. Doze anos depois, com a implantação do Sistema Único de Saúde (SUS) e sob a premissa da descentralização, teve a coleta de dados repassada à atribuição

dos Estados e Municípios, através das suas respectivas Secretarias de Saúde. Com a finalidade de reunir dados quantitativos e qualitativos sobre óbitos ocorridos no Brasil, o SIM é considerado uma importante ferramenta de gestão na área da saúde (SIM, 2018; disponível em: http://svs.aids.gov.br/cgiae/sim/).

A partir de sua criação foi possível a obtenção de dados sobre óbitos ocorridos no território nacional, subsidiando os diversos níveis de gerenciamento e planejamento de ações de saúde. De acordo com a Portaria nº 20 de 3 de outubro de 2003, o SIM consiste no conjunto de ações relativas à coleta, processamento, fluxo e divulgação de informações sobre os óbitos ocorridos no país (FAJARDO, AERTS, BASSANESI, 2009).

O documento básico e essencial à coleta de dados da mortalidade no Brasil é a Declaração de Óbito (DO) que, consequentemente, alimenta o SIM (Anexo I). A DO é impressa e preenchida em três vias pré-numeradas sequencialmente. Sua emissão e distribuição para os estados são de competência exclusiva do Ministério da Saúde. A distribuição para os municípios fica a cargo das Secretarias Estaduais de Saúde. Às Secretarias Municipais de Saúde cabe o controle na distribuição das DO entre os estabelecimentos de saúde, Institutos de Medicina Legal, Serviços de Verificação de Óbitos, Cartórios do Registro Civil, profissionais médicos e outras instituições que dela façam uso legal e permitido. Compete às Secretarias de Saúde (Estado e Municípios) o recolhimento das primeiras vias da Declaração de Óbito, junto aos Estabelecimentos de Saúde e aos cartórios (SIM, 2018; disponível em: http://svs.aids.gov.br/cgiae/sim/).

Segundo orientações do Centro Brasileiro de Classificação de Doenças, os dados referentes às condições e causas de óbito (anexo II) devem ser preenchidas a partir da “linha d” que representa a afecção que iniciou a sequência de eventos que determinaram o óbito. A seguir, a “linha c” e a “linha “b” que indicam as afecções consequenciais. Por fim, a afecção terminal ou imediata que levou ao óbito deve ser registrada na “linha a”. Entre as atividades desenvolvidas pelo SIM, encontra-se a codificação e a seleção da causa de óbito. Além dessas, a pesquisa de dados complementares faz-se necessária quando a doença ou lesão que iniciou a cadeia

de acontecimentos patológicos que conduziram diretamente à morte não é clara e em casos de campos não preenchidos ou incongruentes. Essa atividade faz parte da rotina de trabalho da Secretaria Municipal de Saúde de Porto Alegre, Rio Grande do Sul, Brasil (FAJARDO, AERTS, BASSANESI, 2009).

Os dados secundários do SIM são de grande importância para análises epidemiológicas no país. Um estudo de 2009 estimou a prevalência de defeitos congênitos em uma coorte de nascidos vivos no município de São Paulo entre 01/01/2006 e 30/06/2006 a partir de dados do SIM e do Sistema de Informação sobre Nascidos Vivos (SINASC). Além das declarações de nascidos vivos, o estudo avaliou as DO com base na CID10 e que apresentavam códigos Q00 a Q99 (defeitos congênitos). A partir dos dados associados desses dois bancos, foi possível estimar que a taxa de prevalência ao nascimento de defeitos congênitos na coorte foi de 86,2:10.000 nascidos vivos (GEREMIAS, ALMEIDA e FLORES, 2009). Em 2010, Telles e Schüler-Faccini realizaram um estudo sobre a frequência de malformações congênitas no RS no período entre 2001 e 2005. Foram incluídos todos os recém-nascidos vivos registrados ao nascimento como portadores de uma ou mais anomalias no campo 34 da Declaração de Nascido Vivo, sendo que esses dados foram confrontados com crianças falecidas com menos de um ano, com *causa mortis* defeito congênito e com os óbitos fetais. Nos casos avaliados, 25 malformações congênitas ou grupos de malformações corresponderam a mais de 80% dos defeitos congênitos apresentados pela população em estudo (TELLES e SCHULER-FACCINI, 2010).

Considerando que a DO é o documento oficial que atesta a morte de um indivíduo, que o SIM é o sistema oficial do MS para a informação de óbito em todo o território nacional e que a Portaria nº 1172/GM, de 15 de junho de 2004, regulamenta competências da União, dos estados, do município e do Distrito Federal, na área de Vigilância em Saúde, a Portaria n º72 do MS, de 11 de janeiro de 2010, estabelece que a vigilância do óbito infantil e fetal é obrigatória nos serviços de saúde (públicos e privados) que integram o SUS, sendo que a vigilância desses óbitos é atribuição das Unidades de Vigilância Epidemiológica (UVE) das Secretarias Estaduais, Municipais e do Distrito Federal e, no âmbito federal, do

Sistema Nacional de Vigilância Epidemiológica. Para fins dessa portaria, define-se como óbito infantil aquele ocorrido em crianças nascidas vivas desde o momento do nascimento até um ano de idade incompleto, ou seja, 364 dias (BRASIL, 2010).

A portaria nº 72 do MS prevê também que os óbitos infantis e fetais são considerados eventos de investigação obrigatória por profissinais de saúde, sendo que o instrumento base para o desencadeamento do processo de investigação é a DO, que deverá ser adequadamente preenchida. Os intrumentos base que servirão como roteiro para a investigação, por sua vez, deverão ser aqueles padronizados para uso no Estado ou Município, ou os recomendados pela publicação “Manual de Vigilância do Óbito Infantil e Fetal” do MS ou outros que venham a ser recomendados pela pela Secretaria de Vigilância em Saúde (SVS/MS).

1.6 Triagem Neonatal

A triagem neonatal é um programa de saúde populacional que tem como objetivo geral identificar distúrbios e doenças no recém-nascido, em tempo oportuno, para intervenção adequada, garantindo tratamento e acompanhamento contínuo às pessoas com diagnóstico positivo, com vistas a reduzir a morbimortalidade e melhorar a qualidade de vida das pessoas. A triagem neonatal iniciou-se no mundo na década de 60 com o rastreio de apenas uma única doença: a fenilcetonúria. Um diagnóstico precoce e um tratamento pré-sintomático pode efetivamente evitar o desenvolvimento de retardo mental em crianças com fenilcetonúria clássica. Dessa forma, a fenilcetonúria clássica foi considerado o modelo tradicional de doença passível de ser triada por triagem neonatal (WILSON e JUNGNER, 1968).

A Triagem Neonatal – Teste do Pezinho – foi incorporada ao Sistema Único de Saúde (SUS) no ano de 1992 (Portaria GM/MS n.º 22, de 15 de Janeiro de 1992) com uma legislação que determinava a obrigatoriedade do teste em todos os recém-nascidos vivos e incluía a avaliação para Fenilcetonúria e Hipotireoidismo Congênito. O procedimento foi então incluído na tabela SIA/SUS na seção de Patologia Clínica, podendo ser cobrado por todos os laboratórios credenciados que

realizassem o procedimento. No ano de 2001, o Ministério da Saúde, através da Secretaria de Assistência à Saúde, empenhou-se na reavaliação da Triagem Neonatal no SUS, o que culminou na publicação da portaria ministerial (Portaria GM/MS n.º 822, de 6 de junho de 2001) que criou o Programa Nacional de Triagem neonatal (PNTN) (BRASIL, 2001).

O PNTN é um programa de rastreamento populacional que visa promover, implantar e implementar a triagem neonatal no âmbito do SUS, visando ao acesso universal, integral e equânime, com foco na prevenção, na intervenção precoce e no acompanhamento permanente das pessoas com as doenças incluídas na triagem (BRASIL, 2001).

No Brasil, a triagem neonatal no SUS é oferecida para seis doenças: fenilcetonúria, hipotireoidismo congênito primário, anemia falciforme e outras hemoglobinopatias, fibrose cística, hiperplasia adrenal congênita e deficiência de biotinidase (BRASIL, 2001). Uma exceção à regra é o Distrito Federal. Por ter legislação própria, o Distrito Federal faz a triagem por meio da espectrometria de massa em *tandem* 100% coberta pelo SUS. Nele, são diagnosticadas 27 doenças, incluindo os defeitos de beta-oxidação de ácidos graxos, galactosemia, leucinose, entre outras (BRASIL, 2008). No âmbito privado, também é possível a realização desta “triagem neonatal ampliada”.

O Uruguai, que na década de 90 apresentava uma taxa de mortalidade infantil de 37:1000 nascidos vivos, atualmente apresenta uma taxa inferior ao Brasil (aproximadamente 8,8:1000). Isto é explicado, em grande parte, pela criação do programa nacional de triagem neonatal (PNPNL), instaurado há quase duas décadas, e do Plano Integral de Defeitos Congênitos e Doenças Raras (PIDCER). O PNPNL é de caráter obrigatório e gratuito. Apesar da triagem por espectrometria de massas em *tandem* ainda ser um estudo piloto, a triagem de fenilcetonúria e MCADD é obrigatória. Se o diagnóstico é confirmado, os pacientes recebem tratamento adequado e vitalício, incluindo acompanhamento médico, farmacológico, nutricional, cirúrgico e fonoaudiológico. Um estudo realizado em 2015, mostrou que

de janeiro de 2010 a dezembro de 2013, o PNPNL permitiu o diagnóstico de 3 casos de MCADD, bem como 10 casos de fenilcetonúria (LARRANDABURU *et al.,* 2015).

Atualmente nos EUA, o Departamento de Saúde trabalha de forma a estabelecer um *guideline* nacional para a triagem neonatal, conhecido como *Recommended Uniform Screening Panel* e, até o final de 2016, o mesmo recomenda que cada estado trie pelo menos 34 doenças principais (incluindo a MACDD e outros três defeitos de β-oxidação de ácidos graxos) e 26 condições secundárias. A maioria dos painéis inclui doenças mais tradicionais e que apresentam grande benefício ao serem detectadas precocemente, como a fenilcetonúria, até aquelas que apresentam benefício duvidoso ou aquelas que não possuem tratamento disponível. Atualmente, a triagem norte-americana é realizada por espectrometria de massas em *tandem* (MS/MS) (KELLY, MAKAREN e WASSERSTEIN, 2016).

HIPÓTESE DO ESTUDO

2 HIPÓTESE DO ESTUDO

O baixo número de registros de óbito de crianças menores de um ano de idade no Brasil por EIM associados à SUDI é resultado não da raridade das doenças, mas da subnotificação das mesmas. A falta de diagnóstico ou o diagnóstico errôneo estão associados a um tratamento inadequado, à falta de aconselhamento genético, e, possivelmente, a um maior número de óbitos erroneamente classificados como SUDI.

JUSTIFICATIVA

3 JUSTIFICATIVA

O Rio Grande do Sul (RS) apresentou em 2015 a menor taxa de mortalidade infantil (10,1:1.000 nascidos vivos) desde a década de 70, superando, inclusive, a meta dos Objetivos do Desenvolvimento do Milênio em nível nacional (15,7:1.000 nascimentos) (ODM Brasil, 2016). Entretanto, apesar dos avanços, ela ainda é semelhante a dos países desenvolvidos no final dos anos 70/início dos anos 80, e cerca de duas a cinco vezes maior do que a de países como o Japão (2,62:1000 nascidos vivos), Finlândia (2,81:1000 nascidos vivos), Cuba (5,12:1.000 nascidos vivos), Chile (7,19:1.000 nascidos vivos) e Suécia (2,56:1000 nascidos vivos).

De acordo com a OMS, anomalias congênitas, nas quais estão incluídos os erros inatos do metabolismo, são a segunda causa de óbito infantil no Brasil, nas quais estão incluídos os erros inatos do metabolismo (EIM; OMS, 2017). No RS, as anomalias congênitas desde 2015 são a primeira causa de óbito infantil (FRANÇA *et al.,* 2017). As razões que levaram o país a controlar as afecções perinatais e, por consequência, diminuir as taxas de mortalidade infantil incluem: mudanças socioeconômicas e demográficas (crescimento econômico, redução da disparidade entre ricos e pobres, urbanização, aumento do nível de educação das mulheres e diminuição das taxas de fertilidade), intervenções fora do setor de saúde (como, por exemplo, melhorias nas redes de água e esgoto), programas de saúde (promoção do aleitamento materno e campanhas de vacinação) e implementação de outros programas em nível nacional e estadual para melhorar a saúde e a nutrição infantil, além de promoverem a saúde da mulher (VICTORA *et al.,* 2011). No momento em que há um controle das afecções perinatais, as anomalias congênitas despontam como uma preocupação em Saúde Pública (ODM Brasil, 2016). A prevenção das anomalias congênitas pode ser feita por meio do diagnóstico correto das mesmas e consequente aconselhamento genético.

A incidência isolada de cada um dos EIM é pequena, uma vez que se tratam, em sua maioria, de doenças com padrão de herança autossômica recessiva. A incidência cumulativa de todos os EIM é de aproximadamente 1:800 nascidos vivos (MARK, 2013). Entretanto, o baixo registro de mortes associadas a EIM pode

representar não a raridade dos distúrbios, mas sim a subestimação do seu diagnóstico. A falta de diagnóstico ou diagnóstico errôneo está associada a um tratamento inadequado e, possivelmente, a uma maior morbidade e mortalidade desse conjunto de doenças.

Os EIM constituem potenciais causas de morte súbita, quer pelas crises que ocasionam com intoxicação e comprometimento da sobrevivência do indivíduo, quer por provocarem alterações que aumentam o risco de falência de determinados órgãos. Vários foram os elementos que geraram suspeita entre a relação de EIM e SUDI.

A taxa de morte súbita em crianças relacionada a transtorno metabólico é estimada em 3% (LABAYRU ECHEVERRIA, 2001). Uma das manifestações clínicas mais frequentes da MCADD é a SUDI (ROE e DING, 2001). Quando os sintomas não são detectados, aproximadamente 20-25% dos bebês irão à óbito ou sofrerão de danos neurológicos permanentes como consequência de primeira descompensação metabólica aguda (ARNOLD *et al.,* 2010). O principal EIM associado à SUDI é a MCADD (gene *ACADM*). Quase 80% dos pacientes com MCADD clinicamente identificados são homozigotos para c.985A>G no gene *ACADM*, 18% são heterozigotos para c.985A>G e 2% apresentam ambos os alelos com variantes gênicas raras associadas à doença (GREGERSEN, BROSS E ANDRESEN, 2004; ANDRESEN *et al.,* 2001). Somente um artigo avalia a incidência da mutação c.985A>G na população brasileira, sendo que o mesmo foi realizado na região sudeste do Brasil (FERREIRA *et al.,* 2009). Além da variante patogênica previamente descrita, cerca de 6% dos indivíduos com MCADD apresentam a variante patogênica c.199T>C (p.Tyr42His), a qual é associada com uma atividade residual da enzima MCAD (ANDRESEN *et al.,* 2001).

Tendo em vista que as mortes precoces por MCADD podem ser consideradas evitáveis, em sua maioria, desde que garantido o acesso em tempo oportuno a serviços qualificados de saúde e que não há incidência da doença no RS, esse trabalho faz-se necessário para avaliar a prevalência da mutação c.985A>G e c.199C>T no gene *ACADM* na população do estado.

Por fim, levando em consideração que muitos dos EIM são tratáveis, que o seu diagnóstico e tratamento precoces poderiam prevenir a ocorrência de SUDI, essas mortes podem ser consideradas evitáveis, em sua maioria, desde que garantido o acesso em tempo oportuno a serviços qualificados de saúde. Sendo assim, este trabalho objtivou avaliar a ocorrência de SUDI e de mortes por EIM registrados no estado do RS e em todo o Brasil entre 2002 e 2014, no sentido de averiguar a importância relativa das doenças metabólicas e dos deficitis energéticos nas situações de SUDI, além de contribuir para o esclarecimento da causa morte de alguns casos, bem como a gerar a possível identificação de *clusters* de EIM.

4 OBJETIVOS

4.1 Objetivos gerais

a) Estimar e caracterizar por região do Brasil, os óbitos relacionados a EIM associados a SUDI em neonatos e lactentes com idade inferior a um ano no período de 2002 a 2014.

b) Avaliar a fração atribuível dos EIM nas situações de SUDI em neonatos e lactentes com idade inferior a um ano na população do RS no período de 2002 a 2014.

4.2 Objetivos específicos

a) Estimar a taxa de mortalidade mínima por SUDI em neonatos e lactentes menores de um ano no RS e no Brasil no período entre 2002 e 2014.

b) Estimar a ocorrência de óbitos por SUDI no RS possivelmente associados a EIM no período entre 2002 e 2014.

c) Realizar análise de georreferenciamento dos casos de EIM associados a SUDI e dos casos de SUDI buscando identificar possíveis *clusters* de EIM no RS.

d) Traçar o perfil dos óbitos dos neonatos e lactentes a partir das declarações de óbito por SUDI e EIM associados a SUDI no RS no período de 2002 a 2014.

e) Estimar a frequência das variantes patogênicas c.985A>G e c.199T>C associadas à MCADD em uma população saudável do RS.

f) Estimar a incidência mínima de MCADD no RS.

5 CAPÍTULO 1 – ARTIGO 1

Título do manuscrito: Infant mortality in Brazil attributable to inborn errors of metabolism with sudden death: a time-series study (2002-2014).

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INFANT MORTALITY IN BRAZIL ATTRIBUTABLE TO INBORN ERRORS OF METABOLISM ASSOCIATED WITH SUDDEN DEATH: A TIME-SERIES STUDY (2002–2014).

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ABSTRACT: The literature suggests that 0.9% to 6% of infants who die unexpectedly may have had a metabolic disorder. At least 43 different inborn errors of metabolism (IEMs) have been associated with sudden death (SUDI). To date, the frequency of IEM-associated SUDI has not been studied in Brazil. The present study sought to characterize infant mortality related to IEMs known to cause SUDI disaggregated by each of the regions of Brazil. Methods: This was a descriptive, cross-sectional, population-based study of data obtained from the Brazilian Ministry of Health Mortality Information System (SIM). Death records were obtained for all infants (age <1 year) who died in Brazil in 2002–2014 in whom the underlying cause of death was listed as ICD-10 codes E70 (Disorders of aromatic amino-acid metabolism), E71 (Disorders of branched-chain amino-acid metabolism and fatty-acid metabolism), E72 (Other disorders of amino-acid metabolism), or E74 (Other disorders of carbohydrate metabolism), which are known to be associated with SUDI. Results: From 2002 to 2014, 199 deaths of infants aged <1 year were recorded in the SIM with an underlying cause corresponding to one of the IEMs of interest. The prevalence of IEM-related deaths was 0.67 per 10,000 live births (0.58–0.77). Of these 199 deaths, 18 (9.0%) occurred in the North of Brazil, 43 (21.6%) in the Northeast, 80 (40.2%) in the Southeast, 46 (23.1%) in the South, and 12 (6.0%) in the Center-West region. Across all regions of the country, ICD10-E74 was predominant.

BACKGROUND

Inborn errors of metabolism (IEMs) are rare genetic diseases often caused by a deficiency in activity of a certain enzyme, which leads to partial or complete blockade of a metabolic pathway in the body and, consequently, buildup of the enzyme substrate and lack of the final product. The symptoms of IEMs vary widely, and the clinical severity of each patient depends on the metabolic pathway affected and on the accumulated or deficient metabolite (GOMES[1]). Most IEMs are serious diseases associated with significant morbidity and mortality, particularly in childhood (PAMPOLS[2]). More than 700 IEMs are known to science, with a cumulative incidence of approximately 1 per 800 live births (MARK[3]).

Sudden unexpected death in infancy (SUDI) is one of the most common causes of postneonatal death in the first year of life. The literature suggests that 0.9% to 6% of infants who die unexpectedly may have had a metabolic disorder (BOLES[4]; CHACE[5]; GREEN[6]). A recent systematic review showed that at least 32 different IEMs are associated with sudden death, the most common being medium-chain acyl-CoA dehydrogenase deficiency (MCADD) (van RIJT[7]).

Despite recent decline, infant mortality remains a major public health concern in Brazil. As of 2014, the infant mortality rate was 14.4 per 1,000 live births, far higher than the rates reported by countries such as Canada, Cuba, Japan, and most European nations, in which rates range from 3 to 10 per 1,000 live births (UNICEF[8]). To date, the frequency of IEM-associated sudden death has not been studied in Brazil.

The present study sought to characterize neonatal and infant mortality related to IEMs known to cause SUDI disaggregated by each of the regions of Brazil.

METHODS

This was a descriptive, cross-sectional, population-based study of data obtained from the Brazilian Mortality Information System of the Ministry of Health (SIM, available online at www.saude.gov.br/sim). Birth rates were obtained from the Live Births Information System (SINASC, available at http://www2.datasus.gov.br/DATASUS).

The SIM is the oldest health information system in the country. Established by the Ministry of Health in 1975, it has stored nationally consolidated data since 1979. The mortality information system is universal, provides high coverage, and involves the following set of actions: a) collection of the death certificate (DC); b) cause-of-death coding; c) data processing; and d) flow and dissemination of information on deaths occurring in the country. The DC is an essential document from the legal and epidemiological standpoint, and must be completed for all deaths, including fetal deaths. In principle, responsibility for completing the DC lies with the medical doctor, as enshrined in Article 84, Chapter 10, of the Brazilian Code of Medical Ethics: “A

physician may not fail to attest the death of a patient he or she had been attending to, except when there is evidence of violent death" (BRASIL[9]).

DCs are pre-numbered consecutively and printed in triplicate by the Ministry of Health and distributed free of charge to the State Departments of Health, which will subsequently supply them to the Municipal Departments of Health for distribution to health facilities, medical examiner's offices, death verification services, physicians, and notaries public. The disposition of each of the three copies of a DC is as follows: the first is collected by the Municipal Department of Health; the second is delivered by the decedent's family to the office of vital records, where it will be stored for legal purposes; and the third remains in the health facility from which death was notified, to be attached to the decedent's medical record. The DC is composed of nine blocks covering 59 variables, with one (block V) solely for recording the conditions and causes of death. It is compliant with the international death certificate template adopted by the World Health Organization (WHO) since 1948, and is particularly important as a data source for the underlying (primary) and contributing (secondary) causes of death (BRASIL[10]).

The SINASC was designed by analogy with the SIM and implemented gradually by the Ministry of Health from 1990 onward. It has contained nationally consolidated data since 2004, although the degree of coverage varied during the first few years of implementation. The SINASC registry includes information on all live births in the country, with data on the pregnancy, the delivery, and the child's condition at birth. The system's basic document is the Live Birth Certificate (BRASIL[11]), registration of which has been compulsory since 1999.

To collect data on IEM-related deaths, we selected all infant deaths recorded in Brazil in which the underlying cause was assigned an ICD-10 code (OMS[12]) corresponding to the list of 43 IEMs potentially associated with SUDI and/or Reye Syndrome, as described by van Rijt et al. (Box 1) (van RIJT[7]).

Death records were obtained for all infants (age <1 year) who died in Brazil in 2002–2014 in whom the underlying cause of death was listed as ICD-10 codes E70 (Disorders of aromatic amino-acid metabolism), E71 (Disorders of branched-chain

amino-acid metabolism and fatty-acid metabolism), E72 (Other disorders of amino-acid metabolism), or E74 (Other disorders of carbohydrate metabolism), which are known to be associated with sudden death. Although mitochondrial respiratory chain disorders do feature in the list, these disorders are clustered under a highly heterogeneous ICD category: E88 (Other metabolic disorders). Due to this heterogeneity and to the fact that not all diseases covered by this ICD code are associated with sudden death, we chose not to include them in analyses. The study period was established taking into account that pre-2002 data are highly incomplete, and that the most recent year for which information was available is 2014.

The underlying cause of death was defined according to the International Classification of Diseases, Sixth Version (1948), which adopted the International Form of the Medical Certificate of Cause of Death, used from 1950 to the present day. The WHO defines the underlying cause of death as “the disease or injury which initiated the train of morbid events leading directly to death, or the circumstances of the accident or violence which produced the fatal injury” (OMS[13]).

The frequencies of the variables of interest were calculated and used to obtain crude IEM rates, by year and location, per 1,000 live births in the same area and period. Then, 95% confidence intervals were calculated for the estimated rates.

The project was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

RESULTS

From 2002–2014, the deaths of 598,734 children under 1 year old were recorded in Brazil. Over the same period, according to the SIM, there were 199 deaths of infants under 1 attributed to the IEMs of interest, which corresponds to a median 17 deaths per year (IQR: 12-18) (Figure 1). The infant mortality rate attributable to the selected IEMs in the period of analysis was 0.67 per 10,000 live births.

Of these 199 deaths, 18 (9.0%) occurred in the North of Brazil, 43 (21.6%) in the Northeast, 80 (40.2%) in the Southeast, 46 (23.1%) in the South, and 12 (6.0%)

in the Center-West region. Across all five regions of the country, ICD-10 code E74 (Other disorders of carbohydrate metabolism) was predominant; of all IEM-related infant deaths recorded in the study period, 80 (37.2%) were assigned this ICD code as the underlying cause. In the North and Southeast regions, the second leading cause was ICD-10 code E72 (Other disorders of amino-acid metabolism), whereas in the South and Northeast regions, code E70 (Disorders of aromatic amino-acid metabolism) was the second leading cause. In the Center-West region of Brazil alone, disorders classified under ICD-10 code E71 (Disorders of branched-chain amino-acid metabolism and fatty-acid metabolism) were the second leading cause of death (Table 1).

According to the latest demographic census at the time of writing, the population of Brazil was 202,768,562, with 2,979,259 live births in 2014 and an infant mortality rate of 14.4 per 1,000. Table 2 provides infant mortality rates attributable to the IEMs of interest, using these data as a baseline.

DISCUSSION

According to the WHO, congenital anomalies are the second leading cause of neonatal and infant death, and they contribute to increased risk of chronic diseases and disability in many countries. Congenital anomalies, also known as birth defects, congenital disorders, or congenital malformations, can be defined as structural or functional anomalies (such as metabolic disorders) that occur during intrauterine life and can be identified prenatally, at birth or later in life. An estimated 94% of severe congenital anomalies occur in low- and middle-income countries (UN[14]. Available at: www.who.int). Stratification of infant mortality by causes reveals that the overall mortality rate is declining in many regions worldwide, particularly that attributable to infectious causes; as a result, the proportion of such deaths attributable to congenital malformations is on the rise (HOROVITZ[15]). However, it bears stressing that structural anomalies account for the majority of congenital disorders; although metabolic derangements are considered within the definition of congenital anomalies, they are rarely reported in global statistics. Within this context, the present study was the first to evaluate infant mortality attributable to IEMs in Brazil. The data obtained

show that IEM-related infant deaths may be underreported in the Center-West, North, and Northeast regions of the country, while a higher mortality rate was observed in the South.

As infectious diseases and nutrient deficiencies are being addressed, congenital and hereditary disorders are becoming increasingly pertinent in public health, and must be the object of specific official actions (HOROVITZ[16]; GOMES[17])

Despite recent decline in Brazil, infant mortality remains a major public health concern. Current levels are considered high and incompatible with country development; many serious issues must be addressed to tackle this, such as persistent, notorious regional and urban inequalities (UNICEF[8]).

In September 2000, the United Nations convened the Millennium Summit, a meeting of heads of state and government which saw the adoption of the Millennium Declaration, which sets out eight general goals to solve most of the problems faced by poor countries. Among these goals is a reduction in child mortality. In Brazil, the goal was to reduce by two thirds, by 2015, the mortality rate among children under 5. Indicators show that the infant mortality rate per 1,000 live births decreased from 29.7 in 2000 to 15.6 in 2010. The most marked decline occurred in the North region, which nonetheless still has the highest rate in Brazil. The under-5 child mortality rate also declined 65% between 1990 and 2010 (ODM Brasil[18]. Available at: www.odmbrasil.gov.br).

Disorders of beta-oxidation (included in ICD-10 code E71) appear to account for 1 to 3% of all neonatal sudden deaths (SIM[19]; WILCOX[20]; YAMAMOTO[21]). Most fatty-acid oxidation disorders prevent the use of fat or protein as an alternative energy source during periods of fasting and/or increased metabolic demand. These conditions can lead to hypoglycemia and metabolic crisis, which can rapidly result in sudden death (CÔTÉ[22]). Contradicting reports in the literature, we found that ICD-10 code E71 was least prevalent as a cause of death. This may be associated with the fact that the complexity involved in diagnosis of these diseases, combined with a lack of expertise and resources for metabolic investigation in SUDI cases, leads to under-

investigation and underdiagnosis (LOUGHREY[23]). Furthermore, metabolic autopsy is not performed in cases of sudden death in Brazil.

Neonatal screening, also known as the heel-stick test, is a preventive action designed to diagnose a variety of neonatal and infectious diseases which are asymptomatic in the neonatal period, thus allowing early intervention and disease modification through specific treatment to mitigate or altogether prevent any associated clinical sequelae. Neonatal screening has been mandatory throughout Brazil since the 1990s. In 2001, the Brazilian Ministry of Health implemented the National Neonatal Screening Program, seeking to expand existing screening opportunities and include early detection of other congenital diseases (BRASIL[24]). The conditions currently included are phenylketonuria, congenital hypothyroidism, sickle-cell disease, hemoglobinopathies, cystic fibrosis, congenital adrenal hyperplasia, and biotinidase deficiency.

A review of the literature conducted by van Rijt et al. shows that at least 43 IEMs are associated with SUDI and/or Reye Syndrome, 26 of which can cause symptoms as early as the neonatal period. At least 32 of these IEMs are treatable, and 26 can be detected by tandem mass spectrometry screening (van RIJT[7]). Of the IEMs associated with sudden death according to van Rijt et al., only biotinidase deficiency (ICD-10 E71) is part of the Brazilian neonatal screening program (van RIJT[7]; BRASIL[25]). We found that ICD-10 code E71 was the least prevalent cause of IEM-related infant death. Inclusion of this disease in the neonatal screening program probably leads to early diagnosis and, consequently, rapid initiation of appropriate treatment, thereby reducing mortality.

The isolated incidence of each of the IEMs of interest was very small, which is consistent with the fact that most are inherited in an autosomal recessive pattern. However, the cumulative incidence of all IEMs is approximately 1 in 800 live births (MARK[3]). The small number of IEM-related deaths recorded in the period of analysis (199 cases in 13 years; 0.67 deaths per 10,000 live births) may represent not the rarity of the underlying disorders, but rather their underdiagnosis. Failure to enter a death into vital records, whether due to difficulty in doing so, lack of guidance, burial in irregular cemeteries, or simple lack of knowledge of the importance of death

certificates among the population makes it difficult to measure the true magnitude of the problem and identify health interventions that might reduce mortality rates (BRASIL[26]).

It is important to highlight that Brazil is politically and geographically divided into five regions: North, Northeast, Southeast, South, and Center-West, each of which has distinct physical, demographic, and socioeconomic characteristics. The Southeast is the most populated region, while the Center-West is least populated.

The low information quality of DCs, represented by a large contingent of poorly defined or imprecise causes of death—so-called “junk codes”—and unfilled fields, hinders analysis of the factors that contribute to mortality and, consequently, makes it difficult to implement interventions (BRASIL[26]). A 2010 Brazilian study showed that physicians often found it difficult to establish the underlying cause of death, an essential piece of information that allows SIM coding. In the same study, 68% of respondents reported general difficulty in completing DCs. The large number of fields in the document and the lack of information on the patient were also reported as factors that hinder DC completion (MENDONÇA[27]). This low quality of death registration may be an additional possible cause for the low rate of IEM-related deaths during the study period. Furthermore, the growing investment in and improvement of the SIM notwithstanding, underreporting of death is still a significant issue, especially in North and Northeast Brazil (PAES[28]).

In 2013, the Office of the General Coordination for Epidemiological Analyses published the first and only document consolidating SIM data for the period 2005–2011. According to this publication, the SIM coverage rate—defined as the ratio of deaths recorded in SIM to the number of deaths predicted by the Brazilian Institute of Geography and Statistics—was 96.1%. Coverage approached 100% in nearly all states in the South, Southeast and Center-West regions. In the North and Northeast regions, some states reported >90% coverage, while others still had rates in the 80–90% range (BRASIL[29]). Underreporting of events and the high rate of poorly defined causes of death (approximately 7.0%), in addition to improperly completed or incomplete DCs, lead to variation in the quality of available mortality data (LAURENTI[30]; PEDROSA[31]; BRASIL[29]).

According to the Brazilian Society of Medical Genetics and Horovitz et al., the Southeast and South regions of the country also have the largest number of specialized medical genetics centers (SBGM[32] available at www.sbgm.org.br). Most of these facilities are located in the Southeast region, particularly in the state of São Paulo. In the South region, clinical and laboratory coverage is available across all three states. Except in the state of São Paulo, the vast majority of medical genetics centers in Brazil are located in state capitals (HOROVITZ[16]). This geographical distribution of specialized centers may be associated with a greater number of diagnoses and, consequently, of reported deaths in the Southeast and South regions. Furthermore, considering that the Southeast region has the highest rate of live births in the country, it would be expected to account for a larger number of deaths overall and, consequently, of IEM-related deaths.

Consanguinity increases the prevalence of congenital rare diseases and approximately doubles the risk of neonatal and infant death (UN[14]. Available at: www.un.org). Bronberg et al. established the rate and spatial distribution of consanguinity in South America through analysis of information from 126,213 live births of infants without congenital malformations, delivered between 1967 and 2011 at 204 hospitals affiliated with the ECLAMC (Latin American Collaborative Study of Congenital Malformations) across 10 countries in the region. Their results show that Brazil has one cluster of high consanguinity rates (1.59%, consanguinity coefficient 0.00063), which includes 9 cities predominantly in the Southeast region of the country; and two clusters of medium consanguinity rates (0.76%, consanguinity coefficient 0.00055; and 1.22%, consanguinity coefficient 0.00043), which include 7 municipalities in the Northeast and South regions, respectively (BRONBERG[33]). Another study reported finding several genetic isolates in different cities across the Southeast region, such as spinocerebellar ataxia type 1 in São Paulo and spinocerebellar ataxia type 3 in Rio de Janeiro (CASTILLA[34]). These data corroborate the findings of the present study, in which the highest IEM-related infant mortality rates during the period of analysis were reported in the South and Southeast regions. However, it bears stressing that most published studies on consanguinity in Brazil have focused precisely on the South and Southeast regions of the country.

Although studies have shown very high rates of consanguinity in rural areas in the Northeast region (6 to 41%) (FREIRE-MAIA[35]; WELLER[36]), our study detected underdiagnosis of IEMs in this region, as the proportion of IEM-related deaths recorded during the study period was lower than the proportion of live births in the region and the regional IEM mortality rate was lower than the overall countrywide rate. One plausible explanation for this finding is that, despite growing investment in and improvement of the SIM, underreporting of death is still a significant issue in North and Northeast Brazil (PAES[28]).

Likewise, our findings suggest that IEMs are underdiagnosed in the Center-West region of Brazil as well. According to the Brazilian Society of Medical Genetics, there are only seven specialized medical genetics centers across the entire region, two of which operate exclusively in the field of oncology (SBGM[32]; available at www.sbgm.org). Possibly, the smaller number of records from this region may be due to the scarcity of specialized centers, which may hinder access to diagnosis.

Some particular difficulties related to the study of IEM-related infant mortality in developing countries must be mentioned. First, there is the difficulty of classifying IEMs within the ICD-10 framework. Diseases associated with sudden death, such as mitochondrial chain disorders, are classified under highly heterogeneous categories that include different IEM groups. Another point to consider in Brazil is the SIM search function. The search strategy is restricted to main ICD-10 categories, and does not allow stratification by subgroups. For instance, although tyrosinemia corresponds to ICD-10 code E70.2 (Disorders of tyrosine metabolism) and classical phenylketonuria to ICD-10 code E70.0, the SIM would only allow searching for code E70. As SIM searches are excessively broad, we may have included deaths in our sample that were not necessarily caused by IEMs known to be associated with sudden death.

Another relevant issue is that autopsy is not always available or performed. When available, as in Brazil (during the study period, autopsy was mandatory for all infants who died at home), it is usually performed by a general pathologist for legal purpose and does not include microscopic studies and tests geared specifically to diagnosis of IEM, which may explain the underdiagnosis of IEMs as a primary cause

of death in the assessed cases. Furthermore, lack of knowledge and limited training of medical practitioners in completion of death certificates may contribute to under-registration of IEM-related deaths (BRASIL[26]). In addition, although Brazilian Ministry of Health Ordinance No. 199 established the National Policy for Comprehensive Care of Persons with Rare Diseases, neither expanded neonatal screening (which would allow early diagnosis of some IEMs) nor diagnostic confirmation of such disorders are available through the unified health system (BRASIL[37]).

The limitations of this study notwithstanding, it should be noted that SUDI remains a major cause of infant mortality, and the present investigation was the first to evaluate infant mortality caused by IEMs known to be associated with sudden death. This article also provides a comprehensive panorama of the last 13 years of operation of the SIM, an essential tool for collection of mortality data recorded in Brazil.

CONCLUSIONS

This 13-year time-series study provides the first analysis of the number of infant deaths in Brazil attributable to IEMs known to be associated with sudden death. The low death rate observed is thought to denote not only the rarity of these conditions, but rather underreporting. Studies of infant mortality are essential for health surveillance activities and to support decision-making by health managers, and serve as essential inputs for the public policy-making process and to assess the outcomes and impacts of such policies.

Underreporting may be associated with the scarcity of specialized medical genetics centers, as well as to insufficient training of health providers in proper completion of death certificates. There is a clear unmet need for strategies targeting the incidence of IEMs, which should allow not only estimation of the true impact of these disorders on infant mortality but also development of prevention strategies.

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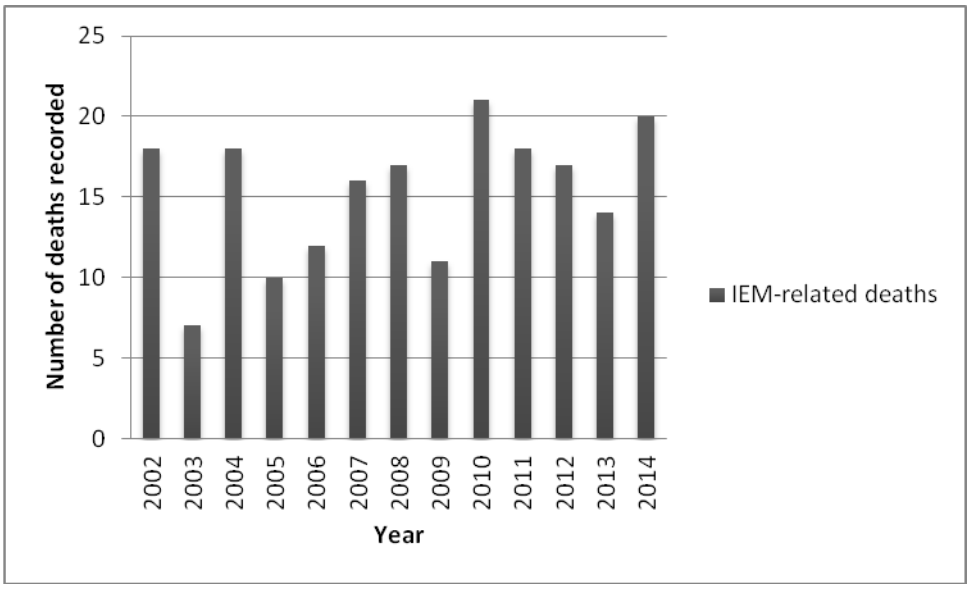

Figure 1. Distribution of the number of infant deaths due to IEMs recorded in Brazil, 2002-2014.

Table 1. Distribution of deaths due to IEMs according to ICD-10 classification, stratified by region of Brazil, 2002–2014

ICD-10	**Region**					
	North	Northeast	Southeast	South	Center-West	**Total**
E70	2	12	10	11	1	36 (18.1%)
E71	1	7	9	10	4	31 (15.6%)
E72	8	9	26	9	-	52 (26.1%)
E74	7	15	35	16	7	80 (40.2%)
Total (%)	18 (9.0%)	43 (21.6%)	80 (40.2%)	46 (23.1%)	12 (6.0%)	**199 (100%)**

Table 2. Infant mortality attributable to IEMs, stratified by region of Brazil, 2002–2014

Region of Brazil	**Cases**	**Births per case**	**Relative frequency (%)**	**Rate***	**95%CI**
South	46	396,105	23.1	1.20	0.9-1.5
Southeast	80	1,183,689	40.2	0.68	0.54-0.84
Center-West	12	245,199	6.0	0.49	2.50-0.85
Northeast	43	833,562	21.6	0.52	0.37-0.69
North	18	320,674	9.0	0.56	0.33-0.89
Brazil	199	2,979,259	100	0.67	0.58-0.77

*Per 10,000 births.

Box 1. Inborn errors of metabolism associated with sudden death. After van Rijt[7]

Metabolic diseases associated with sudden death	ICD-10
Amino-acid and peptide metabolism	
Urea cycle disorders	E72
Carbamoylphosphate synthetase deficiency	
Ornithine transcarbamylase deficiency	
Citrullinemia type I	
Argininosuccinic aciduria	
Organic acidemias	E71
Glutaric acidemia type I	
Methylmalonic acidemia	
Isovaleric acidemia	
Methylglutaconic acidemia type I	
L-2-hydroxyglutaric acidemia	
Disorders of biotin metabolism	
Biotinidase deficiency	E71
Disorders of phenylalanine or tyrosine metabolism	E70
Tyrosinemia type I	
Disorders of glycine or serine metabolism	E72
Nonketotic hyperglycinemia	
Disorders of amino-acid transport	E72
Lysinuric protein intolerance	
Carbohydrate metabolism	
Disorders of gluconeogenesis	E74
Phosphoenolpyruvate carboxykinase deficiency	
Glycogen storage disorders	E74
Glycogen storage disease type Ia	
Glycogen storage disease type Ib	
Glycogen storage disease type II	
Fatty acid and ketone body metabolism	
Disorders of carnitine transport and the carnitine cycle	E71
Carnitine transporter deficiency	
Carnitine palmitoyltransferase I deficiency	
Carnitine-acylcarnitine translocase deficiency	
Carnitine palmitoyltransferase II deficiency	
Disorders of mitochondrial fatty acid oxidation	E71
Very long-chain acyl-CoA dehydrogenase deficiency	
Medium-chain acyl-CoA dehydrogenase deficiency	
Multiple acyl-CoA dehydrogenase deficiency	
Energy metabolism	
Mitochondrial respiratory chain disorders	E88

6 CAPÍTULO 2 – ARTIGO 2

Título do manuscrito: Inborn errors of metabolism and sudden unexpected death in infancy: a panorama of the state of Rio Grande do Sul, Brazil.

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INBORN ERRORS OF METABOLISM AND SUDDEN UNEXPECTED DEATH IN INFANCY: A PANORAMA OF THE STATE OF RIO GRANDE DO SUL, BRAZIL

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Short title: IEM and SUDI in Rio Grande do Sul

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ABSTRACT: From 0.9 to 6% of cases of sudden unexpected death in infancy (SUDI) involve inborn errors of metabolism (IEM). This study aimed at evaluating the infant mortality related to IEM SUDI and SUDI in Rio Grande do Sul (RS), Southern Brazil. *Methods*: A cross-sectional, descriptive, population-based study of data obtained from the public Mortality Information System and in death certificates (DC) for RS. We obtained all DCs of children <1 year old in the period between 2002 and 2014 and whose cause of death, coded by ICD-10, was ICD-10 E70 (disorders of aromatic amino acid metabolism), E71 (disorders of branched-chain amino acid metabolism and of fatty acid metabolism), E72 (other disorders of amino acid metabolism), and E74 (other disorders of carbohydrate metabolism), R95 (sudden infant death syndrome), R96 (other sudden deaths of unknown cause) and R99 (other ill-defined and unspecified causes of mortality). Variables contained in the DC were evaluated, and the distribution of the cases in the state were georeferenced to identify clusters. *Results*: In the period evaluated, 21 and 650 children <1 year old died in RS due to IEM and SUDI, respectively. The infant mortality rate for IEM was of 1.5:10,000 live births (95%CI 0.33-0.81) and for SUDI of 45.0:10,000 live births (95%CI 15.0-18.0). The spatial analysis of infant mortality rates for SUDI, corrected per year and infant deaths (up to 1 year old), revealed Paraí and Nova Brescia as the municipalities that showed higher than expected rates for ICD-10 R99, and the municipality of Pejuçara for ICD-10 R95. The georeferencing of infant deaths due to SUDI (ICD-10 R95, R96 and R99) revealed that Paraí and Nova Brescia again had high infant death rates for these ICD. *Conclusion*: The low number of deaths due to IEM in the region might have been due to underreporting or underdiagnosis. The high rate of infant deaths due to ICD-10 R99 and SUDI (ICD-10 R95, R96 and E99) in Paraí and Nova Brescia due to ICD-10 R95 in Pejuçara may be an indicator of IEM in these localities, which should be analyzed in more detail in later studies.

INTRODUCTION

In 2015, the state of Rio Grande do Sul (RS) showed the lowest infant mortality rate in Brazil (10.1:1,000 live births) since the 1970s, even surpassing the goal of Objectives of Millennium Development at the national level (15.7:1,000 births)

(ODM Brasil, 2016). According to the World Health Organization (WHO), perinatal conditions (mainly infectious and parasitic diseases) are the leading cause of infant death in Brazil as a whole since the 1990s. In the second position, there are the congenital anomalies, which include inborn errors of metabolism. In RS, congenital anomalies appear as the first cause of infant death (FRANÇA *et al*., 2017). At the moment when there is control of the perinatal conditions, congenital anomalies emerge as a concern in public health (ODM Brasil, 2016). IEM are genetic diseases often caused by deficient activity of a given enzyme. The decrease of the enzyme activity leads to a total or partial blockage of a metabolic pathway that results in the accumulation of the substrate and the lack of the final product (CHUANG and SHIH, 2001). IEM represent a vast, diverse and heterogeneous group of genetic diseases that are a significant cause of morbidity and mortality, especially in childhood (PAMPOLS, 2010).

Although individual IEM is rare, the cumulative incidence has been shown to be upwards of 1 in 800. the incidence of overall IEMs is high and varies dramatically in different countries and regions. For example, the incidence of IEMs was reported to be 1/667 in Saudi Arabia, 1/784 in United Kingdom, 1/2500 in Canada, 1/2900 in Germany, 1/1944 in Egypt. The incidence of IEMs is much lower in Japan, approximately 1/9000 (GUO *et al.,* 2018). The cumulative incidence of IEM has been shown to be upwards of 1 in 800 (MAK *et al.,* 2013).

Although the incidence of sudden unexpected death in infancy (SUDI) in unknown, fom 0.9 to 6% of cases SUDI involve IEM (EMERY *et al*., Van RIJT *et al*., 2016). To date, approximately 43 IEM have been associated with SUDI and/or Reye's syndrome (Van RIJT *et al*., 2016), with beta-oxidation defects of fatty acids being the main cause of SUDI in childhood (SAUDUBRAY and GARCIA CAZORLA, 2018). Among fatty acid beta-oxidation defects, the main one is medium-chain acyl-CoA dehydrogenase deficiency (MCADD). SUDI is the main symptom in at least a quarter of cases of MCADD (LOUGHREY, PREECE, GREEN, 2014). When diagnosed early and with adequate treatment, it is possible to prevent the development of intellectual disability, brain abnormalities and SUDI (KOMPARE and RIZZO, 2008). Table 1 shows the IEM associated with SUDI and their

classification according to the International Classification of Diseases and Related Problems - 10th revision (ICD-10).

Considering that IEM is one of the causes of SUDI and that the true impact of this group of diseases on infant mortality in RS is not recognized, this study evaluated infant mortality related to IEM associated with SUDI in RS, taking into account the spatial distribution of cases.

METHODS

We conducted a cross-sectional, descriptive and population-based study of data obtained from the Mortality Information System (SIM) of the Brazilian Ministry of Health and from death certificates (DC). SIM, created in 1975, has variables that allow, from the *causa mortis* attested by the physician, to construct indicators and process epidemiological analyses for the purpose of collecting quantitative and qualitative data on deaths occurring throughout Brazil. The basic and essential document for the data collection of mortality in Brazil is the DC, which consequently supplies the SIM with data (available at www.saude.gov/sim).

According to the guidelines of the Brazilian Center for Disease Classification, the data referring to the ailments and causes of death (part I of the DC) should be filled starting on "line d" representing the ailments that initiated the sequence of events that determined death. Next, "line c" and "line b" indicate the consequential ailments. Finally, the terminal or immediate condition that led to death should be recorded on "line a" (FAJARDO, AERTS, BASSANESI, 2009). To obtain the "death by IEM associated with SUDI" outcome, deaths of children less than one year old who died in RS between 2002 and 2014 were selected and whose underlying cause, coded by ICD-10, was ICD-10 E70 (disorders of aromatic amino acid metabolism, ICD-10 E71 (disorders of branched-chain amino acid metabolism and fatty acid metabolism), ICD-10 E72 (other disorders of amino acid metabolism) and ICD-10 E74 (other disorders of carbohydrate metabolism) (Supplementary Table 1). Although mitochondrial respiratory chain disorders appear, these diseases are grouped into a very heterogeneous category of ICD: E88 (other metabolic disorders).

This heterogeneity and the fact that not all the diseases included in this ICD are associated with sudden death, we chose not to include them in our analysis.

In ICD-10 and according to Shapiro-Mendonza *et al*., SUDI is not a homogeneous and unique group, and may be included in the following codes: ICD-10 code R95 - "sudden infant death syndrome" (after diagnosis of exclusion); ICD-10 code 96 includes "other sudden deaths of unknown cause," including instantaneous death (R96.0) and death occurring within 24 hours after the onset of symptoms and which cannot be explained (R96.1); and ICD-10 code R99 - "Other ill-defined and unspecified causes of mortality" - in which the cause of death remains unknown even after investigation. The only deaths included in this category are those in which only this cause is described on DC (SHAPIRO-MENDONZA *et al*., 2006). Accordingly, all death records of children under one year old who died in RS, where the primary cause of death was ICD-10 R95, R96 and R99 in the period from 2002 to 2014, were included in the study.

The period from 2002 to 2014 was selected because death records in the SIM were not very robust in years prior to 2002 and that, at the time of beginning the analyses, the last year available for consultation and with consolidated data was 2014.

From the information in the SIM, a database was constructed with variables related to the child (sex, age, skin color and birth weight), maternal attributes (maternal age and maternal schooling), pregnancy and delivery (type of delivery) and at the moment of death (place of occurrence, medical care, and death confirmed by autopsy). The frequencies of the variables were calculated and, subsequently, the crude rates of IEM were obtained per year and place of occurrence, per 10,000 live births in that same area and period. 95%CI was established for the calculated rates.

For the georeferencing analyses, the total number of deaths due to ICD-10 R95, R96 and R99 was compared to the total number of deaths of children under one year old by their residence. For the calculation of smoothed rates, we used the *EBlocal* function of the *spdep* package, which is based on the empirical Bayesian model of local smoothing, which includes in its analysis, spatial effects for obtaining

the estimates, taking into account the geographic neighbors of the areas. This analysis allowed us to test if neighboring areas showed greater similarity in observed mortality compared to what would be expected in a pattern of complete spatial randomness. From the smoothed IEM rates per year and place of occurrence, the values were corrected for 100 deaths in that same area and period. Next, we performed a hierarchical cluster analysis of the smoothed and corrected rates using the *hclust* function for the visualization and definition of the IEM ranges in RS. Five smoothed rate ranges were defined for ICD-10 R95 and R99 and total SUDI (ICD-10 R95, R96 and R99). The *maptools* and *maps* packages were used to compile the case distribution maps. All analyses were performed in R version 3.4.2 software (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). The project was approved by the Ethics Committees of the Hospital de Clínicas de Porto Alegre and Municipal Health Department of Porto Alegre, under No. 16-0055.

RESULTS

Between 2002 and 2014, 21 children under one year old died in RS due to the IEM surveyed. In the same period, 650 died due to SUDI. Considering that the number of live births in RS in 2014 was 143,315 (SINASC, 2014), infant mortality rates by IEM and SUDI were calculated and shown in Table 2. In the same period in Brazil, there were 14,127 deaths due to SUDI (R95, R96 and R99) and 199 deaths due to IEM (E70, E71, E72 and E74), where infant death rate was 47/10,000 live births (95%CI 47-48) and 0.067/10,000 live births (95%CI 0.58-0.77), respectively, considering that the number of live births in 2014 was 2,979,259. The DC data of the individuals included in the study are presented in the supplementary tables.

The georeferencing of the municipalities of children under one year old who died due to ICD-10 R95 is shown in Figure 1a. The analysis of spatial distribution identified Pejuçara as a geographical isolate with a high crude death rate (1 death due to ICD-10 R95 in 2 total deaths) and a smoothed rate of 23.04, with a range of 15-24. The other municipalities showed smoothed rates of 0-2.5, 2.5-5, 5-10 and 10-15. The analysis of DC showed no particularity regarding maternal and perinatal data. In adddition, there was no information on the provision of medical care and

autopsy. In relation to the cause of death, this Dc reported unspecified congenital pneumonia as an initial condition.

The georeferencing by ICD-10 R99 identified two geographical isolates with high crude death rates in the 13 years of evaluation: Paraí (2 infant deaths due to ICD-10 R99 in 7 total infant deaths) and Nova Bréscia (1 infant death due to ICD-10 R99 in 2 total infant deaths), where the smoothed rate for Paraí was 18.57 and the one for Nova Brescia was 16.25, both in the range of 15-19. The other municipalities showed smoothed rates of 0- 2.5, 2.5-5, 5-10 and 10-15 (Figure 1b).

The DC of the baby who died in Paraí due to ICD10-R99 did not contain information on the provision of medical assistance at the time of death, but it indicated the autopsy. No initial, sequential, or terminal ailments were identified that led to the conclusion of the underlying cause of death (ICD-10 R99). Autopsy was reported. On the other hand, the DC of the baby who died in Nova Brescia included information on the provision of medical care at the time of death (at home) and the lack of autopsy as well. There had been medical assistance, but autopsy was not performed and there was no information on other conditions that could indicate the underlying cause of death.

In relation to deaths due to SUDI (including all cases of death due to ICD-10 R95, R96 and R99), georeferencing analysis again pointed to Paraí and Nova Brescia as geographical isolates with high smoothed death rates, i.e., 18.9 and 16.3, respectively, both in the range of 15-19 (Figure 1c).

The georeferencing of deaths rates due to R96 and IEM (ICD-10 E70, E71, E72 and E74) was not performed due to the low number of death records in the period analyzed (which does not allow clusterization): 13 and 21 deaths, respectively.

For children less than one year old who died of IEM, in addition to the chapter of ICD-10 that designates the underlying cause of death, it was possible to identify the category to which each belonged (Table 3).

In addition, in 19 of the 21 cases of death due to the selected IEM, the DC showed records of some initial, sequential or terminal ailments (also coded by ICD-

10): septicemia, unspecified chronic hepatitis, unspecified pneumonia, convulsions, unspecified shock, epilepsy, congestive heart failure, unspecified coma, post-viral fatigue syndrome, cardiomyopathy, respiratory failure, hepatic insufficiency, unspecified anemia and unspecified hypoglycemia.

On the other hand, for the cases of death by SUDI, only seven (1.0% of the total sample) had records of initial, sequential or terminal ailments. The following ailments were reported: inhalation of gastric juice (at home), unspecified sepsis, unspecified encephalopathy, respiratory arrest, inhalation and ingestion of food causing obstruction of the respiratory tract (at home), unspecified congenital pneumonia and convulsions. In no DC indicating SUDI were ailments found whose ICD-10 related to IEM. Likewise, no death due to IEM showed any ICD-10 associated with SUDI in the list of initial, sequential or terminal ailments.

In relation to the municipalities where the deaths occurred, our data showed that of the 21 deaths, 16 (76.2%) occurred in Porto Alegre, 2 (9.5%), in Santa Maria, 1 (4.8%) in Pelotas, 1 (4.8%) in Passo Fundo and 1 (4.8%) in Ijuí (data not shown).

DISCUSSION

Infant mortality is an important indicator of maternal and child health. Brazil, one of the largest economies in Latin America and a country historically characterized by substantial inequities in health, has taken a series of measures to improve maternal, child, and neonatal health (Barros *et al*., 2010; VICTORA *et al*., 2011). In 2015, RS achieved the lowest infant mortality rate in history (10.1: 1,000 live births). Despite advances, it remains a public health problem.

RS is the most southern state of the 27 federative units in Brazil. It is a state divided into 497 municipalities (where the emancipation of the municipality of Pinto Bandeira occurred in 2013), and its total area is 281,730,223 km^2, which is equivalent to approximately 3% of the country. With a population of 11.29 million, it is the most populous state in the southern region and the fifth most populous in the country (IBGE, 2016).

One of the vehicles for monitoring infant mortality rates in Brazil and the state is through data from the SIM. At the national level, the implementation of the SIM

allowed the continuous collection of information on the social, demographic and epidemiological characteristics of infant deaths, which allows monitoring, greater detailing and understanding of infant mortality and its determinants (HARAKI, GOTILIEB and LAURENTI, 2005). Among the activities developed by SIM, there is the codification and selection of the cause of death. In addition, the investigation of complementary data is necessary when the disease or injury that started the chain of pathological events that led directly to death is not clear and in cases of unfilled or incongruent fields. This activity is part of the surveillance of the Municipal Health Department of Porto Alegre, RS, Brazil (FAJARDO, AERTS and BASSANESI, 2009).

However, the system needs regularity and that all DC must be filled in correctly so that the conclusions drawn from the analysis are close to the local reality (SOARES, HORTA and CALDEIRA, 2007). As of 2010, the Ministry of Health of Brazil instituted mandatory monitoring of infant and fetal death throughout the country. Thanks to a defined standard, published by Administrative Order GM/MS No.72, dated January 11, 2010, the investigation of deaths became institutionally recognized as a tool for understanding the chain of determinants of deaths, especially those that are preventable. However, the number of infant deaths investigated in the country still needs to be expanded (BRASIL, 2010).

To follow Ordinance GM No. 1,172, of June 15, 2004, which recommends that a municipality investigate infant and maternal deaths, verbal autopsy (VA) forms have been established in the country as a complementary instrument of the home investigation of deaths when the cause of death is poorly defined (Chapter XVIII of ICD-10 R95 to R99). In these cases, the interviewer uses the home investigation form and, in the same home visit, applies the corresponding VA. During the investigation of the cause of death, the two forms should be used and the information obtained from the various sources will be of great value not only to determine the cause of each of the deaths but also to improve the quality of the SIM and contribute to the understanding of the changes in mortality patterns and the impact these changes have on different population groups (MINISTERIO DA SAÚDE, 2008).

This is the first study to evaluate infant mortality rate for IEM-associated SUDI and SUDI in RS. The data showed that the infant mortality rate for IEM in the state is very close to the national rate calculated for the same period (0.67/10,000 live births)

(de BITENCOURT, SCHWARTZ and VIANNA, 2018 – not published). On the other hand, the infant mortality rate for SUDI is around 30 times higher (16/10,000 live births), representing approximately 15% of the total infant mortality rate in RS in 2014 (IBGE, 2013). The main causes of SUDI described are: asphyxia or suffocation (caused by inability to breathe, where the main cause is accidental suffocation and strangulation in bed); injury or trauma; sudden infant death syndrome (SIDS, which is a diagnosis of exclusion and occurs unexpectedly and usually during sleep, not caused by suffocation, aspiration, abuse or neglect); and IEM, in addition to unknown or unclassifiable causes (when there is no investigation or the cause of death is not evident or specific) (SHAPIRO-MENDONZA, 2007).

The georeferencing of death cases by SUDI identified the municipalities of Paraí and Nova Brescia as geographical isolates with high rates of infant deaths due to ICD-10 R99 and SUDI (ICD-10 R95, R96 and R99). Paraí is located in the northeast region of RS, and has an area of 121.28 km^2 and a population of 7,357 inhabitants (IBGE, 2016). Until the year 1880, the land that today forms the municipality belonged to farmers of the municipality of Lagoa Vermelha. At the beginning of the 20th century, colonizers acquired part of these lands, dividing them into smaller lots, which were sold to immigrants, mainly Italians, but also to Portuguese and some Germans and Poles. The municipality of Nova Brescia, in turn, has a population of 3337 inhabitants and an area of 102.18 km^2 (IBGE, 2010). Like Paraí, it was colonized predominantly by Italians.

With regard to deaths due to R95, there was a high infant death rate in Pejuçara (geographical isolate), with an area of 404.78 km^2 and a population of 3,973 inhabitants (IBGE, 2010). Despite having received a large number of black slaves and having also been inhabited by indigenous people, during World War II, Pejuçara was colonized by Italian immigrants.

The occurrence of gegraphical isolates with high infant death rates for SUDI in municipalities as small as Paraí, Nova Brescia and Pejuçara, raises some hypotheses about the high occurrence of SUDI in the regions. The first one leads to a possible association between IEM and SUDI. The small population size, coupled with

the fact that the municipality originated from the territory of a few farmers, can lead to a possibility of greater consanguinity in the region. Bronberg and colleagues, through the analysis of 126,213 births of children with congenital malformations, established the rate and spatial distribution of consanguinity in South America. According to the study, Brazil has one cluster of high consanguinity rate, which includes nine cities in the southeastern region and two clusters with moderate consanguinity rate, which includes 8 municipalities in the northeastern and southern regions (BRONBERG *et al*., 2016). Because the IEM evaluated here are all autosomal recessive diseases, consanguinity may increase the prevalence of these diseases and, consequently, the risk of infant and neonatal death.

Van Rijt *et al*. showed that 0.9 to 6% of cases of SUDI involve EIM, where fatty acid beta-oxidation defects, including MCADD, appear to account for about 5% of cases of sudden death in newborns (Van RIJT *et al*., 2016; Yamamoto *et al*., 2015). SUDI is the main symptom shown by at least a quarter of cases of MCADD (LOUGHREY, PREECE, GREEN, 2005). Almost 80% of patients of European ancestry clinically identified with MCADD are homozygous for c.985A>G in the *ACADM* gene (ANDRESEN *et al.,* 2001). According to the literature, there is a founding effect of this pathogenic variant in northern Europe and the incidence of MCADD varies according to geographic location (LEAL *et al*., 2014). The Iberian Peninsula has an incidence of 1:11,945 live births (one of the highest incidences ever described in the world population), while Portugal has a rate of 1:8380 live births (ROCHA *et al*., 2014). In Germany, the incidence depends on the geographic location, ranging from 1:4900 live births in the northern region to 1:13,400 in the southwestern region of the country (SANDER *et al*., 2001; LINDNER *et al*., 2011). In the central part of Italy, the incidence of MCADD is 1:23,000 live births (CATARZI *et al*., 2013).

Given that MCADD is the main IEM associated with SUDI and that the populations of Paraí, Nova Brescia and even Pejuçara had European colonization, it is possible that the frequency of heterozygotes in the region is greater, which would lead to an increase in the incidence of the disease. Due to the complexity of the disease and the difficulty of diagnosis, which is potentiated by not including it in the

National Neonatal Screening Program, it is possible that cases of sudden death by MCADD can be classified as deaths due to SUDI, among them, ICD-10 R99 .

In addition, for an adequate diagnosis of IEM, it is crucial that physicians receive appropriate training in relation to these conditions, especially in regard to the group of IEM that causes energy deficiency, in which the clinical symptoms are not obvious, showing varied severity and long periods with no symptoms (SAUDUBRAY and GARCIA-CAZORLA, 2018). In RS, medical geneticists practice mainly in the capital (Porto Alegre), metropolitan regions and university cities (SBMG, 2018). The lack of trained professionals makes it difficult to make an early diagnose and may contribute to the increase in cases of death erroneously classified as SUDI, in the same way that the lack of metabolic investigation during autopsy may conceal a postmortem diagnosis of IEM. In our study, in the case of death by R95 (SIDS) in the municipality of Pejuçara there was no information about the autopsy. Finally, in a period of thirteen years, only 21 deaths due to IEM were recorded in RS. These data may not reflect the rarity of the disorders but their underdiagnosis and underreporting. This picture is in line with the national panorama. Although the Ministry of Health has approved Directive 199 establishing the National Policy for Comprehensive Care for Persons with Rare Diseases, neither expanded neonatal screening (which could diagnose early many of the SUDI-causing IEMs evaluated here, such as fatty acid metabolism defects) nor confirmatory diagnosis is available from the National Health System (SUS) (de BITENCOURT, SCHWARTZ and VIANNA , 2018 – not published).

In the DC, there is important information for understanding the main determinants of infant mortality. However, studies show a high proportion of incompleteness, especially with regard to infant deaths (SOARES, HORTA and CALDEIRA, 2007).

The data from our study showed that there is very low notification (about 1.0% only) of initial, sequential and/or terminal ailments, also coded by ICD-10, in SUDI DC. In addition, when present, none are included in the group of IEM studied (ICD-10 E70, E71, E72 and E74). The ailments listed are less specific, such as pneumonia or

convulsions. It has been reported that, in general, there are many problems in the DC regarding the correct logical sequence of causes of death (MENDONÇA, DRUMOND and CARDOSO, 2010). However, when these records are present, it is noted that some ailments are characteristic of SUDI, namely suffocation or inhalation , such as inhalation of gastric juice (at home) and inhalation and ingestion of food causing obstruction of the respiratory tract (at home). These ailments were reported in two DC whose underlying cause of death was coded as ICD-10 R95 (SIDS). On the other hand, clinical findings in neonates and infants with IEM are nonspecific and include vomiting, dehydration, lethargy, respiratory distress, hepatomegaly, hypotonia, and convulsions (SEASHMORE and RINALDO, 1993; SAUDUBRAY and CAZORLA, 2018). Among the ailments reported in the DC for SUDI, only convulsions are on the list of classic symptoms of IEM. Therefore, it is not feasible to evaluate whether the other cases of SUDI could be due to IEM.

Regarding race/color, our data are in agreement with the expected ethnical distribution in RS. According to the latest IBGE census, in the state, due to its European ancestry, 84.7% of the population are white, while 5.2% are black, 10.4% brown and 0.4% yellow (IBGE, 2010).

Age of death and birth weight did not show large variations between the IEM and SUDI groups or between the different ICD. Likewise, almost 50% of the children who died due to IEM and SUDI had mothers who had completed high school. A high maternal educational level is associated with an increase in infant survival rates, since it fosters effective knowledge about mechanisms to prevent, recognize and treat childhood illnesses (ATRASH, 2011).

For the cases of SUDI, we found that a third occurred without the provision of medical care at the time of death. This is in line with expectations, since SUDI occurs suddenly in previously healthy children (REYES, SOMERS and CHIASSON, 2018). For the cases of death due to IEM, in turn, most of the children received some type of medical care at the time of death, all of whom died in the hospital. This may be due to the fact that IEM are multisystemic complex diseases that show worsening over time, which means that medical follow-up is regular and that death occurs in a

hospital environment, possibly due to previous hospitalization. In addition, IEM deaths occurred in municipalities with major university centers. This can be explained by the fact that these regions have medical geneticists. Another important factor is the autopsy question as an aid in determining the underlying cause of death. Defining the underlying cause of death as ICD-10 R95 (SIDS) requires intensive investigation, including complete autopsy, detailed analysis of the place of death and review of the medical history (GOLDSTEIN, KINNEY and WILLINGERM 2016). Of the 79 cases of death by R95, only 30% were submitted to autopsy, and this information is not indicated in the same proportion of DC. In addition, in Brazil and RS, there is no ordinance that requires autopsy in cases of infant death due to ill-defined cause or SUDI. It is also important to note that, when performed, there is no metabolic investigation to diagnose IEM. Our findings showed that in almost 60% of cases of SUDI, autopsy was not performed or this data were ignored on the DC.

Some difficulties related to the study of infant mortality due to SUDI and IEM-associated SUDI should be mentioned. First, there is a difficulty in classifying IEM according to ICD-10. Diseases associated with SUDI, such as mitochondrial respiratory chain disorders, are in very heterogeneous categories and include different IEM groups. In addition, the SIM search was restricted to the main category of the ICD-10 and did not allow stratification by subgroups. This was observed when the DC were analyzed. For example, one of the causes of death cited was albinism (ICD-10 E70.3). Although disorders of aromatic amino acid metabolism (ICD-10 E70) are associated with SUDI, albinism is not one of the causative diseases of SUDI. Accordingly, the study became broad, and we might have included deaths that were not necessarily caused by IEM associated with sudden death.

Finally, it is necessary to carry out future studies in the municipalities of Paraí, Pejuçara and Nova Brescia to verify if the high rates of infant deaths due to SUDI recorded in these locations are associated with undiagnosed IEM.

CONCLUSIONS

This is the first study to evaluate the infant mortality rate for IEM-associated SUDI and SUDI in RS over a period of 13 years. The low number of IEM deaths in

the region may be due to underreporting or underdiagnosis. The incompleteness of DC with respect to initial, sequential and terminal ailments makes it difficult to establish a relationship between classic IEM symptoms in neonates and infants and SUDI. In addition, important data such as medical care at the time of death and autopsy are not indicated in a considerable number of DC, which complicates the coding of the underlying cause of death according to ICD-10. The presence of geographical isolates with high SUDI death rates among children under one year old in the municipalities of Paraí, Nova Brescia and Pejuçara may be an indicator of the presence of IEM in the region. This hypothesis is supported by factors such as the difficulty of diagnosing such complex diseases as IEM, the lack of metabolic autopsy, and the European ancestry of the region, possibly associated with a higher incidence of MCADD, the major IEM related to SUDI.

Recognition of the cause of death is of extreme importance not only in terms of public health, but at the family level, where it helps in the grieving process and provides adequate information regarding medical and genetic implications.

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Table 1. Inborn errors of metabolism associated with SUDI and their classification according to ICD-10. Adapted from van Rijt *et al*., 2016.

Inborn errors of metabolism associated with sudden death	ICD-10
Amino acid and peptide metabolism	
Urea cycle disorders	E72
Carbamoylphosphate synthetase deficiency	
Ornithine transcarbamylase deficiency	
Citrullinemia type I	
Argininosuccinic aciduria	
Organic acidemias	E71
Glutaric acidemia type I	
Methylmalonic acidemia	
Isovaleric acidemia	
Methylglutamic acidemia type II	
L-2-hydroxyglutaric acidemia	
Holocarboxylase synthetase deficiency	
Disorders of biotin metabolism	E71
Biotinidase deficiency	
Disorders of phenylalanine and tyrosine metabolism	E70
Tyrosinemia	
Disorders of glycine and serine metabolism	E72
Non-ketotic hyperglycinemia	
Amino acid transport disorders	E72
Lysinuric protein intolerance	
Carbohydrate metabolism	
Disorders of gluconeogenesis	E74
Phosphoenolpyruvate carboxykinase deficiency	
Glycogen storage disease	E74
Glycogenosis type Ia	
Glycogenosis type Ib	
Glycogenosis type II	
Metabolism of fatty acids and ketone bodies	
Disorders of carnitine transport and carnitine cycle	E71
Carnitine transport deficiency	
Carnitine palmitoyltransferase I deficiency	
Carnitine-acylcarnitine translocase deficiency	
Carnitine palmitoyltransferase II deficiency	
Defects of fatty acid oxidation	E71
Very-long-chain acyl-CoA dehydrogenase deficiency	
Medium-chain acyl-CoA dehydrogenase deficiency	
Multiple acyl-CoA dehydrogenase deficiency	
Energy metabolism	
Mitochondrial respiratory chain disorders	E88

Table 2. Infant mortality rate due to inborn errors of metabolism and sudden death in Rio Grande do Sul, Brazil (2002-2014).

ICD-10	CASES (n)	INFANT MORTALITY RATE*	95%CI
E70 – disorders of aromatic amino acid metabolism	4	0.28	0.01-0.64
E71 - disorders of branched-chain amino acid metabolism and fatty acid metabolism	6	0.42	0.018-0.83
E72 - other disorders of amino acid metabolism	3	0.21	0.06-0.54
E74 – other disorders of carbohydrate metabolism	8	0.56	0.024-1.10
E GROUPED (E70, E71, E72 and E74)	**21**	**1.5**	**0.9-2.2**
R95 – sudden infant death syndrome	79	5.5	4.4-6.9
R96 – other sudden deaths of unknown cause	13	0.98	0.53-1.6
R99 - other ill-defined and unspecified causes of mortality	558	39.0	36.0-42.0
R GROUPED (R95, R96 and R99)	**650**	**45.0**	**42.0-49.0**

*Expressed as per 10,000 live births, considering the total live births in the state in 2014 (143.315)

Table 3. Classification of initial, sequential or terminal ailments presented in death certificates involving IEM in Rio Grande do Sul, Brazil (2002-2014).

Underlying cause of death (groups)	Initial, sequential or terminal ailments
E70 (n=4)	E70.2 - disorders of tyrosine metabolism (n=3)
	E70.3 – albinism (n=1)
E71 (n=6)	E71.1 – maple syrup urine disease (n=1)
	E71.1 - other disorders of amino acido metabolism and branched-chain fatty acid metabolism (n=5)
E72 (n=3)	E72.2 - urea cycle disorders (n=1)
	E72.3 – disorders of lysine and hydroxylysine metabolism (n=1)
	E72.9 – unspecified disorder of amino acido metabolism (n=1)
E74 (n=8)	E74.0 – glycogen storage disease (n=3)
	E74.1 - disorders of fructose metabolism (n=1)
	E74.2 - disorders of galactose metabolism (n=1)
	E74.4 - disorders of pyruvate metabolism and gluconeogenesis (n=2)
	E74.9 – unspecified disorder of carbohydrate metabolism (n=1)

Supplementary Table 1. Characteristics of death certificates of children <1 year old who died due to inborn errors of metabolism associated with sudden death in Rio Grande do Sul, Brazil (2002-2014).

	E70	E71	E72	E74
CASES (n; %)	4 (19.0)	6 (28.6)	3 (14.3)	8 (38.1)
GENDER				
Female (n; %)	2 (50)	5 (83.3)	2 (67.7)	4 (50)
SKIN COLOR				
White (n; %)	3 (75)	6 (100)	3 (100)	5 (62.5)
Not white (n; %)	1 (25.0)	----	----	3 (37.5)
AGE OF DEATH (days; Mean±SD)	282.8±51.9	289.7±38.2	306.0±4.6	305.9±3.4
MATERNAL AGE (years; Mean ±SD)	25.0± 1.4 (n=3)	29.2±8.8 (n=6)	32.5±7.8 (n=2)	23.2±7.8 (n=8)
WEIGHT AT BIRTH(grams; Mean ±SD)	2765.0± 591.0 (n=3)	2965.8± 396.2 (n=6)	2490.0±381.8 (n=2)	2982.5±523.1 (n=8)
MATERNAL EDUCATION				
Middle (n; %)	1 (25)	2 (33.3)	1 (33.3)	6 (75)
Incomplete secondary (n; %)	1 (25)	1 (16.7)	----	1 (12.5)
Complete secondary (n; %)	1 (25)	1 (16.7)	----	----
Not reported (n; %)	1 (25)	2 (33.3)	2 (66.7)	1 (12.5)
MEDICAL CARE*				
Yes (n; %)	2 (50)	5 (83.3)	2 (66.7)	4 (50)
No (n; %)	----	1 (16.7)	----	4 (50)
Not reported (n; %)	2 (50)	----	1 (33.3)	----
AUTOPSY				
Yes (n; %)	2 (50)	5 (83.3)	----	1 (12.5)
No (n; %)	----	1 (16.7)	2 (66.7)	3 (37.5)
Not reported (n; %)	2 (50)	----	1 (33.3)	4 (50)
TYPE OF DELIVERY				
Vaginal (n; %)	2 (50)	3 (50)	----	6 (75)
Cesarean (n; %)	1 (25)	3 (50)	2 (66.7)	2 (25)
Not reported (n; %)	1 (25)	----	1 (33.3)	----
PLACE OF DEATH				
Hospital (n; %)	4 (100)	6 (100)	3 (100)	8 (100)

*Medical care: Indicates any assistance by medical staff
Not reported: no information in the death certificate

Supplementary Table 2. Characteristics of death certificates of children who died from sudden death in Rio Grande do Sul, Brazil (2002-2014).

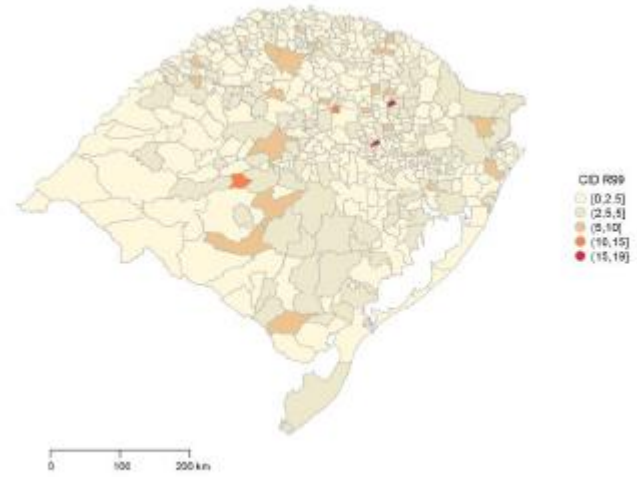
	R95	R96	R99
CASES (n; %)	79 (12.2)	13 (2.0)	558 (85.8)
GENDER			
Male (n; %)	45 (57)	8 (61.5)	306 (54.8)
SKIN COLOR			
White (n; %)	63 (79.7)	10 (76.9)	454 (81.4)
Not white (n; %)	16 (20.3)	3 (23.1)	69 (18.6)
AGE OF DEATH (days; Mean±SD)	278.6±47.0	291.2±29.9	294.9±32.1
MATERNAL AGE (years; Mean±SD)	24.0±6.6 (n=76)	23.8±5.8 (n=12)	23.1±6.4(n=478)
WEIGHT AT BIRTH(gramas; Mean±SD)	2813.7±626.6 (n=74)	2960.0±721.5 (n=12)	2772.8±669.0 (n=475)
MATERNAL EDUCATION			
No schooling (n; %)	----	----	2 (0.4)
Elementary - 1 to 4 (n; %)	3 (3.8)	----	23 (4.1)
Elementary - 5 to 8 (n; %)	9 (11.4)	2 (15.4)	47 (8.4)
Middle (n; %)	38 (48.1)	7 (53.8)	251 (45)
Incomplete secondary (n; %)	18 (22.8)	3 (23.1)	127 (22.8)
Complete secondary (n; %)	3 (3.8)	----	12 (2.2)
Not reported (n; %)	8 (10.1)	1 (7.7)	96 (17.2)
MEDICAL CARE*			
Yes (n; %)	13 (16.5)	4 (30.8)	106 (19)
No (n; %)	14 (17.7)	3 (23.1)	104 (18.6)
Not reported (n; %)	52 (65.8)	6 (46.2)	348 (62.4)
AUTOPSY			
Yes (n; %)	24 (30.4)	2 (15.4)	251 (45)
No (n; %)	26 (32.9)	8 (61.5)	151 (27.1)
Not reported (n; %)	29 (36.7)	3 (23.1)	156 (28)
TYPE OF DELIVERY			
Vaginal (n; %)	42 (53.2)	8 (61.5)	303 (54.3)
Cesarean (n; %)	32 (40.5)	2 (15.4)	172 (30.8)
Not reported (n; %)	5 (6.3)	3 (23.1)	83 (14.9)
PLACE OF DEATH			
Hospital (n; %)	25 (31.6)	8 (61.5)	236 (42.3)
Other health establishments (n; %)	----	----	22 (3.9)
Home (n; %)	46 (58.2)	3 (23.1)	241 (43.2)
Public road (n; %)	2 (2.5)	1 (7.7)	11 (2)
Others (n; %)	6 (7.6)	1 (7.7)	43 (7.7)
Not reported (n; %)	----	----	5 (0.9)

*Medical care: Indicates any assistance by medical staff

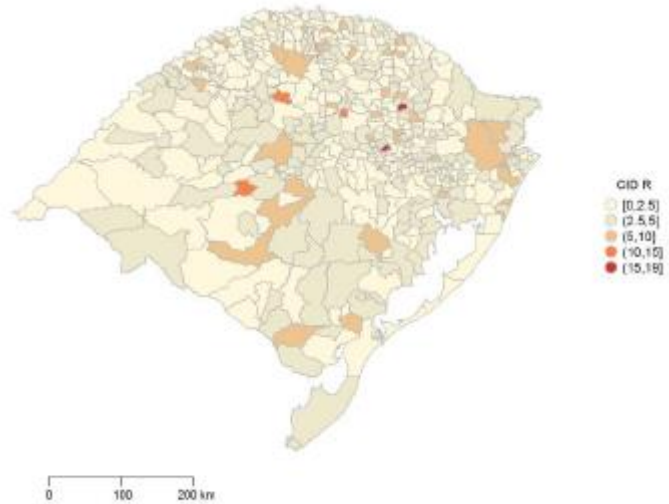
Not reported: no information in the death certificate

(a)

7



(b)



(c)

Figure 1 Map of RS showing the georeferencing of deaths for ICD-10 R95 (a), R99 (b) and R (c)

CAPÍTULO 3 – ARTIGO 3

8 CAPÍTULO 3 – ARTIGO 3

Título do manuscrito: Prevalência das variantes c.985A>G e c.19T>C do gene *ACADM* em uma população saudável do Rio Grande do Sul, Brasil.

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PREVALÊNCIA DAS VARIANTES c.985A>G E c.199T>C DO GENE *ACADM* EM UMA POPULAÇÃO SAUDÁVEL DO RIO GRANDE DO SUL, BRASIL.

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RESUMO

Introdução: Morte súbita inesperada e inexplicada na infância (do inglês, *Sudden unexpected death in infant* – SUDI) é um segmento da mortalidade infantil no qual a morte ocorre de forma súbita e inesperada. Os defeitos de beta-oxidação de ácidos graxos, principalmente a deficiência de acil-CoA desidrogenase de cadeia média (MCADD) são erros inatos do metabolismo (EIM) frequentemente associados à SUDI. Quase 80% dos pacientes de ascendência europeia, clinicamente identificados como possuindo MCADD, são homozigotos para c.985A>G no gene *ACADM*, enquanto que cerca de 6% apresentam a mutação patogênica c.199T>C. *Objetivos:* estimar a frequência de heterozigotos e a prevalência de MCADD na população do Rio Grande do Sul (RS), este estudo tem como objetivo estimar a prevalência das mutações c.985A>G e c.199T>C no estado. *Metodologia:* Estudo observacional e transversal para estimar a prevalência das variantes patogênicas mais frequentes no gene *ACADM* (c.985A>G e c.199T>C) em uma amostra de 1.000 indivíduos. A genotipagem foi realizada por PCR em tempo real. *Resultados:* Até o momento, foram analisados 300 indivíduos com relação à variante patogênica c.199T>C. Não foi encontrada a variante patogênica pesquisada. *Conclusões:* Os resultados parciais mostram que até o momento mostram que não foi encontrada a variante c.199T>C na população estudada (n=300), o que pode ser resultado de alguns fatores, como o pequeno tamanho amostral e a grande heterogeneidade alélica associada a MCADD.

INTRODUÇÃO

Morte súbita inesperada e inexplicada na infância (do inglês, *Sudden unexpected death in infant* – SUDI) é um segmento da mortalidade infantil (óbitos de crianças menores de 1 ano de idade) no qual a morte ocorre de forma súbita e inesperada. Os defeitos de beta-oxidação de ácidos graxos, principalmente a deficiência de acil-CoA desidrogenase de cadeia média (MCADD; OMIM #201450), são erros inatos do metabolismo (EIM) frequentemente associados à SUDI; estimativas mostram que os EIM podem ser responsáveis de 0,9% a 6% dos casos

de morte súbita em neonatos (YAMAMOTO *et al.,* 2015, VAN RIJT *et al.,* 2016; WILCOX *et al.,* 2002).

MCADD é uma doença autossômica recessiva causada por mutações no gene *ACADM* (acil-coenzima A desidrogenase, C-4 de cadeia de C-12 em linha reta) (ZHANG *et al.,* 1992). Até o momento, de acordo com o Human Gene Mutation Database, cerca de 160 mutações já foram identificadas nesse gene, sendo a maioria delas mutações *missense* (HGMD, disponível em http://www.hgmd.org). A mutação mais comum na MCADD é a c.985A> (p.Lys304Gly), localizada no éxon 11 do gene *ACADM* (MATSUBARA *et al.,* 1992). Quase 80% dos pacientes de ascendência europeia, clinicamente identificados como possuindo MCADD, são homozigotos para c.985A>G. Além disso, cerca de 6% dos indivíduos com MCADD apresentam a mutação patogênica c.199T>C (p.Tyr42His) no éxon 3, a qual é associada com uma atividade residual da enzima MCAD (ANDRESEN *et al.,* 2001). A literatura aponta um efeito fundador para a mutação c.985A>G no Norte da Europa (entre 1:40 e 1:100 indivíduos), sendo que a incidência mundial da MCADD varia entre 1:6.500 a 1:20.000 nascidos vivos (LEAL *et al.,* 2014; GREGERSEN *et al.,* 1993)

Evidências anedóticas sugerem que a MCADD é pouco frequente ou pouco diagnosticada no Brasil e no Rio Grande do Sul (de BITENCOURT, VIANNA e SCHWARTZ*,* 2018 – artigo não publicado). Tendo em consideração que MCADD é um EIM tratável, que o seu diagnóstico e tratamento precoces poderiam prevenir a ocorrência de SUDI, que o Programa Nacional Brasileiro de Triagem Neonatal nacional (PNTN) não inclui a pesquisa de defeitos de beta-oxidação de ácidos graxos e que é desconhecida a frequência de heterozigotos e a prevalência de MCADD na população do Rio Grande do Sul, este estudo tem como objetivo estimar a prevalência das mutações c.985A>G e c.199T>C no RS .

MATERIAIS E MÉTODOS

Trata-se de um estudo observacional e transversal para estimar a prevalência das variantes patogênicas mais frequentes no gene *ACADM* (c.985A>G e c.199T>C). O tamanho amostral estimado foi calculado em 1.000 indivíduos para

detectar uma prevalência mínima de 0,41%. A amostragem foi por conveniência, sendo que os dados aqui apresentados são preliminares e incluem 300/1000 indivíduos saudáveis, doadores voluntários de sangue, atendidos no Banco de Sangue do Hospital de Clínicas de Porto Alegre, Rio Grande do Sul entre setembro de 2017 e maio de 2018. Além da coleta de sangue em EDTA, para cada participante foi preenchida uma ficha de coleta de dados com informações básicas, como local de nascimento, presença de ancestralidade europeia, consanguinidade na família, histórico familiar de morte súbita e a presença de algum problema de saúde.

Todos os participantes assinaram Termo de Consentimento Livre e Esclarecido e o projeto está aprovado no Comitê de Ética local de acordo as regulamentações brasileiras de pesquisa em seres humanos.

Amostra biológica

O DNA genômico foi extraído com Easy-DNA™ Kit (Invitrogen™, Carlsbad, CA, USA) de acordo com o protocolo fornecido pelo fabricante. A análise da variante c.199T>C foi realizadas por PCR em tempo real através de genotipagem pelo sistema Taqman (Thermo Fisher), em um equipamento QuantStudio 3 (Thermo Fisher), de acordo com as instruções do fabricante.

Análise estatística

A análise estatística foi realizada através do Programa *SPSS*, versão 22.0 (SPSS® Inc, Chicago, IL). A prevalência estimada das variantes patogênicas foi apresentada por 10.000 habitantes e com intervalo de confiança de 95% (IC 95%). As frequências alélicas e genotípicas foram calculadas por teste qui-quadrado (ou teste exato de Fisher), tendo-se em consideração que a população está em Equilíbrio de Hardy-Weinberg. O nível de significância foi de 5%.

RESULTADOS

As características da população incluída no estudo estão representadas nas Tabelas 1 e 2.

Até o momento, foram analisados 300 indivíduos com relação à variante patogênica c.199T>C. Não foi encontrada a variante patogênica c.199T>C.

DISCUSSÃO

A primeira referência à associação entre uma doença metabólica (hiperplasia das supra-renais congênita) e a SUDI data de 1962 (CLEVELAND; GREEN; WILNKINS, 1962). No entanto, só em meados dos anos 80 é que surgiram numerosos estudos dando especial atenção à associação entre EIM e SUDI (BONHAM e DOWNING, 1992; BENNETT, VARIEND, POLLIT, 1986; HOWAT *et al.,* 1984), os quais se focaram predominantemente nas alterações da beta-oxidação mitocondrial de ácidos graxos e mais concretamente na MCADD (HOWAT *et al.,* 1984; ROE *et al.,* 1986).

Pacientes com MCADD que não são diagnosticados quando bebês, mas sim quando há surgimento dos primeiros sintomas, que ocorre por volta das primeiras semanas de vida. Dentre todas as manifestações da doença, a mais grave representa a morte súbita, sendo que a taxa de mortalidade varia em torno de 25% devido às manifestações agudas da doença. Uma detecção precoce da MCADD melhora o desfecho desses pacientes e diminui a chance de SUDI (WILCKEN *et al.,* 2007).

Mutações no gene *ACADM* podem resultar em atividade enzimática reduzida ou nula. A variante patogênica c.985A>G é a mais prevalente em descendentes de europeus e em indivíduos sintomáticos e pode ser detectada em 80% desses indivíduos em homozigose e em 18% em heterozigose (WADDELL *et al.,* 2006; YOKOTA *et al.,* 1991). Essa variante patogênica é considerada menos frequente em indivíduos diagnosticados por triagem neonatal, onde inúmeras outras variantes, incluindo a segunda mais frequente c.199T>C, são detectadas nesse grupo de pacientes (ANDRESEN *et al.,* 2001; MAIER *et al,* 2005). Aproximadamente 6% dos indivíduos com MCADD apresentam a variante c.199T>C (ANDRESEN *et al.,* 2001).

Além disso, cerca de 160 variantes patogênicas já foram identificadas no gene *ACADM* (HGMD, disponível em: www.hgmd.cf.ac.uk/). A possível não identificação de heterozigotos para a variante patogênica c.199T>C pode ser

consequência do pequeno tamanho amostral (n=300) ou pode ser resultado da grande heterogeneidade alélica associada a MCADD. Em suma, é possível que outras variantes diferentes das pesquisadas sejam mais prevalentes no RS.

Um estudo brasileiro avaliou retrospectivamente 1722 indivíduos no período entre 2003 e 2007 no Instituto Hermes Pardini da cidade de Belo Horizonte, Minas Gerais (sendo apenas 84 da região Sul do país) e mostrou que a frequência de heterozigotos para a variante patogênica c.985A>G é de 1:246, enquanto que a incidência estimada da MCADD é de 1:250.000 indivíduos. Não houve diferença significativa nas frequências genotípicas entre as diferentes regiões brasileiras, o que pode refletir a necessidade de aumentar o tamanho amostral para representar significativamente cada uma das regiões (FERREIRA *et al.,* 2009).

Outro estudo realizado no RS mostrou que, para os 243 indivíduos avaliados, não foram encontrados alelos mutados para a variante c.985A>G, o que também pode ser resultado do pequeno tamanho amostral (NETTO, 1997). Por outro lado, nenhum estudo avalia a prevalência da variante patogênica c.199T>C na população brasileira, o que torna esse estudo pioneiro nesse campo.

Estudos mostram que a incidência de MCADD varia de região para região. Na península Ibérica, por exemplo, a incidência é de 1:11.945 nascidos vivos, enquanto que Portugal, 1:8.380 (ROCHA *et al.,* 2014). Na Alemanha, por sua vez, a incidência depende da localização geográfica, variando de 1:4.900 nascidos vivos na região norte para 1:13.400 na região sudoeste do país (SANDER *et al.,* 2001; LINDNER *et al.,* 2011). Na parte central da Itália, a incidência de MCADD é de 1:23.000 nascidos vivos (CATARZI *et al.,* 2013).

A incidência encontrada nesses estudos não pode ser extrapolada para o Brasil como um todo, uma vez que o país apresenta uma população bastante heterogênea, onde a variabilidade genética é resultante de populações indígena, europeia e africana, além da constante migração interna do país (PARRA *et al.,* 2003). Consequentemente, pode haver diferenças entre as regiões, sendo necessária a realização de estudos específicos para as diferentes populações, o que faz o nosso estudo ser de grande importância, além de incluir um número maior de indivíduos provenientes do RS do que o estudo de Ferreria e colaboradores (2009).

Além disso, apesar da maioria dos indivíduos analisados até o momento para a variante c.199T>C serem de Porto Alegre (48,7%), observa-se que indivíduos provenientes de outros 63 municípios também foram avaliados, o que garante uma maior representatividade do estado como um todo.

Nossos dados mostram que 62% dos indivíduos avaliados (n=300) apresentam ancestralidade europeia, a maioria sendo descendentes de alemães (25%) e italianos (15,7%). Isso pode refletir numa diferente frequência de heterozigotos da encontrada por Ferreria e colaboradores (2009).

Outro fator a ser considerado é a presença de consanguinidade nas famílias dos indivíduos avaliados. A literatura aponta associação entre consanguinidade e a frequência de EIM (AFZAL, LUND e SKOVBY, 2018). Tanto a MCADD, quanto a maioria dos EIM associados a SUDI, apresentam padrão de herança autossômico recessivo, o que faz com que a taxa de recorrência para famílias afetadas seja de 25% (van RIJT *et al,* 2016).

Um estudo do nosso grupo mostrou que a taxa de mortalidade infantil por SUDI no Brasil e no RS é de 47/10.000 nascidos vivos e de 16/10.000 nascidos vivos, respectivamente (de Bitencourt *et al.,* 2018 – artigo não publicado). Considerando-se o número de indivíduos avaliados até o momento (n=300), 9% (n=27) relatam ter ter algum caso de morte súbita de crianças menores de um ano de idade na família, um percentual bastante elevado em comparação com as taxas de óbitos infantis por SUDI nacional e regional. Apesar de ser um dado indireto, de não se ter acesso às declarações de óbitos dessas crianças e/ou acesso ao diagnóstico formal, esses dados podem, novamente, sugerir a presença de EIM erroneamente classificados como SUDI. Além disso, para um diagnóstico adequado de EIM, é crucial que os médicos recebam um treinamento adequado em relação a essas condições, principalmente no que diz respeito ao grupo de EIM que causam deficiência energética, onde se encontra a MCADD, no qual os sintomas clínicos são pouco óbvios, apresentam gravidade variada, e longos períodos com ausência de sintomas (SAUDUBRAY e GARCIA-CAZORLA, 2018).

Outro importante fator a ser considerado é que o RS apresenta três isolados geográficos nos quais a taxa de óbitos infantis por SUDI é superior ao esperado: os

municípios de Paraí, Pejuçara e Nova Bréscia. Isso pode ser um indicador da presença de EIM nessas regiões. Essa hipótese é apoiada por fatores tais como a dificuldade de diagnóstico de doenças tão complexas como os EIM, a ausência de necropsia metabólica, a ancestralidade europeia da região, a qual pode estar associada a uma maior incidência de MCADD, principal EIM relacionado a SUDI (de Bitencourt *et al.,* 2018 – artigo não publicado). Até o momento, nenhum dos indivíduos incluídos no estudo (n=300) são provenientes dessas três regiões. A grande maioria é proveniente de capital do RS, Porto Alegre, e da região metropolitana, o que pode contribuir para o fato de não ter sido encontrado nenhum heterozigotos para as variantes patogênicas c.985A>G e c.199T>C.

CONCLUSÕES

Este é o primeiro estudo brasileiro a avaliar a frequência de heterozigotos para as variantes patogênicas c.985A>G e c.199T>C no gene *ACADM* na população do RS. Os dados obtidos mostram que há relatos de casos de morte súbita em 9,0% das famílias dos indivíduos avaliados, o que é um percentual bastante considerável e pode indicar a presença de EIM erroneamente classificados como SUDI. Além disso, a taxa de consanguinidade, a qual está associada a uma maior frequência de EIM, relatada na amostra é um pouco superior a esperada na população da América do Sul.

Os resultados parciais mostram que até o momento mostram que não foi encontrada a variante c.199T>C na população estudada (n=300), o que pode ser resultado de alguns fatores. Primeiramente, do pequeno tamanho amostral ou da grande heterogeneidade alélica associada a MCADD. Em segundo lugar, apesar de existências de três isolados geográficos no estado onde a taxa de óbitos por SUDI é maior que o esperado para região, nenhum dos indivíduos avaliados é proveniente desses municípios.

PERSPECTIVAS

O estudo está em curso e, como perspectivas, será realizada a análise genética dos 1.000 indivíduos para ambas as variantes patogênicas c.985A>G e

c.199T>C. Com estes dados, será possível estimar a frequência de heterozigotos e a incidência de MCADD na população saudável do RS.

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Tabela 1. Características dos indivíduos incluídos no estudo (n=300).

	Sim	**Não**	**Não sabe**
Ancestralidade europeia	186 (62,0%)	87 (29,0%)	27 (9,0%)
Alemã	84 (28,0%)	------	------
Italiana	47 (15,7%)	------	------
Portuguesa	39 (13,0%)	------	------
Outra	16 (5,3%)	------	------
Consangüinidade na família	38 (12,7%)	239 (79,6%)	23 (7,7%)
Morte súbita na família (<1ano)	27 (9,0%)	263 (87,7%)	10 (3,3%)

Tabela 2. Local de nascimento dos indivíduos incluídos no estudo (n=300).

Local de Nascimento	Número de indivíduos (%)
Alegrete	1 (0,3)
Alvorada	3 (1,0)
Bagé	1 (0,3)
Barra do Ribeiro	1 (0,3)
Butiá	1 (0,3)
Caçapava do Sul	1 (0,3)
Cachoeira do Sul	3 (1,0)
Cachoeirinha	1 (0,3)
Caibaté	1 (0,3)
Camaquã	3 (1,0)
Canguçu	1 (0,3)
Canoas	11 (3,7)
Cerro Grande do Sul	1 (0,3)
Charqueadas	1 (0,3)
Cruz Alta	1 (0,3)
Dois Irmãos	2 (0,7)
Dom Pedrito	1 (0,3)
Encantado	1 (0,3)
Encruzilhada do Sul	1 (0,3)
Esteio	4 (1,3)
Gravataí	9 (3,0)
Guaíba	3 (1,0)
Horizontina	1 (0,3)
Igrejinha	1 (0,3)
Ijuí	4 (1,3)
Ivoti	2 (0,7)
Lagoa Vermelha	2 (0,7)
Lagoão	1 (0,3)
Monte Belo do Sul	1 (0,3)
Nova Palma	1 (0,3)
Novo Hamburgo	4 (1,3)

Tabela 2 (continuação).

Local de Nascimento	Número de indivíduos (%)
Osório	1 (0,3)
Paim Filho	1 (0,3)
Palmares do Sul	1 (0,3)
Palmeira das Missões	1 (0,3)
Passo Fundo	2 (0,7)
Pedro Osório	2 (0,7)
Pelotas	3 (1,0)
Pinheiro Machado	1 (0,3)
Poço das Antas	1 (0,3)
Porto Algre	146 (48,7)
Putinga	1 (0,3)
Rosário do Sul	1 (0,3)
Santa Cruz do Sul	1 (0,3)
Santa Maria	8 (2,7)
Santo Ângelo	3 (1,0)
Santo Antônio da Patrulha	2 (0,7)
São Borja	1 (0,3)
São Francisco de Paula	1 (0,3)
São Gabriel	1 (0,3)
São Jerônimo	1 (0,3)
São José do Ouro	1 (0,3)
São Leopoldo	5 (1,7)
São Lourenço do Sul	4 (1,3)
São Miguel das Missões	1 (0,3)
São Sebastião do Caí	1 (0,3)
São Sebastião do Passé	1 (0,3)
Sapucaia do Sul	1 (0,3)
Seberi	1 (0,3)
Taquari	1 (0,3)
Uruguaina	1 (0,3)
Vacaria	1 (0,3)
Viamão	7 (2,3)
Victor Graeff	1 (0,3)
OUTROS ESTADOS	24 (8,0)
OUTROS PAISES	2 (0,7)
Total	**300 (100)**

DISCUSSÃO

9 DISCUSSÃO

A mortalidade infantil é um importante indicador da saúde de uma população. O Brasil, uma das nações com maior economia da América Latina e um país historicamente caracterizado por iniquidades substanciais na área da saúde, durante os últimos 20 anos fez uma série de ações para melhorar os indicadores de saúde brasileiros, o que acarretou numa redução da taxa de mortalidade infantil (BARROS *et al.,* 2010; VICTORA *et al.,* 2011). A taxa de mortalidade infantil é utilizada como ferramenta para avaliar a qualidade de vida da população e os cuidados de saúde dispensando às crianças menores de um ano de idade. Os óbitos infantis estão associados a uma grande variedade de fatores socioeconômicos, comportamentais e biológicos/fisiológicos (GARCIA, FERNANDES e TRAEBERT, 2018).

A mortalidade infantil apresentou mundialmente um decréscimo importante, o qual foi atribuído ao progresso das condições sociais e ambientais e às melhorias nos serviços de saúde. Apesar do Brasil e do RS terem apresentado em 2015 as menores taxas de mortalidade infantil (13,82:1.000 nascidos vivos e 10,1:1000 nascidos vivos, respectivamente) desde a década de 70, superando, inclusive, a meta dos Objetivos do Desenvolvimento do Milênio (ODM) em nível nacional (15,7:1000 nascimentos), a taxa de óbitos infantis no país ainda é extremamente superior ao de países desenvolvidos, como Noruega, Suécia, França e Itália, onde as taxas de óbitos infantis são de 2,48; 2,70; 3,28 e 3,29:1.000 nascidos vivos, respectivamente (ODM Brasil, 2016; IBGE, 2015).

Quando comparamos as causas de óbito infantil no Brasil nos anos 90 com 2015, verificamos que houve uma diminuição do número de óbitos por doenças causadoras de diarreia que, em 1990 ocupava o segundo lugar no *ranking*, e que em 2015 deslocou-se para a sétima posição, com expressiva redução de taxa. Esse tipo de mudança indica que houve melhoras nas condições sanitárias e nutricionais no país, assim como melhoria de acesso aos cuidados básicos de saúde. As condições respiratórias apresentam resultados semelhantes. Em contraste, as anomalias congênitas (grupo no qual se encontram os EIM), apresentam taxas relativamente

estáveis no período, sendo, inclusive, a principal causa de óbito infantil na maioria dos estados, incluindo o RS (FRANÇA *et al.,* 2017).

SUDI é uma categoria utilizada para descrever casos de óbito súbito e inesperado em crianças <1 ano de idade, nas quais a causa de óbito não pode ser imediatamente identificada. A distinção entre as causas de SUDI é bastante complexa. Depois de uma adequada investigação, as causas de SUDI podem ser determinadas como sendo de causas médicas (por exemplo, EIM) ou lesão acidental ou não acidental. Entretanto, em 50% dos casos de SUDI nenhuma causa de óbito é identificada, e esses casos podem ser diagnosticados como SIDS (quando o óbito acontece durante o período de sono) ou causa desconhecida (GARSTANG, ELLIS e SIDEBOTHAM, 2015).

Diversos EIM que causam deficiência de energia e/ou intoxicação estão associados com SUDI. Baseado na literatura, até 6% dos casos de SUDI envolvem alguma doença metabólica. Ao menos 32 EIM estão associados à SUDI, sendo que a maioria é passível de alguma forma de tratamento. Destes, 22 podem ser detectados em sua fase pré-assintomática por triagem neonatal, utilizando-se espectrometria de massas em *tandem* (Van RIJT *et al.,* 2016). No Brasil, a triagem neonatal é realizada há três décadas. Entretanto, somente em 2001, o MS implantou o PNTN pelo SUS, por meio da Portaria GM/MS nº 822, de 6 de junho de 2001. O Programa tem como objetivo promover a detecção de doenças congênitas em fase pré-sintomática em todos os nascidos vivos, permitindo o tratamento precoce (BRASIL, 2001).

No contexto de mortalidade infantil, SUDI, por sua vez, também é uma importante causa para as taxas de óbito em crianças. De acordo com França e colaboradores (2017), só os casos de SIDS subiram da 43ª posição nas causas de óbito infantil no Brasil em 1990 para a 19ª posição em 2015, isso sem mencionar os casos em que a causa de SUDI não consegue ser estabelecida (FRANÇA *et al.,* 2007).

Os dados na literatura sobre a mortalidade infantil causada por EIM associados à SUDI são bastante limitados. Da mesma forma, há uma escassez de

trabalhos sobre o panorama de SUDI e de EIM associados a ela no nosso estado. Além disso, apenas dois trabalhos avaliam a frequência de heterozigotos para a principal mutação no gene *ACADM*, sendo que um deles foi realizado na região sudeste do país e com um pequeno tamanho amostral de indivíduos provenientes da região sul, sendo que o segundo não encontrou a variante c.985A>G talvez como consequência do pequeno tamanho amostral (243 indivíduos) (FERREIRA *et al.,* 2009; NETTO, 1997).

De acordo com a CID10, os EIM associados à SUDI pertencem aos grupos E70, E71, E72, E74 e E88, sendo que esse último é formado por uma grande heterogeneidade de doenças, sendo muitas delas não causadoras de SUDI. Os defeitos de beta-oxidação de ácidos graxos parecem ser responsáveis por cerca de 5% dos casos de SUDI em neonatos, sendo que MCADD é o principal representante desse grupo (YAMAMOTO *et al.,* 2015). De acordo com a CID10, esse EIM está incluído no grupo E71.

Os dados obtidos no Capítulo 1 demonstram que há um baixo registro de óbitos de crianças <1 ano de idade no Brasil por EIM associados à morte súbita. Possivelmente isso é um reflexo da subdiganósitco ou subnotificação desses óbitos e, não da raridade dos EIM. Alguns fatores podem explicar esse fato. Primeiramente, os EIM associados à SUDI não estão incluídos no PNTN (com exceção da deficiência de biotinidase incorporada a partir de 2014), o que não permite a triagem gratuita em nível de SUS e dificulta o diagnóstico (BRASIL, 2012). Além disso, algumas regiões do Brasil, tais como Norte e Nordeste, apresentam uma cobertura do SIM abaixo do ideal, o que gera problemas de subnotificação (BRASIL, 2013). Por fim, a baixa qualidade das informações nas DOs, representada pelo grande contingente de causas mal definidas de óbito – imprecisões na declaração da “causa da morte” – e campos não preenchidos, prejudica a análise dos fatores que influenciam a mortalidade e, consequentemente, podem levar a uma subnotificação dos óbitos infantis por EIM no país (BRASIL, 2009).

Nessa mesma linha e ao contrário do mencionado na literatura, nossos achados mostram que o registro de óbitos por CID10-E71 é o menos frequente. Isso

pode estar associado ao fato que a complexidade do diagnóstico dessas doenças, combinada com a falta de expertise e de recursos para proceder com a investigação metabólica de SUDI, resulta em subinvestigação e subdiagnóstico (LOUGHREY, PREECE, GREEN, 2014). Da mesma forma, a deficiência de biotinidase está incluída no grupo E71. Uma vez que um EIM passa a ser incorporado no teste do pezinho, como o caso da deficiência de biotinidase em 2014, o diagnóstico e, consequentemente o tratamento, são precoces, o que pode prevenir SUDI. Este fator também pode ter contribuído para menores taxas de óbito infantil por CID10-E71.

Tendo em vista que a primeira causa de óbito infantil no RS em 2015 foram as anomalias congênitas, onde estão incluídos os EIM, realizamos uma análise da mortalidade infantil por EIM associados à SUDI e por SUDI propriamente dita no estado. O baixo registro de óbitos por EIM associados à SUDI no RS ser consequência da subnotificação ou subdiagnóstico, o que parecer ser um problema recorrente no Brasil como um todo, de acordo com os resultados do capítulo 1. Os dados encontrados mostram que há uma alta taxa de óbitos infantis por CID10-R95 no município de Pejuçara e de óbitos infantis por CID10-R99 nos municípios de Paraí e Nova Bréscia. O mesmo padrão é encontrado quando todos os casos de SUDI são georreferenciados juntos (CID10-R95, R96 e R99): Paraí e Nova Bréscia. O georreferenciamento dos casos de R96 e dos EIM selecionados não foi possível devido ao baixo número de registros de óbitos por essas causas básicas no período avaliado, o que pode ser consequência da subnotificação ou subdiagnóstico, o que parecer ser um problema recorrente no Brasil como um todo (VIANNA *et al.,* 2015; FAJARDO, AERTS, BASSANESI, 2009; COSTA e FRIAS, 2011).

Os municípios de Paraí, Pejuçara e Nova Bréscia apresentam pequeno tamanho populacional e pequena área, sendo que foram colonizados por imigrantes europeus, principalmente italianos. Esses dados podem indicar uma possível presença de EIM nessas regiões, principalmente no que diz respeito a doenças mais prevalentes em população europeia, como MCADD, a qual está associada a SUDI. Além disso, devido à complexidade de diagnóstico de EIM e do fato de não haver necropsia metabólica e nem a incorporação de todos os EIM associados a SUDI no

PNTN, é possível que óbitos por EIM estejam erroneamente classificados como SUDI ou como sintomas associados a ela, especialmente CID10-R99. Os dados resultantes do capítulo 2 também mostram que há baixíssima notificação de afecções iniciais, sequenciais e terminais, o que dificulta o estabelecimento entre sintomas típicos de EIM e óbitos por SUDI. Associado a isso, observa-se que apenas um terço dos casos de SUDI foram submetidos à necropsia, uma importante ferramenta para determinação da causa de óbito por trás da morte súbita. É importante enfatizar também que não há qualquer portaria que exija a realização de necropsia em casos de óbito infantil por causa mal definida ou SUDI e que, quando realizada, não há uma investigação metabólica que permita diagnosticar EIM.

Levando em consideração o cenário do RS, onde observamos uma baixa taxa de mortalidade infantil por EIM associados à SUDI, a presença de isolados geográficos com altas taxas de óbitos infantis por CID10-R95, R99 e SUDI como um todo (R95, R96 e R99) em regiões específicas do estado e de o CID10-E71 ser uma das causas de óbito menos prevalentes no país, a análise da frequência de heterozigotos para as variantes patogênicas do gene *ACADM* no RS faz-se necessária para estabelecer se MCADD, principal defeito de beta-oxidação relacionado à SUDI, é realmente raro ou se há subdiagnóstico.

A variante patogênica c.985A>G no gene *ACADM* é a mais prevalente em indivíduos sintomáticos e pode ser detectada em 80% desses indivíduos em homozigose e em 18% em heterozigose (WADDELL *et al.,* 2006; YOKOTA *et al.,* 1991). A detecção precoce de recém-nascidos assintomáticos pode se dar através da triagem neonatal onde inúmeras outras variantes associadas a atividades residuais da enzima MCAD e, consequentemente a individuos assintomáticos, incluindo a segunda mais frequente c.199T>C, são detectadas nesse grupo de pacientes (ANDRESEN *et al.,* 2001; MAIER *et al,* 2005). Aproximadamente 6% dos indivíduos com MCADD apresentam a variante c.199T>C em heterozigose (ANDRESEN *et al.,* 2001). Além disso, cerca de 160 variantes patogênicas já foram identificadas no gene *ACADM* (STENSON *et al.,* 2003).

Somente dois estudos brasileiros avaliaram a frequência de heterozigotos para a variante c.985A>G, sendo que um deles, realizado em Porto Alegre-RS, não

encontrou nenhum heterozigoto para esta variante, o que pode ser resultante do pequeno tamanho amostral (n=243) (NETTO, 1997). O outro estudo, no qual incluiu 1772 indivíduos, sendo que menos de 5% era proveniente da região sul, encontrou uma frequência de heterozigotos de 0,41% (1:246 indivíduos estudados) (GREGERSEN, BROSS E ANDRESEN, 2004; ANDRESEN *et al.,* 2001; FERREIRA *et al.,* 2009). Por outro lado, nenhum estudo avalia a frequência de heterozigotos para a variante patogênica c.199T>C na população brasileira.

Os resultados apresentados no capítulo 3 ainda são parciais: 300 indivíduos foram analisados apenas para a variante patogênica c.199T>C. A possível não identificação de heterozigotos para a variante patogênica c.199T>C pode ser consequência do pequeno tamanho amostral ou pode ser resultado da grande heterogeneidade alélica associada a MCADD ou até mesmo de uma frequêcia maiks baixa na população do RS. Em suma, é possível que outras variantes diferentes possam ser mais prevalentes no RS. Além disso, 62% dos indivíduos avaliados apresentam ancestralidade europeia, a maioria sendo descendentes de alemães e italianos. Sabe-se que há um efeito fundador da variante patogênica c.985A>G no norte da Europa e que a incidência de MCADD varia de região para região baseado na constituição genética. Assim, a ancestralidade europeia relatada pelos indivíduos incluídos no estudo pode levar à uma incidência estimada da doença diferente do encontrado por Ferreira e colaboradores (2009). Além disso, a variante c.199T>C, ao contrário da c.985A>G, é frequente em indivíduos assintomáticos, como o caso da população aqui avaliada.

Por fim, o percentual de consanguinidade relatado pelo indivíduos incluídos no estudo é um pouco superior à taxa de consanguinidade estabelecida para a América do Sul (BITTLES, 2001). Por a MCADD ser uma doença autossômica recessiva, assim como a maioria dos EIM associados a SUDI, é possível que haja uma maior incidência da doença na população do RS.

Até o momento, nenhum indivíduo avaliado na etapa 3 é originário dos municípios de Paraí, Pejuçara ou Nova Bréscia. Entretanto, 9% relatam ter histórico familiar de morte súbita em crianças menores de um ano. Esses dados podem ser um indício da presença de EIM erroneamente classificados como SUDI no estado.

O fato de terem sido avaliados 300 indivíduos somente para a variante patogênica c.985A>G é resultado de alguns fatores, como o atraso nas compras dos reagentes, devido a um atraso no recebimento de verba proveniente do edital FAPERGS, da sonda necessária para a análise da variante patogênica c.985A>G ter apresentado defeito, sendo necessária nova compra e do fato do número de doadores voluntários do Banco de Sangue do Hospital de Clínicas de Porto Alegre ter sofrido uma queda considerável nos últimos meses, o que gera dificuldade na captação de indivíduos para pesquisa.

Outra questão a ser considerada é que todas as amostras de sangue foram coletadas de forma não anônima, conforme orientação do Comitê de Ética local. Uma vez que a MCADD é uma doença rara, a chance de que heterozigotos sejam encontrados é muito baixa. No entanto, foi oferecida aos participantes a opção de serem contatados e de tomarem conhecimento dos resultados, caso alguma alteração seja encontrada. Para aqueles identificados como heterozigotos para as variantes será oferecido aconselhamento genético. As coletas não anônimas, por outro lado, impossibilitaram a utilização de amostras de DNA de controles saudáveis previamente coletadas por outros projetos.

O estabelecimento da frequência de heterozigotos para as variantes patogênicas c.985A>G e c.199T>C no gene *ACADM,* bem como o cálculo da incidência de MCADD no estado, permitiriam estabelecer um paralelo com a taxa de mortalidade infantil por EIM, podendo-se, em ultima estância, verificar-se se há subdiagnóstico ou subnotificação dos óbitos infantis. Por fim, os dados produzidos por esse projeto de mortalidade infantil são fundamentais para o desenvolvimento de ações de vigilância em saúde e para o processo de tomada de decisões por parte dos gestores, especialmente no que diz respeito a uma possível incorporação dos EIM associados à SUDI no PNTN, o que permitiria um diagnóstico precoce e tratamento adequado, possibilitando, assim, a prevenção de SUDI no país.

10 CONCLUSÕES

Este estudo permitiu avaliar o número de registros de óbito por EIM associados a SUDI no território brasileiro num período de 13 anos, sendo que a taxa de mortalidade infantil por EIM calculada para o país foi de 0,67 a cada 10.000 nascidos vivos. Dos 199 óbitos registrados, 18 (9,0%) ocorreram na região Norte, 43 (21,6%) na região Nordeste, 80 (40,2%) na região Sudeste, 46 (23,1%) na região Sul e 12 (6,0%) na região Centro-Oeste do país, sendo que o CID10 menos frequente foi o E71, o qual inclui os defeitos de beta-oxidação de ácidos graxos, os EIM mais frequentemente associados à SUDI. A variação das taxas de óbito entre as regiões do Brasil pode ser explicada por diferentes coberturas do SIM, por maiores concentrações de centros especializados em genética em alguns estados e pela baixa qualidade das informações provenientes das DOs. A taxa de óbitos encontrada pode sugerir não apenas a raridade das doenças, mas a subnotificação das mesmas.

Com relação aos **objetivos específicos**:

a) Estimar a taxa de mortalidade mínima por SUDI em neonatos e lactentes menores de um ano no RS e no Brasil no período entre 2002 e 2014.

No período avaliado (2002-2014), a taxa de mortalidade infantil por SUDI, incluindo todos os casos de óbitos de crianças <1ano devido aos CIDs R95, R96 e R99, no país foi de 47,0:10.000 nascidos vivos (IC95% 47,0-48,0), enquanto que no RS foi de 45,0:10.000 nascidos vivos (IC95% 15,0-18,0).

b) Estimar a prevalência de óbitos por SUDI no RS possivelmente associados a EIM no período entre 2002 e 2014.

Devido à incomplitude das DOs no bloco referente às afecções iniciais, sequenciais ou terminais que levaram a causa básica de óbito (todas codificadas pela CID10) e a ausência de descrição de qualquer descrição de CID10-E70, E71, E72 e E74 nas listas de afecções, não foi possível estabelecer associação entre óbitos classificados como SUDI e EIM potencialmente associados a SUDI.

c) Realizar análise de georreferenciamento dos casos de EIM associados a SUDI e dos casos de SUDI buscando identificar possíveis *clusters* de EIM no RS.

Não foi possível realizar o georreferenciamento de registros de óbitos infantis por EIM associados a SUDI (CID10-E70, E71, E72 e E74) e nem por CID10-R96. Isso se deve ao fato do pequeno do baixo registro de óbitos registrados por esses CIDs no período (21 óbitos por E70, E71, E72 e E74 juntos e apenas 13 óbitos por R96). Em contrapartida, há dois isolados geográficos com altas taxas de óbitos infantis por CID10-R99 nos municípios de Paraí e de Nova Bréscia. Os mesmos dois municípios também são isolados geográficos quando são georreferenciados todos os casos de SUDI (R95, R96 e R99) no estado. Por outro lado, os dados mostram que há um isolado geográfico com alta taxa de óbitos infantis por CID10-R95 no município de Pejuçara. Devido às características geográficas e históricas desses locais (municípios pequenos, com baixo tamanho populacional e colonizados por europeus, principalmente italianos), é possível que os óbitos relatados possam ser devido a EIM não diagnosticados.

d) Traçar o perfil dos óbitos dos neonatos e lactentes a partir das declarações de óbito por SUDI e EIM associados a SUDI no RS no período de 2002 a 2014.

Os resultados aqui apresentados mostram um panorama bastante completo das informações disponíveis nas DOs das crianças <1ano que faleceram no RS por EIM e SUDI. No que diz respeito à cor de pele, os dados estão de acordo com a distribuição étnica da população do RS. A idade e peso ao óbito são semelhantes entre os grupos de EIM e SUDI, assim como o nível educacional materno.

Com relação aos óbitos por SUDI, apenas 1% das DOs apresentam descrições das afecções iniciais, sequenciais e/ou terminais que levaram ao estabelecimento da causa básica de óbito e, quando presentes, não estão incluídos CIDs associados a EIM. Além disso, um terço dos casos de óbito por SUDI ocorreu sem prestação de assistência médica, ao contrário do encontrado no grupo de óbitos por EIM, em que todos tiveram assistência e faleceram em ambiente hospitalar.

Outro ponto a ser considerado é em 60% dos casos de óbito por SUDI, não foi realizada necropsia.

e) Estimar a frequência das variantes patogênicas c.985A>G e c.199T>C associadas à MCADD em uma população saudável do RS.

Tendo em consideração que a etapa 2 ainda não foi concluída, não foi possível estimar a prevalência de heterozigotos na população avaliada. Até o momento, nenhum alelo com a variante patogênica c.199T>C no gene *ACADM* foi encontrado. Além disso, é necessária a análise da variante patogênica c.985A>G na mesma população. A frequência de heterozigotos no RS será calculada após a análise de toda a amostra.

f) Estimar a incidência mínima de MCADD no RS

Os resultados da análise genética do gene *ACADM* ainda são parciais. Após a análise completa dos 1.000 indivíduos e a verificação da frequência de heterozigotos para as variantes patogênicas pesquisadas, será possível estimar a incidência mínima de MCADD no estado.

11 PERSPECTIVAS

Esta tese foi apoiada pela Sociedade Brasileira de Genética Médica e pelo INAGEMP-Instituto Nacional de Genética Médica Populacional, e está de acordo com a Política Nacional de Atenção Integral às Doenças Raras. Além disso, ele foi contemplado com o **Edital Chamada FAPERGS/MS/CNPQ/SESRS n. 03/2017 – Programa Pesquisa para o SUS: Gestão Compartilhada em Saúde PPSUS – 2017**.

Como primeira etapa, seguiremos com a análise dos 1.000 indivíduos incluídos no estudo para se determinar a frequência das variantes patogênicas c.985A>G e c.199T>C no gene *ACADM* e, consequentemente, a incidência mínima de MCADD na população saudável do RS. Levando em consideração nossos achados, pretendemos ampliar a análise molecular de forma a estimar a frequência para as variantes patogênicas associadas a outros dez EIM associados à SUDI e não incluídos no PNTN: deficiência de 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHADD), deficiência de acil-CoA desidrogenase de cadeia muito longa (VLCADD), tirosinemia tipo I, glicogenose tipo Ia (GSDIa), glicogenose tipo Ib (GSDIb), glicogenose tipo II (GSD II), deficiência de carnitina palmitoiltransferase II (CPT II), citrulinemia tipo I (CTLN1), acidemia glutárica tipo I (GA I) e acidemia metilmalônica isolada do tipo mut0 ou mut-. A partir do conhecimento da frequência alélica, poderemos estimar a incidência mínima desses EIM e a possibilidade de inclusão dos mesmos no PNTN.

Também está prevista a elaboração de uma cartilha específica sobre o processo de investigação de morte súbita, enfatizando-se a contribuição dos EIM nesse contexto, a ser distribuída em Unidades Básicas de Saúde, Serviços de Emergência Pediátrica e Neonatal no RS e mídia eletrônica. Essa cartilha tem como objetivo ampliar o conhecimento sobre a investigação de SUDI e a associação de SUDI com EIM, auxiliando, assim, no diagnóstico e tratamento precoces dessas doenças.

O georreferenciamento dos óbitos infantis no RS demonstra a existência isolados geográficos com altas taxas de óbito infantil por SUDI (CID10-R95, R96 e R99) e por CID10-R99 nos municípios de Nova Bréscia e Paraí. Da mesma forma, o georreferenciamento dos óbitos por CID10-R95 também mostrou que o município de Pejuçara é um isolado geográfico com alta taxa de óbitos infantis. Tendo em consideração esse achado, estamos avaliando a possibilidade de realizarmos um estudo nesses locais, de forma a avaliar o contributo dos EIM associados à SUDI nessa região e, consequente estabelecimento da incidência desses EIM. Uma das formas de realizar essa análise é através de um estudo prospectivo de análise genética das variantes patogênicas previamente descritas a partir de amostras de sangue impregnado em papel filtro provenientes da triagem neonatal dos bebês nascidos em Paraí, Pejuçara e Nova Bréscia, o qual será realizado em parceria com o INAGEMP. Após a realização do estudo, prevemos a distribuição da cartilha sobre investigação de morte súbita nos diferentes ambientes vinculados à saúde pública, bem como consultas de aconselhamento genético em caso de identificação de indivíduos heterozigotos para os EIM avaliados.

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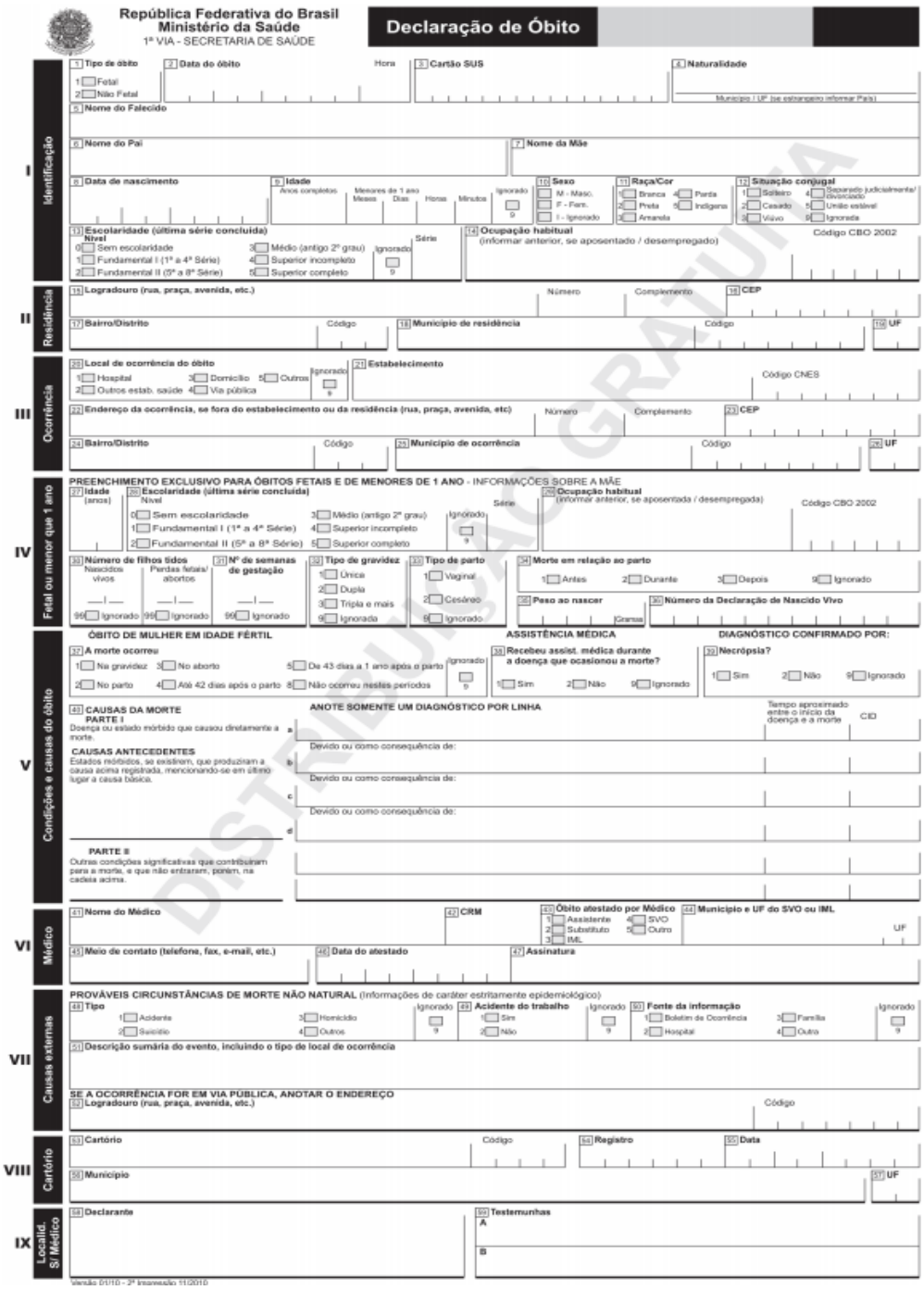
Anexo I Modelo de Declaração de Óbito. Disponibilizado pelas Secretarias Estaduais de Saúde do Brasil. Adaptado do Manual de Instruções para Preenchimento da Declaração de Óbito. MS, 2011

Anexo A – Modelo da Declaração de Óbito



Anexo II Campo 40 da Declaração de Óbito. Correspondente à definição da causa da morte. Adaptado do Manual de Instruções para Preenchimento da Declaração de Óbito. MS, 2011

ÓBITO DE MULHER EM IDADE FÉRTIL

37 A morte ocorreu
1 Na gravidez 3 No aborto 5 De 43 dias a 1 ano após o parto
2 No parto 4 Até 42 dias após o parto 8 Não ocorreu nestes períodos
Ignorado 9

ASSISTÊNCIA MÉDICA

38 Recebeu assist. médica durante a doença que ocasionou a morte?
1 Sim 2 Não 9 Ignorado

DIAGNÓSTICO CONFIRMADO POR:

39 Necrópsia ?
1 Sim 2 Não 9 Ignorado

40 CAUSAS DA MORTE

ANOTE SOMENTE UM DIAGNÓSTICO POR LINHA

		Tempo aproximado entre o início da doença e a morte	CID
PARTE I – Doença ou estado mórbido que causou diretamente a morte.	a		
CAUSAS ANTECEDENTES – Estados mórbidos, se existirem, que produziram a causa acima registrada, mencionando-se em último lugar a causa básica.	Devido ou como conseqüência de: b		
	Devido ou como conseqüência de: c		
	Devido ou como conseqüência de: d		
PARTE II – Outras condições significativas que contribuíram para a morte, e que não entraram, porém, na cadeia acima.			

Anexo III Carta de aprovação do projeto

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA

A Comissão Científica do Hospital de Clínicas de Porto Alegre analisou o projeto:

Projeto: 160055
Data da Versão do Projeto: 31/01/2016

Pesquisadores:
FERNANDA HENDGES DE BITENCOURT
FERNANDA SALES LUIZ VIANNA

Título: MORTALIDADE INFANTIL NO BRASIL ASSOCIADA A ERROS INATOS DO METABOLISMO COM ÊNFASE EM MORTE SÚBITA

Este projeto foi APROVADO em seus aspectos éticos, metodológicos, logísticos e financeiros para ser realizado no Hospital de Clínicas de Porto Alegre.
Esta aprovação está baseada nos pareceres dos respectivos Comitês de Ética e do Serviço de Gestão em Pesquisa.

- Os pesquisadores vinculados ao projeto não participaram de qualquer etapa do processo de avaliação de seus projetos.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao Grupo de Pesquisa e Pós-Graduação (GPPG)

Porto Alegre, 16 de fevereiro de 2016.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

Anexo IV Carta de aprovação do projeto

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA

A Comissão Científica do Hospital de Clínicas de Porto Alegre analisou o projeto:

Projeto: 170249
Data da Versão do Projeto: 11/05/2017

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
FERNANDA SPERB LUDWIG
FERNANDA SALES LUIZ VIANNA

Título: Identificação de heterozigotos para a Erros Inatos do Metabolismo associados à morte súbita: investigação da prevalência de mutações em doadores de sangue voluntários no Hospital de Clínicas de Porto Alegre

Este projeto foi APROVADO em seus aspectos éticos, metodológicos, logísticos e financeiros para ser realizado no Hospital de Clínicas de Porto Alegre.
Esta aprovação está baseada nos pareceres dos respectivos Comitês de Ética e do Serviço de Gestão em Pesquisa.

- Os pesquisadores vinculados ao projeto não participaram de qualquer etapa do processo de avalição de seus projetos.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao Grupo de Pesquisa e Pós Graduação (GPPG)

Porto Alegre, 23 de maio de 2017.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

Anexo V Comprovante de submissão do artigo 1

BMC Public Health

INFANT MORTALITY IN BRAZIL ATTRIBUTABLE TO INBORN ERRORS OF METABOLISM ASSOCIATED WITH SUDDEN DEATH: A TIME-SERIES STUDY (2002-2014).

--Manuscript Draft--

Manuscript Number:	PUBH-D-18-01787
Full Title:	INFANT MORTALITY IN BRAZIL ATTRIBUTABLE TO INBORN ERRORS OF METABOLISM ASSOCIATED WITH SUDDEN DEATH: A TIME-SERIES STUDY (2002-2014).
Article Type:	Research article
Section/Category:	I don't know (Editor will assign section)
Funding Information:	Graduate Programe in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Brazil — Not applicable
Abstract:	Background: The literature suggests that 0.9% to 6% of infants who die unexpectedly may have had a metabolic disorder. At least 43 different inborn errors of metabolism (IEMs) have been associated with sudden death (SUDI). To date, the frequency of IEM-associated SUDI has not been studied in Brazil. The present study sought to characterize infant mortality related to IEMs known to cause SUDI disaggregated by each of the regions of Brazil. Methods: This was a descriptive, cross-sectional, population-based study of data obtained from the Brazilian Ministry of Health Mortality Information System (SIM). Death records were obtained for all infants (age <1 year) who died in Brazil in 2002-2014 in whom the underlying cause of death was listed as ICD-10 codes E70 (Disorders of aromatic amino-acid metabolism), E71 (Disorders of branched-chain amino-acid metabolism and fatty-acid metabolism), E72 (Other disorders of amino-acid metabolism), or E74 (Other disorders of carbohydrate metabolism), which are known to be associated with SUDI. Results: From 2002 to 2014, 199 deaths of infants aged <1 year were recorded in the SIM with an underlying cause corresponding to one of the IEMs of interest. The prevalence of IEM-related deaths was 0.67 per 10,000 live births (0.58-0.77). Of these 199 deaths, 18 (9.0%) occurred in the North of Brazil, 43 (21.6%) in the Northeast, 80 (40.2%) in the Southeast, 46 (23.1%) in the South, and 12 (6.0%) in the Center-West region. Across all regions of the country, ICD10-E74 was predominant. Conclusions: This 13-year time-series study provides the first analysis of the number of infant deaths in Brazil attributable to IEMs known to be associated with sudden death.
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Order of Authors Secondary Information:	
Opposed Reviewers:	

APÊNDICES

13 APÊNDICES

13.1 Ficha de coleta

FICHA DE COLETA DE DADOS

Título do Projeto: Identificação de heterozigotos para a Erros Inatos do Metabolismo associados à morte súbita: investigação da prevalência de mutações em doadores de sangue voluntários no Hospital de Clínicas de Porto Alegre

1. **Nome:**
2. **Sexo** ( ) Feminino ( ) Masculino
3. **Data de nascimento:**
4. **Local de nascimento:**
5. **Ancestralidade europeia** ( ) SIM ( ) NÃO ( ) não sabe

   Qual? ( ) Alemã

   ( ) Italiana

   ( ) Portuguesa

   ( ) Outra _____

6. **Consanguinidade na família**

( ) Não sabe ( ) NÃO ( ) SIM

7. **Histórico familiar de morte súbita**

( ) Não sabe ( ) NÃO ( ) SIM

8. **Algum problema de saúde**

( ) NÃO ( ) SIM. Qual? _____

9. **Contato**

   Telefone residencial:

   Celular:

   Email:

Rubrica do pesquisador _____

13.2 Artigo 1

BITENCOURT FH, VIEIRA TA, STEINER CE, NETO JC, BOY R, SCHWARTZ IVD. Medical costs related to enzyme replacement therapy for Mucopolysaccharidosis types I, II, and VI in Brazil: a multicenter study. **Value Health Reg Issues.** 2015 Dec(8):99-106.

13.3 Artigo 2

JACQUES CE, DONIDA B, MESCKA CP, RODRIGUES DG, MARCHETTI DP, BITENCOURT FH, BURIN MG, DE SOUZA CF, GIUGLIANI R, VARGAS CR. Oxidative and nitrative stress and pro-inflammatory cytokines in Mucopolysaccharidosis type II patients: effect of long-term enzyme replacement theapy and relation with glycosaminoglycan accumulation. **Biochim Biophys Acta.** 2016 Sep; 1862(9): 1608-1616.

13.4 Artigo 3

BRAVO H, NETO EC, SCHULTE J, PEREIRA J, FILHO CS, BITTENCOURT F, SEBASTIÃO F, BENDER F *et al.* Investigation of newborn with abnormal results in a newborn screening program for four lysosomal storage diseases in Brazil. **Mol Genet Metab Rep.** 2017 Jul 4; 12:92-97.

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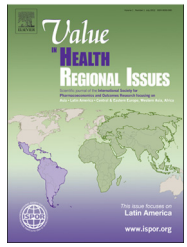

Medical Costs Related to Enzyme Replacement Therapy for Mucopolysaccharidosis Types I, II, and VI in Brazil: A Multicenter Study

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ABSTRACT

Background: Mucopolysaccharidosis (MPS) type I (MPS I), MPS type II (MPS II), and MPS type VI (MPS VI) are lysosomal storage disorders for which enzyme replacement therapy (ERT) is available. **Objective:** The objective of this study was to evaluate the frequency of medical interventions in a cohort of patients with MPS I, II, and VI on ERT to estimate the impact of direct medical costs associated with the treatment of MPS and compare its frequency with that observed among patients not on ERT. **Methods:** This was a multicenter study using a retrospective design including a convenience sampling of Brazilian patients with MPS I, II, and VI. Data on the number and type of medical appointments, hospital admissions, medications used, and surgical procedures performed per patient were obtained through a review of medical records, as were data on ERT. These variables were then compared between patients undergoing ERT and those not on ERT. **Results:** Thirty-four patients (27 on ERT) were included in the study. Overall, between-group differences were found in median absolute frequencies of hospital admissions and surgical procedures per year, both of which were higher in the non-ERT group. Furthermore, we observed a high rate of failure to record medication dosage regimens. **Conclusions:** Our findings suggest that Brazilian patients with MPS I, II, and VI who are on ERT undergo fewer medical interventions, which can lead to a reduction in direct medical costs to the publicly funded health care system. The cost of ERT, however, is extremely high and probably outweighs this reduction.

Keywords: enzyme replacement therapy, health technology assessment, mucopolysaccharidosis type I, mucopolysaccharidosis type II, mucopolysaccharidosis type VI, rare disorders.

Introduction

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by deficient activity of enzymes involved in the degradation of glycosaminoglycans (GAGs). These disorders are characterized by intralysosomal buildup and increased urinary excretion of GAGs, which ultimately leads to cell, tissue, and organ dysfunction [1]. The GAG catabolism pathway involves different enzymes; deficiency of each of these 11 enzymes is associated with a specific type of MPSs [2] (see Appendix Table in Supplemental Material found at http://dx.doi.org/10.1016/j.vhri.2015.08.002). From a clinical standpoint, the MPS share many features, and so enzyme assays or DNA analyses are required for diagnostic confirmation. All are progressive disorders, characterized by childhood onset, and usually lead to death at an early age; the severity of clinical presentation is extremely variable, but predominantly comprises complications due to the buildup of GAGs in the respiratory system (recurrent respiratory tract infections, obstructive sleep apnea, restrictive lung disease), heart (valve disease), and joints/bone (*dysostosis multiplex*, decreased joint range of motion). Neurological involvement, is common; mucopolysaccharidosis type IV-A (MPS IV-A) and mucopolysaccharidosis type VI (MPS VI) were originally believed to be "protected" from cognitive involvement, but an article suggests that it may occur even in MPS VI [3]. Mucopolysaccharidosis type III-D, mucopolysaccharidosis type IV-B, mucopolysaccharidosis type VII, and mucopolysaccharidosis type IX are the rarest types. Mucopolysaccharidosis type I (MPS I) is the leading type of MPSs in the United Kingdom [4], mucopolysaccharidosis type II (MPS II) is the most common type in Brazil [5] and Japan

Conflict of interest: The authors have indicated that they have no conflicts of interest with regard to the content of this article.

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http://dx.doi.org/10.1016/j.vhri.2015.08.002

[6], and MPS III-A is the most common type found in Germany [7]. According to the São Paulo State MPS Association (Associação Paulista de MPS), as of 2012, there were 645 patients living with MPSs in Brazil: 160 with MPS I, 191 with MPS II, 33 with MPS III, 79 with MPS IV, and 182 with MPS VI (Nilton Próspero, personal communication, 2012). MPS II is the only form that presents an X-linked recessive pattern of inheritance; hence, it is found almost exclusively in male patients [6].

There is no curative treatment for MPSs. Currently available treatment options include interventions that target the clinical phenotype (supportive care or symptomatic therapy) and those that target the mutant protein (specific treatments, such as hematopoietic stem cell transplantation [HSCT] and enzyme replacement therapy [ERT]) [8]. Growing research interest in MPSs from a health technology assessment standpoint is explained by the advent of ERT for MPS I, II, IV-A, and VI, a high-cost treatment modality [8] that has been approved by regulatory agencies within the framework of so-called orphan drugs (see Appendix Table). Notably, no such framework exists in Brazil. ERT for MPS I consists of intravenous administration of laronidase, a protein analogue of human α-L-iduronidase produced from Chinese hamster ovary (CHO) cells [9]. ERT for MPS II consists of intravenous administration of idursulfase, an analogue of human iduronate-2-sulfatase produced from human cell lines [10,11], whereas therapy for MPS VI consists of intravenous administration of galsulfase, an analogue of *N*-acetylgalactosamine 4-sulfatase (arylsulfatase B) also produced from CHO cells [12,13]. ERT with recombinant human GALNS (elosulfase alfa) represents a new treatment option for patients with MPS IVA that was approved in the United States by the Food and Drug Administration in February 2014. Elosulfase alfa is produced in a genetically engineered CHO mutant cell line that expresses the cDNA encoding for the full human GALNS protein [14].

The high cost of orphan drug development for the treatment of these diseases, compounded by the difficulty of conducting clinical trials in an extremely low population, shows that it can be characterized as a public health problem [15]. The present study constitutes the first stage of a larger project that seeks to estimate the economic impact of ERT for MPS I, II, and VI on the Brazilian publicly funded health care system, the Unified Health System (Sistema Único de Saúde [SUS]). As well as test the data collection instrument, our objective was to evaluate the frequency of medical interventions performed in Brazilian patients with MPS I, II, and VI regularly followed up at SUS hospitals so as to estimate the impact of ERT on direct medical resources, and to establish whether they are related to the severity and duration of the disease.

Methods

Study Design

This was a retrospective, hospital-based, cohort study designed to collect data on variables of interest to a pharmacoeconomic assessment of ERT for MPS I, II, and VI. MPS IV-A was not included because ERT with elosulfase alfa was not approved at the time of the study. The study was approved by all involved ethics committees.

Data Collection Instrument

The data collection instrument (available on request) consisted of 15 questions organized in two sections. The first section was designed to obtain general data on each patient: center of origin, severity of phenotype (with or without cognitive involvement, according to the registries), ERT status, history of HSCT, date of birth, date of diagnosis, date of first medical geneticist appointment, date of first infusion (in case of ERT), and date of death (when applicable). The second section of the instrument was designed to collect data on variables directly associated with the cost of MPS treatment. Data recorded included the date, specialty, and reason for each medical appointment; the date, type, and indication for each test; the date, type, and length of stay for each surgical procedure; the type, cause, and length of stay for each hospital admission; and the type, dosage regimen, duration of use, and indication for each medication prescribed. Data were also collected on ancillary therapies, such as physical therapy, speech and language therapy, occupational therapy, social services, and psychology or counseling (duration, frequency, and indication for visits), and on the use of medical devices, including eyeglasses, hearing aids, wheelchairs, and continuous positive airway pressure/bilevel positive airway pressure devices. For patients on ERT, a separate data collection sheet was specifically designed to record the date and number of infusions performed, the number of infusions lost, the number of vials used (dose calculated according to body weight), premedication, and infusion duration. Throughout 2011, one of the investigators (F.H.B.) reviewed the records of all the patients included in the study and completed the data collection instrument.

Sample

A convenience sampling strategy was used. Four Brazilian MPS treatment centers were included in this study: the Medical Genetics Service of Hospital de Clínicas de Porto Alegre, state of Rio Grande do Sul (SGM-HCPA); the Department of Medical Genetics of Universidade Estadual de Campinas, state of São Paulo; Pontifícia Universidade Católica de Campinas, state of São Paulo; and the Department of Pediatrics at Universidade Estadual do Rio de Janeiro, state of Rio de Janeiro. These centers were chosen because all kept high-quality patient records, all are part of the SUS, all are affiliated with universities in the south or southeast regions of Brazil, and all monitored both patients who were on ERT and those not on ERT (the exceptions were Department of Medical Genetics of Universidade Estadual de Campinas, state of São Paulo, which followed patients not on ERT, and Pontifícia Universidade Católica de Campinas, state of São Paulo, which monitored only those on ERT). Furthermore, all are led by medical geneticists. This would ensure good record-keeping and a measure of consistency with respect to follow-up protocols. It bears noting that of the four participating centers, only one (SGM-HCPA) had electronic medical records.

Patient Inclusion/Exclusion Criteria

The patient inclusion criteria were as follows:

1. Diagnosis of MPS I, II, or VI confirmed by enzyme assay in plasma/leukocytes or fibroblasts and/or DNA analysis;
2. Not being on a clinical trial involving ERT;
3. No history of HSCT;
4. Treatment duration, according to presence or absence of ERT:
   a. Patients in the ERT group were required to undergo regular follow-up and to be on ERT for at least 12 months before the start of data collection (January 2011).
   b. Patients in the no ERT group were required to undergo regular follow-up for at least 12 months before the start of data collection (January 2011).

For purposes of analysis, patients aged 18 years or older were considered adults. All others were classified as children/adolescents.

Assessment of the Study Instrument and Data Collection Method

The data collection instrument was designed by the multidisciplinary team of study investigators, which comprises physicians, pharmacists, health technology assessment experts, and economists.

The data collection allowed the evaluation of the study instrument regarding its layout. The quality of data records was appraised qualitatively and quantitatively by analysis of the number of missing variables in the instrument (due to missing data or inadequate record-keeping) and of the quality of information obtained. For the variables medical appointments, tests, hospital admissions, and surgical procedures, records were considered adequate if they contained information on the type of intervention, the date of intervention, the duration or frequency of the intervention, and the indication(s) for the intervention. Records were considered adequate for ancillary therapies if they contained information on the type, frequency, and duration of therapy; for medications (pharmacotherapy), if they contained data on the active pharmaceutical ingredients used, duration, dosage regimen, and indications for each medication; for chronic-use medical devices, if they contained information on the date use of each device was started; and finally, regarding ERT, records were considered adequate if they contained data on time on ERT, number of vials used, number of scheduled infusions, and number of completed infusions.

Estimation of the Effect of ERT, Cognitive Involvement, and Duration of the Disease on the Frequency of Medical Interventions

All data were collected by a chart review.

The following data were taken into account for this analysis: date of birth, age at diagnosis, presence of cognitive involvement (according to medical records, even in the absence of IQ testing), date ERT was started, and frequency of medical interventions (number and type of hospital admissions, tests, surgical procedures, medical appointments, and medications prescribed/used) performed between January and December 2010.

Medications used as part of ERT (laronidase, idursulfase, or galsulfase, premedications and medications used in the treatment of infusion adverse reactions) and those not directly related to MPS management (such as oral contraceptives) were not tallied. For the purposes of this study, the number of medications used was defined as the number of different active pharmaceutical ingredients used during the study period; for example, if a patient received two courses of plain amoxicillin and one course of amoxicillin/clavulanate, these would be tallied as "two medications used during the study period."

Tests were tallied as a single instance when performed on the same day; for example, complete blood cell count alone or complete blood cell count plus platelet count were both counted as a single test for statistical purposes as long as both tests were performed at a single visit. This practice was used for hematology and biochemistry tests alike, including urea, creatinine, bilirubin, Alanine transaminase (ALT), aspartate transaminase (AST), Gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), alkaline phosphatase, cholesterol, glucose, sodium, potassium, chloride, magnesium, calcium, phosphorus, albumin, globulins, and total protein. Conversely, imaging tests of different body segments were counted separately even when performed on the same day.

Variables (medians) were initially assessed with regard to disease duration (equivalent to the age of the patient; in this analysis, data only from patients in the non-ERT group were taken into account) and presence/absence of cognitive involvement. To assess the influence of time on ERT on the variables of interest, data from the ERT group were included.

Statistical Analysis

Databases were constructed in Microsoft Office Excel 2010, and statistical analyses were performed in the SPSS 20.0 software environment. Descriptive data were described as frequencies, means and SDs, and medians and quartiles.

For evaluating the effect of the duration of disease on the frequency of medical intervention, a Pearson correlation was performed. The influence of the presence of cognitive involvement on other variables was then assessed using the Kruskal-Wallis test. The Mann-Whitney *U* test was used for comparison of the median number of medications, medical appointments, hospital admissions, tests, and surgical procedures in the ERT and non-ERT groups. To assess the influence of time on ERT on the variables of interest, data from the ERT group were included and Pearson correlation coefficients were calculated.

For all analyses, *P* values of less than 0.05 were considered statistically significant.

All monetary values that are expressed in pounds sterling were obtained through the exchange rate provided by the Central Bank of Brazil (Banco Central do Brasil) on July 15, 2014. Because the data collected are prior to 2010, the monetary values determined may have suffered variations because of annual inflation rates in Brazil (around 6%).

Results

Forty-three patients with MPSs (I = 15, II = 23, VI = 5) were alive and registered at the four participating centers in 2010. Of these, only 35 met the inclusion criteria because 8 did not have any appointments in 2010 (e.g., they were not regularly seen at the center). The medical records of one patient were not available for review. Therefore, the sample comprised 34 patients: 27 on ERT ("ERT group") and 7 receiving supportive care only ("non-ERT group"). The reasons why patients from the non-ERT group were not receiving ERT were not clearly stated in medical records. Table 1 describes the profile of the patients included in the sample.

Appraisal of the Data Collection Instrument

Table 2 lists the variables associated with the cost of MPS treatment and describes our appraisal of the adequacy of record-keeping of these variables in patient charts.

Data on the chronic use of medical devices and information on ancillary therapies are presented in Table 3.

Influence of Disease Duration on the Variables of Interest

There were no significant correlations between length of disease and any of the variables of interest (data not shown).

Comparison between the ERT and Non-ERT Groups

There were significant between-group differences in the median number of hospital admissions and surgical procedures, both of which were higher in the non-ERT group (Table 4).

There were no significant between-group differences when only children and adolescents were taken into account (data not shown). Because there were no patients older than 18 years in the non-ERT group, no such analysis could be conducted for adult patients.

Table 1 – Profile of Brazilian MPS I, II, and VI patients included in the sample.

Patients profile	No ERT (n = 7)	ERT (n = 27)
MPS type		
I	1	11
II	5	12
VI	1	4
Sex		
Female-to-male ratio (n)	2:5	8:19
Center of origin		
HCPA	6	17
Other participating centers	1	10
Median age at diagnosis (y) (IQR 25–75)	2.2 (1.6–5.8)	6.2 (6.8–12.3)
Median time elapsed between diagnosis and study enrollment (y) (IQR 25–75)	4.5 (3.9–5.7)	9.4 (9.6–12.2)
Age at study enrollment (y), n (%)		
Children/adolescents (<18y)	7 (100)	16 (59.2)
Adults (≥18y)	0	11 (40.8)
Median (IQR 25–75)	6.8 (4.8–9.4)	12.8 (9.4–22.6)
Cognitive involvement		
Present, n (%)	6 (85.7)	9 (33.3)
MPS I	1	4
MPS II	4	5
MPSVI	1	0
Median age at ERT onset (y) (IQR 25–75)		10.5 (7.7–19.0)
MPS I	–	17.8 (14.8–25.3)
MPS II	–	10.1 (7.3–17.2)
MPS VI	–	9.8 (8.7–10.6)
Median time on ERT (y) (IQR 25–75)	–	2.7 (1.56–5.3)
MPS I		3.7 (2.3–5.8)
MPS II		2.6 (1.2–3.8)
MPS VI		3.2 (2.6–4.1)
Scheduled infusions per year	–	48
Median infusions performed (IQR 25–75)	–	46 (45–47)

ERT, enzyme replacement therapy; HCPA, Hospital de Clínicas de Porto Alegre; IQR, interquartile range; MPS I, mucopolysaccharidosis type I; MPS II, mucopolysaccharidosis type II; MPS VI, mucopolysaccharidosis type VI.

Influence of Cognitive Involvement on the Variables of Interest

Comparison between patients with cognitive involvement and those with no cognitive involvement showed that the former used a significantly greater number of medications, both in the ERT ($P = 0.032$) and in the non-ERT ($P = 0.024$) groups.

Influence of Time on ERT on the Variables of Interest

A correlation was found between time on ERT and the median number of hospital admissions ($r = -0.504$; $P = 0.007$). Time on ERT did not correlate with any other variables (data not shown).

When only patients younger than 18 years were considered, there was also a correlation between time on ERT and the median number of hospital admissions ($r = -0.674$; $P = 0.004$). No such correlation was found in adult patients.

Table 2 – Adequacy of medical record information in relation to variables analyzed in the present study.

Variable	No. of patients with intervention reported in medical records	No. (%) of patients with adequate records*
Medical appointments	33	
Date		33 (100)
Type		33 (100)
Indication		17 (51.5)
Hospital admissions	12	
Length of stay		11 (91.7)
Indication		12 (100)
Surgical procedures	6	
Date		5 (83.3)
Type		6 (100)
Length of stay		6 (100)
Tests	32	
Date		32 (100)
Type		32 (100)
Indication		15 (46.9)
Medications	19	
Type		19 (100)
Dosage regimen		7 (36.8)
Indications for use		7 (36.8)
Duration of use		18 (94.7)
ERT	27	
Number of vials used		27 (100)
Number of scheduled and missed infusions		27 (100)

ERT, enzyme replacement therapy.

* *Adequate record-keeping* was defined as the presence of information pertaining to the variables listed in the leftmost column of the table in the medical records of each patient.

Discussion

The importance of this article is based on the worldwide scientific, economic, and social relevance of rare diseases and orphan drugs.

The present study used an exploratory and retrospective design and was based exclusively on data contained in patient records before the implementation of Ordinance No. 199, January 30, 2014, from the Brazilian Ministry of Health, which establishes the National Policy on Comprehensive Care for People with Rare Diseases in the SUS. The policy forecasts the incorporation and use of technologies for the promotion, prevention, and comprehensive care, including drugs and nutritional formulas as specified in the SUS, which will change the panorama of rare diseases in the county. For purposes of this ordinance, a disease that affects up to 65 people in every 100,000 individuals, or 1.3 people per 2000 individuals [16], is considered a rare disease.

Unfortunately, most participating centers did not have electronic medical records. Furthermore, some centers use different records or forms for different departments or sectors in which patients are seen over the course of their treatment, thus making data collection a complex, extensive, and eventually incomplete endeavor.

However, one of the advantages of chart review studies is that medical records provide very precise information on the date and type of medical appointments, surgical procedures, hospital admissions, and tests performed at the hospital in which patients receive follow-up, as well as the type and duration of pharmacotherapy.

Table 3 – Adequacy of medical record information in relation to the use of medical devices and ancillary therapies by Brazilian patients with MPS I, II, and VI.

Medical devices/ Ancillary therapies	No. (%) of patients with intervention reported in medical records	No. (%) of patients with adequate records*
Medical devices for long-term use	16†/34 (47.0)	13/16 (81.2)
Eyeglasses	6	5
Hearing aid	5	3
CPAP	3	2
BiPAP	1	0
Walker	1	1
Wheelchair	1	0
Leg orthosis	1	1
Neck brace	1	1
Ancillary therapies	15‡/34(44.1)	9/34 (60)
Physical therapy	10	4
Speech-language pathology	4	1
Occupational therapy	0	0
Social services	4	4
Psychologist/ counselor	0	0

BiPAP, bilevel positive airway pressure; CPAP, continuous positive airway pressure; MPS I, mucopolysaccharidosis type I; MPS II, mucopolysaccharidosis type II; MPS VI, mucopolysaccharidosis type VI.

* *Adequate record-keeping* was defined as the presence of information pertaining to the type of device and date device use began (for medical devices) or duration, frequency, and type of therapy (for ancillary therapies).

† Three patients used more than one device.

‡ Three patients were undergoing more than one type of therapy.

At least in this study, however, data on use of medical devices and ancillary therapies apparently were not adequately recorded.

In 2012, Wyatt et al. [17] conducted a pioneering study of the costs of MPS I and MPS II treatment in the United Kingdom. All cost estimates in their investigation were based on data collected by means of questionnaires designed to obtain information on the last 12 months of patient follow-up. Questionnaires were administered directly to patients whenever possible or to their caregivers otherwise. One of the advantages of this mode of data collection is that patients and their caregivers are more likely to have reliable information on ancillary therapies, such as physical therapy, speech and language pathology, and occupational therapy, as well as on the duration of use of medical devices. Therefore, we suggest that future studies on this topic use both designs and data collection methods, so as to ensure collection of reliable data.

We detected a high rate of missing data in relation to the reasons or indications for medical appointments and tests. Although this circumstance hinders assessment of the natural history of MPS, it has little impact on pharmacoeconomic analysis of the treatment of MPS. Missing data pertaining to medication dosage regimens, however, will certainly affect the calculation of MPS treatment costs in subsequent stages of our project. Another point worth considering concerns collection of data on chronic use of medical devices and use of ancillary therapies, as mentioned above. In addition to the issue of inadequate record-keeping, we found that very few patients actually used these interventions—fewer than expected. Children with severe MPS I, for instance, have limited development of language skills [1] and monitoring by a speech-language pathologist is essential. From a behavioral standpoint, children with MPS I tend to be placid, whereas those with MPS II tend to exhibit aggressive behavior [1]; therefore, psychological treatment can play a very important role. Hearing loss is quite common in MPS [1], and many patients require hearing aids. Obviously, the low rate of use of these services and devices in our sample may be secondary to failure to record these interventions in patient charts, or may reflect difficulty obtaining access to these therapies. Brazilian MPS treatment centers do not always make ancillary therapies available to all patients; when provided, they are often extramural, which may explain, at least partly, why their use was not reported in patient records. Previous studies by our group [19,20] suggest, however, that many patients with MPS actually did not have access to ancillary therapies. Turra and Schwartz [18] conducted a multicenter study including 78 Brazilian patients with MPS (17 presenting with mental retardation) who underwent an interview with a speech and language therapist and physical examination. Of these patients, only 18 were undergoing speech therapy intervention at the time of the study [18]. In 2012, Guarany et al. [19] conducted a prospective, longitudinal study of 21 Brazilian patients with MPSs. Of these, only seven reported that they have been treated at rehabilitation clinics or institutions; physical therapy, speech therapy, and psychotherapy were among the treatments provided.

Despite missing data, the study instrument was able to collect reliable records on the frequency and type of medical interventions in this patient sample, thus providing data for future analysis of the treatment costs of MPS I, MPS II, and MPS VI.

Descriptive Analysis of Treatment Costs in MPS I, II, and VI

Since its establishment in 1990, the SUS has ensured the right to care—including medical appointments, tests, hospital admission, and treatment—at all affiliated health facilities to all Brazilian citizens [20]. Nevertheless, the demand for ERT is on the rise, with patient requests many times supported by court orders that conflict directly with Brazil's National Medicines Policy and with evidence-based medicine. Furthermore, the influence of pharmaceutical industry lobbying for registration and marketing of new drugs in Brazil cannot be ruled out [21]. A Brazilian article published in 2012 showed that in the case of MPSs, litigation results from the lack of a clear policy in the health system for rare diseases in general, thereby leading to excessive expenditures for MPS treatment. The authors reviewed files from 196 court rulings ordering the Brazilian Ministry of Health to provide medicines, in addition to Ministry of Health administrative records. Overall, 195 patients sued to secure their access to laronidase, idursulfase, and galsulfase between 2006 and 2010, at a total cost of £57,112,763.76 to the public purse, distributed as follows: £2,408,375.06 for laronidase (24 patients with MPS I), £22,616,218.82 for idursulfase (68 patients with MPS II), and £32,088,170.14 for galsulfase (103 patients with MPS VI) [15].

Despite the high cost of recombinant enzymes for the treatment of MPS I, II, and VI, the current state of the evidence provides only limited information on the overall cost burden of these conditions [15]. Within this context, the present study was the first Latin American investigation to assess the economic impact of ERT on the cost burden of disease as represented by medical interventions.

The only similar study in the international literature is the aforementioned investigation by Wyatt et al. [17], which consisted of a retrospective assessment of 68 patients with MPS I (20 adults and 48 children) and 39 patients with MPS II (3 adults and

Table 4 – Comparison between the ERT and non-ERT groups.

Medical interventions	No ERT (n = 7)	ERT (n = 27)	P
Medical appointments			
Number/patient, median (IQR 25–75)	8 (2–13)	7 (3–10)	0.915
Patients who attended visits in the period, n (%)	7 (100)	26 (96.2)	
Most commonly seen specialists*	Ear-nose-throat, medical geneticists, surgeons	Geneticists, ear-nose-throat, pulmonologists	
Hospital admissions			
Number/patient, median (IQR 25–75)	1 (0–2)	0 (0–1)	0.015
Patients who were hospitalized in the period, n (%)	5 (71)	7 (25.9)	
Most common reasons for hospital admission*	Asthma, respiratory insufficiency	Respiratory insufficiency, surgery	
Surgeries			
Number/patient, median (IQR 25–75)	0 (0–2)	0 (0–0)	0.040
Patients who underwent surgery in the period, n (%)	3 (42.8)	3 (11.1)	
Most commonly performed surgeries*	Adenoidectomy, umbilical hernia repair, tonsillectomy	Adenoidectomy, inguinal hernia repair, myringotomy	
Tests			
Number/patient, median (IQR 25–75)	13 (6–44)	8 (2–13)	0.096
Patients who had tests in the period, n (%)	7 (100)	25 (92.5)	
Most commonly performed tests*	Blood counts/chemistry panels, chest X-ray, echocardiogram	Blood counts/chemistry panels, urinary GAG measurement, echocardiogram	
Medications†			
Number/patient, median (IQR 25–75)	2 (0–22)	3 (0–3)	0.735
Patients who used medications in the period, n (%)	4 (57–1)	15 (55.5)	
Most commolnly prescribed medications*	Antibiotics, analgesics, corticosteroids	Analgesics, antibiotics, antihistamines	

ERT, enzyme replacement therapy; GAG, glycosaminoglycan; IQR, interquartile range.
* Listed in order of frequency.
† Median refers to the number of different active pharmaceutical ingredients used during the study period.

36 children), recruited from several UK centers. In the MPS I group, 24 patients with no cognitive involvement (12 children and 12 adults) were on ERT, with a median time on ERT of 4.68 years, slightly higher than that found in our sample. The remaining patients in the Wyatt et al. [17] sample had undergone HSCT, whereas the other patient with MPS I in our sample was on supportive care alone. Wyatt et al. [18] estimated the annual cost of MPS I treatment to the National Health Service (NHS) and publicly funded social-care services, including the costs of hospital services (hospital admissions, medical appointments, etc.) and extramural services (occupational therapy and other therapies) at £2000 for adult patients and £5300 for children. Furthermore, the annual cost of laronidase ERT was estimated as £258,201 for an adult patient versus £139,563 for a child. Thirty-seven patients with MPS II were on ERT, with a median time on ERT similar to that found in our sample. The two other patients with MPS II included in the sample were receiving supportive care alone [17]. Wyatt et al. [17] estimated the annual cost to the NHS and publicly funded social-care services at £15,500 for an adult patient with MPS II and £3500 for a child with MPS II. The annual cost of idursulfase ERT was £537,605 for an adult patient versus £314,004 for a child with the condition.

In the Wyatt et al. [17] sample, median age at ERT onset was 18.7 years for adults and 3.38 years for children with MPS I and 16.6 years for adults and 6.96 years for children with MPS II. In our sample, onset of ERT was later in both forms of MPSs, and in children and adults alike (data not shown). Many factors may have contributed to this late onset of therapy, including delays in diagnosis and the lack of reimbursement of ERT in Brazil. A study of 113 Brazilian patients with several forms of MPSs showed a 4.8-year delay between symptom onset and diagnostic confirmation [22]. Because of the progressive course of MPSs, early diagnosis and immediate institution of therapy are paramount [23], and may even lead to a reduction in treatment costs. Case studies of nontwin siblings with MPS I, II, and VI have reported much better outcomes in siblings who are diagnosed at birth and begin ERT within the first 6 months of life [24–26].

When analysis was restricted to children and adolescents, we found no difference between the ERT and non-ERT groups in terms of the frequency of medical interventions. These results are in line with those of Wyatt et al. [17], who found that in children with MPS I, there was no association between time on ERT and total NHS and social-care costs, hospital-care costs, or non–hospital-care costs, and in children with MPS II, there was no statistically significant association between time on ERT and total NHS and social-care costs or non–hospital-care costs. The authors, however, did find an association between time on ERT and hospital costs (hospital admissions, accident, and emergency visits, etc.) (costs 3.78 times

higher) in children with MPS II. One major factor that should be taken into account is the difference in profile between the two groups of the present study: the non-ERT group was composed exclusively of children and adolescents, most with MPS II, whereas only 59.3% of the patients in the ERT group were children. Contrary to the suggestion by Wyatt et al. [17] that children with MPS II generate a lower cost burden than do adults, our data suggest higher costs for patients not on ERT; consequently, the higher cost burden of this group must be attributable to children and adolescents.

Analysis of all patients regardless of age showed that the frequency of medical interventions was essentially similar in the ERT and non-ERT groups, with the exception of surgical procedures and hospital admissions, which were less frequent in the ERT group. In our sample, the leading causes of hospitalization in both groups were respiratory tract infections and surgery. According to the SUS coding database, Sistema de Gerenciamento da Tabela de Procedimentos, Medicamentos e Órteses e Próteses e Materiais do Sistema Único de Saúde (SIGTAP), the total cost (including hospital charges and provider fees) of an adenoidectomy, bilateral inguinal hernia repair, or umbilical hernia repair—the most common surgical procedures in both groups—is £90.53, £90.01, and £93.77, respectively [27]. Despite the cost of ERT, the impact of these costs appears to be lower in the ERT group, due to the relatively low frequency of surgical procedures and hospital admissions. According to SIGTAP, the cost of 1 day in an intensive care unit (adult or pediatric) is £36.14 [27].

Our findings also suggest that the most common medical procedures in both groups are physician appointments (medical appointments) and tests. Within the SUS, according to SIGTAP [27], the reimbursement rate for a visit to a primary care physician (e.g., to a pediatrician or general surgeon) is £0.53, whereas the rate for a specialist physician visit (e.g., geneticist or cardiologist) is £2.60. Since the creation of Ordinance No. 199, SIGTAP began to incorporate the procedure of Clinical Diagnostic Evaluation for Rare Disorders – Inborn Errors of Metabolism with a total cost of £156.00. Among the list of inborn errors of metabolism contemplated by this assessment, there are MPS I and MPS II. MPS VI, in turn, does not fit in this procedure [27]. Therefore, we believe that the costs of physician appointments will not be a major burden on the total treatment costs of patients with MPS I, II, or VI. This finding is consistent with the results reported by Wyatt et al. [17].

The most common tests undergone by patients included complete blood cell counts/blood chemistry panels, chest radiographs, echocardiography, and urinary GAG quantitation. The SUS covers the costs associated with these interventions, with a reimbursement rate of £1.07 per sample for complete blood cell counts, £0.48 to £0.91 for each blood chemistry test (urea, creatinine, bilirubin, Alanine transaminase (ALT), aspartate transaminase (AST), Gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), alkaline phosphatase, cholesterol, glucose, sodium, potassium, chloride, magnesium, calcium, phosphorus, albumin, globulins, and total protein), £1.79 per plain chest radiograph, and £10.38 for echocardiography [27]. We observed an increase in the frequency of urinary GAG quantitation orders in the ERT group. This is attributable to three factors. First, urinary GAG levels are used extensively in clinical trials of ERT for MPS I, II, and VI as a pharmacodynamic marker of in vivo enzyme activity and a purported surrogate biomarker of treatment response [28]. Second, because ERT is a novel treatment modality and has been the subject of relatively little study, attending physicians are understandably concerned about its effects; this leads to an increase in test orders. Third, according to the protocol for follow-up of patients with MPS I, II, and VI developed by Rede MPS Brasil (a network of Brazilian medical genetics services supported by public and private funding), GAG quantitation should be performed every 3 months [5]. Although currently the identification of urinary GAGs, as well as enzymatic assays in plasma and leukocytes for the diagnosis of EIM, is still to be covered by the SUS [27] at the time of conducting the study, urinary GAG quantitation was available only through the SUS for diagnostic purposes, with a reimbursement list rate of £0.96 per sample. In addition to not listing which assays are included in this test (toluidine blue, GAG chromatography or electrophoresis, or GAG quantification), it does not cover any form of GAG quantitation for monitoring purposes [27]. Therefore, at all participating centers, all GAG quantitation tests performed during the pre-ERT period (for diagnostic purposes) and during ERT are covered by MPS Brazil Network.

Thus far, no studies have been published on the cost of galsulfase ERT in patients with MPS VI. Although the worldwide incidence of MPS VI is estimated at only 1:250,000 live births [29], it seems to be more common in Brazil. A study conducted in the southern region of the country found a high frequency of MPSs among inborn errors of metabolism, with MPS I and MPS VI being the most frequently diagnosed forms [30]. Monte Santo, a municipality in northeastern Brazil, features a markedly elevated incidence of several genetic conditions, including MPS VI. The incidence of MPS VI in the area is estimated at 1 in 5000 live births [31]. Within this context, it needs to be noted that this was the first study to address the pharmacoeconomics of ERT for MPS VI.

A previous study conducted by our group assessed the effects of ERT in a sample of patients with MPS I ($n = 9$) throughout their follow-up at SGM-HCPA. Variables were compared between the pre-ERT and post-ERT periods within the same group of patients. Our findings suggested that ERT does not alter the natural history of MPS I (according to the medical interventions analyzed), and that—contrary to the findings of the present study—the treatment costs of patients with MPS I increase during ERT. Comparison between these two retrospective, hospital-based studies clearly shows the superior design and larger sample size of the present investigation [32]. Furthermore, the previous study was restricted to patients with MPS I, whereas the present study included patients with MPS II and MPS VI as well. In view of the sample size, we chose not to conduct subgroup analyses by MPSs type. Hence, there may be differences in terms of cost and treatment efficacy among patients with each of these three types of MPSs.

Conclusions

Our findings suggest that excluding the cost of recombinant enzymes, Brazilian patients with MPS I, II, and VI who receive disease-specific treatment undergo fewer medical interventions than do patients in supportive care. This seems to be associated with lower SUS expenditures with direct medical costs for patients with ERT. Despite some missing data, particularly regarding medication dosage and administration regimens, the study instrument appears adequate for collection of data on the costs associated with treatment of MPSs. Longitudinal studies will be useful in the evaluation of the long-term costs associated with ERT and its impact on the SUS.

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Supplemental Materials

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Oxidative and nitrative stress and pro-inflammatory cytokines in Mucopolysaccharidosis type II patients: effect of long-term enzyme replacement therapy and relation with glycosaminoglycan accumulation

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ABSTRACT

Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disease caused by a deficient activity of iduronate-2-sulfatase, leading to abnormal accumulation of glycosaminoglycans (GAG). The main treatment for MPS II is enzyme replacement therapy (ERT). Previous studies described potential benefits of six months of ERT against oxidative stress in patients. Thus, the aim of this study was to investigate oxidative, nitrative and inflammatory biomarkers in MPS II patients submitted to long term ERT. It were analyzed urine and blood samples from patients on ERT (mean time: 5.2 years) and healthy controls. Patients presented increased levels of lipid peroxidation, assessed by urinary 15-F2t-isoprostane and plasmatic thiobarbituric acid-reactive substances. Concerning to protein damage, urinary di-tyrosine (di-Tyr) was increased in patients; however, sulfhydryl and carbonyl groups in plasma were not altered. It were also verified increased levels of urinary nitrate + nitrite and plasmatic nitric oxide (NO) in MPS II patients. Pro-inflammatory cytokines IL-1β and TNF-α were increased in treated patients. GAG levels were correlated to di-Tyr and nitrate + nitrite. Furthermore, IL-1β was positively correlated with TNF-α and NO. Contrastingly, we did not observed alterations in erythrocyte superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities, in reduced glutathione content and in the plasmatic antioxidant capacity. Although some parameters were still altered in MPS II patients, these results may suggest a protective role of long-term ERT against oxidative stress, especially upon oxidative damage to protein and enzymatic and non-enzymatic defenses. Moreover, the redox imbalance observed in treated patients seems to be GAG- and pro-inflammatory cytokine-related.

Abbreviations: 3-NT, 3-nitrotyrosine; ABTS®, 2,2′-azino-di-3-(ethylbenzthiazoline sulfonate); AChE:Fab′, acetylcholinesterase:Fab′; CAT, catalase; Cr, creatinine; di-Tyr, di-tyrosine; DNPH, 2,4-dinitrophenylhydrazine; DS, dermatan sulfate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; ERT, enzyme replacement therapy; FU, fluorescence units; GAG, glycosaminoglycans; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; HCT, hematopoietic cell transplantation; HS, heparan sulfate; IDS, iduronate-2-sulfatase; IEM, inborn errors of metabolism; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LSD, lysosomal storage disorders; MDA, malondialdehyde; MPS II, Mucopolysaccharidosis type II; MPS, mucopolysaccharidoses; NF-κB, nuclear factor-kappa B; NO, nitric oxide; $NO_2^- + NO_3^-$, nitrate + nitrite; NO_2•, nitrogen dioxide radical; Nox, NAD(P)H oxidase; O_2•$^-$, radical superoxide anion; OH•, hydroxyl radical; $ONOO^-$, peroxynitrite; PAC, plasmatic antioxidant capacity; RNS, reactive nitrogen species; ROS, reactive oxygen species; SD, standard deviation; SEM, standard error of the mean; SOD, superoxide dismutase; TAS, total antioxidant status; TBARS, thiobarbituric acid-reactive substances; TNB, 2-nitro-5-thiobenzoic acid; TNF-α, tumor necrosis factor-alpha.

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1. Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders (LSD) caused by deficiency of one of the enzymes involved in the lysosomal degradation of glycosaminoglycans (GAG) [1]. MPS type II (MPS II) – or Hunter syndrome –, is biochemically characterized by the deficient activity of iduronate-2-sulfatase (IDS; EC 3.1.6.13), which removes *O*-linked sulfates moieties from heparan sulfate (HS) and dermatan sulfate (DS). Among all types of MPS, MPS II is the only with an X-linked recessive inheritance pattern Although almost all MPS II patients are males, some cases of women presenting the full-blown phenotype have been reported, most of them because of a skewed X-chromosome inactivation or a *de novo* mutation on the IDS codifying gene [2–4].

The age of onset, the progression and the phenotype are variable in MPS II, but the common signs and symptoms include claw-like hands, coarse face features, general skeletal deformities, thickened and inelastic-appearing skin, cardiac and pulmonary dysfunctions and, in two-thirds of the cases, neurological impairment [5–7]. The incidence of MPS II is estimated at 1:232,000 to 1:92,000 live births, depending on the analyzed population [8–12]. In Brazil, MPS II is known to be among the most frequent types of MPS [5,13], although there are no official data about its incidence in the country.

The enzyme replacement therapy (ERT) developed for MPS II was approved for use in Brazil in 2008. By administering a recombinant enzyme, this therapeutic strategy aims to reduce the abnormal storage of GAG in the lysosome, thus decreasing the cellular and tissue dysfunctions associated with the GAG accumulation. MPS II patients submitted to ERT show improvements in growth rate, in the 6-minute-walk test and other functional capacity outcomes, as well as a decrease in GAG excretion and a reduction of spleen and liver volumes [14–16]. ERT is generally safe and infusion-related reactions are as frequent as other protein-based therapies [17,18]. Although ERT is not fully effective against some important disease features – especially on central nervous system and skeletal system – it is thought to elongate patients' life expectancy and ameliorate their life quality. Currently, ERT is administered to MPS II patients since their early childhood, being well tolerated and producing important somatic improvements [19,20].

Several studies have described oxidative stress in patients affected by inborn errors of metabolism (IEM), including MPS II and other LSD [21–23]. Even though the relation between oxidative stress and the pathophysiology of IEM is not well established, the abnormal accumulation of toxic metabolites in cells, tissues and body fluids is thought to be the main cause of the increased generation of reactive species [24,25]. In LSD, the imbalance of redox status may play an even more important role in the pathophysiology, since lysosomes are very susceptible to oxidative stress. The reactive oxygen and nitrogen species (ROS and RNS, respectively) generated due to the lysosomal overload may initiate peroxidative cascades, leading to a destabilization of lysosomal membranes and, ultimately, causing an overflow of its contents into the cytoplasm [26,27]. The underlying mechanisms – including oxidative stress and inflammation – of MPS pathology have been studied mainly in animal models. Villani et al. [28] have demonstrated an upregulation of NAD(P)H oxidase (Nox) and pro-inflammatory cytokines in MPS IIIB knockout mice due to microglia activation. An intense production of the radical superoxide anion ($O_2^{\bullet-}$) – whose main source during inflammatory conditions is Nox – triggers the production of other ROS, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), enhancing the oxidant environment [29]. In this context, there are other well-based evidences showing the microglia activation as a first step in MPS-related neurodegeneration. As a result, an increase of ROS and RNS occurs due to microglial Nox and inducible nitric oxide synthase (iNOS) activation [30,31].

In patients, studies involving MPS I, II and IVA have demonstrated alterations in parameters of oxidative stress and inflammation [21–23,32]. Regarding to MPS II patients, Filippon et al. [21] investigated the effect of ERT on several biomarkers of oxidative stress. Compared to controls, non-treated MPS II patients showed a global impairment in the redox status, evidenced by an increase in lipid and protein oxidation, as well as alterations in superoxide dismutase (SOD) and catalase (CAT) activities. After just 15 days of treatment, plasmatic levels of malondialdehyde (MDA) – an end product of lipid oxidation – were significantly diminished compared to moment before the ERT beginning. Despite that, after six months of ERT, MDA did not reach control levels in treated MPS II patients. In this line, content of sulfhydryl groups in plasma was decreased in patients before treatment and improved throughout ERT. Nevertheless, the sulfhydryl levels in patients were still lower than in the control group up to the sixth month of ERT.

Considering that oxidative seems to be involved in MPS II, and that ERT can have beneficial effects in these processes, the aim of this study was to evaluate and correlate oxidative damage to biomolecules, levels of RNS, pro-inflammatory cytokines and GAG levels in MPS II patients submitted to long-term ERT.

2. Materials and methods

2.1. Subjects

The study was performed with eight MPS II male patients with ages varying between 7.5 and 30.1 years [17.1 ± 8.1 years-old; mean ± standard deviation (SD)] and with 10 healthy male individuals with ages ranging between 3.1 and 30.1 years (22.3 ± 7.5 years-old; mean ± SD). All MPS II patients were receiving ERT treatment (idursulfase – Elaprase® 0.5 mg/kg of body weight) for about 5.2 years (mean; range 1.5–7.0 years). Detailed patients' data were described in Table 1. ERT was administered by intravenous infusion on a weekly frequency. Diagnosis of all patients was established by deficient activity of IDS in plasma and/or leukocytes and by the identification of a molecular abnormality in the IDS gene. In all cases, GAG measurement in urine indicated high levels of total GAG excretion and presence of increased amounts of HS and DS. Informed consent was obtained from all participants. The study was approved by The Ethics Committee of the *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil, and registered under the number 14-0506.

2.2. Sample collection and preparation

Heparinized blood and occasional urine samples were obtained from patients right before the ERT session. Urine samples were collected in sterile flask, aliquoted and frozen at −80 °C until analysis. Whole blood was centrifuged at 1000 ×*g* for 10 min and plasma was separated by aspiration, aliquoted and frozen at −80 °C until the biochemical determinations. After removal of the buffy coat, erythrocytes were washed three times with cold solution of 0.153 mol/L NaCl and the lysates were

Table 1
Data of studied MPS II patients.

Patient	Age (years)	Time on ERT (years)	Leukocyte IDS activity at diagnosis[a] (nmol/4 h/mg protein)	Urinary GAG in the analyzed samples[b] (μg/mg Cr)
1	21.3	4.0	3.4	130
2	16.9	1.5	1.3	123
3	11.0	5.0	12.0	212
4	11.4	5.5	5.4	113
5	12.2	5.5	1.4	127
6	30.1	7.0	6.2	122
7	26.3	7.0	1.6	157
8	7.5	6.0	4.3	ND
Mean ± SD	17.1 ± 8.1	5.2 ± 1.8	4.5 ± 3.6	140.6 ± 34.4

ND: not determined.
[a] Normal range: 31–110 nmol/4 h/mg protein.
[b] Normal ranges: 7–9 years-old = 44–106 μg/mg Cr; 9–14 years-old = 26–97 μg/mg Cr; 14–18 years-old = 13–59 μg/mg Cr and >18 years-old = 13–45 μg/mg Cr.

prepared by the addition of 1 mL of distilled water to 0.1 mL of washed erythrocytes. The lysates were also frozen at −80 °C until the measurement of antioxidant enzymes activities and reduced glutathione (GSH) content. For these assays, the supernatant – obtained after centrifugation at 13,500 ×*g* for 10 min – was diluted in order to contain approximately 0.5 mg/mL of protein. Samples from MPS II patients and controls were obtained concomitantly.

2.3. Biochemical determinations

2.3.1. Plasmatic thiobarbituric acid-reactive substances (TBARS) determination

TBARS content – a biomarker of lipid peroxidation – was determined according to the method described by Ohkawa et al. [33]. To tubes containing 100 μL of plasma it was added, in the following order: 50 μL of 8% sodium dodecyl sulfate, 375 μL of 20% acetic acid in aqueous solution (V/V) pH 3.5 and 375 μL of 0.8% thiobarbituric acid. The test tubes were vortexed and the reaction was carried out in 100 °C for 60 min. After cooling on water for 5 min, tubes were centrifuged at 1000 ×*g* for 10 min. The resulting pink-stained TBARS were determined in a spectrophotometer at 535 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. TBARS were calculated as nmol TBARS/mg protein.

2.3.2. Urinary 15-F2t-isoprostane levels

15-F2t-isoprostane, a product of arachidonic acid metabolism and a biomarker of lipid peroxidation, was measured by *Urinary Isoprostane ELISA kit* (Oxford Biomedical Research, Inc., Oxford, MI, USA) according the kit's instructions. In this assay, the analyte present in the urine samples competes with the horseradish peroxidase-conjugated 15-F2t-isoprostane for the binding to a specific antibody fixed on the microplate. 15-F2t-isoprostane concentration was determined spectrophotometrically at 630 nm, by the intensity of color developed after the substrate was added to the wells. Results were expressed as ng of isoprostanes per mg of urinary creatinine (ng/mg Cr).

2.3.3. Total plasmatic levels of carbonyl groups

The content of plasmatic carbonyl groups – which is directly correlated to the levels of protein oxidation – was measured based on the reaction with 2,4-dinitrophenylhydrazine (DNPH), as previously described by Levine et al. [34]. Oxidatively modified proteins present a reinforcement of carbonyl content. These groups react with DNPH, producing a correspondent dinitrophenylhydrazone, which is yellow and absorbs at 370 nm. Results were expressed as nmol/mg protein.

2.3.4. Total plasmatic levels of sulfhydryl groups

Plasmatic concentration of sulfhydryl groups was determined as described by Aksenov and Markesbery [35]. The method is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups into 2-nitro-5-thiobenzoic acid (TNB), a yellow derivative whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.3.5. Urinary di-tyrosine (di-Tyr) levels

In order to determine the levels of protein oxidation in urine, the intensity of di-Tyr fluorescence was measured according to the method described by Kirschbaum [36]. For this assay, 50 μL of thawed urine was added to 950 μL of 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 min, di-Tyr concentration was measured using a fluorimeter (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg of urinary creatinine (FU/mg Cr).

2.3.6. Erythrocyte superoxide dismutase (SOD) activity

SOD activity was measured using the *RANSOD*® kit (Randox Lab, Antrim, UK). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and $O_2^{\bullet-}$, which is produced by the incubation with the xanthine–xanthine oxidase reaction system. The absorbance of the product is measured spectrophotometrically at 505 nm. One unit of SOD corresponds to a 50% inhibition of red formazan formation. The specific activity of SOD was expressed as U/mg protein.

2.3.7. Erythrocyte catalase (CAT) activity

CAT activity in erythrocytes was determined by observing the rate of decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm, as previously described by Aebi [37]. One unit of the enzyme is described as 1 μmol of H_2O_2 consumed per minute; the specific activity is reported as U/mg protein.

2.3.8. Erythrocyte glutathione peroxidase (GPx) activity

Erythrocyte GPx activity was measured by using a commercially available kit (RANSEL®; Randox Lab, Antrim, UK). GPx catalyzes the oxidation of glutathione (GSH) to GSSG (oxidized glutathione). In the presence of glutathione reductase (GR) and NADPH, the oxidized GSSG is converted to its reduced form, with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance of NAPDH was measured at 340 nm after one and 2 min. The results were expressed in U/mg protein.

2.3.9. Erythrocyte glutathione reductase (GR) activity

GR activity was determined by the method of Carlberg and Mannervik [38], which is based on GSSG reduction using NADPH as a cofactor. Erythrocyte samples were added to a reaction medium containing 0.2 mol/L sodium phosphate buffer, pH 7.5, 6.3 mmol/L EDTA and 0.4 mmol/L NADPH. At 340 nm, the consumption of NADPH was monitored for 10 min. One unit of GR represents one μmol of NADPH consumed per minute, and its specific activity is expressed as mU/mg protein.

2.3.10. Erythrocyte reduced glutathione (GSH) content

In order to measure GSH levels, the main intracellular antioxidant, lysates of erythrocytes were processed as described by Browne and Armstrong [39] and the fluorescence measured (excitation = 350 nm, emission = 420 nm) was compared to a calibration curve prepared with GSH solutions. Results were expressed as nmol/mg protein.

2.3.11. Plasmatic antioxidant capacity (PAC) determination

PAC was determined using the *Antioxidant Assay Kit* from Cayman Chemical® (Cayman Chemical Company, Ann Arbor, MI, USA). This assay measures the capacity of antioxidants in the sample to inhibit the oxidation of 2,2′-azino-di-3-(ethylbenzthiazoline sulfonate) (ABTS®) by metmyglobin. This reaction can be monitored by detecting the absorbance at 750 nm and the inhibition of the oxidation is proportional to antioxidants concentration. A standard curve using Trolox – a water-soluble tocopherol analogue – is used to calculate the capacity of the antioxidants in preventing ABTS® oxidation. PAC was expressed as nmol Trolox/mg protein.

2.3.12. Urinary nitrate/nitrite ($NO_3^- + NO_2^-$) levels

Total levels of $NO_3^- + NO_2^-$ in urine were determined using the *Nitrate/Nitrite Colorimetric Assay Kit* (*LDH Method*) from Cayman Chemical® (Cayman Chemical Company, Ann Arbor, MI, USA). This method is based on the reduction of NO_3^- to NO_2^- using nitrate reductase. The amounts of NO_2^- generated by NO_3^- reduction, plus the NO_2^- already present in the sample react with sulfanilamide producing a cationic intermediate, which reacts with *N*-(1-naphtyl)ethylenediamine. This reaction results in an azo product whose absorption can be measured at 540 nm. Results were reported as μmol/mg Cr.

2.3.13. Plasmatic nitric oxide (NO) production levels

The quantification of NO equivalents in plasma samples was performed using the *Non-Enzymatic Nitric Oxide Assay Kit* (Oxford Biomedical Research, Inc., Oxford, MI, USA), according to the manufacturer's instructions. The NO produced in biological systems rapidly degrades to its stable products NO_3^- and NO_2^-. In samples with high protein content, the conversion of NO_3^- to NO_2^- is improved using metallic cadmium for an overnight period. Subsequently, the formed NO_2^- is quantified using the Griess reaction, which results in a colored product, read at 540 nm. Results were expressed as log of nmol NO/mg protein.

2.3.14. Plasmatic pro-inflammatory cytokines (IL-1β and TNF-α) concentration

IL-1β and TNF-α concentrations both were determined in plasma using commercial immunometric assay kits from Cayman Chemical® (Cayman Chemical Company, Ann Arbor, MI, USA). Samples are disposed into wells pre-coated with the anti-IL-1β or anti-TNF-α capture antibodies. Using a sandwich technique, the cytokine is detected by the addition of an acetylcholinesterase:Fab′ (AChE:Fab′) conjugate, which binds selectively to a different epitope on the cytokine molecule. After five washing cycles, acetylthiocholine – a substrate of AChE – is added to the wells, producing thiocholine. This intermediate reacts with DNTB resulting in TNB, which is spectrophotometrically quantified at 412 nm. TNF-α concentrations were expressed as pg/mL and IL-1β levels as log of pg/mL.

2.3.15. Urinary glycosaminoglycan (GAG)

The measurement of urinary GAG was performed according to the technique described by de Jong et al. [40]. The method principle is based on formation of a complex between sulfated GAG present in urine with stain 1,9-dimethylmethylene blue. This complex can be detected in a spectrophotometer at 520 nm. The results were expressed as log of μg/mg Cr.

2.3.16. Urinary creatinine (Cr)

Creatinine was determined using *Creatinine Kinetic* kit of Bioclin® (Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil), by reaction with picric acid under alkaline conditions, producing an orange colored derivative, whose absorbance was determined in a spectrophotometer at 510 nm. Results were expressed as mg Cr/dL.

2.3.17. Plasmatic protein determination

Plasma and erythrocyte protein concentrations were determined, respectively, by Biuret method — using the commercial kit of Labtest® (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil) — and by the method of Lowry et al. [41].

2.4. Statistical analysis

All experiments were performed in triplicate. Results were expressed as mean ± standard error of the mean (SEM). Normal distribution was tested by the Shapiro-Wilk test. Logarithmic (log) transformation was done in data not normally distributed in order to transform them in parametric. Unpaired Student's *t*-test was used for all comparisons between the two groups. Statistical results are expressed as [t(degrees of freedom) = *t*-value, significance of *p*-value]. Correlations between parameters were performed by Pearson's correlation test. Differences were considered significant when $p < 0.05$. All analyses and graphs were done using the GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA — version 5.0) software.

3. Results

3.1. Oxidative damage to lipids

Results showed that MPS II patients who were undergoing long-term ERT had increased levels of TBARS in plasma (Fig. 1A) [$t(14) = 3.038$, $p < 0.01$] as well as 15-F2t-isoprostanes in urine (Fig. 1B) [$t(15) = 2.743$, $p < 0.05$] compared to controls.

3.2. Oxidative damage to proteins

It was observed an increase of di-Tyr excretion in urine from MPS II patients when compared to controls (Fig. 2A) [$t(13) = 2.240$, $p < 0.05$], evidencing some degree of protein oxidation. On the other hand, there were no differences between the two groups regarding plasmatic carbonyl (Fig. 2B) and sulfhydryl contents (Fig. 2C) [$t(14) = 0.712$, $p > 0.05$; $t(13) = 1.247$, $p > 0.05$, respectively].

3.3. Enzymatic and non-enzymatic defenses

No significant differences between patients and controls were observed in GSH content, SOD, CAT, GPx and GR activities in erythrocytes [GSH: Control = 0.601 ± 0.078; MPS II ERT = 0.504 ± 0.072 (nmol/mg protein, mean ± SEM); $t(13) = 0.932$, $p > 0.05$]; [SOD: Control = 2.449 ± 0.083; MPS II ERT = 2.325 ± 0.110 (U/mg protein, mean ± SEM); $t(14) = 0.921$, $p > 0.05$]; [CAT: Control = 0.519 ± 0.023; MPS II ERT = 0.560 ± 0.050 (U/mg protein, mean ± SEM); $t(14) = 0.811$, $p > 0.05$]; [GPx: Control = 0.052 ± 0.005; MPS II ERT = 0.051 ± 0.004 (U/mg protein, mean ± SEM); $t(14) = 0.011$, $p > 0.05$]; [GR: Control = 3.158 ± 0.129; MPS II ERT = 3.211 ± 0.269 (mU/mg protein, mean ± SEM); $t(11) = 0.189$, $p > 0.05$]. Plasmatic non-enzymatic defenses – assessed through the PAC measurement – were not found altered in MPS II patients in comparison to healthy controls [Control = 16.50 ± 1.256; MPS II ERT = 17.47 ± 1.408 (nmol Trolox/mg protein, mean ± SEM); $t(13) = 0.515$, $p > 0.05$]. (n = 7–9 per group).

3.4. Reactive nitrogen species (RNS) analysis

Plasmatic concentration of NO (Fig. 3A) and urinary content of $NO_3^- + NO_2^-$ (Fig. 3B) were significantly increased in patients compared to controls [$t(14) = 2.610$, $p < 0.05$; $t(14) = 2.519$, $p < 0.05$, respectively], evidencing higher levels of RNS in treated MPS II patients.

3.5. Pro-inflammatory cytokines

Pro-inflammatory cytokines measured in this study, IL-1β (Fig. 4A) and TNF-α (Fig. 4B), were increased in plasma from MPS II patients submitted to ERT compared to controls [$t(14) = 2.167$, $p < 0.05$; $t(13) = 2.204$, $p < 0.05$, respectively].

3.6. GAG levels

Despite the fact that ERT was administered to the MPS II patients on a weekly frequency, it was observed a higher GAG urinary excretion in patients compared to controls [$t(14) = 8.431$, $p < 0.0001$] (Fig. 5).

3.7. Correlations between RNS, oxidative stress, pro-inflammatory cytokines and GAG levels

GAG levels in MPS II were positively correlated with urine di-Tyr and $NO_3^- + NO_2^-$ concentrations (Fig. 6A and B; $r = 0.761$, $p < 0.05$; $r = 0.797$, $p < 0.05$, respectively). Concentrations of plasmatic pro-inflammatory cytokines IL-1β and TNF-α were correlated with each other in MPS II patients (Fig. 6C; $r = 0.917$, $p < 0.01$). It was also verified a positive correlation between IL-1β and plasmatic concentrations of

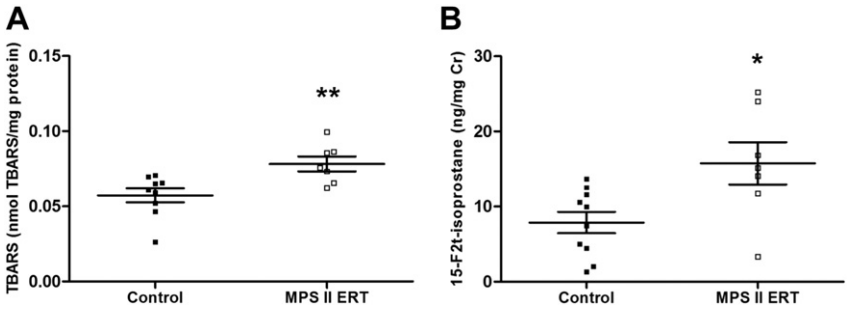

Fig. 1. Oxidative damage to lipids [plasmatic content of thiobarbituric acid-reactive substances - TBARS (A) and urinary levels of 15-F2t-isoprostane (B)] in MPS II patients under ERT (n = 7) and controls (n = 9–10). Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test for unpaired samples) compared to the control group.

NO (Fig. 6D; r = 0.755, $p < 0.05$). No significant correlations were observed between other parameters.

4. Discussion

In LSD, the primary cause of cell and tissue dysfunction is the lysosomal overload resultant from the enzymatic deficiency. In order to deal with the growing concentrations of the accumulated substrates, the cell manages to increase size and number of lysosomes. Despite the fact that the pathogenic mechanisms of LSD have been extensively studied, those are not fully understood yet [42,43]. Reactive species are constantly formed during cellular respiration. In fact, they play important roles in global functioning of cell, since some ROS and RNS are directly involved in cellular signaling pathways [44–46]. However, if the reactive species increase above a sustainable level, a state of oxidative stress takes place, resulting in damage to biomolecules, altering their function and eventually leading to cell and tissue failure [29,47,48].

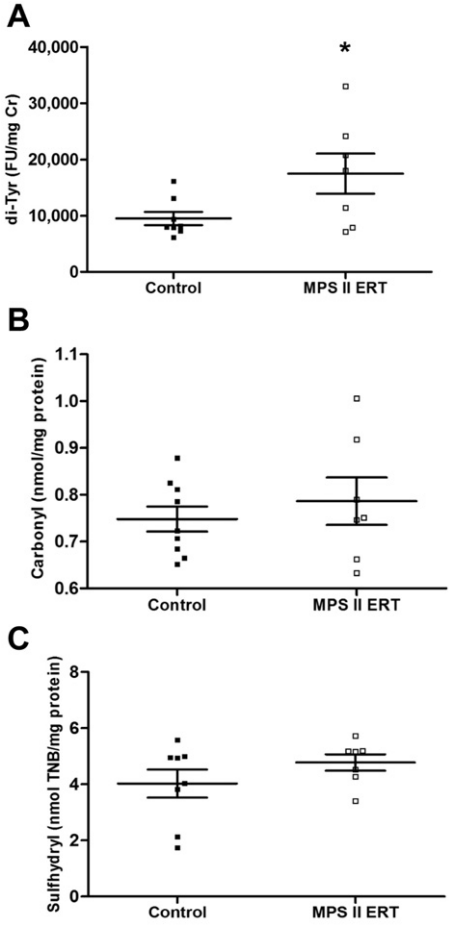

Fig. 2. Oxidative damage to proteins [di-tyrosine in urine – di-Tyr (A), carbonyl groups (B) and sulfhydryl groups (C) in plasma] in MPS II patients under ERT (n = 7) and controls (n = 8–9). Data represent mean ± SEM. $^{*}p < 0.05$ (Student's *t*-test for unpaired samples) compared to the control group.

The involvement of oxidative stress and reactive species is described in more than 100 human diseases, including MPS and other IEM [21–25,49–51]. In lysosomal disorders, such as MPS, oxidative stress can actually imply in more alterations and dysfunctions. Lysosomes are quite susceptible to oxidative stress; one of the reasons is its high iron content. Iron catalyzes Fenton reaction, which is a homolytic splitting of H_2O_2 and yields the extremely reactive hydroxyl radical (OH•). This ROS formed within the lysosome attacks biomolecules, including lipids from lysosomal membrane, resulting in its rupture and subsequent iron efflux to cytoplasm. Therefore, the liberation of lysosomal contents induces secondary ROS and RNS production in cytoplasm, suggesting that lysosomal damage causes further oxidative stress in a loop process [26,27,52–54].

Recent studies from our group evidenced the involvement of oxidative stress in LSD patients. Donida et al. [23] showed that MPS IVA patients treated with ERT presented reduction in non-enzymatic antioxidants contents as well as oxidative damage to lipids, proteins and DNA. In the same way, Fabry disease patients also submitted to ERT had high levels of oxidized proteins and lipids and increased concentrations of plasmatic pro-inflammatory cytokines [55]. Regarding to MPS II, Filippon et al. [21] studied patients before and during the first six months of ERT and found that the treatment may have a protective effect against oxidative stress. Nevertheless, almost all of the evaluated parameters remained altered at the end of the study.

Considering this scenario, the aim of this study was to evaluate oxidative stress parameters and inflammation biomarkers in MPS II patients under long-term ERT, in order to better understand the mechanisms related to the pathophysiology of this disease and the effects of ERT upon these processes.

MPS II patients undergoing long-term ERT (about 5.2 years) showed increased levels of urinary and plasmatic biomarkers of lipid oxidation, assessed by the TBARS measurement in plasma and the concentrations of 15-F2t-isoprostane in urine. Isoprostanes are formed by the reaction of reactive species with arachidonyl moieties, most of them present in arachidonic-esteres of membrane phospholipids [56]. After their formation, isoprostanes are rapidly metabolized and excreted; hence the determination of isoprostane amounts in urine is a well-established biomarker of oxidative stress [57]. TBARS assay quantifies MDA and MDA-like substances, and both are byproducts from lipid peroxidation [33]. The continued oxidation and fragmentation of fatty acid side chains can produce substances like isoprostanes, MDA and 4-hydroxynonenal. These aldehydes may cause alterations in permeability as well as rupture of lysosomal (and other organelles) membranes. Compared to controls, MPS II patients exhibited high concentrations of both biomarkers, even after long time of treatment. Filippon et al. [21] have demonstrated that MPS II patients exhibit high levels of MDA in

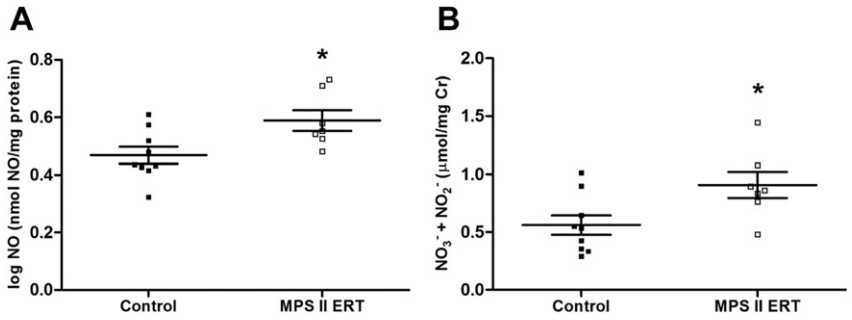

Fig. 3. Reactive nitrogen species [nitric oxide in plasma – NO (A) and nitrate + nitrite content in urine – $NO_3^- + NO_2^-$ (B)] in MPS II patients under ERT (n = 7) and controls (n = 9). Data represent mean ± SEM. *$p < 0.05$ (Student's *t*-test for unpaired samples) compared to the control group.

plasma before and during six months of ERT, even though a substantial decrease in its concentrations could be observed throughout the time of the study. Considering the susceptibility of the lysosomal membrane, as well as the constant lysosomal storage and subsequent rupture of its membrane, we suggest that the increased lipid peroxidation in the patients observed in the present study can be, at least in part, involved in the pathophysiology of MPS II disease, even under long-term ERT.

With respect to oxidative damage to proteins, we found an increase of urinary di-Tyr levels in patients and no alteration of sulfhydryl and carbonyl contents in plasma. A previous study relating oxidative stress to MPS II patients undergoing treatment showed that patients presented protein damage up to the sixth month of ERT, evidenced by low levels of sulfhydryl and high levels of carbonyl in plasma [21]. Sulfhydryl moieties in thiol-containing amino acids (cysteine and cystine) can be reversibly oxidized by reactive species [58]. Similarly, carbonyl content in proteins are specially generated by oxidative attack and can indicate extreme damage to proteins since heavily carbonylated proteins tend to form high-molecular-weight aggregates that are resistant to degradation by the proteasomal system [59]. Our data suggest a beneficial effect of long-term ERT on protein oxidation, once these parameters were not found altered in patients. Nevertheless, the increase of urine di-Tyr levels shows us some degree of protein oxidation in MPS II patients, since di-Tyr is formed by the oxidation of adjacent protein tyrosine residues leading to the formation of a highly stable inter-phenolic bond that does not undergo further metabolism [60]. Another product of tyrosine degradation is 3-nitrotyrosine (3-NT), which unlike di-Tyr, is metabolized and excreted in urine as 3-nitro-4-hydroxyphenylacetic acid [61]. It is described that di-Tyr formation is particularly enhanced by certain reactive species, such as $O_2^{\bullet -}$, OH•, and nitrogen dioxide radical (NO_2•) [62]. Unlike thiol-containing amino acids, tyrosine cannot react directly with $ONOO^-$ [63–65]. di-Tyr and 3-NT formation is indeed enhanced when exposed to $ONOO^-$, but this occurs through a radical mechanism, in which 3-NT and di-Tyr (a dimerization product of tyrosyl radical) are formed by the reaction of tyrosine with radicals, like NO_2• and OH• [63,66]. Likewise, histidine, leucine and phenylalanine do not directly react with $ONOO^-$, but rather with its secondary radicals, such as NO_2• [63,66,67]. In our study, we verified that long-term treated MPS II patients present increased levels of RNS in both urine and plasma. We also verified that these patients did not present alterations in SOD activity, and hence no increase of $O_2^{\bullet -}$ contents. When combined, these results allow to suggest that there is not enhanced $ONOO^-$ formation in MPS II patients, but only increased amounts of NO, NO_2• and $NO_3^- + NO_2^-$. These facts may explain the differences observed in sulfhydryl, carbonyl and di-Tyr results: while the cysteine, glutathione, and other thiol-containing molecules are oxidized by $ONOO^-$ – as well as carbonyl groups are formed –, di-Tyr and 3-NT production does not need $ONOO^-$, but mostly stable NO intermediates, such as NO_2•, which is probably increased in the studied patients. Therefore, the high contents of NO and $NO_3^- + NO_2^-$ found in patients indicate an imbalance of nitrative state, and this may be associated with the high contents of di-Tyr observed in urine. Still regarding protein damage and RNS, GAG levels were found correlated to $NO_2^- + NO_3^-$ and di-Tyr contents. These findings suggest the involvement of the accumulated metabolite in the production of reactive species, as well as in the protein damage.

The consistent enhancement of lipid peroxidation found in MPS II patients – assessed by TBARS and 15-F2t-isoprostane measurements – seems to be in contrast with the moderate protein damage that was observed. This fact might be due to inherent etiology of MPS II. The intralysosomal GAG accumulation leads to an increase of size and number of lysosomes, and eventually conducts to their breakage [27,42]. Subsequently, the release of lysosomal contents into cytosol activates several cascades which may cause different types of damage and also culminate in cell death [26,27,52–54]. In addition to the release of GAG, acid hydrolases and others, lysosomal disruption causes a high efflux of iron into cytosol. Besides catalyzing the Fenton reaction, iron *per se* is able to initiate and propagate lipid peroxidation reactions, by converting lipid hydroperoxides into other highly reactive lipid radicals, such as peroxyl and alcohoxyl radicals [47,68,69]. Therefore, the abnormal lysosomal storage observed in MPS II – even under long-term ERT, once GAG values are not within the normal range – may lead to intense and persistent lipid damage, possibly due to iron efflux into cytosol.

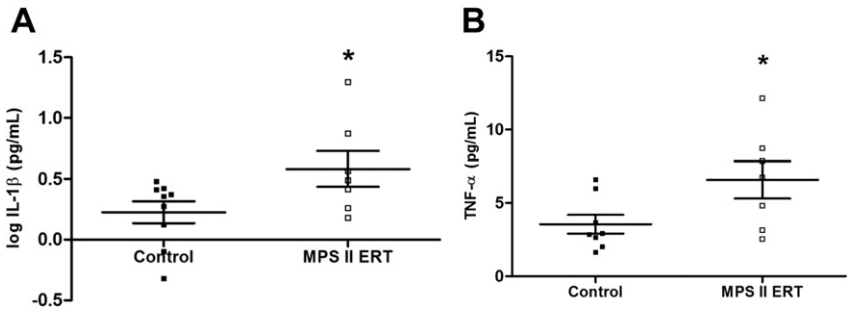

Fig. 4. Pro-inflammatory cytokines [interleukin-1 beta – IL-1β (A) and tumor necrosis factor alpha – TNF-α (B)] in MPS II patients under ERT (n = 7) and controls (n = 8–9). Data represent mean ± SEM. *$p < 0.05$ (Student's *t*-test for unpaired samples) compared to the control group.

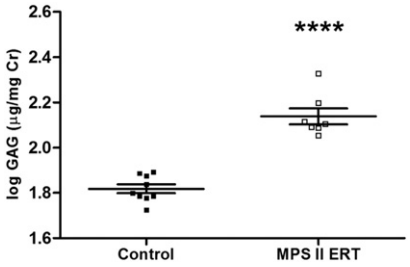

Fig. 5. Glycosaminoglycans (GAG) urinary levels in MPS II patients under ERT (n = 7) and controls (n = 9). Data represent mean ± SEM. ****$p < 0.0001$ (Student's *t*-test for unpaired samples) compared to the control group.

In this study, we assessed several biomarkers of antioxidant defenses, and none of them was found altered in long-term treated MPS II patients compared to controls. Erythrocyte SOD, CAT, GPx and GR activities were no different from healthy individuals. In a study involving MPS I patients before and during the first 24 weeks of treatment, it was observed a decrease in SOD activity at 8 weeks of ERT, but not at 4 or 24 weeks [22]. Similarly, Filippon et al. [21] verified a transient increase of CAT activity in treated MPS II patients during the first six months of ERT. These time-dependent alterations of enzymatic activities found in patients during treatment may be due to erythrocyte lifespan, which lasts approximately 120 days. After maturation, these cells are not able to synthesize new enzymes. Thus, the secondary biochemical alterations provoked by ERT might need some time to reflect in erythrocyte antioxidant enzymes activities [21]. Most importantly, our results provide evidence that long-term ERT can stabilize these pivotal enzymatic antioxidant defenses in MPS II patients. Moreover, we verified that neither GSH content nor PAC was reduced in patients, evidencing no depletion of important non-enzymatic defenses. GSH is the main reductant biomolecule in biological systems, and it is preferentially oxidized by reactive species, then preserving more important biomolecules [29]. Likewise, PAC is a measurement of the antioxidant substances – both macro- and small molecules, such as ferritin, ceruloplasmin, α-tocopherol and ascorbic acid – present in the sample [70,71]. Corroborating with these findings, the study performed during the first six months of ERT in MPS II patients showed a reduction in the total antioxidant status (TAS) in plasma before ERT and up to the fifth month of treatment; at the sixth month, TAS was restored at control levels [21]. Therefore, it is conceivable to state that non-enzymatic antioxidant defenses demand longer times of treatment to reestablish its levels.

Our findings concerning to the pro-inflammatory cytokines IL-1β and TNF-α, both increased in MPS II patients provide evidence that an inflammatory state indeed occurs in these patients. Several studies – mainly in animal models – have assessed levels of pro-inflammatory mediators in MPS, indicating a high expression of inflammatory molecules and cytokines secondary to the accumulation of GAG [28,30,72–74]. A recent study conducted in MPS I, II and VI patients submitted to ERT and/or hematopoietic cell transplantation (HCT) showed that TNF-α levels are positively associated with increased pain, decreased physical functions and social limitations generated by an impaired physical health. Besides that, plasmatic TNF-α did not decrease over ERT treatment and/or after HCT [75]. Furthermore, we observed that both cytokines were correlated with each other – which is concordant with a global pro-inflammatory state – and that plasmatic IL-1β was correlated with NO levels. The enhanced production of NO is more likely to occur under inflammation, and the NO role on cytokines overexpression has already been studied in several conditions, such as lung infections [76], sepsis [77], and also in Gaucher disease [78]. Some studies show that, among other intricate pathways, NO can upregulate pro-inflammatory cytokines – such as IL-1β and TNF-α – *via* nuclear

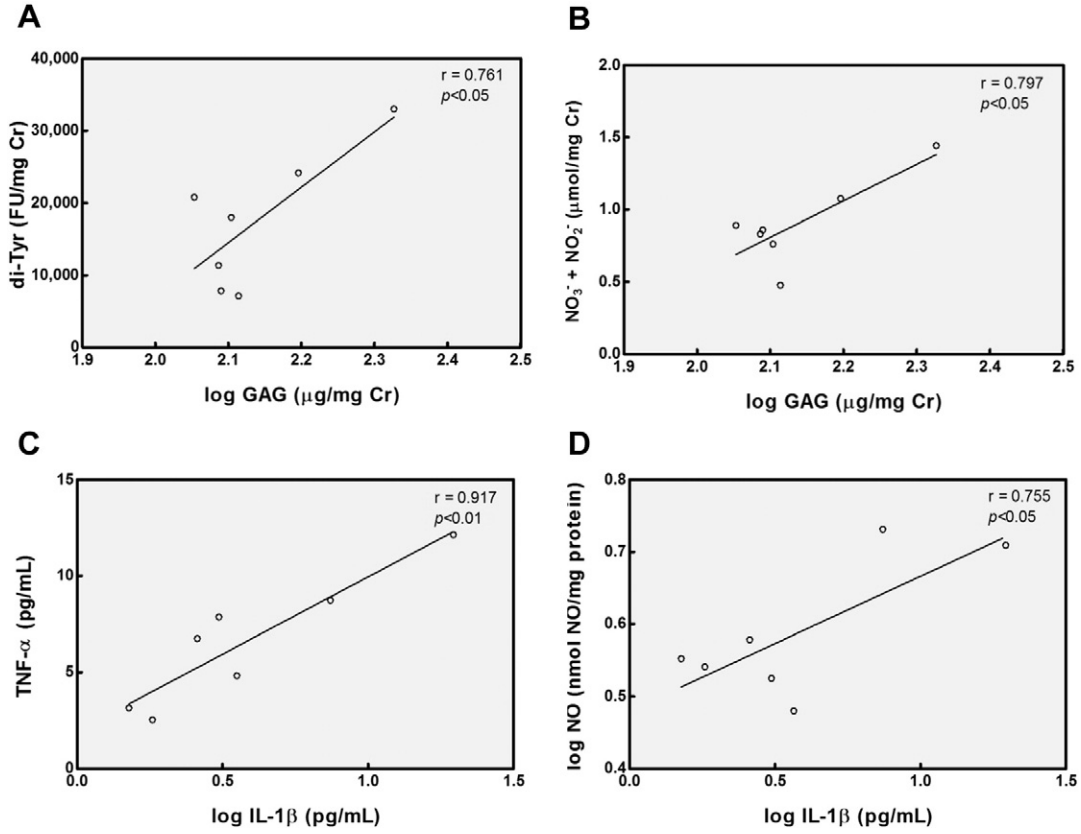

Fig. 6. Correlations between GAG and biomarkers of oxidative and nitrosative stress [urinary di-Tyr (A) and urinary $NO_3^- + NO_2^-$ (B)]; between IL-1β and TNF-α (C) and between IL-1β and plasmatic NO (D) in MPS II patients under ERT (Pearson's correlation).

factor-κB (NF-κB) activation [79–81]. The correlation observed in our study suggests a possible involvement of NO in the induction and maintenance of inflammatory states in MPS II patients.

Despite the fact that ERT significantly reduces GAG excretion in MPS II patients [14–16,82], GAG levels in urine were higher in treated patients compared to controls. Therefore, looking upon the altered parameters and the found correlations, we propose that GAG are related, directly or indirectly, to the alterations found in the redox balance and possibly in the inflammatory process verified in MPS II patients.

In summary, this study indicates that, at some degree, inflammatory processes, oxidative and nitrative imbalances occur in MPS II patients even during long-term ERT. Besides, these alterations seem to be induced by GAG accumulation and by pro-inflammatory cytokines. Notwithstanding these findings, ERT is known to reduce GAG levels and also was efficient in improving several biomarkers of oxidative stress. Therefore, these results reinforce ERT as an important therapeutic strategy in MPS II treatment, once inflammation and oxidative and nitrative stress seem to take part in pathophysiology of this severe disease.

Conflict of interest

R. Giugliani received grants and/or personal fees from Actelion, Alexion, Amicus, Biomarin, Genzyme, Pfizer, Protalix, Shire and Ultragenyx. The other authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Samples

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

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

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

2.2. Enzyme activity analyses

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

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

2.3. Urinary glycosaminoglycans (GAGs) analysis

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

2.4. Gene analysis

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

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

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

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

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

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

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

3. Results

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

3.1. Case 1: suspicion of MPS I

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

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

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

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

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

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

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

3.2. Case 2: suspicion of Pompe disease

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

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

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

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

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

3.3. Case 3: suspicion of Gaucher disease

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

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

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

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

3.4. Case 4: suspicion of MPS I

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

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

biochemical results were considered inconclusive.

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

Author contributions

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

Conflicts of interest

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

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