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ESTUDO ABRANGENTE SOBRE ASPECTOS CLÍNICOS E DIAGNÓSTICOS DA DEFICIÊNCIA DE GLC-NAC-FOSFOTRANSFERASE (MUCOLIPIDOSES II E III)

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LISTA DE ABREVIATURAS E SIGLAS

DNA (*Deoxyribonucleic Acid*) – Ácido Desoxirribonucléico

DLD; Doenças lisossômicas de depósito

ERT (*Enzyme replacement therapy)*: terapia de reposição enzimática

EIM: erro inato do metabolismo

FOS (*Free oligosaccharides):* oligossacarídeos livres

GAG: Glicosaminoglicanos

GNPTAB *N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits):* N-acetilglucosamina-1- transferase, subunidades alfa e beta

GNPTG *(N-Acetylglucosamine-1-Phosphate Transferase, Gamma Subunit):* N-acetilglucosamina-1- transferase, subunidade gama

HERC2 (*HECT and RLD domain containing E3 ubiquitin protein ligase 2*) – Domínio HECT e RLD contendo a proteína ligase 2 ubiquitina E3

LEIM-HCPA: Laboratório de erros inatos do Metabolismo do Hospital de Clínicas de Porto Alegre

LSD (*Lysosomal storage disorders): Doenças lisossômicas de depósito*

MALDI-TOF/TOF: (*Matrix-assisted laser desorption/ionization time-of-flight*):Dessorção / ionização por laser tempo de vôo

MATP (*Membrane-associated Transporter Protein*) – Proteína Transportadora Associada à membrana

ML: mucolipidose

MPS: Mucopolissacaridoses

OCA2 (*Oculocutaneous Albinism II*) – Albinismo Óculo-Cutâneo II

SGM-HCPA: Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre

SLC24A4 (*Solute Carrier Family 24, Member 4*) – Família Transportadora de Soluto 24, Membro 4

SLC45A2 (*Solute Carrier Family 45, Member 2*) - Família Transportadora de Soluto 45, Membro 2

SNPs (*Single nucleotide polymorphisms*) - Polimorfismos de Nucleotídeo Único

SPSS (*Statistical Package for the Social Sciences)*; pacote estatístico para ciências sociais

TYR (Tyrosinase) – Tirosinase

UFRGS: Universidade Federal do Rio Grande do Sul

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Resumo

Introdução: As Mucolipidoses (ML) II e III são doenças lisossômicas raras, causadas pela atividade deficiente da GlcNAc-1-fosfotransferase, codificada pelos genes *GNPTAB* e *GNPTG*, causando alteração na atividade das hidrolases lisossomais (aumentadas em plasma, reduzidas em fibroblastos e normal em leucócitos). O padrão-ouro para o diagnóstico de ML II/III é a demonstração da deficiência intracelular da atividade da GlcNAc-1-fosfotransferase e/ou identificação de mutações patogênicas em *GNPTAB* ou *GNPTG*, que estão disponíveis em poucos centros. Deste modo, a confirmação diagnóstica geralmente é baseada na medida da atividade hidrolases lisossomais, tanto em plasma como em fibroblastos. Entretanto não há consenso sobre quais enzimas devem ser pesquisadas e diferentes painéis são randomicamente utilizados, com fracas evidências para embasar esta prática. Há poucos estudos sobre a história natural das ML II/III, não existindo estudos brasileiros sobre o tema.

Objetivos Gerais: 1) Identificar manifestações clínicas e caracterizar a funcionalidade em uma amostra de pacientes brasileiros com ML II e III. 2) Caracterizar a atividade das enzimas lisossomais em pacientes brasileiros com diagnóstico confirmado de ML II/III, visando propor um protocolo diagnóstico custo-efetivo. **Objetivos Específicos:** 1) Estudar as diferenças clÍnicas entre os subtipos de mucolipidose III (gama e alfa/beta); 2) Caracterizar a funcionalidade dos pacientes com ML II e III no Brasil; 3) Classificar os fototipos dos pacientes com ML e compará-los com seus pais e com controles (indivíduos saudáveis), a fim de ser detectada possível tendência de hipomelanogênese; 4) Estudar a associação entre cor de pele, olhos e cabelos e os seguintes SNPs: rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) and rs1129038 (*HERC2* gene).

Metodologia: Estudo transversal, amostragem por conveniência, incluindo pacientes com diagnóstico clínico e bioquímico ou genético de ML II/III. Os dados clínicos foram obtidos de forma retrospectiva, por entrevista com familiares e revisão de prontuário. Pacientes com MLII/III, >1 ano e seus pais, assim como controles saudáveis e pais destes,avaliados sobre características de pele, cabelo e olhos usando a escala de Fitzpatrick e classificação visual.

SNPs [rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) e rs1129038 (*HERC2* gene)] foram genotipados por KASP (LGC Genomics: www.lgcgenomics.com). Para o protocolo bioquímico, desenvolvemos uma pontuação baseada em 11 itens para escolher quais enzimas deveriam ser inclusas no painel do protocolo (quanto maior a pontuação, mais adequada).

Resultados: 1) Para caracterização clínica, inclusos 27 pacientes (ML II=15, ML III alfa/beta= 9, ML IIII gama=3). Sintomas relacionados ao sistema esquelético foram predominantes em ambos grupos. Pacientes ML II demonstraram maior gravidade do envolvimento cognitivo e somático. Pacientes ML III alfa/beta apresentaram atraso nos marcos iniciais do desenvolvimento e possuíam comprometimento somático maior que ML III gama. Os nossos dados também sugerem que pacientes ML II apresentam hiperparatireoidismo transitório no início da infância, sem evidência de necessidade de tratamento. **2)** Para caracterização bioquímica, incluídos 26 pacientes (ML II=14, ML III alfa/beta=9, ML III gama=3). Destes, todos possuíam atividade enzimática avaliada no plasma e 15 em fibroblastos. Em plasma, todas as enzimas exibiram média da atividade elevada, com exceção da quitotriosidase; em fibroblastos apenas a média da atividade da beta-glicosidase foi normal, enquanto todas as outras foram reduzidas. As pontuações mais altas foram atingidas por alfa-manosidase (29 pontos), alfa–L-iduronidase (28 pontos), beta-hexosaminidase total (27 pontos), beta-glicuronidase (26 pontos), e alfa–N-acetilglicosaminidase (25 pontos). **3)** No estudo de pigmentação, 17 pacientes (MLII=7, MLIII alfa/beta=7, ML III gama=3) e 29 pais foram incluídos, assim como 20 controles saudáveis e 34 pais de controles. A maioria dos pacientes apresentou fototipo (Fitzpatrick) I–III (n=14/17, 82%) taxa discrepante do fototipo de seus pais (n=19/29, 66%), dos controles saudáveis (n=14/20, 70%) e pais de controles (n=25/34, 74%). Quanto à associação genótipo-fenótipo, as diferenças entre fenótipo e cor esperada como suposto pelo fenótipo foram: para rs1126809, 2/17 pacientes tiveram cabelos mais claros; para rs16891982, 4/17, 6/17 e 10/17 pacientes apresentavam, respectivamente, olhos, cabelos e da pele e 3/17, 4/17 and 2/17 pacientes, respectivamente, olhos, cabelo e pele mais escuros; e para rs1426654, 2/17,

1/17, 1/17 pacientes tiveram olhos, cabelos e pele mais clara e 9/17 e 3/17 h tinham cabelo e pele mais escuro; para rs1129038, 6/16 pacientes apresentavam olhos mais escuros que o esperado. **3)** Em relação à testagem genética, os nossos dados sugerem que a análise de *GNPTAB* e *GNPTG* pode ser feita por seqüenciamento de nova geração, e que a a identificação das mutações patogênicas que segregam na famíia é de suma importância para a identificação de heterozigotos e diagnóstico pré-natal.

Conclusão: Esta é a primeira série de casos de pacientes ML II/III no Brasil, onde a MLII parece ser o tipo mais comum. Além disso, as ML II/III tem diagnóstico tardio, sintomas multissistêmicos e aparentam exibir modificações na melanogênese (mais comum hipomelanose), porém estudos adicionais são necessários para confirmar estes achados. Levando em consideração as enzimas avaliadas neste estudo, sugerimos aferir a atividade enzimática em ao menos três enzimas que obtiveram pontuação mais alta na nossa análise. Este painel enzimático propõe ser um método mais rápido, simples e com menor custo para o diagnóstico bioquímico. O seqüenciamento de nova geração parece ser uma boa alternativa para a confirmação diagnóstica desses pacientes.

Abstract

Introduction: Mucolipidosis (ML) II and III are rare lysosomal diseases caused by GlcNAc-1-phosphotransferase deficiency, which is encoded by the GNPTAB and GNPTG genes, leading to altered activity of lysosomal hydrolases (increased in plasma, reduced in fibroblasts, and normal in leukocytes). Gold-standard method for diagnosis of ML II / III is the demonstration of intracellular deficiency of GlcNAc-1-phosphotransferase activity and/or identification of pathogenic mutations in GNPTAB or GNPTG, which are available in few centers. Thus, the diagnostic confirmation of ML II / III is usually based on measurement of many lysosomal hydrolases activities, both in fibroblasts and in plasma. However, there is no consensus about which enzymes should be investigated, and different random panels are used, with weak evidence to support this practice. Few studies about the natural history of ML II/III are available, besides that, there are no Brazilian studies about this theme. General **Objectives: 1)** Identify clinical manifestations and characterize functionality in a sample of Brazilian patients with ML II and III; and **2)** To characterize the activity of lysosomal enzymes in Brazilian patients with confirmed diagnosis of ML II / III, aiming to propose a cost-effective diagnostic protocol. **Specific Objectives. 1)** Study clinical differences between mucolipidosis subtype III (alpha/beta and gamma; **2)** To characterize functionality of ML II and III patients in Brazil; **3)** To classify phototypes of ML II / III patients and compare them to their parents and to controls (healthy individuals), to identify possible trends to hypomelanogenesis; and **4)** To study the association between skin, eyes, and hair color and the following SNPs: rs1126809 (TYR gene), rs16891982 (SLC45A2 gene), rs1426654 (SLC24A5 gene), and rs1129038 (HERC2 gene). **Methods:** Cross-sectional study, with convenience sample, including patients with a clinical and biochemical, or genetic, diagnosis of ML II/III. Clinical and demographic data were obtained retrospectively by chart review and / or interview with the family. Brazilian patients with ML II/III, >1yo, and parents, as well as healthy controls and their parents, were evaluated on characteristics of skin, hair, and eyes using the Fitzpatrick scale and visual classification. SNPs [rs1126809 (TYR gene), rs16891982 (SLC45A2 gene), rs1426654 (SLC24A5 gene), and rs1129038 (HERC2 gene)] were genotyped with KASP genotyping

assay (LGC Genomics: www.lgcgenomics.com). For biochemical protocol, we developed a score based on 11 items to choose from which enzymes should be included in the protocol panel (the higher the score, the better suited). **Results: 1)** For clinical characterization we included 27 patients (ML II= 15, ML III alpha/beta= 9, ML III gamma=3), Skeletal system-related symptoms were predominant in both groups. ML II patients demonstrated greater physical and cognitive severity, ML III alpha / beta had delay in initial developmental milestones and somatic impairments were greater than ML III gamma. Our data also suggest that the ML II patients presented transient hyperparathyroidism in early infancy without apparent need of treatment; **2)** For biochemical characterization, a total of 26 patients were included (ML II=14, ML III alpha/beta=9, ML III gamma=3). Of these, all 26 had lysosomal enzyme activity evaluated in plasma and 15 in fibroblasts. In plasma, all enzymes exhibited high mean activity, except for chitotriosidase; in fibroblasts, only mean beta-glucosidase activity was normal, while all others were reduced. The highest scores were assigned to α-mannosidase (29 points), α–L-iduronidase (28 points), total β-hexosaminidase (27 points), β-glucuronidase (26 points), and α–N-acetylglucosaminidase (25 points). **3)** In the pigmentation study, 17 patients (ML II=7, ML III alpha/beta=7, ML III gamma=3) and 29 parents were included in the study, as well as 20 healthy controls and 34 control parents. Most patients had Fitzpatrick skin types I–III (n=14/17, 82%), discrepant rate of their parents' skin type (n=19/29, 66%), of healthy controls (n=14/20, 70%), and control parents (n=25/34, 74%). Regarding genotype-phenotype association, for rs1126809, 2/17 had lighter hair than expected; for rs16891982, 4/17, 6/17, and 10/17 patients had, respectively, lighter eyes, hair, and skin color, and 3/17, 4/17, and 2/17 patients had, respectively, darker eyes, hair, and skin color than expected; and for rs1426654, 2/17, 1/17, and 1/17 had lighter eyes, hair, and skin and 9/17 and 3/17 had darker hair and skin than expected; for rs1129038, 6/16 patients had darker eyes than expected. **4)** Regarding genetic analysis, our data suggest that analysis of GNPTG and GNPTAB can be done by next generation sequencing, and that the identification of pathogenic mutations which segregate in family is of paramount importance for the identification of heterozygotes and for conducting prenatal diagnosis. **Conclusion**: This is the

first case series of patients ML II / III in Brazil, where ML II seems to be the most common type. Moreover, the ML II / III has delayed diagnosis, multisystemic symptoms, and appear to display changes in melanogenesis (as most common, Hypomelanosis), but further studies are needed to confirm these findings. Taking into consideration the enzymes evaluated in this study, we suggest measurement of plasma and fibroblast levels of at least three of the enzymes which had the highest scores in our analysis. This enzyme panel proposes to be a faster, simpler, and less expensive method for biochemical diagnosis. The next generation sequencing seems to be a good alternative for diagnostic confirmation of these patients

1. INTRODUÇÃO

1.1 Mucolipidoses

Os erros inatos do metabolismo (EIM) englobam um grupo heterogêneo de defeitos genéticos que afetam a síntese, degradação, processamento e transporte de moléculas no organismo. As doenças lisossômicas (DL) são um grupo de EIM que engloba aproximadamente 50 doenças que podem ser classificadas, de acordo com o substrato acumulado, em esfingolipidoses, mucopolissacaridoses (MPS), glicoproteinoses e outras DL (Gieselmann, 1995; Wraith *et al*, 2002). Dentro do grupo das glicoproteinoses (ou oligossacaridoses), situam-se as Mucolipidoses tipo II (ML II, doença das células de inclusão ou doença das células I, MIM#252500) ou tipo III (ML III, polidistrofia pseudo-Hurler, MIM# 252600 e 252605), doenças autossômicas recessivas, causadas por deficiência da enzima UDP-N-acetil-glicosamina-1-fosfotransferase (GlcNAc-fosfotransferase; EC 2.7.8.17) (Hasilik *et al*, 1981; Reitman *et al*, 1981). Esta enzima cataliza o passo inicial na síntese do marcador manose 6-fosfato (M6P), essencial para o endereçamento das hidrolases da rede *trans*-Golgi e da membrana celular até os lisossomos (Hasilik & Neufeld, 1980; Kornfeld & Sly, 2010). Com a atividade deficiente da GlcNAc-fosfotransferase, as enzimas não possuem o marcador M6P e não podem se ligar aos receptores na superfície do lisossomo; desta forma, as hidrolases não são internalizadas pelo lissosomo, sendo liberadas no espaço intracelular e nos fluidos corporais (Cathey *et al*, 2008). É válido observar que vários tipos celulares e órgão apresentam níveis de enzimas lisossomais próximos do normal, o que sugere a existência de uma via alternativa e independente de M6P (Braulke e Bonifacio*,* 2009).

Outras duas doenças eram denominadas como mucolipidose tipo I e tipo IV, mas posteriormente concluiu-se que eram bioquímica e clinicadmente diferentes da deficiência da GlcNAc-fosfotransferase (mucolipidoses II e III). A mucolipidose tipo I decorre da deficiência da neuraminidase e atualmente é denominada sialidose, caracteriza-se por displasia esquelética, mancha vermelho-cereja macular e sinais de neurodegeneração envolvendo células neurais e mielina (Souza *et. al*, 2009). A mucolipidose IV é uma doença

neurodegenerativa causada por mutações no gene *MCOLN1*, que codifica a mucolipina-1, uma proteína de membrana pertencente a família do canais iônicos conhecidos como receptores de potencial trasitório; clinicamente cursa com grave atraso do desenvolvimento neuropsicomotor, deficiência visual progressiva e acloridia (Souza *et. al*, 2009).

1.1.2 Epidemiologia

Individualmente raros, os EIM em seu conjunto representam uma situação freqüente, com prevalência estimada de 1:2.500 indivíduos (Blau *et al*, 2006). A incidência da ML II e III varia de acordo com a população estudada. Considerando os recém-nascido vivos, estima-se em 1:120.000 em Portugal (Tiede *et al*, 2006), 1:252.000 no Japão (Okada *et al,* 1985), 1:422.000 na Austrália (incluindo diagnóstico pré e pós-natal de doenças lisossômicas) (Meikle *et a*l, 1999) e 1:642.000 na Holanda (Poorthuis *et al*, 1999). Acredita-se que em algumas populações esta a incidência seja maior, como na região de Quebec, no Canadá, onde estima-se que a incidência de MLII seja de 1:6.184 recém-nascidos vivos e que a frequência de heterozigotos seja 1:39 (De Braekeleer, 1991). Não foram encontrados estudos na população geral. Não há estudos avaliando a prevalência de ML III alfa/beta, mas alguns autores acreditam que a prevalência pode ser na mesma magnitude que ML II (Cathey & Leroy, 2012ab); já sobre a ML III sabe-se apenas que é muito rara mas (Cathey & Leroy, 2012b). No Brasil, não existem dados relativos à incidência das ML II e III.

1.1.3 Bases moleculares das mucolipidoses II e III

As hidrolases lisossômicas catalisam a degradação de diversas macromoléculas incluindo proteínas, carboidratos, ácido nucleico e lipídios. Elas são sintetizadas no retículo endoplasmático e transportadas especificamente através do aparato de Golgi, de onde são transportadas em vesículas e entregues ao compartimento endossomal/lisossomal (Coutinho, Prata, Alves, 2011).

A explicação de como as enzimas lisossômicas são especificamente reconhecidas e selecionadas, entre muitas outras proteínas, na rede trans-Golgi, depende da identificação por um marcador único: o grupo M6P, que é adicionado às hidrolases lisossômicas. A geração de marcador de M6P depende de uma reação envolvendo a atividade seqüencial de duas enzimas: inicialmente, a GlcNAc-fosfotransferase catalisa a transferência do GlcNAc 1-fosfato do UDP-GlcNAc aos resíduos terminais de manose das cadeias de oligossacarídeos ligadas às asparaginas das hidrolases lisossômicas; posteriormente, a GlcNAc-fosfodiesterase remove o GlcNAc adicionado, expondo os resíduos de M6P terminais (Kang *et al,* 2010; Kornfeld & Sly, 2010). O grupos de M6P são reconhecidos por receptores transmembrana, presente na rede trans-Golgi, essas proteínas ligam-se às hidrolases lisossômicas no lado luminal da membrana e auxiliam a empacotar as hidrolases em vesículas que brotam da rede trans-Golgi e levam seu conteúdo aos endossomos que irão desenvolver-se em lisossomos maduros, onde as hidrolases começarão digerir o material endocitado (Coutinho, Prata, Alves, 2011).

Deste modo, os pacientes com ML II e III, manifestam deficiência de diversas enzimas lisossomais em vários tipos celulares, juntamente com níveis elevados de atividade enzimática em fluídos extracelulares, tais como soro e plasma. Esta condução inadequada de múltiplas hidrolases lisossômicas também resulta no acúmulo de seus substratos que então aparecem como corpos de inclusão intracitoplasmática, que constituem um dos marcos da MLII e MLIII (Kornfeld & Sly, 2010).

A análise de células e tecidos de pacientes com ML II que não possuem enzimas marcadas com resíduos de M6P, devido ausência de atividade da GlcNAc-fosfotransferase, demonstra níveis de enzimas lisossômicas normais em muitos órgãos, como, por exemplo, hepatócitos, células de Kupffer, leucócitos, fígado, baço, rim e cérebro; este achado que sugere a existência, nestas células, de uma via independente do M6P para o direcionamento das enzimas lisossômicas (Braulke e Bonifacio*,* 2009; Kornfeld & Sly, 2010)

1.1.4 Alterações bioquímicas nas mucolipidoses II e III

O direcionamento inadequado das enzimas ao lisossomos leva a complicações clínicas. A degradação adequada de glicoproteínas tem importante relevância médica, uma vez que ocorre dentro de lisossomos e requer hidrolases específicas, as glicosidases. Nos humanos, foram identificadas várias afecções hereditárias, conhecidas como doenças lisossômicas, causadas por defeitos em genes que codificam as glicosidases, levando ao acúmulo excessivo de glicoproteínas parcialmente degradadas. Os fenótipos de muitas dessas doenças se sobrepõem em algumas características.

Os pacientes com ML II geralmente apresentam da atividade residual da enzima GlcNAc-fosfotransferase ausente ou quase ausente, e os com ML III detém uma atividade residual em níveis superiores (Kornfeld & Sly, 2010). Em decorrência desta anormalidade, os pacientes apresentam alta concentração das enzimas lisossômicas no plasma ou em outros fluidos biológicos (hipersecreção para o meio extracelular), e baixa concentração destas enzimas no meio intracelular (Kornfeld & Sly, 2010). Da mesma forma, observa-se aumento na excreção urinária de oligossacarídeos (Cathey *et al*, 2008). Entretanto, nem todas as enzimas lisossômicas são afetadas do mesmo modo (Brooks *et al*, 2007), abaixo estão sintetizadas algumas das descrições disponíveis na literatura sobre as alterações bioquímicas na ML (Tabela 1). A quitotriosidase encontra-se aumentada em muitas doenças lisossômicas (Hollak *et.al,* 1994).

A concentração das hidrolases lisossômicas em papel filtro foi avaliada por dois autores. Chamoles *et al,* em 2001, demonstraram aumento na atividade da beta-hexosaminidase em amostras de cinco pacientes ML II/III. Fuller *et al*, em 2011, estudaram a concentração da sulfaminidase, esfingomielinidase ácida, alfa-iduronidase, LAMP-1, alfa-glicosidase, beta-glicosidase, saposina C, alfa-galactosidase, arilsulfatase A, iduronato-2-sulfatase, N-acetilgalactosamina 4-sulfastase (arilsulfatase B), quitotriosidase, CD 45 e N-acetilglicosaminidase em fibroblastos de 10 pacientes ML II/III e encontraram aumento expressivo da arilsulfatase-A, esfingomielinidase ácida, iduronato-2-sulfatase, N-

acetilgalactosamina 4-sulfastase e N-sulfaminidase, em relação aos controles; já a enzima alfa-N-acetilglicosaminidase estava aumentanda em menor intensidade que as anteriores. As outras hidrolases avaliadas apresentaram comportamento semelhante aos controles.

Tabela 1: Síntese da literatura, enzimas com atividade aumentada em plasma e reduzidas em fibroblastos em pacientes com ML II ou III.

Autor	Tipo celular	Enzimas alteradas
Hultberg & Sjöblad, 1980	Fibroblastos	alfa-fucosidase, alfa-manosidase, alfa-galactosidase, beta-glicuronidase, beta-galactosidase, N-acetil-beta-glicosaminidase
Hultberg *et al*, 1980	Soro	alfa-manosidase, alfa-fucosidase, alfa-galactosidase, beta-galactosidase, beta-glicuronidase e alfa -N-acetilglicosaminidase
Potier *et al*, 1979	Fibroblastos	neuraminidase
Natowicz & Wang, 1996	Plasma	alfa-manosidase, beta-glicuronidase e beta-hexosaminidase.
Song *et al*, 2003	Leucócitos, Plasma e Fibroblastos	N-acetilglicosaminidase, beta-hexosaminidase, alfa-galactosidase, beta-galactosidase, beta-glicosidase e beta-glicuronidase (normal em leucócitos, aumentadas em plasma e reduzida em fibroblastos). Apenas beta-glicosidase foi normal em fibroblastos
Takashi *et al*, em 2005	Fibroblastos	esfingomielinase
Bargal *et al*, em 2006	Plasma e Fibroblastos	Arilsulfatase A, beta-hexosaminidase e alfa-manosidase
Tappino *et al*, em 2009	Plasma e fibroblastos	Alfa-fucosidase, alfa manosidase e beta-hexosaminidase total
Cathey *et al*, em 2010	Plasma	alfa-fucosidase, arilsulfatase A, beta-galactosidase, beta-glicuronidase e N-acetilglicosaminidase

O anexo VI demonstra os valores de referência para a atividade das enzimas estudadas neste projeto, de acordo com o LEIM-HCPA.

1.1.5 Genoma

A GlcNAc-fosfotransferase é uma enzima hexamérica, formada por três subunidades (alfa2, beta2, gama2) (Bao *et al*, 1996); sugere-se que a atividade catalítica da enzima esteja nas subunidades alfa e beta, enquanto a subunidade gama seja responsável pelo

reconhecimento das proteínas lisossômicas (Raas-Rothschild *et al,* 2000; Kornfeld & Sly, 2010). As subunidades são codificadas por dois genes separadamente: alfa e beta por *GNPTAB* e a gama por *GNPTG (Raas-Rothschild et al, 2000; Tiede et al, 2005; Kudo et al, 2005).*

GNPTAB localiza-se no braço longo do cromossomo 12, na posição 23.2 (12q23,2) e é composto por 22 éxons, 85.442 bases e 1256 aminoácidos. *GNPTG* encontra-se no cromossomo 16p13.3, composto por 11 éxons, 11,453 bases e 305 aminoácidos (http://www.ncbi.nlm.nih.gov/gene).

A classificação da ML ocorre de acordo com as alterações moleculares, sabe-se que os pacientes com ML II e a maioria dos pacientes com ML III são homozigotos ou heterozigotos compostos para as mutações no *GNPTAB* e, por consequência, alterações nas subunidades alfa e beta, sendo denominados como ML II alfa/beta e ML III alfa/beta. Já um segundo grupo de pacientes ML III são homozigotos ou heterozigotos compostos para mutações no *GNPTG*, com modificações na subunidade gama, sendo denominados ML III gama (Cathey *et al*, 2008).

1.1. 6 Fenótipo

A ML II originalmente foi denominada de doença das células de inclusão ("I-cell disease") devido inclusões citoplasmáticas características observadas em pacientes com apresentação semelhante à MPS I (síndrome de Hurler) (Leroy e DeMars, 1967) O quadro clínico das ML II e III, especialmente da ML II, é bastante grave e parecido, respectivamente, com os das formas mais graves e mais brandas das MPS, especialmente da MPS I (DL caracterizada pela excreção urinária aumentada de GAGs e atividade deficiente da alfa-L-iduronidase somente) (Souza *et al,* 2009; OMIM, 2014). Os pacientes com ML II costumam apresentar sintomatologia mais precoce e não costumam apresentar opacificação de córnea importante (achado típico da MPS I) (Souza *et al,* 2009). A doença é clinicamente evidente ao nascimento, apresenta curso progressivo e geralmente apresenta desfecho fatal na

infância (Cathey *et al*, 2010; Kornfeld & Sly, 2010). Os casos “mais leves” podem apresentar sobrevida normal e ausência de envolvimento cognitivo, mas o comprometimento somático associado acaba por diminuir a qualidade de vida dos pacientes.

A ML III alfa/beta, originalmente descrita como polidistrofia pseudo-Hurler (Maroteaux & Lamy 1966; Taylor *et al*, 1973), é de patogênese semelhante à ML II , mas apresenta início dos sintomas na infância, progressão mais lenta e geralmente é fatal no início da vida adulta. A ML III gama é muito rara (Raas-Rothschild *et al*, 2000; Raas-Rothschild *et al*, 2004). Na ausência de bons marcadores genéticos e bioquímicos, as ML II e III são distinguidas com base em critérios clínicos (Souza *et al,* 2009; Cathey *et. al,* 2010; Kornfeld & Sly, 2010).

As manifestações clínicas da ML II e III são multissistêmicas e apresentam amplo espectro, levando a grande variabilidade entre os indivíduos afetados. A tabela 1 apresenta algumas das principais características fenotípicas descritas para as ML II e III (Okada *et al,* 2006; Encarnação *et* al, 2009; Otomo *et al*, 2009; Persichetti *et al*, 2009; Pohl *et al*, 2010)

Tabela 2: Principais características fenotípicas descritas para as mucolipidoses. (Okada *et al*, 1985; Traboulsi *et al*, 1986; Toscano *et al*, 1998; Bargal *et al,* 2006; Encarnação *et* al, 2009; Otomo *et al*, 2009; Persichetti *et al*, 2009; Cathey et.al, 2010; Pohl *et al*, 2010; Kornfeld & Sly, 2010, Raas-Rothschild & Spiegel, 2010)

Manifestação /Sistema	Mucolipidose II	Mucolipidose III alfa/beta	Mucolipidose III gamma
Idade de aparecimento dos sintomas	Do nascimento à infância precoce (por volta de 1 ano de idade)	Infância precoce a tardia (entre 2 a 4 anos)	Por volta dos 7- 10 anos de idade
Curso clínico	"Failure to thrive"; grave atraso do desenvolvimento,	Lentamente progressivo	Rigidez articular progressiva
Aparência geral	Hiperplasia gengival, face infiltrada, dobras epicânticas proeminentes, ponte nasal plana, macroglossia	Características faciais gradualmente grosseiras. Leve hiperplasia gengival e macroglossia.	Sem dismorfias faciais. Pescoço e tronco curto
Crescimento /estatura	Crescimento intra-uterino restrito à normal; baixa velocidade de crescimento linear e ganho ponderal. Estatura final média <80cm.	Crescimento intra-uterino normal; redução do crescimento linear após os 4 anos de idade, estatura final em média>115cm; puberdade normal.	Estatura baixa a normal
Desenvolvimento	Atraso motor e da linguagem grave, raramente caminham sozinhos e apresentam linguagem limitada. Atraso cognitivo leve à moderado, raramente grave	Desenvolvimento normal da linguagem; motor normal a atraso mínimo. Podem ter dificuldade de aprendizado à retardo mental	Podem ter dificuldade de aprendizado.
Envolvimento neurológico	EEG normal, ausência de crises convulsivas, hipotonia	Anormalidades subclínicas do SNC. Síndrome do túnel do carpo pode ocorrer	Sem descrição
Sistema esquelético	Mãos curtas, em garra. Cifoescoliose, vértebras bicôncavas, contornos irregulares, deformidade na coluna lombar, contraturas e restrição articular, displasia congênita do quadril. Podem ocorrer fraturas intra-útero e hiperparatireoidismo neonatal	Mãos normais à longas. Hipoplasia anterior dos corpos vertebrais lombares, escoliose. Destruição progressiva da articulação do quadril, hipoplasia do ilíaco. Osteopenia e aumento do turnover e reabsorção óssea podem estar presentes	Rigidez articular progressivas, principalmente mãos, quadril e coluna. Hiperlordose, joelho valgo, pés chatos, contraturas dos ombros, displasia espôndilo-epifisária
Sistema Respiratório	Infecções repetidas de vias aéreas superiores, pneumonia menos frequente.	Sem alterações descritas	Sem alterações descritas
Sistema Cardiovascular	Hipertrofia de septo e ventrículo esquerdo, espessamento das valvas mitral e aórtica, sopro cardíaco, insuficiência cardíaca	Envolvimento valvular, insuficiência aórtica e/ou mitral; insuficiência cardíaca sintomática é rara	Doença cardíaca valvular pode estar presente
Manifestações oftalmológicas	Córnea: raros casos de opacificação, granularidades estromais difusas	Córneas geralmente claras, granularidades estromais; pode ocorrer retinopatia leve.	Miopia pode ocorrer
Abdômen	Protuso, hérnia umbilical e inguinal, diátese de retos abdominais	Hérnia umbilical, inguinal, hepatoesplenomegalia pode ocorrer	Ausência de hepatoesplenomegalia
Prognóstico	Sobrevivência até os 5 a 8 anos de idade, relatos até adolescência.	Sobrevivência até a idade adulta, pouco se sabe após a terceira década de vida.	Pouco se sabe sobre o curso natural

1.1.7 Relação genótipo-fenótipo

O espectro mutacional do *GNPTAB* é caracterizado por uma alterações que causam impacto no processamento *GNPTAB*. Variabilidade clínica intra-familial é descrita, mais ainda não está estabelecida uma relação clara genótipo-fenótipo (Encarnação *et al*, 2009; Tappino *et al*, 2009; Otomo *et al*, 2009; Cathey *et al*, 2010). Este achado pode ser explicado, entre outros, pela existência de genes modificadores e pelo diferente fluxo através de vias metabólicas relacionadas ("dinâmica dos sistemas"). Estudos em pacientes de diferentes etnias e buscaram demonstram relação entre as mutações no gene *GNPTAB* e o fenótipo. A síntese de alguns dos maiores estudos de asociação encontram-se abaixo.

Otomo *et al*, em 2009 estudaram o gene G*NPTAB* em 40 pacientes japoneses não relacionados. Demonstraram que homozigotos ou heterozigotos compostos de mutações sem sentido ou que alteram a matriz de leitura apresentavam fenótipo mais grave (ML II).

Encarnação *et al*, em 2009, identificaram mutações *GNPTAB* em nove pacientes não-relacionados, de origem principalmente portuguesa. Encontraram mutações sem sentido em homozigose num paciente com ML II e em heterozigose em três pacientes com um fenótipo menos grave (ML III). Concluíram que o fenótipo de ML II está mais associado a presença de mutações sem sentido ou que alteram a matriz de leitura em homozigose, enquanto a presença de pelo menos uma mutação leve no gene GNPTAB está associada com a ML III.

Tappino *et al*, em 2009, estudaram 46 pacientes, em sua maioria italianos, demonstrarando que todos os pacientes foram homozigotos para mutações sem sentido ou que alteram a matriz de leitura em homozigose manifestaram MLII, sendo que homozigose para tais mutações não foram vistas em pacientes ML III. Além disso, encontraram mutações sem sentido associadas com ML III, sugerindo que na presença destas permanece um atividade enzimática residual.

Cathey *et al*, em 2010, identificaram alterações patogênicas no gene *GNPTAB* de 63 pacientes (61 probandos) provenientes principalmente dos EUA. Aqueles com ML II tinha um fenótipo mais grave, com evidência de problemas craniofaciais e ortopédicos ao nascer,

retardo psicomotor grave e atividade enzimática de menos de 1% dos valores controle. Crescimento, deambulação, fala e função cognitiva estavam prejudicadas. A atividade da enzima GlcNAc-1-fosfotransferase em leucócitos, comparada a controles, completamente abolida em paciente ML II, variava ente 0,84 e 10% em 4 amostras de paciente ML III e era 3% dos controles em um paciente ML intermediário. A ML II estava associada com mutações alteram a matriz de leitura, enquanto ML III foi associado com mutações hipomórficas.

Cury *et al*, em 2013 descreveram as mutações de *GNPTAB* em pacientes brasileiros com MLII e MLIII alfa/beta. Foram inclusos 12 pacientes de várias regiões brasileiras, sendo demonstrado que a mutação patogênica mais comum foi a c.3503_3504delTC, localizada no éxon 19.

Velho *et al*, em 2014 descrevem um paciente brasileiro com ML III gama e mutação patogênica em *GNPTG,* sendo um alelo mutante paterno e outro proveniente de uma mutação germinativa materna ou uma mutação *de novo.*

1.1.8 Diagnóstico

O passo inicial no diagnóstico da ML envolve a suspeita clínica. O diagnóstico definitivo envolve a identificação da deficiência na atividade da GlcNAc-fosfotransferase (Varki *et al*, 1981), porém esta análise é complexa não se encontra amplamente disponível, atualmente sendo realizada apenas em centros de pesquisa. Não existe relato, na literatura, de pacientes que apresentem ML II e III devido à atividade deficiente da GlcNAc-fosfodiesterase (todos os casos relatados são secundários à deficiência de GlcNAc-fosfotransferase) (Kornfeld & Sly, 2010).

Como o diagnóstico definitivo não encontra-se amplamente acessível, frente a suspeita diagnóstica, são realizados vários ensaios enzimáticos: medida dos níveis das enzimas lisossomais no plasma e em fibroblastos. Em decorrência da atividade deficiente da GlcNac-fosfotransferase, os pacientes com ML II e III apresentam alta concentração das

enzimas lisossômicas no plasma ou em outros fluidos biológicos (ocorre hipersecreção das enzimas para o meio extracelular), e baixa concentração destas enzimas no meio intracelular (principalmente em fibroblastos), desta forma, nem todas as enzimas são afetadas com a mesma intensidade na ML (Brooks *et al*, 2007). A atividade intracelular de algumas destas enzimas, entretanto, parece ser normal em leucócitos e tecidos de origem não-mesenquimal, sugerindo a existência de uma via independente do M6P para sua internalização (Kornfeld & Sly, 2010). Na literatura não há um consenso sobre as enzimas a serem pesquisadas para o diagnóstico bioquímico de ML, seja em fibroblastos ou plasma. Muitos dos autores citam "hidrolases lisossômicas", sem relatar a sensibilidade e especificidade da enzima ou ainda quantas enzimas seriam suficientes para estabelecer o diagnóstico. A tabela 3 sintetiza as recomendações dos principais autores para o diagnóstico bioquímico da ML.

O diagnóstico pré-natal pode ser feito através da identificação de uma atividade aumentada das enzimas lisossômicas em líquido amniótico e sua diminuição nos amniócitos cultivados, ou, mais recentemente, por meio da atividade da GlcNac-fosfotransferase em células fetais cultivadas ou pesquisa da mutação específica em amostra de DNA fetal (Leroy & Cathey, 2012ab; Alegra & Koppe, 2014).

Destacam-se novos meios de rastreio diagnóstico das ML II/III, ainda em fase experimental, como a triagem de oligossacarídeos livres em urina e análise de glicoaminoácidos por matriz assistida por desadsorção a laser tempo de voo de ionização ("*glycoaminoacid analyses by matrix-assisted laser desorption ionization time-of-flight" MALDI-TOF/TOF)* (Xia *et al*, 2013; Bonesso *et al*, 2014). O sequenciamento de nova geração (*next-generation sequencing)* também tem sido utilizado como método diagnóstico principalmente das ML III-gamma, uma vez que o quadro clínico de alguns pacientes pode ser tão atenuado a ponto da hipótese diagnóstica de MLIII não ser aventada clinicamente (Yang *et al*, 2013).

Tabela 3: Enzimas sugeridas por diferentes autores para o diagnóstico bioquímico das ML II e III em plasma e fibroblastos.

Referência	Enzimas em plasma	Enzimas em Fibroblastos
Livro: *Lysosomal storage disorders* (Brooks *et al,* 2007)	Alfa-L-iduronidase Arilsulfatase A Esfingomielinase Iduronato-sulfatase Sulfaminidase	Alfa-L-iduronidase Dosagem da GlcNac-fosfotransferase é o padrão-ouro, mas não encontra-se facilmente disponível
Laboratório Australiano de Referência Nacional para diagnósticos de doenças lisossômicas (Adelaide – Australia) (Brooks *et al,* 2007)	Alfa-L-fucosidase Alfa-N-acetilglicosaminidase Alfa-manosidase Arilsulfatase A Beta-hexosaminidase A	Alfa-fucosidase Alfa-galactosidase Arilsulfatase A Beta-Glicosidase Beta-Hexosaminidase A e B Beta-Manosidase.
Livro: *The Metabolic and Molecular Bases of Inherited Disease* (Kornfeld & Sly,2010)	Arilsulfatase A Hexosaminidase total Iduronato sulfatase	Sugere usar o padrão característico de deficiência das hidrolases lisossômicas, não cita nenhuma enzima especificamente. Dosagem da GlcNac-fosfotransferase é o padrão-ouro, mas não encontra-se facilmente disponível
Livro Eletrônico: *Gene Reviews* (Leroy & Cathey 2012ab)	Alfa-manosidase Arilsulfatase A Beta-Galactosidase Beta-Glicuronidase Hexosaminidase total	Não cita quais enzimas avaliar. Dosagem da GlcNac-fosfotransferase é o padrão-ouro, mas não encontra-se facilmente disponível

1.1.9 Tratamento

O único tratamento específico disponível é o transplante, seja de células-tronco hematopoiéticas (TCH), medula óssea (TMO) ou cordão umbilical. Tais procedimentos foram realizados em um número relativamente pequeno de pacientes com ML II e sua eficácia não está ainda comprovada (Kurobane *et al*,1986; Peters *et al*, 2003; Grewal *et al*, 2003; Li *et al*, 2004; Martin *et al*, 2006). Os estudos disponíveis na literatura relatam sobretudo variáveis relativas ao sucesso do transplante e seus efeitos adversos, mas poucos avaliam o impacto dessa intervenção nas complicações da doença, modificação da história natural e sobrevida.

Uma menina transplantada aos 8 meses de idade demonstrou normalização da atividade enzimática em plasma das enzimas alfa-manosidase, N-acetil-glicosaminidase, alfa-fucosidase, arilsulfatase A e beta-galactosidase; além disso, três meses após o tratamento, houve redução progressiva da hepatomegalia, melhora da elasticidade da pele e a opacificação de córnea não evoluiu (Kurobane *et al*, 1986). Três pacientes transplantados pelo grupo da universidade de Minnesota, EUA, apesar de terem sido transplantados em idade precoce (0,3-1,7 ano de idade), mostraram evolução heterogênea do ponto de vista cardio-respiratório (1/3 desenvolveu hipertensão pulmonar, 2/3 permaneceram estáveis) (Peters *et al*, 2003) Entretanto, o TCH permanece como alternativa para os pacientes com ML II diagnosticados em idade precoce. Grewal *et al*, em 2003 relatam o seguimento de uma menina ML II submetida TCH com 19 meses de idade. Cinco anos após o TCH ela não apresentava história de infecções respiratórias, permanecia com função cardíaca normal, continuava lentamente ganhando marcos do neurodesenvolvimento, entretanto as deformidades esqueléticas pioraram apesar do TCH . Não há relatos de TCH para ML III (Leroy & Cathey, 2012b; OMIM, 2014).

O transplante de células-tronco provenientes de cordão umbilical também está descrito. Uma menina de 12 meses de idade recebeu o transplante com células de cordão de doador não-relacionado e, apesar do sucesso do transplante, no seguimento a curto prazo não houve redução da atividade enzimática em plama (os autores não relatam o tempo exato nem enzimas pesquisadas) (Li *et al*, 2004). Em outro centro, 36 crianças ML II receberam transplante de células do cordão umbilical, houve pequena incidência de efeitos adversos, entretanto os autores não relatam se houveram modificações na história natural da ML (Martin *et al*, 2006).

Assim, a intervenção a ser adotada é essencialmente sintomática e paliativa, baseada no atendimento global ao paciente e no aconselhamento genético (Souza et. al, 2009). As alterações do metabolismo ósseo podem ser tratadas, quando indicado, com bisfosfonados. Robinson *et al* (2002) relataram dois irmãos com ML III, aventando a presença de um distúrbio distinto do metabolismo ósseo. Os marcadores bioquímicos de

remodelação óssea estavam aumentados e a biópsia óssea transilíaca demonstrou tanto osteopenia trabecular, quanto reabsorção óssea subperiosteal. Tais autores propõem que os bifosfonatos teriam um papel importante no manejo da dor óssea nos pacientes com ML III, como acontece na doença de Gaucher.

O êxito da TRE na doença de Gaucher e o desenvolvimento da TRE nas MPS I, II e VI e na doença de Fabry sinalizam que as ML seriam candidatas potenciais para este tipo de terapia (Beutler & Grabowski, 2001; Harmatz *et al*, 2006; Muenzer *et al*, 2006; Connock *et al,* 2006). Todavia, como não estão claros quais os mecanismos celulares mais importantes para o fenótipo das ML II e III, não se pode propor um protocolo com o uso concomitante das enzimas recombinantes atualmente existentes (a alfa-L-iduronidase, a iduronato-sulfatase, a arilsulfatase B e a alfa-galactosidase A).

Uma alternativa terapêutica que vem sendo estudada *in vitro* em fibroblastos humanos de mucolipidose II é a genisteína, uma isoflavona de soja. Em um estudo ela demonstrou-se capaz de reduzir heparan sulfato no meio intracelular (Otomo *et al,* 2011).

Alguns autores recomendam, após o diagnóstico de ML, avaliações do esqueleto através de radiografias, avaliação cardíaca e oftalmológica. Entretanto ainda não está claramente descrito na literatura a melhor forma de seguimento a longo prazo destes pacientes, não há consenso sobre periodicidade dos exames laboratoriais e de imagem, ou detalhes sobre estratégias para melhor acompanhamento da evolução da doença.

1.2 Melanogênese e função lisossômica

Os lisossomos são organelas ricas em hidrolases ácidas, capazes de degradar a maioria das macromoléculas biológicas, oriundas de diversas vias endocíticas e biossintéticas; além disso, em muitas células, os lisossomos também contém proteínas secretoras, que podem ser liberadas em respostas a estímulos externos, proporcionando uma gama de funções em diferentes tipos celulares (Stinchcombe *et al,* 2004). Estudos

genéticos, bioquímicos e estruturais demonstram que certos tipos celulares especializados podem conter organelas relacionadas aos lisossomos (Huizing *et al,* 2008), tais como melanossomos, grânulos líticos, compartimentos do complexo de histocompatibilidade classe da II (MHC), dentre outros (Huizing *et al,* 2008). Disfunções nos lisossomos e nos melanossomos tem sido observadas em certas doenças genéticas humanas, tais como algumas formas de albinismo, dado defeitos na pigmentação, e doenças com disfunção imune; no entanto ainda há muitas características clínicas a desvendar como estando relacionadas a estas organelas (Huizing *et al,* 2008). É interessante observar que o acúmulo de substratos pode interferir em outros processos celulares, tais como tráfego vesicular e biogênese dos lisossomos, apenas para citar a fisiopatologia diversificada neste conjunto complexo de transtornos (Parkinson-Lawrence *et al,* 2010).

A ML II e III está relacionada ao direcionamento inadequado das hidrolases lisossômicas, mas ainda é uma doença cuja fisiopatologia ainda está sendo desvendada; deste modo pouco sabe-se a respeito da função dos melanossomos nesta afecção. Há descrição de um único paciente chinês com uma coloração atípica do cabelo: loiro ao nascimento, em contraste com os cabelos negros de seus pais, que escureceram aos dois meses de vida. Os autores salientam que não há relatos prévios semelhantes e aventam a possibilidade de que a modificação na cor do cabelo esteja ocorrendo por redução temporária da produção de melanina, relacionada à algumas mutações do *GNPTAB*, mas comentam que ainda não está claro de que forma isto ocorreria (Ma *et al,* 2011). Assim, frente aos estudos de fisiopatologia das doenças com alterações no tráfego vesicular intracelular, somados às nossas observações clínicas, sugerimos que os pacientes com mucolipidose II e III possam ter menor pigmentação da pele e cabelos; hipótese inédita até o momento.

A pigmentação em humanos é influenciado por uma série de genes. Destes diversos SNPs relacionados à variação na cor dos olhos, cabelo e pele foram escolhidos e sequenciados numa amostra de pacientes com ML II e III [rs1126809 (gene *TYR*), rs16891982 (gene *SLC45A2*), rs1426654 (gene *SLC24A5*) e rs1129038 (gene *HERC2*)] O

gene *TYR* é responsável pela tirosinase, uma enzima que desempenha um papel-chave na síntese de melanina, influenciando variações na cor da pele, olhos e cabelo; de fato, alguns polimorfismos estão associados com risco de câncer de pele (Sturm, 2009). *HERC2* influencia a expressão do *OCA2*, que codifica um transportador específico nos melanócitos, uma proteína de membrana integral envolvidas no transporte de pequenas moléculas, especificamente tirosina - um precursor da melanina que atua na via de maturação de melanossomos na íris (Donnelly et al, 2012; Visser et al, 2012). *SLC45A2* codifica a proteína associada à membrana de transportador (MATP), também conhecidas como a família transportadora de soluto 45 membro 2 (*SLC45A2*) ou antigénio de melanoma aim1, um transportador que medeia a síntese de melanina (Sturm, 2009). *SLC24A5* codifica o trocador de sódio/potássio/cálcio 5 (NCKX5), também conhecida como família carregadora de soluto 24 membro 5 (*SLC24A5*). Sugere-se que a melanogênese e desenvolvimento dos melanossomas possam ser dependente dos níveis de cálcio no melanossomo, regulada pelo gene *SLC24A5* (Lamason et al 2005;. Sturm, 2009); além disso, alguns estudos demonstraram que o gene desempenha papel importante na pigmentação em várias populações humanas (Norton et al., 2007). O alelo A rs1126809 (gene *TYR*) está relacionada com os olhos azuis, e com maior sensibilidade da pele à luz solar (Sulem et al, 2008), e statetes heterozigotos favorece fenótipo mais leve, bem como (Cerqueira et al, 2014). O 374Phe (C) alelo de rs16891982 (gene *SLC45A2*) está associada com pele clara, e o 374Leu (L) alelo, com pele cor de oliva.

1.3 Classificação de fototipos

A cor natural da pele é multifatorial, ou seja, controlada por fatores genéticos que atuam em todas as etapas da melanogênese e fornecem características específicas aos melanossomas e por fatores ambientais, tais como exposição ao sol, processo de envelhecimento, ação de hormônios, tabagismo, alcoolismo e a poluição. (Fitzpatrick, 1988).

Com intuito de classificar as diferentes características físicas e respostas da pele à radiação solar e – portanto – as diferentes colorações da pele, Fitzpatrick (1988) propôs uma classificação em categorias, que abrange um espectro variável desde o tipo mais sensível ao sol, até aqueles que apresentam grande tolerância. Esta classificação é amplamente utilizada no meio dermatológico tanto na assistência quanto na pesquisa e, além da queimadura solar, considera características como cor dos cabelos e olhos, conforme demonstrado na tabela 4.

Tabela 4: Classificação da pele de acordo com a sua cor, proposta por Fitzpatrick

Fototipos	**Descrição**	**Sensibilidade ao Sol**
I - Branca	Queima com facilidade, nunca bronzeia	Muito sensível
II - Branca	Queima com facilidade, bronzeia pouco	Sensível
III - Morena Clara	Queima e bronzeia moderadamente	Normal
IV - Morena Moderada	Queima pouco, bronzeia com facilidade	Normal
V - Morena Escura	Queima raramente, bronzeia bastante	Pouco sensível
VI - Negra	Nunca queima, totamente pigmentada	Insensível

2. JUSTIFICATIVA

O conhecimento acerca da ML II e III na literatura nacional e internacional ainda é restrito, embora tenha evoluido significativamente nos últimos anos. No Brasil ainda não existe estudo sobre o perfil clínico das ML II e III em pacientes brasileiros. Algumas questões práticas da assistência ainda são controversas entre os autores internacionais e necessitam de resposta, tais como a melhor estratégia para diagnóstico bioquímico dos indivíduos sob suspeita, como deve ser o seguimento após este período ou como é a qualidade de vida ou funcionalidade dos afetados. Além disso, como a ML II/III decorre de alterações complexas na rota dos endossomos/lisossomos, levantamos a hipótese de que a melanogênese destes pacientes possa estar alterada, como já descrita para outras doenças lisossômicas. Assim exploramos esta hipótese já que não há nenhum estudo em ML II/III avaliando pigmentação de pele, olhos e cabelos.

O presente trabalho é um dos seguimentos de uma ampla pesquisa já em andamento, coordenada pela Profa. Dra. Ida D. Schwartz (“Estudo abrangente sobre as mucolipidoses II e III no Brasil: uma oportunidade para a compreensão dos processos genéticos que controlam o tráfego intracelular de proteínas”). Esperamos gerar dados consistentes sobre a ML no Brasil, contribuindo tanto para o delineamento clínico e molecular destas doenças quanto para o desenvolvimento de estratégias que permitam um diagnóstico mais rápido e econômico, bem como um acompanhamento adequado das mesmas. A caracterização molecular dos pacientes está sendo realizada por pesquisadores sob orientação da professora supracitada. O conjunto destas informações poderão ser utilizadas para o entendimento e tratamento não somente da ML II/III, mas de outras DLDs. Acreditamos que o melhor entendimento da história natural da doença poderá contribuir para as terapias que estão em andamento e para futuros tratamentos que possam vir a surgir.

O laboratório do SGM-HCPA (LEIM-HCPA) é um serviço de referência internacional para o diagnóstico dos Erros Inatos do Metabolismo (EIM), além disso, está integrado com o Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (SGM-HCPA) e

Departamento de Genética da Universidade Federal do Rio Grande do Sul (UFRGS), propiciando aos pacientes atendimento multidisciplinar por uma equipe experiente em EIM. O LEIM-HCPA, com auxílio da Rede MPS Brasil, concentra uma casuística significativa (se não a maior do país) em pacientes com diagnóstico de ML, porém ainda não há nenhum estudo publicado sobre esta coorte de pacientes. Além disso enfrentamos dificuldades práticas no diagnóstico bioquímico destes pacientes, já que na literatura não há consenso sobre a forma eficaz e com menor custo para investigação.

3. OBJETIVOS

Objetivos primários:

1) Caracterizar aspectos clínicos de uma amostra de pacientes brasileiros com ML II e III.
2) Caracterizar o fenótipo bioquímico de pacientes brasileiros com ML II e III, visando a elaboração de um protocolo laboratorial custo-efetivo para o diagnóstico das ML.

Objetivos secundários:

1) Estudar as diferenças cllínicas entre os subtipos de mucolipidose III (gama e alfa/beta);
2) Classificar os fototipos dos pacientes com ML e compará-los com seus pais e controles (indivíduos saudáveis), a fim de ser detectada possível tendência de hipomelanogênese;
3) Estudar a associação entre a classificação de Fitzpatrick para cor da pele e os seguintes SNPs: [rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) and rs1129038 (*HERC2* gene)]

4. RESULTADOS

Os resultados serão apresentados na forma de artigos científicos já publicados (n=2), em processo de elaboração (n=1) ou em fase final de preparação e revisão para ser submetido à publicação (n=3).

4.1 Capítulo 1

Biochemical characterization of a sample of Brazilian patients with mucolipidosis II/ III: towards a more cost-effective protocol for diagnosis

Artigo em fase final de preparação, será submetido à Revista *Molecular Genetics and Metabolism*

Biochemical characterization of a sample of Brazilian patients with mucolipidosis II/ III: towards a more cost-effective protocol for diagnosis

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Abstract

Introduction: Mucolipidosis (ML) II and III are rare lysosomal diseases caused by deficiency of GlcNAc-1-phosphotransferase, which is encoded by the *GNPTAB* and *GNPTG* genes, leading to altered activity of lysosomal hydrolases (increased in plasma, reduced in fibroblasts, and normal in leukocytes). The golden standard methods for diagnosis of ML II/II are the demonstration of intracellular deficiency of GlcNAc-1-phosphotransferase activity and/or identification of pathogenic mutations in *GNPTAB* or *GNPTG*, which are available in few centers. Thus, the confirmation of diagnosis of MLII/II is usually based on measurement of many lysosomal hydrolases activities both broblasts and in plasma. However, there is no consensus as to which enzymes should be investigated, and different random panels are used in different centers, with weak evidence to support this practice. **Objective:** To characterize activity of lysosomal enzymes in a sample of Brazilian patients with confirmed diagnosis of ML II/III so as to propose a more cost-effective panel for diagnosis. **Methods:** Retrospective, cross-sectional study including patients with a clinical and biochemical or genetic diagnosis of ML II/III established at a referral center for diagnosis of lysosomal disorders in Southern Brazil. We developed a score based on 11 items to choose which enzymes should be included in the panel (the higher the score, the better suited the enzyme). We only analyzed enzymes investigated in at least four patients. **Results:** A total of 26 patients were included (14 with ML II, 9 with ML III alpha/beta, and 3 with ML III gamma). Of these, all 26 had lysosomal enzyme activity evaluated in plasma and 15 in fibroblasts. In plasma, all enzymes exhibited high mean activity, except for chitotriosidase; in fibroblasts, only mean beta-glucosidase activity was normal, with all others reduced. On comparison between ML II and ML III, levels of α-N-acetylglucosaminidase were higher in ML II, and levels of β-glucosidase in fibroblasts were lower in ML II. The enzyme which exhibited the greatest magnitude of increase in plasma and the greatest reduction in fibroblasts was α-mannosidase. The highest scores were assigned to α-mannosidase (29 points), α–L-iduronidase (28 points), total β-hexosaminidase (27 points), β-glucuronidase (26 points), and α–N-acetylglucosaminidase (25 points). **Discussion/Conclusion:** Our data suggest that differences exist in the biochemical phenotypes of ML II and ML III. Taking into consideration the enzymes evaluated in this study, we suggest measurement of plasma and fibroblast levels of at least three of the enzymes which had the highest scores in our analysis. This panel would be a faster, simpler, and less expensive method for biochemical diagnosis of ML II/III.

Keywords: mucolipidosis II/III, biochemical diagnosis, lysosomal enzymes, diagnostic protocol

Abbreviations:
LSD: lysosomal storage disorders; ML: mucolipidosis,

1. Introduction

Mucolipidosis (ML) II and ML III (OMIM##252500, #252600, #252605) are rare, autosomal recessive genetic diseases caused by deficient activity of N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase, EC 2.7.8.17) (Hasilik *et al*, 1981; Reitman *et al*, 1981). This enzyme catalyzes the synthesis of mannose 6-phosphate (M6P), a recognition marker that plays an essential role in directing hydrolases from the *trans*-Golgi network and cell membrane to the lysosomes; if this process fails, acid hydrolases are not internalized by the lysosomes, and are instead released into the intracellular space and body fluids (Hasilik & Neufeld, 1980; Kornfeld & Sly, 2010). However, several cell types, such as leukocytes, exhibit near-normal lysosomal enzyme levels, which suggests the existence of an alternative, M6P-independent pathway (Braulke & Bonifacio*,* 2009). GlcNAc-1-phosphotransferase is a hexameric enzyme composed of three subunits (two alpha, two beta, and two gamma) (Bao *et al*, 1996). The alpha and beta subunits are encoded by the *GNPTAB* gene, and the gamma subunit, by the *GNPTG* gene (Raas-Rothschild *et al*, 2000; Tiede *et al,* 2005; Kudo *et al*, 2005, Cury et.al, 2013).

ML II/II are classified according to the clinical and genetic manifestations present. Although there is a phenotypic spectrum, ML II (MIM #252500) is more severe, clinically evident at birth, and generally fatal in childhood; ML III (MIM #252600 and #252605) presents with a later onset of symptoms and slower disease progression (Cathey *et al* 2010). Symptoms include skeletal changes similar to *dysostosis multiplex*, failure to thrive, and coarse facial features (Cathey *et al*, 2010, Leroy & Cathey, 2012b). In milder cases, the

lifespan may be normal and cognitive involvement may be absent (Okada et al, 2006; Encarnação et al, 2009; Otomo et al, 2009; Persichetti et al, 2009; Pohl et al, 2010; Cathey *et al*, 2010, Leroy & Cathey, 2012ab). Patients with ML II (and most with ML III) are homozygous or compound heterozygous for mutations in *GNPTAB*, which lead to changes in the alpha and beta subunits of the enzyme (the resulting disease is known as ML II or III alpha/beta), whereas a minority of patients with ML III are homozygous or compound heterozygous for *GNPTG* mutations, which lead to changes in the gamma subunit of the enzyme and, consequently, to ML III gamma (Cathey *et al*, 2008).

The variable phenotypic spectrum of the ML and the overlap of their signs and symptoms with those of other lysosomal storage disorders (LSD), particularly the mucopolysaccharidoses (MPS), may hinder or delay diagnosis. Clinical suspicion is the first step for diagnosis of ML, but definitive diagnosis requires identification of a pathogenic *GNPTAB* or *GNPTG* mutation or demonstration of deficient intracellular GlcNAc-1-phosphotransferase activity (Kornfeld & Sly, 2010), which is a complex test available only in a select few research centers. Thus, GlcNAc-1-phosphotransferase activity is usually assessed indirectly by measuring the activity of lysosomal hydrolases in plasma, fibroblasts, and leukocytes, as patients with ML II and III have elevated levels of hydrolases in plasma and other body fluids and decreased levels of these enzymes in the intracellular compartment (particularly in fibroblasts). However, there is no consensus in the literature as to which enzymes should be tested for biochemical diagnosis of ML II/III (Brooks *et al,* 2007, Kornfeld & Sly, 2010, Leroy & Cathey, 2012ab). The present study sought to evaluate the activity of

various lysosomal hydrolases in plasma, fibroblasts, and leukocytes in a sample of Brazilian patients with ML II and III, all of whom presenting a confirmed biochemical or molecular diagnosis of ML established at the same laboratory, so as to propose a more cost-effective enzyme panel for diagnosis of these conditions.

2. Patients and methods

This cross-sectional, retrospective study was carried out at a nationwide reference center for LSD diagnosis and management located in Southern Brazil. The sample comprised patients with a clinical and biochemical/DNA diagnosis of ML II or III established at the Inborn Errors of Metabolism Laboratory of the Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil (LEIM-HCPA), between 1983 and 2013. As direct measurement of GlcNAc-1-phosphotransferase is not available in Brazil, diagnosis of ML is confirmed indirectly by the finding of altered lysosomal enzyme activities (elevated in plasma and reduced in fibroblasts). Until 2007, routine practice at LEIM-HCPA involved diagnostic confirmation of ML II/III only after analysis of, on average, 4.9±2 lysosomal hydrolases in plasma and 3.50±7 in fibroblasts, but the choice of which enzymes to include in the panel was not standardized. Between 2007 and 2013, a panel including measurement of, on average, 5.4 ±2 hydrolases in plasma and 6±3 in fibroblasts was used, but there was still no standard guideline as to which enzymes should be included. Furthermore, since 2007, the

panel sometimes has included measurement of plasma chitotriosidase levels (which are elevated in some LSDs), and the analysis of *GNPTAB* and *GNPTG* mutations (Cury et al., 2013).

In the present study, subjects for whom no fibroblasts were available and whose workup had consisted solely of measurement of enzyme activity in plasma were included only if they had DNA analysis results confirming the presence of pathogenic mutations in *GNPTAB* or *GNPTG*. The diagnostic algorithm used at LEIM-HCPA is shown in Figure 1.

FIGURE 1

Only those enzymes measured in at least four patients in the sample were included for analysis in the present study. To systematize the decision process, we created a score based on the characteristics of the enzyme assays employed and on methodological aspects of our sample (such as the number of patients available) (Table 1). This score included 11 items, scored on a scale of 0 to 3 each for a maximum of 33 points (the higher the score, the greater the suitability of the assay for inclusion in the diagnostic panel). At the authors' discretion, enzyme assay results were considered abnormal if ≥20% above or below the upper or lower limit of the reference range.

Clinical data were collected by means of a specific form, which was completed by each patient's attending physician. Patients were classified as having ML II or ML III at their physician's discretion. Subclassification of ML III into alpha/beta or gamma was based on

genotype. When available, the results of any urine screens for LSDs (thin-layer chromatography of glycosaminoglycans [GAGs], direct GAG measurement, or thin-layer chromatography of sialylated oligosaccharides) were also analyzed.

Data were analyzed in the Statistical Package for the Social Sciences (SPSS) 22.0 software environment. Data were expressed as means and standard deviations when normally distributed or as medians otherwise. The significance level was set at 5% ($p<0.05$). Student's *t*-test for independent samples was used for pairwise comparison of the mean difference in enzyme activity between patients with ML II, ML III, and ML III gamma.

The ethical and methodological aspects of this study were approved by the HCPA Research Ethics Committee.

3. Results

Twenty-six patients were included in the study. All 26 had undergone enzyme activity measurement in plasma, 19 in leukocytes, and 15 in fibroblasts (Table 2). Twenty-two patients had also undergone *GNPTAB* and *GNPTG* analysis. The sister of one patient whose ML II diagnosis had been confirmed by biochemical analysis (enzyme activity in plasma and fibroblasts) and genetic testing had a clinical picture consistent with ML II and enzyme assays only in leukocytes and plasma; she was also included in the study.

3.1 Plasma

Plasma activities of eight enzymes were included in the study. All enzymes had mean activity above the upper level of normal, except for chitotriosidase (Table 3), which was normal in 19 patients, high in two patients (169 and 160 nmol/h/mL, respectively; reference range, 8.8-132), and low in one with chitotriosidase deficiency (1.4 nmol/h/mL; reference range, 8.8–132; DNA analysis of *CHIT1* showed he was homozygous for dup24 mutation). The enzymes that diverged most from their reference ranges were, in decreasing order: α-mannosidase; α-L-iduronidase; α-N-acetylgalactosaminidase; α-N-acetylglucosaminidase; and total β-hexosaminidase.

3.2 Fibroblasts

Fibroblast activities of 12 enzymes were included in the study (Table 3). Most had a mean activity below the lower bound of the reference range, except for β-glucosidase; this enzyme was analysed in 7 patients and had low activity in only one patient with ML II (10 nmol/h/mg protein; reference range, 257-668). The enzymes that diverged most from their reference ranges were: neuraminidase; α-mannosidase; α-L-iduronidase; sphingomyelinase; and total β-hexosaminidase.

3.3 Other samples

3.3.1 Leukocytes

Leukocyte activities of 11 enzymes were included in the study. Although most patients had normal activity levels of these enzymes, the following abnormalities were observed: α-L-iduronidase was increased in 1/13 patients and reduced (28%) in 1/13 patients (23 nmol/h/mL; reference range: 32-56); arylsulfatase B was increased in 2/7 patients (80% and 140% respectively); β-glucosidase was reduced (70%) in one patient (2.9 nmol/h/mL; reference range:10-45); iduronate 2-sulfatase was increased (160%) in 1/8 patients; β-glucuronidase was increased in 4/5 patients (25%, 30%, 75% and 114% respectively); sphingomyelinase was reduced (25%) in 1/7 patients (0.56 nmol/h/mL; reference range:0.74-4.9); and total β-hexosaminidase was increased in 3 of 6 patients (74%, 76%, and 240% respectively). It is worth noting that, in patients with reduced enzyme activity in leukocytes, this abnormality was found in only one enzyme per patient, and activity levels still remained higher than those found in patients with MPS I and Niemann-Pick type A/B disease, but not for Gaucher disease. The patient with low β-glucosidase activity has a clinical and biochemical picture typical of ML III, normal levels of chitotriosidase in plasma, normal levels of β-glucosidase in fibroblasts (526 nmol/h/mg protein; reference range, 257-668), and the DNA analysis confirmed the diagnosis of ML III gamma. She was described by our group elsewhere (Velho *et. al,* 2014).

3.3.2 Urine

Eighteen patients underwent urine screening for LSDs; however, not all patients underwent all three urine assays available. GAG measurement was normal in 17 of 18 patients; Thin layer GAG chromatography revealed elevated levels of keratan sulfate in 1 of 18 patients, traces of both heparan and dermatan sulfate in 4 of 18 patients, and traces of dermatan sulfate alone in 2 of 18 patients. Thin layer chromatography for sialoligosaccharides was normal in 15 of 18 patients.

3.4 Comparison between mucolipidosis types

3.4.1 ML II versus ML III (both alpha/beta and gamma)

Age at diagnosis was significantly younger in patients with ML II than in patients with ML III alpha/beta and ML III gamma ($p<0.05$ for all comparisons) (Table 2). Plasma activity levels of α-N-acetylglucosaminidase were significantly higher in ML type II; and activity of β-glucosidase in fibroblasts was lower in ML II (Table 4). There were no significant differences in activity of other enzymes, whether in plasma or fibroblasts (data not shown).

3.4.2 ML III alpha/beta versus ML III gamma

The only statistically significant difference between the alpha/beta and gamma subtypes of ML III was chitotriosidase activity in plasma, which was higher in ML III gamma (mean ± SD: 26.2±19 vs. 87.3±29 nmol/h/mL; reference values= 8.8-132; $p=0.004$); for this analysis, we excluded the patient with chitotriosidase deficiency.

4. Proposed biochemical diagnosis panel

The analyzed enzymes were grouped into three clusters by score: high-scoring enzymes (25 to 29 points – α-L-iduronidase, α-mannosidase, total β-hexosaminidase, β-glucuronidase, and α-N-acetylglucosaminidase), intermediate-scoring enzymes (17 to 22 points – Arylsulfatase A, Iduronate sulfatase, β–Galactosidase, Neuraminidase), and low-scoring enzymes (11 to 13 points – Arylsulfatase B, β–Glucosidase, Sphingomyelinase)

4. Discussion

As the literature is lacking in evidence-based information about the biochemical diagnosis of ML II/III (Brooks *et al,* 2007, Kornfeld & Sly,2010, Leroy & Cathey, 2012 ab), we assessed patients at a referral center for inborn errors of metabolism in an attempt to identify the most suitable enzymes for inclusion in a diagnostic panel, with a view to optimization of the diagnostic process and reduction of the costs and time involved. As expected, enzyme activity in leukocytes was normal in the majority of patients and for the majority of the tested enzymes, and chitotriosidase activity in plasma was normal in most patients (Hollak *et al*, 1994).

Therefore, taking into account the cost and complexity of enzyme assays, the sample size of the present study, the magnitude of the differences from normal observed in the tested enzymes, and the possibility of carrying out the same enzyme assay in plasma and

fibroblasts, we propose a diagnostic protocol that involves assessment of three of the five enzymes in the highest-scoring cluster. It bears stressing that indirect biochemical diagnosis is not the golden standard for identification of patients with ML II/III, but it is still more accessible than other methods (such as measurement of GlcNAc-1-phosphotransferase activity of screening for *GNPTAB* or *GNPTG* mutations). Potential novel methods for ML II/III screening still in the experimental stage include screening of free oligosaccharides (FOS) in urine and glycoamino-acid analyses by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry (Xia *et al*, 2013; Bonesso *et al*, 2014). Next-generation sequencing has also been used as a diagnostic method for ML II/III (Yang *et al*, 2013).

ML II and ML III still pose a challenge in terms of pathogenesis and management. Although we detected some differences in biochemical phenotype between the severe (II) and mild (III) forms of the disease, and even between ML III gamma and ML III alpha/beta, these differences may not reflect the distinct clinical manifestations and natural history of these forms, as we did not test enzyme activity in truly affected tissues (such as bone). From this perspective, it is interesting to note that α-L-iduronidase was one of the enzymes that most diverged from its reference range in our sample, both when measured in plasma and when assessed in fibroblasts. Isolated α-L-iduronidase deficiency characterizes MPS I, a disease that usually involves corneal opacity – which, in turn, is a very infrequent finding in ML II/III. Nevertheless, our findings suggest metabolic pathways that may be better explored in terms of treatment potential. For instance, if enzyme replacement therapy (ERT) for ML

II/III were a possibility, the first treatment to be tested could be a “cocktail” of the enzymes most affected in our patients (α-L-iduronidase, α-mannosidase, total β-hexosaminidase, and neuraminidase). As GlcNAc-1-phosphotransferase is not only hexameric but insoluble (it is located in the Golgi transmembrane region), its replacement by currently existing ERT methods is a very remote possibility.

It is also very interesting our findings regarding β-glucosidase and chitotriosidase activities. Unexpectedly, since β-glucosidase is not an M6P-dependent enzyme, a patient with ML III gamma presented very low levels of this enzyme in leukocytes but normal in fibroblasts; unfortunately, we have not been able to study the *GBA1* gene sequence of this patient. Aditionally, our data suggest β-glucosidase activity in fibroblasts is lower in ML II, and that ML III gamma patients present higher levels of plasma chitotriosidase than ML III alpha/beta (chitotriosidase is a biomarker of Gaucher disease, and it is found to be higher in most patients presenting this disease). Low levels of β-glucosidase in leukocytes havelalso been described by Lo et al., 2013 in Niemann-Pick C disease (Lo et al., 2013). We suggest the β-glucosidase metabolic pathway be better characterized in ML III gamma.

6. Conclusion

According to the data obtained herein, we suggest that measurement in plasma and fibroblasts of at least three of the following enzymes – α-L-iduronidase, α-mannosidase, total β-hexosaminidase, β-glucuronidase, and α-N-acetylglucosaminidase – be performed as a panel for indirect biochemical diagnosis of ML II/III. Our findings suggest that differences in

biochemical phenotype exist between ML II and ML III, with the former being more severe in terms of biochemical abnormalities.

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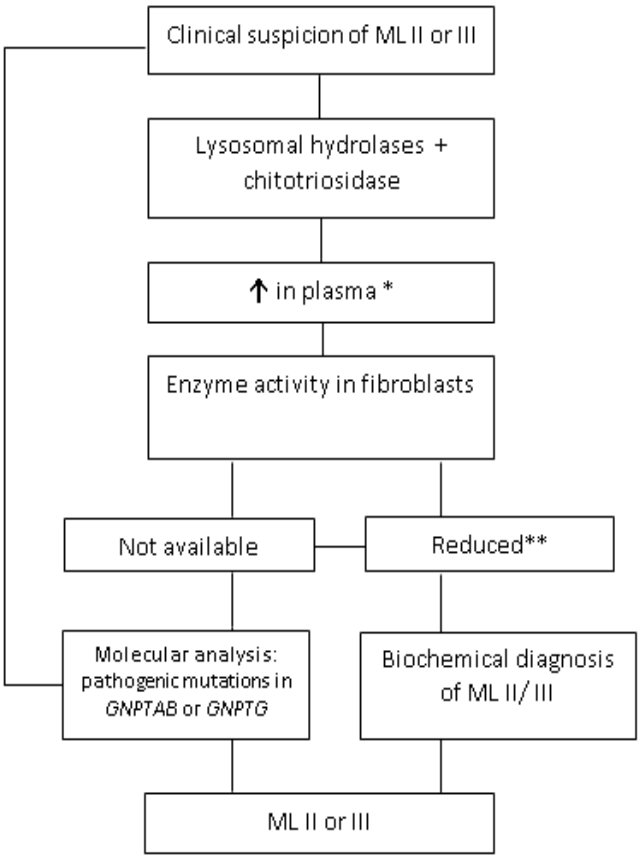

Figure 1: Algorithm for diagnosis of mucolipidoses II/III employed at the Inborn Errors of Metabolism Reference Laboratory, Hospital de Clínicas de Porto Alegre, Brazil.

↑, *elevated; *activity at least 20% above the upper reference value; ** activity at least 20% under the lower reference value.*

Table 1. Score created by our research group for assessment of enzyme assays used in the indirect biochemical diagnosis of mucolipidosis II/II

Incubation time	**3 points 0.25-2 hours**	**2 points 3-5 hours**	**1 point >5 hours**
Tissue types	Plasma Leukocytes Fibroblasts	Plasma Fibroblasts	Only one
Assay method	Colorimetric	Fluorometric	Radioisotopic
Difficulty	1 (easiest)	2	3 (hardest)
Cost	$	$$	$$$
Fold increase from normal – plasma	>10x	5-10x	<5x
Fold reduction from normal – fibroblasts	>10x	5-10x	<5x
Sensitivity – plasma	100%	90-100%	<90%
Sensitivity – fibroblasts	100%	90-100%	<90%
No. of patients – plasma	>20	10-20	<10
No. of patients – fibroblasts	>20	10-20	<10

Table 2. Sample profile

	Overall	Type II	Type III alpha/beta	Type III gamma
Number	26	13	10	3
Mean age at diagnosis±SD(y)	7±10	1.6±1.2*	10.5±7.6*	27.6±17
Samples available				
Plasma	26	13	10	3
Fibroblasts	15	9	3	3
Leukocytes	19	7	9	3
Urine	18	7	8	3
Region of origin (Brazil)				
North	2	1	0	1
Northeast	8	4	4	0
Center-West	3	3	0	0
Southeast	10	5	5	0
South	3	0	1	2

y, years old; IQR, interquartile range; *p=0.04 for comparison of age at diagnosis between ML II e III.

Table 3. Summary of characteristics of the enzyme assays tested in this study

Biochemical investigation	Sample	Time* (hours)	Assay	Cost	Difficulty
α–L-Iduronidase (*EC* 3.2.1.76)	Plasma	1	Fluorometric	$$	1
α–Mannosidase (*EC* 3.2.1.24)	Plasma	1	Fluorometric	$	1
α–N-Acetylglucosaminidase (*EC* 3.2.1.50)	Plasma	2	Fluorometric	$	1
Arylsulfatase A (*EC* 3.1.6.8)	Plasma	1	Colorimetric	$	1
β–Glucuronidase (*EC* 3.2.1.31)	Plasma	1	Fluorometric	$	1
β–Hexosaminidase, total (*EC* 3.2.1.30)	Plasma	4	Fluorometric	$	1
Iduronate 2-sulfatase (*EC* 3.1.6.12)	Plasma	28	Fluorometric	$$	2
Chitotriosidase (*EC* 3.2.1.14)	Plasma	0.25	Fluorometric	$	1
α–L-Iduronidase (*EC* 3.2.1.76)	Leukocyte	1	Fluorometric	$$	1
α–Mannosidase (*EC* 3.2.1.24)	Leukocyte	1	Fluorometric	$	1
Arylsulfatase A (*EC* 3.1.6.8)	Leukocyte	5	Colorimetric	$	1
Arylsulfatase B (*EC* 3.1.6.12)	Leukocyte	1.5	Colorimetric	$	1
β–Galactosidase (*EC* 3.2.1.23)	Leukocyte	1	Fluorometric	$	1
β–Glucosidase (*EC* 3.2.1.21	Leukocyte	1	Fluorometric	$$	1
β–Glucuronidase (*EC* 3.2.1.31)	Leukocyte	1	Fluorometric	$	1
β–Hexosaminidases (*E.C.*3.2.1.30)	Leukocyte	4	Fluorometric	$	1
Iduronate 2-sulfatase (*EC* 3.1.6.12)	Leukocyte	28	Fluorometric	$$	2
Sphingomyelinase *EC* 3.1.4.12)	Leukocyte	4	Radioisotopic	$$	3
α–Fucosidase (*EC* 3.2.1.51)	Fibroblasts	1	Fluorometric	$	1
α–L-Iduronidase (*EC* 3.2.1.76)	Fibroblasts	1	Fluorometric	$$	1
α–Mannosidase (*EC* 3.2.1.24)	Fibroblasts	1	Fluorometric	$	1
α–N-Acetylglucosaminidase (*EC* 3.2.1.50)	Fibroblasts	1	Fluorometric	$	1
Arylsulfatase A (*EC* 3.1.6.8)	Fibroblasts	5	Colorimetric	$	1
β–Glucuronidase (*EC* 3.2.1.31)	Fibroblasts	1	Fluorometric	$	1
β–Hexosaminidases, total (*EC* 3.2.1.30)	Fibroblasts	4	Fluorometric	$	1
β–Galactosidase (*EC* 3.2.1.23)	Fibroblasts	1	Fluorometric	$	1
β–Glucosidase (*EC* 3.2.1.21	Fibroblasts	1	Fluorometric	$$	1
Iduronate 2-sulfatase (*EC* 3.1.6.12)	Fibroblasts	28	Fluorometric	$$	2
Neuraminidase (*EC* 3.2.1.18)	Fibroblasts	0.5	Fluorometric	$	1
Sphingomyelinase (*EC* 3.1.4.12)	Fibroblasts	1	Radioisotopic	$$	3

*Incubation time

Table 4. Lysosomal hydrolases and chitotriosidase: summary of activity measurements in patients with mucolipidosis II/III.

		Leukocytes		Plasma			Fibroblasts			
Enzyme	**Associated LSD**	**Mean ± SD** (RV nmol/h/mL)	**Observation**	**Mean ± SD** (RV nmol/h/mL)	**Fold elevation**	**Sensitivity**	**Mean ±SD** (RV nmol/h/mg protein)	**Fold reduction**	**Sensitivity**	**Total score (0-33)**
α–N-Acetylglucosaminidase (*EC* 3.2.1.50)	MPS III-B	NP	NP	277±117 (11-37)	7.5	12/12	19.3±11 (54-217)	2.8	4/4	25
α–Mannosidase (*EC* 3.2.1.24)	Alpha-mannosidosis	132±42.4 (60-400)	Normal (n=4)	1978±527 (17-56)	35.3	20/20	16±8.2 (93-359)	5.8	9/9	29
α–L-Iduronidase (*EC* 3.2.1.76)	MPS I	47±15 (32-56)	Reduced in 1/13 patient (28%)	190±83 (6.8-13.7)	14		14±20 (6.8-13.7)	5.3	7/11	28
Arylsulfatase A (*EC* 3.1.6.8)	Metachromatic leukodystrophy	13±3.2 (5-20)	Normal (n=4)	NP	NP	Positive in 21/26*	NP	NP	NP	22
Arylsulfatase B (*EC* 3.1.6.12)	MPS VI	199±123 (72-176)	Normal (n=7)	NP	NP	NP	NP	NP	NP	13
β–Glucosidase (*EC* 3.2.1.21)	Gaucher disease	11.4±4.5 (10 -45)	Reduced in 1/8 patient (70%)	NP	NP	NP	415.4±210 (257-668)	0.6	1/7	11
β–Galactosidase (*EC* 3.2.1.23)	Gangliosidosis GM1, MPS IV-B, galactosialidosis	108±37 (78-280)	Normal (n=15)	NP	NP	NP	132.8±194 (394-1440)	3	12/13	18
β–Hexosaminidase, total (*E.C.*3.2.1.30)	Tay-Sachs disease, Sandhoff disease	2714±1610 (552-1662)	Elevated in 3/6 patients (74, 76, 240%)	17438±7423 (1000-2857)	6.1	26/26	856.3 ±509 (3000-13480)	3.5	11/11	27
β–Glucuronidase (*EC* 3.2.1.31)	MPS VII	223.2±71 (23-151)	Elevated in 4/5 patients (25,30,75, 114%)	924±460 (30-300)	3.1	25/26	17.1±11 (62-361)	3.6	10/10	26
Iduronate 2-sulfatase (*EC* 3.1.6.12)	MPS II	300±633 (31-110)	Elevated in 1/8 patients (160%)	1959±543 (122-463)	4	12/12	10.7±4.9 (35-90)	3.3	7/7	20
Neuraminidase (*EC* 3.2.1.18)	Sialidosis	NP	NP	NP	NP	NP	3.4±4.6 (30-38)	8.8	11/11	17

Chitotriosidase (*EC* 3.2.1.14)	Activity increased in several LSDs	NP	NP	65±48 (8.8-132)	Normal	22/23	NP	NP	NP	12
Sphingomyelinase (*EC* 3.1.4.12)	Niemann-Pick disease (A/B)	1.4±0.5 (0.74-4.9)	Reduced in 1/7 patients (24%)	NP	NP	NP	12.4±6 (49-72)	3.9	7/7	12

RV, reference value; MPS, mucopolysaccharidosis, NP, not performed, * qualitative analysis

Table 5. Comparison of lysosomal enzyme activity between mucolipidosis II and III

	Type	n	Mean	SD	Significance (p)
Age at diagnosis	II	13	1.6	1.2	0.04
	III	13	14	12.3	
α-N-Acetylglucosaminidase, plasma	II	6	353.6	106.3	0.014
	III	6	199.8	69.3	
β-Glucosidase, fibroblasts	II	4	276.5	187.7	0.046
	III	3	600.6	106.8	

n, number of patients; NS, not significant; SD, standard deviation.
Only enzymes with differences statistically significant are shown. The P-values refer to comparison of means.

4.2 Capítulo 2

Caracterização clínica das Mucolipidoses II e III no Brasil: um estudo multicêntrico

Artigo em elaboração

Caracterização clínica das Mucolipidoses II e III no Brasil: um estudo multicêntrico

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Resumo

As mucolipidoses (ML) II e III são doenças lisossômicas de depósito, autossômicas recessivas, raras, causadas pela deficiência da GlcNAc-fosfotransferase (fosfotransferase), enzima codificada pelos genes *GNPTAB* (mutado nas ML II ou III alpha/beta) e *GNPTG* (mutado na ML III gamma). A fosfotransferase está envolvida na síntese do marcador manose 6-fosfato, responsável por direcionar as hidrolases lisossômicas ao lisossomo. As MLII/III são caracterizadas por alterações clínicas multissistêmicas, das quais destacam-se as alterações esqueléticas. O fenótipo mais grave é o II. **Objetivo:** caracterizar aspectos clínicos de pacientes brasileiros com ML II e III. **Pacientes e métodos:** estudo transversal, rmulticêntrico, com amostragem por conveniência. Foram incluídos pacientes com ML II/III cujo diagnóstico bioquímico e/ou genético foi confirmado no Laboratório de Erros Inatos do Metabolismo do Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre de 1983 a 2013 (n= 27).Os dados clínicos e demográficos foram obtidos de forma retrospectiva, por revisão de prontuário, e/ou entrevista com familiares. **Resultados**: Os pacientes incluidos (ML II=15, ML III alfa/beta= 9, ML III gama= 3, sexo masculino= 16) eram oriundos de 20 famílias não-relacionadas, sendo que 19 estavam vivos em 2013. Consanguinidade parental foi relatada por 2/25 famílias (8%). Nove pacientes eram provenientes da região sudeste, cinco da região sul, oito do nordeste, dois do norte e três do centro-oeste do Brasil. A mediana de idade ao diagnóstico foi 2,7 anos (intervalo interquartil (1,8-7,3) Houve predomínio de sintomas relacionados ao sistema esquelético tanto em ML II como III. Os pacientes ML II demonstraram maior gravidade física e cognitiva, enquanto que os ML III alfa/beta apresentam comprometimento somático maior que ML III gama e atraso nos marcos iniciais do desenvolvimento. **Conclusão:** Este foi o primeiro estudo clínico, avaliando funcionalidade e explorando características de pacientes brasileiros com ML II e III. Observamos maior frequência dos pacientes ML II no Brasil.

Palavras-chave: mucolipidose II, mucolipidose III alfa/beta, mucolipidose III gama, funcionalidade.

1. Introdução

As mucolipidoses (ML) II (MIM#252500) e III (MIM# 252600 e 252605) são doenças lisossômicas de depósito (DLDs), raras, autossômicas recessivas, causadas por deficiência da GlcNAc-fosfotransferase (EC 2.7.8.17) (Hasilik *et al*, 1981; Reitman *et al*, 1981) . Esta enzima catalisa o passo inicial na síntese do marcador manose 6-fosfato (M6P), essencial para o endereçamento das hidrolases aos lisossomos (Hasilik & Neufeld, 1980; Kornfeld & Sly, 2010), levando à deficiência de diversas enzimas lisossomais no meio intracelular e níveis elevados no extracelular, como soro e plasma; resultando no acúmulo de seus substratos (Kornfeld & Sly, 2010). Porém alguns tipos celulares apresentam níveis de enzimas lisossomais próximos do normal (hepatócitos, células de Kupffer, leucócitos, fígado, baço, rim e cérebro), o que sugere a existência de uma via alternativa e independente de M6P (Braulke e Bonifacio*,* 2009).

A incidência da ML II e III varia de acordo com a população estudada. Considerando os recém-nascido vivos, estima-se 1:120.000 em Portugal (Tiede *et al*, 2006), 1:252.000 no Japão (Okada *et al,* 1985), 1:422.000 na Austrália (incluindo diagnóstico pré e pós-natal de doenças lisossômicas) (Meikle *et a*l, 1999) e 1:642.000 na Holanda (Poorthuis *et al*, 1989) possuam essa doença. As estimativas da prevalência do ML III alpha/beta com base em dados objetivos não estão disponíveis. É, contudo, possível que a prevalência é da mesma ordem de grandeza que a do ML II (Leroy & Cathey, 2012 ab)

A classificação das ML ocorre de acordo com alterações clínicas e moleculares, sabe-se que os pacientes com ML II e a maioria dos pacientes com ML III apresentam mutações patogênicas em *GNPTAB,* sendo denominados ML II e ML III alfa/beta; enquanto outro grupo de pacientes com ML III possuem mutações no *GNPTG*, sendo denominados ML III gamma (Cathey *et al*, 2008). As manifestações clínicas da ML II e III ainda não estão completamente elucidadas, sabe-se que são multissistêmicas, de amplo

espectro e variabilidade entre os indivíduos afetados. Os pacientes com ML II apresentam sintomatologia mais precoce, clinicamente evidente ao nascimento, curso progressivo e geralmente desfecho fatal na infância (Cathey *et al*, 2010, Kornfeld & Sly, 2010, Leroy & Cathey, 2012 a). A ML III alfa/beta apresenta início dos sintomas na infância, progressão mais lenta e geralmente é fatal no início da vida adulta.Os pacientes apresentam face típica, infiltrada, hiperplasia gengival, ponte nasal plana; ocorre atraso grave no desenvolvimento. Em relação ao sistema esquelético, podem apresentar mãos curtas, amplas, em garra, cifoescoliose, disostose múltipla e luxação congênita do quadril (Cathey *et al*, 2010, Kornfeld & Sly, 2010, Leroy & Cathey, 2012 a) . Os pacientes com ML III têm curso lentamente progressivo, características faciais gradualmente grosseiras, leve hiperplasia gengival; o desenvolvimento da linguagem é normal, e o desenvolvimento motor pode ser normal ou mostrar atraso mínimo. Já a ML III gama é muito rara e pouco descrita na literatura (Raas-Rothschild & Spiegel, 2010) . A história natural da ML III é pouco descrita na literatura (Cathey *et al*, 2010, Kornfeld & Sly, 2010, Leroy & Cathey, 2014 b).

O conhecimento acerca da ML II/III na literatura e internacional é crescente, mas ainda é restrito. O objetivo do nosso trabalho é caracterizar as manifestações clínicas de uma amostra de pacientes com ML II e III brasileiros.

2.Pacientes e Métodos

Estudo transversal, multicêntrico, aprovado pelos comitês de ética locais. A amostra foi de conveniência e foram incluídos pacientes ML II/III cujo diagnóstico bioquímico e/ou genético foi confirmado no Laboratório de Erros Inatos do Metabolismo do Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (LEIM-HCPA), Brasil, de 1983-2013 (n= 27). O LEIM-HCPA é o maior centro de referência nacional para diagnóstico e manejo das LSD no Brasil, e provavelmente concentra o maior número de diagnósticos de ML II/III no país. Como a medida direta da atividade da enzima GlcNAc-

fosfotransferase não está disponível no Brasil, o diagnóstico destes pacientes é confirmado de modo indireto, através da atividade de enzimas lisossomais: elevadas em plasma e reduzidas em fibroblastos. Neste estudo, os indivíduos sem fibroblastos disponíveis e que haviam sido investigados somente por meio da medida da atividade enzimática em plasma, foram inclusos somente quando possuíam análise de DNA confirmando a presença de mutações patogênicas em *GNPTAB* ou *GNPTG*.

Os dados clínicos e demográficos foram obtidos preferencialmente pelo primeiro autor deste estudo (n=16) ou pelo médico assistente (n=11) por meio de preenchimento de ficha clínica específica a partir de revisão de prontuário e/ou entrevista com familiares. Baixo peso ao nascimento foi definido como peso<2,5kg. No início da realização do estudo (2011), quatro pacientes já haviam felecido, sendo que outros quatro faleceram no decorrer do mesmo.

A classificação do tipo de ML (II/III) foi feita de acordo com o julgamento do médico assistente, sendo sempre revisada por dois médicos especialistas em ML II/III (TA e IVDS). A classificação das ML III em alpha/beta ou gamma foi realizada de acordo com os resultados da genotipagem de *GNPTAB* e *GNPTG* (Cury et al., 2013).

3. Resultados

Durante o período analisado, 35 pacientes apresentaram suspeita clínica e bioquímica de ML II/III (aumento da atividade das hidrolases lisossômicas em plasma), mas este diagnóstico foi confirmado somente em 27 deles por meio de análise das enzimas em fibroblastos e/ou análise de *GNPTAB* ou *GNPTG* (nos demais, não havia fibroblastos ou DNA disponível para confirmação diagnóstica), sendo estes os pacientes incluídos no estudo (ML II= 15, ML III alfa/beta= 9, ML III gama= 3, sexo masculino= 16). Entre eles, 14 nasceram entre 2000-2010, sendo 5 entre 2000-2005, e 9 entre 2006-2010. Considerando que, neste período, houve no Brasil 32.996.065 nascimentos (Ministério da Saúde do Brasil, Sistema de Informações sobre Nascidos Vivos - SINASC),

estimamos que a incidência mínima das ML II/III no Brasil seja de 1:2.357.000- nascidos vivos no período (ou de 1: 3.696.306 entre 2000-2005, e de1:1.613.000 entre 2006-2010).

Os 27 pacientes incluídos no estudo eram oriundos de 25 famílias não-relacionadas e a taxa relatada de consangüinidade parental foi de 2/25 (8%). Em relação à região brasileira de procedência, nove pacientes eram da região sudeste, cinco da região Sul, oito da nordeste, dois da norte e três da centro-oeste. A média de idade ao diagnóstico foi mediana de idade ao diagnóstico foi 2,7 anos (intervalo interquartil (1,8-7,3)

4. Manifestações Clínicas

4.1 Mucolipidose tipo II (n= 15, tabela 1, figura 1)

Houve relato de oligoidramnio em uma gestação, sem relatos de outras intercorrências durante o pré-natal para as demais gestações. Entretanto, informação sobre o perímetro cefálico e comprimento ao nascimento estavam disponíveis apenas para 6 (32±1,6cm) e 8 (47±4 cm) pacientes, respectivamente Os sintomas iniciais foram observados no primeiro semestre de vida, sendo os principais: face típica (n=13), alterações ósseas (n= 7; giba tóraco-lombar, deformidade torácica com estreitamento e abaulamento do torax) ,restrição articular em membros superiores ou inferiores (n=5), déficit pôndero-estatural (n=3) e atraso do desenvolvimento (n=3). Outros sintomas foram descritos em um numero menor de pacientes: cabelos secos (n=2), hepatoesplenomegalia (n=2), diástase de retos abdominais (n=1), hérnia inguinal bilateral (n=1), infecções respiratórias frequentes (n=1), miocardiopatia (n=1) eoligoidrâmnio (n=1). Em nenhum paciente foi descrita opacidade de córnea ou surdez, através do exame clínico. Apnéia obstrutiva do sono, diagnosticada por polissonografia, estava descrito em n=5/14 pacientes com este exame disponível. Observamos que a maioria dos pacientes possuía atraso no marcos de desenvolvimento, predominantemente do desenvolvimento motor, mas alguns chegaram a caminhar e falar ao redor da idade de 4

anos. (DATA NOT SHOWN). Seis pacientes faleceram todos por complicações respiratórias, alguns com longa estadia em unidade de terapia intensiva.

4.2 Mucolipidose tipo III alfa/beta (n=9, figura 2)

Os principais dados dos pacientes inclusos, estão demonstrados na tabela 1. Não houve relatos de intercorrências durante o pré-natal. Informação sobre o perímetro cefálico e comprimento ao nascimento não estavam disponíveis para todos os pacientes: ao nascimento, o perímetro cefálico apresentou média de 34,5±0,7 (n=2) e o comprimento de 49,6±2 (n=4) cm. Os sintomas iniciais foram observados próximos ou na adolescência, sendo os principais: limitação articular em membros superiores ou inferiores (n=5), dor na coluna (lombar ou torácica) (n=4), contratura articular mãos (n=3) e déficit pôndero-estatural (n=3). Outras alterações esqueléticas (n=6: aumento da cifose dorsal, *pectus carinatum*, pescoço curto) atraso fala (n=1) e abdômen protuso (n=1) foram menos freqüentes. Atraso nos marcos do desenvolvimento foi observado em três pacientes, mas – apesar desta dificuldade no início da vida– todos puderam frequentar a escola regular aparentemente sem maiores dificuldades, alguns chegando ao cursar ensino superior. O paciente da nossa coorte que faleceu durante o estudo tinha 20 anos de idade ao óbito, hipertensão pulmonar e alterações restritivas do tórax, sendo as complicações respiratórias a causa de óbito.

4.3 Mucolipidose tipo III gama (n=3, figura 3)

Não houve relatos de intercorrências durante o pré-natal destes pacientes. Infelizmente, não foram recuperadas informações do nascimento para o perímetro cefálico, já para o comprimento o relato foi de 53cm em um deles e desconhecidos nos demais. Os sintomas iniciais foram observados na adolescência, sendo os principais: limitação articular em membros superiores ou inferiores (n=3), dor ou alterações na coluna (n=1), déficit pôndero-estatural (n=2). Os três pacientes possuíam alterações na

articulação do quadril, dois com prótese de quadril bilateral (aos 35 e 37 anos em um paciente e 40 e41 anos no outro). Todos os indivíduos frequentaram escola regular e não demonstravam déficit cognitivo aparente. Não foi registrado óbito, sendo a idade dos pacientes na avaliaçao: 20, 42 e 44 anos, respectivamente.

4.4 ML III alfa/beta versus ML III gama

Quando comparamos ML III alfa/beta e gama observamos que a idade de início dos sintomas foi significativamente menor em ML III alfa/beta (4,1±4 *versus* 7,6±4,4, p=0,045), mas não houve diferença estatisticamente significativa quanto a idade ao diagnóstico (10,3±7,6 *versus* 29,3±19,1, p=0,2) .

4.5 Alterações clínicas

Quanto à aparência geral, todos os pacientes ML II apresentavam hiperplasia gengival, em ML III alfa/beta 2/8 pacientes, e essa alteração não encontrada nos pacientes com ML III gama; face infiltrada também foi observada em todos os ML II, em 4/8 ML III e 1/8 ML III gama. Além disso, todos os pacientes apresentavam baixa estatura em relação à idade. Como a estatura de ambos os pais não estava disponível na maioria dos casos, não foi realizado o cálculo da estatura alvo.

Em relação ao sistema cardiovascular, ao exame físico os pacientes apresentaram alterações na ausculta cardíaca (sopro sistólico), geralmente relacionado à insuficiência leve valvular; possuíamos ecocardiograma de oito pacientes que demonstravam função sistólica do VE preservada (n=8/8), forame oval patente (n=1/8, 10 meses, MLII), espessamento mitral em (n=4/8, dos quais um MLIII), regurgitação mitral leve (n=7/8), espessamento da valva aórtica (n= 2/8, ambos MLIII, um deles com obstrução leve e aortoesclerose), insuficiência fisiológica da valva pulmonar (n=8/8). Visualizada HVE em dois pacientes MLII: hipertrofia concêntrica em um (4 anos) e discreto aumento de VE em outro (5 anos). PSAP>30mmHg em 3/8 pacientes: 2/8 com

valor limítrofe (MLIII) e 1/8 com HP (MLIII, atualmente em uso de pressão positiva contínua na via aérea – CPAP – e Sildenalfil, com boa resposta); dos demais com PSAP <30mmHg, um paciente (MLII) usava CPAP.

As alterações esqueléticas mais proeminentes foram alterações em quadril (n=23/23), contratura articular em mãos (de intensidade variável) em 18/22 pacientes, sendo que 3/22 eram ML III alfa/beta e um ML III gama. Todos apresentavam contraturas articulares em cotovelos e ombros, com gravidade variável entre os tipos e entre os indivíduos do mesmo tipo. Alterações na coluna lombar e torácica foram as mais presentes.

Alterações oculares foram pouco estudadas, mas avaliação oftalmológica formal estava disponível em 13 pacientes . Destes, o achado mais relevante foi dispersão pigmentar na retina em um paciente ML III gama, não descrita na literatura. Nenhum dos indivíduos apresentou opacidade de córnea ou manchas em fundo de olho.

Apesar do abdômen protuso observados em todos os indivíduos, apenas um paciente ML II apresentou hepatoesplenomegalia ao exame físico. Hérnia umbilical foi relatada em n=1 paciente (ML III gama), hérnia inguinal foi vista em 2/15 pacientes com ML II.

O exame neurológico detalhado não estava descrito para a maioria dos pacientes, mas não houve relato de crises convulsivas ou compressão medular no período. Um paciente com ML III gamma apresentava síndrome do túnel do carpo grave, diagnosticada clinicamente e por eletroneuromiografia. Imagem do encéfalo estava disponível para n=3 pacientes (ML III alfa/beta= 1, ML III gama n=2).

Quanto ao sistema respiratório, a principal causa de morbi-mortalidade dos indivíduos, observamos restrições torácicas em todos os indivíduos, com exceção de um ML III gamma. O número de internações ou de infecções respiratórias ao ano não foi obtido, mas pelo relato subjetivo dos pacientes, parecia ser semelhante à população, mas, quando ocorriam, em geral motivavam internação hospitalar. Apnéia obstrutiva do

sono foi descrita em n=5/14 pacientes, entretanto, a polissonografia para estudo desta alteração nem sempre está disponível para os pacientes, em geral por dificuldade relacionada à estrutura do sistema público de saúde.

5. Discussão

A ML II predominou em nossa amostra em relação aos ML III. Na literatura não está claro qual o tipo de ML mais frequente (Cathey *et al*, 2010, Kornfeld & Sly, 201t0), mas alguns autores sugerem que a frequência de ambos seja semelhante (Leroy & Cathey, 2012 ab); como a ML II é clinicamente mais grave, isto poderia aumentar a busca por diagnóstico e, consequentemente, o número de indivíduos diagnosticados. A taxa geral de consanguinidade em nossa amostra foi maior que o observado na literatura (8% versus 1,5%).Houve alta taxa de cesáreas em ambos grupos (50% no total), que é semelhante a taxa brasileira de 43,8% de partos cesáreos (Fonte: Ministério da Saúde, SVS - Sistema de Informações sobre Nascidos Vivos/2009). Já na comparação entre os subgupos ML II e ML III, houve mais partos cesáreos nos MLII (p=0,006), cujas razões não conhecemos, visto os dados peri-parto não estarem disponíveis. Entre ambos não houve diferença estatisticamente significativa do peso ao nascimento, entretanto predominaram pacientes com baixo peso (<2500g) em pacientes com ML II (55%, n=6) em contraste com os pacientes ML III alfa/beta (13% , n=1) e a população brasileira (8,43%. Fonte: Ministério da Saúde, SVS - Sistema de Informações sobre Nascidos Vivos/2009). Cathey *et al*, em 2010 observou peso médio ao nascimento de 2400g para ML II e 3100g para ML III (alfa/beta); sendo n=8/12 pacientes ML II pequenos para a idade gestacional. Como os nossos dados foram retrospectivos, houve prejuízo de informações do período pré e peri natal, incluindo condições de nascimento, por exemplo. Através da nossa análise observamos que a maioria dos pacientes ML II apresentou peso <2500g ao nascimento (sendo que nenhum com peso <1700g), mas infelizmente não possuímos informações sobre a idade gestacional para correlacionar ao peso e

compreender se os pacientes também era pequenos para a idade gestacional ou se este dado se correlacionava com prematuridade.

A idade dos primeiros sintomas em nossos pacientes foi diferente da literatura, sendo no nosso caso a apresentação notada mais tardiamente; no maior estudo clínico disponível todos os pacientes ML II e 5/21 pacientes com ML III alfa/beta apresentavam sintomas ao nascer (Cathey *et al*, 2010) O sintoma proeminente em todos os indivíduos foi restrição articular, que pudemos observar objetivamente através da goniometria em um pequeno número de pacientes; as articulações mais acometidas desta amostra foram joelhos (flexão), quadril e tornozelo, com pior desemprenho dos pacientes com ML III, provavelmente por apresentarem idade superior aos os ML II. Quando comparamos ML III alfa/beta e gama observamos que a idade de início dos sintomas foi significativamente menor nos primeiros e com comprometimento somático mais grave também nos primeiros. A cognição não foi medida com instrumentos formais, mas os ML II tem o desenvolvimento motor e a linguagem bastante afetados; dado concordante com a literatura (Cathey *et al*, 2010). Já os ML III alfa/beta e gama atingiram escolaridade satisfatória para sua idade, embora os primeiros demonstrem atraso no desenvolvimento motor no início da vida.; como não aferimos estes dados de modo formal (com o quociente de inteliência – QI - por exemplo) não podemos comparar diretamente na literatura; descreve-se para ML III alfa/beta déficit cognitivo em 5/15 pacientes (QI menor que 70) , (Cathey *et al*, 2010)

6. Conclusão

Este foi o primeiro estudo clínico explorando características de pacientes brasileiros com ML II e III. A ML II foi mais frequente e com comprometimento somático e cognitivo mais grave e precoce. Os pacientes ML III alfa/beta apresentam comprometimento somático maior que ML III gama e atraso do desenvolvimento apenas nos primeiros anos de vida. Algumas questões práticas da assistência ainda são

controversas entre os autores internacionais e necessitam de resposta, além disso, acreditamos que o melhor entendimento da história natural da doença poderá contribuir para as terapias que estão em andamento e para futuros tratamentos que possam surgir.

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Tabela 1: Síntese das principais características clínicas dos pacientes com Mucolipidose II/III incluídos no presente estudo.

	Tipo II	Tipo III alfa-beta	Tipo III gamma	Total	Comparação ML II e III (alfa/beta + gama)
n	15	9	3	27	-
Sexo M:F	6:9	8:1	2:1	16:11	-
Consanguinidade (n)	2	0	0	2	-
Idade Diagnóstico - média±DP (anos)	2±1	13,4±13,5	29,2±19	7,9±11	p=0,007
Óbito (média Idade anos±DP)	6 (4±1,9)	1 (20)	0	7 (6,6±6,2)	Idade: p=0,006
Tipo Parto vaginal:cesárea (% cesáreas)	3:7 (70%)	6:3 (33%)	2:1 (33%)	11:11 (50%)	p=0,004
Peso nascimento (média±DP Kg)	2,6±0,5 (n=11)	3±0,6 (n=7)	3,8±0 (n=1)	2,8±0,6 (n=19)	NS
% Pacientes com baixo peso (<2500g)	55% (n=6)	13% (n=1)	0	37% (n=7)	-
Idade início sintomas (média±DP anos)	0,3±0,3	7,4±7	12,5±7,5	3,2±5,6 (mediana= 0,5 anos)	p=0,006

Figura 1 . Espectro fenotípico de pacientes com atividade deficiente da Glc-NAc-fosfotransferase (Mucolipidose II/III).

1A-C. Mucolipidose tipo II (2 anos de idade) Podem ser observados abdômen protruso, giba tóraco-lombar, face típica, hiperplasia gengival e contraturas articulares em mãos.

2A-C.Mucolipidose tipo III alfa/beta (29 anos) Pectus carinatum, contratura articular em joelhos, ombros e discreta em mãos; face infiltrada, mas com pouca intensidade.

3 A-C. Mucolipidose tipo III gama (41 anos). Contraturas articulares cotovelos, ombros e mãos. Este paciente possui prótese de quadril bilateral.

4.3 Capítulo 3

Is melanogenesis disturbed in mucolipidosis II/III? A multicenter study based on clinical and genetic findings

Artigo em fase final de preparação, será submetido no *American Journal of Clinical Genetics*

Is melanogenesis disturbed in mucolipidosis II/III? A multicenter study based on clinical and genetic findings

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Abstract

Mucolipidosis (ML) II and III are inborn errors of metabolism caused by deficient activity of GlcNAc-1-phosphotransferase, an enzyme responsible for correct targeting of lysosomal hydrolases to the lysosomes. We hypothesized that melanogenesis would be altered in patients with ML II/III. **Objectives:** 1) To characterize the skin, hair, and eye color of patients with ML II/III and compare these features to those of their parents and of healthy controls; and 2) to establish a genotype-phenotype association involving SNPs known to be associated with skin, hair, and eye color in normal populations. **Methods:** This multicenter, prospective, controlled, cross-sectional study employed a convenience sampling strategy. Brazilian patients with ML II/III aged >1 year and their parents, as well as healthy controls matched by gender and age to the patients, were examined for skin, hair, and eye characteristics using the Fitzpatrick scale and visual classification. Patients were screened for SNPs [rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) and rs1129038 (*HERC2* gene)] through KASP genotyping assay. **Results/discussion:** Seventeen patients (7 with ML type II, 7 with ML type III alpha/beta, and 3 with ML type III gamma; 13 male, 4 female; mean age, 13±12.5 years) and 29 parents were included in the study, as well as 20 healthy controls and 34 control parents. Most patients had Fitzpatrick skin types I–III (n=14/17, 82%) a rate discrepant with the skin type of their parents (n=19/29, 66%) and with that of healthy controls (n=14/20, 70%) and control parents (n=25/34, 74%). One patient was not genotyped for the SNPs of interest. Regarding genotype-phenotype- association, for rs1126809 2/17 had lighter hair than expected; for rs16891982, 4/17, 6/17 and 10/17 patients had, respectively, lighter eyes, hair and skin color, and 3/17, 4/17 and 2/17 patients had, respectively, darker eyes, hair, and skin color than expected; and for rs1426654, 2/17, 1/17, 1/17 had lighter eyes, hair and skin and 9/17 and 3/17 had darker hair and skin than expected; for rs1129038 6/16 patients had darker eyes than expected. **Conclusion:** Patients with ML II/III appear to exhibit changes in melanogenesis (most commonly hypomelanosis). Further studies are required to corroborate these findings.

Keywords: mucolipidosis II/III, human pigmentation, rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene), rs1129038 (*HERC2* gene)

1. Introduction

Mucolipidosis type II (ML II, MIM#252500) and type III (ML III, MIM# 252600 e 252605) are very rare autosomal recessive lysosomal storage disorders (LSDs) caused by deficient activity of the enzyme UDP-N-acetylglucosamine-1-phosphotransferase (GlcNAc-1- phosphotransferase; EC 2.7.8.17). This enzyme is encoded by two genes – *GNPTAB* and *GNPTG* – and is responsible for catalyzing the addition of mannose 6-phosphate (M6P) residues to lysosomal hydrolases (Kornfeld & Sly, 2010). These residues play an essential role in receptor-mediated transport to the endosomal and prelysosomal compartments. A breakdown in this recognition signal will cause incorrect targeting of lysosomal enzymes. Hence, patients with ML II/III exhibit a deficiency of lysosomal enzymes in several cell types, as well as elevated enzyme activity levels in extracellular fluids such as serum and plasma (Kornfeld & Sly, 2010).

The clinical manifestations of ML II and III are multisystemic and cover a broad spectrum, which is reflected by great variability among affected individuals (Kornfeld & Sly, 2010; Cathey et al, 2010; Leroy et al, 2014ab). In the absence of useful genetic and biochemical markers, the distinction between ML II and III can be made by clinical criteria (Cathey et al, 2008). ML II is caused by mutations in *GNPTAB*; patients experience earlier onset of symptoms and greater disease severity, with a progressive course generally leading to death in childhood (Kornfeld & Sly, 2010; Cathey et al, 2010; Leroy et al, 2014ab). ML III alpha/beta is also caused by *GNPTAB* mutations; symptom onset is also in childhood, but disease progression is slower, with death generally occurring in adulthood (Kornfeld & Sly, 2010; Cathey et al, 2010; Cury et al, 2013; Leroy et al, 2014ab). ML III gamma is due to mutations in *GNPTG*, and its natural history is still underreported in the literature (Kornfeld & Sly, 2010; Cathey et al, 2010; Leroy et al, 2014ab).

LSDs are characterized by progressive intracellular deposition of undigested macromolecules due to gene defects that lead to deficient activity of specific enzymes. Melanosomes are organelles similar to lysosomes (Huizing et al, 2008), and disorders in melanosomal biogenesis and trafficking lead to syndromic albinism in humans (Parkinson-Lawrence et al, 2010). ML II/III is known to be associated with inadequate targeting of lysosomal hydrolases, but its pathophysiology is still being elucidated, and little is known about the potential role of melanosomes in ML. We hypothesize that patients with ML II/III may have abnormal melanosome function and, consequently, alterations in skin, eye, and hair pigmentation. Within this context, the objectives of this study were: 1) to characterize the skin, hair, and eye color of patients with MLII/III and compare these characteristics to those of their parents and of healthy controls; and 2) to establish a genotype-phenotype association involving SNPs known to be associated with skin, hair, and eye color in some populations.

2. Patients and methods

This was a multicenter, prospective, controlled, cross-sectional study with a convenience sampling strategy, approved by the local Ethics Committee. The study sample included patients with a confirmed biochemical and/or genetic diagnosis of ML II/III, as established at the Inborn Errors of Metabolism Laboratory of the Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil (LEIM-HCPA), between 1983 and 2013. As direct measurement of GlcNAc-1-phosphotransferase is not available in Brazil, biochemical diagnosis was confirmed indirectly by the finding of altered lysosomal enzyme activity (normal in leukocytes, elevated in plasma, and reduced in fibroblasts). Subjects for whom no fibroblasts were available were included if they had molecular analysis results confirming the presence of pathogenic mutations in *GNPTAB* or *GNPTG*. As some benign melanocyte-related skin conditions, such as Mongolian spot,

can resolve spontaneously at around age 1 year (Kikuchi, 1982; Gupta & Thappa, 2013), we considered that younger infants can exhibit greater variation in skin pigmentation and thus excluded all patients aged <1 year.

Healthy children were recruited from the HCPA outpatient pediatrics clinic during routine well-child appointments to serve as age-matched controls. Patients, their parents, controls, and controls' parents were examined by the same investigator for skin, hair, and eye characteristics, using the Fitzpatrick scale and visual analysis for classification (Table 1). Perhaps the most famous skin phototype classification system, the Fitzpatrick scale, created in 1976, classifies individuals by sensitivity to sun into six types, ranging from I (white) to VI (black) (Fitzpatrick, 1988). Eye color was classified as blue, green, brown or black; and hair as red, blond, brown or black. Furthermore, SNPs related to these features [rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) and rs1129038 (*HERC2* gene)] were genotyped (KASP, LGC Genomics: www.lgcgenomics.com) in patients and compared to their eye, hair, and skin phenotype (color). These SNPs are commonly related to variation in eye, hair, and skin pigmentation, and a previous study conducted in a Brazilian population indicated that this association is independent of ethnic miscegenation (Cerqueira et al, 2014). The *TYR* gene codes for tyrosinase, an enzyme that plays a key role in melanin synthesis, and thus influences variations in skin, eye, and hair color; indeed, some *TYR* polymorphisms are associated with risk of skin cancer (Sturm, 2009). *HERC2* influences expression of *OCA2*, which codes for the melanocyte-specific transporter protein or pink-eyed dilution protein homolog, a protein that is believed to be an integral membrane protein involved in small-molecule transport, specifically of tyrosine – a precursor of melanin that acts on the melanosome maturation pathway in the iris (Donnelly et al, 2012; Visser et al, 2012). *SLC45A2* codes for membrane-associated transporter protein (MATP), also known as solute carrier family 45 member 2 (SLC45A2) or melanoma antigen AIM1, a transporter

that mediates melanin synthesis (Sturm, 2009). *SLC24A5* codes for sodium/potassium/calcium exchanger 5 (NCKX5), also known as solute carrier family 24 member 5 (SLC24A5). It has been suggested that melanogenesis and development of the melanosomes themselves may be dependent on calcium levels in the melanosome, regulated by the *SLC24A5* gene (Lamason et al. 2005; Sturm, 2009); furthermore, some studies have shown that this gene appears to play a major role in pigmentation in several human populations (Norton et al., 2007). The A allele of rs1126809 (*TYR* gene) is related to blue eyes and greater skin sensitivity to sunlight (Sulem et al, 2008), and heterozygous statetes favours lighter phenotype as well(Cerqueira et al, 2014). The 374Phe (C) allele of rs16891982 (*SLC45A2* gene) is associated with pale skin, and the 374Leu (G) allele, with olive skin and dark hair (Sturm & Duffy, 2012), heterozygotes tend to have darker characteristics (Cerqueira et al, 2014). The A allele of SNP rs1426654 (*SLC24A5* gene) indicates less melanin, therefore, carriers will probably be light-skinned; however, heterozygotes have intermediate phenotype, because there are no dominance between the alleles (Lamason et al, 2005; Cerqueira et al, 2014). The A allele of rs1129038 (HERC2 gene) increases the likelihood of blue eyes even in heterozygotes (Eiberg et al, 2008; Sturm et al, 2008; Cerqueira et al, 2014).

Due to the low sample size and aimming at a better visualization of the relationship genotype-phenotype, we group the characteristics studied into two categories: lighter (for green and blue eyes, red and blond hair, white skin) and darker (for brown and black eyes, brown and black hair, darker skin),

A single investigator (TA), with experience in the management of ML II/III, classified the patients as having ML II or III. Classification of ML III into alpha/beta or gamma was based on the results of *GNPTAB* and *GNPTG* genotyping.

As this was a small exploratory study, no formal statistical analyses were conducted to compare patients and controls.

3. Results

A total of 17 patients from 16 families were included in the study (7 with ML type II, 7 with ML type III alpha/beta, 3 with ML type III gamma), as well as 26 parents of patients, 20 controls, and 34 parents of controls (Table 2). There were patients from all regions of Brazil (North = 1; Northeast = 3; Center-West = 2; South = 3; Southeast = 7).

Patients with ML II/III had a greater frequency of Fitzpatrick skin types I–III as compared with their parents or with healthy controls (Table 3). Among patients with skin types I–III (n=14), 7 of 14 (41%) had a mother and 6 of 10 (60%) had a father with the same skin type. Among patients with skin types IV–V (n=4), 3 of 4 (75%) had a mother and 2 of 2 (100%) had a father with the same skin type. Of the patients who had at least one parent with a different Fitzpatrick skin type (I–III or IV–V) (discordant cases; n=8, 44%), 4 had ML II and 4 had ML III (3 with ML III alpha-beta, 1 with ML III gamma).

Among controls with skin types I–III (n=14), 11 of 14 (79%) had a mother with the same skin type and 5 of 8 (63%) had a father with the same skin type. Among controls with skin type IV–V (n=6), all had a mother with the same skin type and 1 of 6 (17%) had a father with the same skin type. Controls who had at least one parent with a discordant skin type accounted for 11 of 20 cases (55%).

3.1 Frequency of SNPs in patients

The frequency of each SNP is listed in Table 4.

3.2 Genotype vs. phenotype association

We compared the genotype of each patient versus the corresponding phenotype for eye, hair, and skin color separately. Table 4 lists the frequency of genotype-phenotype discordance for the SNPs analyzed in the patients with ML II/III included in the present study.

4. Discussion

Knowledge of ML II/III has been increasing; however, several unanswered questions remain regarding aspects ranging from its pathophysiology to its clinical manifestations. ML causes broad modifications to the lysosomal hydrolase pool, leading to deposition and global impairment of lysosome-dependent pathways, including vesicular trafficking and biogenesis, as well as a building up of substrates that can interfere with other cell processes (Ballabio & Gieselmann, 2009; Parkinson-Lawrence et al, 2010). On the basis of this assumption and of empirical clinical observations, we sought to investigate whether patients with ML might exhibit abnormalities in melanogenesis and phenotypic changes in pigmentation, affecting eye, hair, and skin color. We found that patients with ML II/III are more likely to have Fitzpatrick skin types I–III (i.e., lighter skin) and their parents, which was corroborated by a comparison of control children and the control's parents.

It has been established that dysfunctions in melanosome biogenesis and trafficking, instead of impaired activity of a specific lysosomal hydrolase, leads to syndromes of albinism in humans, such as Chediak-Higashi syndrome (MIM#214500), Griscelli syndrome (MIM#214450), and Hermansky-Pudlak syndrome (MIM#203300, #608233, #614072, #614073, #614074, #614075, #614076, #614077, and #614171). Breaches in the integrity of vesicular trafficking in the endosome-lysosome network may occur at different points along this system, but manifest as similar functional outcomes. The altered vesicular trafficking observed in the aforementioned albinism syndromes is somewhat similar to other LSDs, but in the latter, the effects on biogenesis are secondary to enzyme deficiency and substrate accumulation (Parkinson-Lawrence et al, 2010). Although a significant knowledge base has been constructed about diseases that involve lysosomal dysfunction, we have yet to fully understand the mechanisms behind these

conditions. To the best of our knowledge, these changes have not been studied systematically in patients with ML II/III.

As part of our investigation, we genotyped patients for SNPs known to be associated with pigmentation in several ethnicities and recognized as valid for the Brazilian population (Cerqueira et al, 2014). Rs1126809 is a SNP in the *TYR* gene, and its allele rs1126809(A) has previously been shown to affect eye color and sun sensitivity: it is associated with blue eyes, blond hair, and a slight increase in skin cancer risk (Sulem et al, 2008; Duffy et al, 2010). The most important polymorphism affecting skin and hair color is the rs16891982*Leu374Phe (G/C), SNP located at chromosome 5, Rs16891982*Leu allele being strongly associated with olive skin and dark hair (Sturm & Duffy, 2012). This SNP is also highly involved in variations in eye, skin, and hair pigmentation in humans. The rs1426654(A) allele is indicative of less melanin; therefore, its carriers are likely to be light-skinned (Lamason et al, 2005; Soejima & Koda, 2007). The rs1129038 variant of *HERC2* is found in 97% of all Caucasians with blue eyes, and is relatively common in Caucasians, though rare among other ethnic groups. The A allele increases the likelihood of blue eyes (Eiberg et al, 2008; Sturm et al, 2008). We found discrepancy in the patient group regarding eye color [rs1126809(*TYR*) and rs16891982(*SLC45A2*)] and more prominent differences in skin color [rs16891982(*SLC45A2*), rs1426654(*SLC24A5*) and rs16891982(*SLC45A2*)]. Despite of the most patients exhibit changes in melanogenesis (most commonly hypomelanosis), some patients exhibited darker pigmentation than genotypically predicted, contradicting our expectations.

However, melanocyte-related abnormalities that cause hyperpigmentation, namely large Mongolian spots, have previously been reported in ML II (Su et al, 2010). Mongolian spots are maculae consisting of spindle-shaped melanocytes in the lower dermis that have failed to migrate to the dermal-epidermal junction during fetal life; they are present

at birth in many children, particularly those of African descent, and generally show spontaneous regression during childhood (Jacobs & Walton, 1976). Mongolian spots characterized by extensive distribution may be associated with other LSDs, such as mucopolysaccharidosis (MPS) I, MPS II, MPS VI, gangliosidosis GM1, Niemann-Pick disease type C and alpha-mannosidosis (Ochiai et al, 2007; Su et al, 2010; Okamura et al, 2013; Gupta & Thappa, 2013)

The present study was the first to correlate hypopigmentation changes in a cohort of patients with ML II/III. To our knowledge, only one other such report exists: a case report of a Han Chinese patient whose hair was blond at birth, in contrast with the black hair of his parents, and darkened by age 2 months (Ma et al, 2011). One of our patients (Figure 1) had Fitzpatrick skin type II (blond hair, blue eyes, fair skin) ate age 2 years, while both parents were skin type IV, corroborating the biological plausibility of pigmentation changes as manifestations of ML II/III. The fact that we did not genotype the SNPs of interest in controls is a limitation of the present study. Another potential limitation is that while our patients were drawn from all regions of Brazil, controls were all from the South region of the country – regions with a high degree of miscegenation, but different patterns of ancestry. However, Cerqueira et al (2014) found that such SNPs as rs1426654 (*SLC24A5)* and rs16891982 (*SLC45A2*) were associated with pigmentation even in two distinct regions of Brazil (states of Rio Grande do Sul and Bahia). Another limitation is the fact that we were unable to achieve our *a priori* objective of including controls that could be matched to patients by age and gender; as shown in Table 2, our patients were older than their controls.

On analysis of our findings, it is plausible to consider that GlcNAC-1-phosphotransferase per se may not be the main factor involved in the melanogenesis process, as most patients did not exhibit discrepancies in eye, hair, and skin color. However, the enzyme (and/or the accumulation of its substrate) may influence other

regulatory genes involved in melanogenesis. Corroborating our hypotheses, the greatest discordance observed was in skin phenotype in relation to eyes and hair. This is consistent with the fact that tissues are affected heterogeneously in ML II/III (Kornfeld & Sly, 2010): fibroblasts (which are part of skin connective tissues) are affected, whereas the brain is not (notably, the iris is derived from neural tissue, and little variation in iris color was observed).

5. Conclusion

Our findings suggest that patients with ML II/III may exhibit alterations in melanogenesis. Additional studies using other modalities to assess melanosome function, from electron microscopy studies through clinical trials with larger sample sizes to genotyping of cases and controls, should be conducted to corroborate these findings.

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Table 1: Fitzpatrick skin type classification (Fitzpatrick, 1988).

Skin type	Characteristics	Description	Skin sensitivity
I – White	Pale eyes, blond or red hair	Burns easily, never tans	Very sensitive
II – White	Blue, green, or light brown eyes; blond or red hair	Burns easily, tans minimally	Sensitive
III – Light brown	Brown or black eyes, red to brown hair	Burns moderately, tans moderately	Normal
IV – Moderate brown	Brown or black eyes, brown to black hair. Includes Asians	Burns minimally, tans easily	Normal
V – Dark brown	Black eyes and hair	Rarely burns, tans well	Minimally sensitive
VI – Black	Black eyes and hair	Never burns, deeply pigmented	Insensitive

Table 2. Number and age of cases, controls and parents included in this study.

	Patients (n=18)	Controls (n=20)
Age, years (mean ± SD)	13±12	6.5±9
Male gender (n)	13	8
Fathers tested (n)	12	14
Mothers tested (n)	18	20

Table 3: Fitzpatrick skin types of subjects included in the study.

	I (n)	II (n)	III (n)	IV (n)	V (n)	I–III (n)	Prevalence of I–III (%)
Patients (n=18)	1	7	6	3	1	14	78
Patients' parents (n=30)	0	6	13	8	3	19	63
Controls (n=20)	1	4	9	3	3	14	70
Controls' parents (n=23)	0	6	19	4	5	25	74

Table 4: Discordance between SNP genotypes and eye, hair, and skin phenotype in patients with mucolipidosis II/III included in the sample.

			Genotype-phenotype (n/total with the genotype)								
			Eye color			**Hair color**			**Skin color**		
				Discordant			**Discordant**			**Discordant**	
Gene	**SNP**	**Genotype**	**Concordant**	**Lighter [c]**	**Darker [d]**	**Concordant**	**Lighter[e]**	**Darker[f]**	**Concordant**	**Lighter[a]**	**Darker [b]**
TYR	rs1126809	GG	13/13	0	0	11/13	**2/13**	0	13/13	0	0
		GA	4/4	0	0	4/4	0	0	4/4	0	0
		AA	0	0	0	0	0	0	0	0	0
SLC45A2	rs16891982	CC	0	0	3/3	0	0	3/3	1/3	0	2/3
		CG	5/5	0	0	4/5	1/5	0	2/5	3/5	0
		GG	5/9	4/9	0	3/9	5/9	1/9	2/9	7/9	0
SLC24A5	rs1426654	GG	1/2	1/2	0	1/2	1/2	0	0	1/2	0
		GA	3/5	2/5	0	3/5	2/5	0	4/5	1/5	0
		AA	2/10	0	8/10	1/10	0	9/10	6/10	1/10	3/10
HERC2	rs1129038	GG	6/6	0	0		*	*		*	*
		GA	1/7	0	6/7		*	*		*	*
		AA	3/3	0	0		*	*		*	*

* Not assessed, as the influence of this polymorphism is predominantly on eye color.
[a] I-III in Fitzpatrick's scale means patients with Lighter skin color.
[b] IV-VI in Fitzpatrick's scale means patients with Darker skin color.
[c] Lighter eye color: green and blue eyes
[d] Darker eye color: brown and black eyes
[e] Lighter hair color: red and blond hair
[f] Darker hair color: brown and black hair
in Fitzpatrick's scale means patients with Darker skin color.

Figure 1. A 2-year-old male patient with mucolipidosis type II and his parents. This family is originally from Northeastern Brazil. The patient has Fitzpatrick skin type II, while his parents have skin type IV. (Photo obtained and used with consent.)

4.4 Capítulo 4

Exome sequencing for mucolipidosis III: *GNPTAB* gene novel mutation in a patient with a milder phenotype

Artigo em fase final de preparação para ser submetido

Exome sequencing for mucolipidosis III:
***GNPTAB* gene novel mutation in a patient with a milder phenotype**

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Abstract

Mucolipidosis II and III alpha/beta (ML II/III alpha/beta) is a rare autosomal recessive disease, but commonly misdiagnosed. ML II/III alpha/beta is caused by deficiency of N-acetylglucosamine-1-phosphotransferase, responsible for the essential mannose-6-phosphate recognition marker system in lysosomal hydrolases. A Brazilian patient previously diagnosed as having ML III was investigated through whole next-generation sequencing (NGS). Using this approach, we identified two mutations in the *GNPTAB* gene confirmed to be *in trans* status by parental analysis: c.1208T>C (p.Ile403Thr), previously reported as being pathogenic, and the novel mutation c.1723 G>A (p.Gly575Arg), which is probably pathogenic. This work demonstrates the effectiveness of using whole NGS for the molecular diagnosis of ML II/III alpha/beta.

Keywords: mucolipidosis II/III alpha/beta, whole exome sequencing, molecular diagnosis

Introduction

In recent years, the use of massive parallel sequencing, or next-generation sequencing (NGS), is revolutionizing genetic investigation as well as clinical practice, mostly when dealing with rare diseases. It is particularly useful when the same phenotype can be caused by mutations in different genes. As cost of sequencing is progressively coming down, it is expected that in the near future comprehensive molecular diagnosis will become a standard of care. NGS can be used in several ways: as a panel targeting selected genes, as a test where the whole exome (i.e., the ~2% coding regions of the genome) is captured and sequenced, or as an exam where the whole genome, with its coding and non-coding regions, is sequenced. Even though sequencing a limited number of genes is apparently more rationale than all genes, developing several panels can be expensive and burdensome. Additionally, having a single text (as whole exome or genome sequencing) for thousands of different genetic disorders is practical and gives opportunities for new discoveries.

Mucolipidosis II and III (ML II and III) are autosomal recessive lysosomal disorders (LSD) in which the essential mannose 6-phosphate (M6P) recognition marker system is deficient. ML II and III are caused by mutations in the *GNPTAB* or *GNPTG* genes, which encode the subunits α and β (*GNPTAB*) or γ (*GNPTG*) of the N-acetylglucosamine-1-phosphotransferase (phosphotransferase, EC 2.7.8.17). Phosphotransferase is a hexameric enzyme (2 subunits α, 2 subunits β and 2 subunits γ) that mediates the first step of the synthesis of M6P, but there are other genes involved in this pathway, such as the *NAGPA* gene [which codifies uncovering enzyme (EC 3.1.4.45), reviewed in 1] and the *MBTPS* gene that encodes the S1P enzyme. S1P or “site-1 protease” (EC 3.4.21.112) acts post-translationally and cleaves α and β subunits of phosphotransferase for the correct assembly of hexameric complex [2]. At least theoretically, allelic mutations in the *NAGPA* or *MBTPS* genes could cause ML II/III, even though they have never been reported.

ML is classified in II or III according to clinical alterations associated. Although there is a phenotypic spectrum, ML II (MIM#252500) is more severe, clinically evident at birth, and usually fatal at childhood; on the other hand, ML III (MIM# 252600 and 252605) presents a

later-onset symptomatology and a slower progression. Clinically, skeletal alterations similar to *dysostosis multiplex*, failure to thrive, and infiltrated facies are observed. Milder cases may have a normal survival rate and absence of cognitive involvement. Patients with ML II and most patients with ML III are known to be homozygous or compound heterozygous for mutations in *GNPTAB* and, consequently, show alterations in subunits alpha and beta (being called ML II or III alpha/beta patients). On the other hand, a second group of ML III patients is homozygous or compound heterozygous for mutations in *GNPTG*, with modifications of the gamma subunit (thus being ML III gamma patients) [reviewed in 3].

Schrader *et al* [4] was the group that first demonstrated the effectiveness of molecular diagnosis for ML III using NGS. They were able to show a 6-bp deletion in the *GNPTG* gene in a family with retinitis pigmentosa and skeletal abnormalities, patients who were not previously known to have ML III. In turn, using targeted NGS, Yang *et al* [5] were able to identify two homozygous nonsense mutations in the *GNPTAB* gene in two Chinese families, patients who had previously been diagnosed through biochemical assays as having ML II.

In this work, we evaluated the reliability and feasibility of molecular diagnosis by whole exome sequencing for MLII/III. The study was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Research Ethics Committee.

Case Report

The first child born to a non-consanguineous couple, this male patient started with claudication at the age of 4 years old. Until the age of 4, his development was normal, and he had no history of respiratory infections or other significant comorbidity. Since the age of 8, his weight had been > p95, and his height, between p10 and 25 for his age. At the age of 11, after being followed by an orthopedics service, he was referred, for the first time, to our medical genetics service for suspected multiple epiphyseal dysplasia. His skeletal radiographs were suggestive of a lysosomal disorder (e.g., *dysostosis multiplex* was present) and showed the following: a decrease in intervertebral spaces specially in the thoracolumbar transition, a decrease in the T10 and T11 vertebrae, oval-shaped L1/L2 vertebrae, flattening

of the head of the humerus with an increase in the humerus-acromion distance, as well as bilateral alterations in the femoral head suggestive of Legg-Calvé-Perthes disease. His echocardiogram, complete ophthalmologic exam and abdominal echography were normal. In the physical exam, he presented with atypical facies; varus knees; range of motion restriction of shoulders as well as of wrist and knee; and absence of corneal clouding, claw hands, and hepatosplenomegaly (Figure 1). At the age of 14, his height was 149 cm ($p<5$); he attended regular school and did not present cognitive delay.

Based on the clinical findings, the diagnostic hypothesis initially raised was mucopolysaccharidosis type IV A or Morquio A syndrome (MPS IV-A, MIM#253000), which was ruled out due to the normal activity of N-acetylgalactosamine-6-sulfatase (EC 3.1.6.4) in leukocytes (Table 1). As the patient presented *dysostosis multiplex* at the X-ray, a biochemical investigation for lysosomal storage disorders was requested, which was suggestive of ML II/III (presence of an increased activity of lysosomal hydrolases in plasma and normal activity in leukocytes). As there were no fibroblasts available for biochemical analysis, the presence of phosphorylated residues of M6P in CI-MPR affinity column was investigated, which showed a low presence of those residues in plasma (Table 2) and eventually confirmed the diagnosis of ML II/III.

Due to the atypical picture of the patient and the fact that ML III shows locus heterogeneity, it was decided to perform whole NGS in this patient.

Whole next-generation sequencing

Peripheral blood was extracted using Easy DNA kit (Invitrogen). Whole genome sequencing was performed with Nextera Exome Capture system, followed by NGS with Illumina HiSeq2500 (Mendelics Genomic Analysis).

Whole NGS generated 64,363,166 sequences, each target base was read on average 92X and 94% of the target bases were read at least 10X. Two heterozygous variants in *GNPTAB* were identified: c.1208T>C (p.Ile403Thr), previously reported as deleterious [6], and the novel variant c.1723 G>A (p.Gly575Arg), which was not present in more than 8,000

normal controls (Exome Server Variant and 1,000 Genomes), including 1,000 Brazilians (processed as controls at the NGS laboratory). No pathogenic variants were found in the *GNPTG*, *NAGPA,* and *MBTPS* genes. This result was confirmed by Sanger sequencing, and a segregation study demonstrated that the father was a carrier of the variant c.1208T>C, and the mother, of the transversion c.1723 G>A.

PolyPhen-2 predicted as probably damaging both mutations (scores of 0.993 and 0.974, respectively). Sift v.1.1.3 prediction, however, confirmed as deleterious the first mutation (p.Ile403Thr) and pointed as tolerated/neutral the variant p.Gly575Arg. The MutPred software interpreted the pathogenicity as a confident hypothesis for p.Ile403Thr with score 0.886, supposedly causing the following: loss of stability (p = 0.0146), loss of catalytic residue at I403 (p = 0.0528), gain of sheet (p = 0.0827), gain of loop (p = 0.1069), and loss of ubiquitination at K401 (p = 0.1264). The same software interpreted the pathogenicity of p.Gly575Arg as an actionable hypothesis, with a probability score of deleterious mutation of 0.382, supposedly causing the following: gain of solvent accessibility (p = 0.0171), gain of helix (p = 0.0425), gain of relative solvent accessibility (p = 0.0479), loss of loop (p = 0.0512), and loss of methylation at K573 (p = 0.0536).

Discussion

The aim of this work was to evaluate the molecular diagnosis of ML III using whole NGS, and our results demonstrate the utility of this new technology.

The confirmation of the clinical diagnosis of ML II/III faces several challenges in regards to the performance of biochemical assays, since these assays are not widely available (nor easily done) and depend on the performance of fibroblast biopsy. The phosphotransferase assay, for instance, is radioactive, difficult to implement, and few groups in the world perform this technique.

Currently, biochemical diagnosis of ML II/III is usually performed indirectly, through the measurement of lysosomal hydrolases both in plasma (their activity should be high) and in fibroblasts (their activity should be low). When fibroblasts are not available, other tests are

needed (such as the research of phosphorylated residues, which is also performed in research basis, or DNA analysis only). Moreover, as occurred in the present case, the clinical picture can be so attenuated, sometimes limited to bone disease, that even experienced physicians miss its diagnosis.

Among the available technologies for DNA analysis, exome sequencing is one of the most modern tools available. It is important to point out that ML II/III presents locus heterogeneity and that Sanger sequencing of whole coding *GNPTAB* is made by amplification and sequencing of the 21 exons of the gene, in other words, a situation that requires time and investment and can delay the diagnosis. In this regard, exome sequencing is a reliable alternative for confirmation of the diagnosis of MLII/III.

However, the prediction of the pathogenicity of the rare missense variants found is still a problem. Different sequencing platforms vary in their ability to identify variants, even when sequencing the same genome. In the analysis by NGS, it is important to realize that the mixing of ethnic groups due to the exponential growth of the human population has favored the emergence of new rare variants that are not subject to natural selection. This fact complicates the interpretation of results in NGS [7]. Although the pathogenicity of mutation p.Gly575Arg has not been confirmed by all bioinformatics tools used, the following evidence suggests that it is the second mutation causing the clinical picture of the patient: 1) the patient presents an atypical/mild phenotype and is heterozygous for a mutation that is known to cause a mild phenotype (p.Ile403Thr) [6]; 2) no other mutations causing the phenotype were found in the *GNPTAB*, *GNPTG*, *NAGPA,* and *MBTPS* genes; 3) both mutations found are *in trans* and inherited; 4) p.Gly575Arg was not found in the relatively high number of analyzed controls from the same population as the patient.

We agree with Schrader *et al* [4] and reinforce the idea that the costs of NGS will gradually come down. As soon as this happens, this technique will become the most direct approach for the diagnosis of Mendelian disorders that are phenotypically and genetically heterogeneous, such as ML II/III.

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Table 1 – Mucolipidosis III and Next-Generation Sequencing: Biochemical characterization of patient.

Biochemical Investigation	Sample	Patient	Reference Values
α–L-Iduronidase (*EC* 3.2.1.76)	Plasma	119	6.8-13.7
β–Glucuronidase (*EC* 3.2.1.31)	Plasma	906	30-300
α–N-Acetylglucosaminidase (*EC* 3.2.1.50)	Plasma	875	34-162
α–Mannosidase (*EC* 3.2.1.24)	Plasma	2026	17-56
β– Hexosaminidases A (*E.C.*3.2.1.30)	Plasma	11747	550-1675
β– Hexosaminidases B (*E.C.*3.2.1.30)	Plasma	27535	265-1219
β–Hexosaminidases, total (*E.C.*3.2.1.30)	Plasma	39282	1000-2857
Chitotriosidase (*EC* 3.2.1.14)	Plasma	98	8.8-132
Iduronate 2-sulfatase (*EC* 3.1.6.12)	Plasma	1716	122-463
β–Galactosidase (*EC* 3.2.1.23)	Leukocyte	162	78-280
α–L-Iduronidase (*EC* 3.2.1.76)	Leukocyte	72	32-56
Arylsulfatase B (*EC* 3.1.6.12)	Leukocyte	-	72-176
β–Glucosidase (*EC* 3.2.1.21	Leukocyte	19	10-45
α–N-Acetylglucosaminidase (*EC* 3.2.1.50)	Leukocyte	64	68-352
Iduronate 2-sulfatase (*EC* 3.1.6.12)	Leukocyte	95	31-110
β–Glucuronidase (*EC* 3.2.1.31)	Leukocyte	175	23-151
Sphingomyelinase (*EC* 3.1.4.12)	Leukocyte	1.9	0.74-4.9
Total β–Hexosaminidases (*E.C.*3.2.1.30)	Leukocyte	7384	552-16662
β–hexosaminidase A	Leukocyte	53	150-390
N-acetylgalactosamine-6-sulfatase (EC 3.1.6.4)	Leukocyte	29	14-81
Dosage of GAGs	Urine	128 (79-256)	< 9 years:44-106 mg/L
Thin-layer chromatography of GAGs	Urine	DS+HS+CS/HS	Normal
Thin-layer chromatography of sialoligosaccharide	Urine	Normal	Normal

GAGs: Glycosaminoglycans; - : not available; DS: Dermatan sulfate; HS: Heparan sulfate; CS: Chrondoitin sulfate.

Table 2 – Mucolipidosis III and next-generation sequencing - Presence of phosphorylated residues of mannose-6-phosphate in CI-MPR affinity column

	Control 1	Control 2	Patient
α-Mannosidase	12.5	11.2	0.5
β-Glucuronidase	5.9	14.7	0.1
β-Hexosaminidase	7.7	14.3	0
β-Mannosidase	13.5	19.7	0.4

Figure 1: Male patient with mucolipidosis type III, aged 14 years old, presenting a milder phenotype. A – Absence of hand joint contractures. B – Joint contractures in shoulders, hindering arm elevation above the head; atypical facies with no noticeable infiltrate. C – Whole body posterior image. The obesity associated is evident.

4.5 Capítulo 5

Should neonatal hyperparathyroidism associated with mucolipidosis II/III be treated pharmacologically ?

Artigo publicado no ***Journal of Pediatric Endocrinology and Metabolism(2013)***

Letter to the Editor

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Should neonatal hyperparathyroidism associated with mucolipidosis II/III be treated pharmacologically?[a]

Keywords: Mucolipidosis type II; rickets; transient hyperparathyroidism; vitamin D treatment.

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To the Editor

A recent paper by Lin and Pitukcheewanont (1) published in this journal highlights the therapeutic potential of calcium and vitamin D in the treatment of rickets associated with mucolipidosis type II (ML II). As the authors did not raise the hypothesis that the laboratory findings indicative of rickets in their patients might be merely transient, we would like to report a case that illustrates this possibility.

Our patient was the firstborn son of a young, consanguineous couple from a tropical city in Brazil and has received follow-up at our service since age 14 months. He was born full-term, by cesarean section, with a weight of 2.705 kg, length of 46 cm, and head circumference of 33 cm. The prenatal period was unremarkable. There was no history of maternal vitamin D deficiency. At birth, the patient was diagnosed with a right-sided inguinal hernia, which was repaired at age 2 months. At age 8 months, a tentative diagnosis of mucopolysaccharidosis was considered due to coarse facial features, gingival hyperplasia, pectus carinatum, broad nasal bridge, and delayed psychomotor development. A comprehensive metabolic panel showed increased lysosomal hydrolase (beta-glucuronidase, alfa-mannosidase, and beta-hexosaminidase) activity in plasma and normal alfa-iduronidase, alfa-mannosidase, beta-galactosidase, beta-glucosidase, and beta-hexosaminidase, and iduronate sulfatase activity in white blood cells, as well as normal urine glycosaminoglycan levels. A diagnosis of ML II alpha/beta was later confirmed by sequencing of the *GNPTAB* gene (homozygous for the common mutation c.[3503_3504delTC]). At age 14 months, the patient was referred to our center in southern Brazil for clinical evaluation. Physical examination revealed low weight-for-age and height-for-age Z scores (<-2), coarse facial features, and neuromotor development consistent with the second trimester of life, as well as pectus carinatum, thoracolumbar gibbus deformity, prominent abdomen, no hepatosplenomegaly, slightly restricted joint range of motion (particularly of the elbow), genu varum, claw hands, and asymmetric lower extremities (left<right). Ophthalmological examination revealed no abnormalities of the cornea or lens; funduscopy result was normal. Skeletal radiographs were consistent with dysostosis multiplex, with reduced bone mineral density, brachycephaly, obliteration of the cranial sutures (except for the coronal suture), small sella turcica, relatively thickened long bones, underdevelopment of the distal phalanges of the second to fifth digits, widened ribs, protruding sternum, hypoplasia of the inferior portions of the ilia, and bilateral subluxation of the hip joint. There was marked bowing of the proximal

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Figure 1 Radiographic appearance of a patient with ML II (age 14 months). There is marked bowing of the proximal portion of the left femur.

end of the left femur, with angulation away from the femoral axis, consistent with an old, consolidated fracture (Figure 1). Serum markers of bone metabolism were within normal limits, apart from mildly elevated alkaline phosphatase (ALP) levels: parathyroid hormone (PTH) 32.3 pg/mL [reference values (RV) 15.0–65.0 pg/mL], phosphate 4.4 mg/dL (RR 2.7–4.5 mg/dL), total calcium 9.3 mg/dL (RR 8.6–10.0 mg/dL), alkaline phosphatase 324 U/L (RR <281 U/L), creatinine 0.3 mg/dL, and urea 23 mg/dL. Unfortunately, the measurement of vitamin D [25(OH)D_3] and of other bone turnover markers such as procollagen type I propeptide, bone alkaline phosphatase, and urinary collagen type 1 cross-linked N-telopeptide was not available at our hospital at that time.

The patient's radiographic findings were consistent with dysostosis multiplex, but some aspects – such as the apparent reduction in bone mineral density, the premature cranial suture closure, and the occurrence of fracture – could also suggest the co-existence of other diseases, such as rickets, hyperparathyroidism, or even child abuse. Furthermore, as there was no history of high-impact trauma or falls, the consolidated femoral fracture seen on skeletal radiographs may have occurred in the womb or been due to minor trauma in the first months of extrauterine life. Patients with ML II have been known to exhibit laboratory and radiographic changes suggesting hyperparathyroidism or rickets in the neonatal period (2); however, the laboratorial changes are believed to be transient and resolve spontaneously over the first few months of life (3–5). As there were no data on PTH, ALP, calcium, and phosphate levels during the first year of life in our patient, one may either assume that bone metabolism was never abnormal (which is less probable according to the literature) or that transient, self-limiting alterations occurred in early life. Otomo et al. (6) reported an interesting case of ML II showing decreased mineral density but normal PTH and suggested that apparent reduction in bone mineral density might be related to the elevated bone turnover caused by ML II itself instead of hyperparathyroidism; however, the first PTH evaluation of the patient by Otomo et al. (6) was performed at the age of 2 months, and the values found at that time were at the upper limit of normal range, so there is still the possibility that the patient had presented a transitory hyperparathyroidism.

The laboratory changes described by Lin and Pitukcheewanont (1) were detected in the neonatal period, and the authors attributed the normalization of PTH, vitamin D, calcium, phosphate, and ALP levels to oral vitamin D and calcium supplementation. However, according to the hypothesis of transient hyperparathyroidism in ML II, we believe this improvement may reflect the natural history of the disease rather than the results of vitamin supplementation. Of course, as noted by Lin and Pitukcheewanont (1), ML II and rickets may coexist, which would constitute an additional confounder for therapeutic decision-making. In order to better understand the elevated bone turnover associated with ML II, we suggest that every patient with diagnosis have the bone metabolism markers regularly monitored, especially during the neonatal period and infancy.

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4.6 Capítulo 6

Pitfalls in the prenatal diagnosis of mucolipidosis II alpha/beta: A case report

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Meta Gene

Pitfalls in the prenatal diagnosis of mucolipidosis II alpha/beta: A case report

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ABSTRACT

Mucolipidosis II alpha/beta is an autosomal recessive disorder caused by deficient activity of GlcNAc-1-phosphotransferase. We report the prenatal diagnosis of a fetus who was found to exhibit normal levels of lysosomal enzymes in the amniotic fluid but low levels in amniocytes, and who was found to be heterozygous for the most common *GNPTAB* mutation. As in some carriers of Mucolipidosis II biochemical abnormalities may hinder prenatal diagnosis, we suggest DNA analysis should be performed whenever possible.

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Introduction

Mucolipidosis type II alpha/beta (ML II alpha/beta; OMIM# 252500) is a rare autosomal recessive inborn error of metabolism caused by reduced activity of *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase; EC 2.7.8.17) due to mutations in the *GNPTAB* gene (NG021243; GeneID 79158). GlcNAc-1-phosphotransferease catalyzes the first step in the generation of mannose 6-phosphate recognition marker required for efficient targeting of soluble lysosomal enzymes to lysosomes. Defects in GlcNAc-1-phosphotransferease lead to missorting of several lysosomal enzymes and the lysosomal accumulation of non-degraded macromolecules (Reitman and Kornfeld, 1981). The microscopic pathological findings in these defective cells are cytoplasmic inclusions formed by accumulation of several macromolecules, which culminate in a typical phenotype characterized by coarse facial features, gingival hypertrophy, *dysostosis multiplex*, joint contractures, and death at an early age (Kornfeld and Sly, 2001). There is no specific treatment available for ML II alpha/beta yet, although hematopoietic-cell transplantation can be tried in very early diagnosed cases (Leroy et al., 2008). As expected for autosomal recessive disorders, individuals heterozygous for ML II alpha/beta are not clinically affected, but can exhibit intermediate biochemical abnormalities (Kornfeld and Sly, 2001; Varki et al., 1982; Cathey et al., 2008; Leroy and DeMars, 1967).

Clinical suspicion is the first step in the diagnosis of ML II alpha/beta. Definitive diagnosis depends on demonstration of low GlcNAc-1-phosphotransferase activity using the radioactive [^{32}P]UDP-GlcNAc substrate (Reitman and Kornfeld, 1981). However, as this assay is available in only very few laboratories, an indirect diagnosis is usually established by measurement of lysosomal hydrolases both in mesenchymal cells, where their levels should be low, and their surrounding extracellular environment, where their levels should be high (Kornfeld and Sly, 2001). Although no further steps are usually required and *GNPTAB* gene analysis is generally not necessary to confirm the diagnosis, DNA tests are useful when biochemical testing is inconclusive or to diagnose carriers of the condition (Leroy et al., 2008).

Prenatal diagnosis of ML II alpha/beta can be established by the same biochemical approach, assessing fetal material obtained from chorionic villus biopsy, amniocentesis, or cordocentesis (Kornfeld and Sly, 2001). Likewise, DNA can be extracted and isolated from these fetal materials and analyzed as in the postnatal period, although this prenatal DNA-based approach requires additional information about the parental genotype or the flow/nature of disease-causing alleles in the proband's pedigree (Leroy et al., 2008). There is no report of prenatal diagnosis of ML II using cell free fetal DNA in maternal blood.

Although available in clinical and research grounds (Leroy et al., 2008), fetal DNA analysis of *GNPTAB* has not been described in the literature yet. This is the first reported prenatal identification of a fetus heterozygous for ML II alpha/beta.

Methods

This study, named "Comprehensive study on the Mucolipidosis II and III in Brazil: an opportunity for understanding the genetic processes that control intracellular trafficking of proteins" was approved by the local Ethics Committee (code GPPG 07.244) and the parents signed an informed consent form.

The biochemical and DNA analysis reported herein were performed at the laboratory of the Medical Genetics Service-Hospital de Clínicas de Porto Alegre, Brazil (LRIEM-HCPA), which is an international reference center for diagnosis of lysosomal disorders. The panel of lysosomal enzymes evaluated in the pre and postnatal periods were chosen according to the experience and protocols available at LRIEM-HCPA at the time of the investigation, as well as to the cells/tissue availability for investigation (Burin et al., 2004). Due to the above reasons, the enzymes evaluated in both periods were not the same (Table 1).

The DNA analysis was performed using genomic DNA extracted from leukocytes (first child) and cultured amniocytes (second child). *GNPTAB* analysis in the first child was performed according to Cury et al. (2013), and included the sequencing of all *GNPTAB* exons and exon–intron boundaries. In the second child, only the whole exon 19 was sequenced. Placental tissue was collected at birth of the second child, in order to have the lysosomal enzymes assayed and the glycosaminoglycans (GAGs) measured. It was not possible to assay the enzymes due to the storage conditions, but placental GAGs were extracted and measured using the Dymethyl Blue technique.

Table 1
Mucolipidosis II alpha/beta: biochemical panel of selected lysosomal hydrolases assessed in both siblings.

Enzyme	Enzyme activity (RV)					
	First child (affected)			Second child (heterozygous)		
	Postnatal			Prenatal		Postnatal[a]
	Blood plasma[b]	Leukocytes[c]	Cultured fibroblasts[c]	Amniotic fluid supernatant[b]	Cultured amniocytes[c]	Blood plasma[b]
Total hexosaminidase	27,427 (1000–2875)	NP	696 (3000–13,490)	NP	2318 (2631–2622)	1401 (1000–2857)
β-Glucuronidase	875 (30–300)	196 (23–151)	23 (62–361)	72 (26–86)	97 (40–254)	NP
α-Mannosidase	2640 (17–56)	127 (60–400)	4.6 (93–359)	14 (1.2–21)	NP	59 (17–56)
β-Galactosidase	NP	78 (78–280)	6.5 (394–1440)	NP	314 (521–1783)	NP
α-L-Iduronidase	NP	23 (32–56)	3.4 (74–148)	NP	29 (92–264)	52 (6.8–13.3)

RV, reference values.
[a] Only plasma sample was available.
[b] nmol/h/mL.
[c] nmol/h/mg of protein.

Case report

The case reported herein is the second child of a young consanguineous couple. The firstborn male was diagnosed with ML II alpha/beta at age 1 year due to neurologic and psychomotor impairment, typical coarse facial features, gingival hypertrophy, kyphosis, *pectus carinatum*, and joint contractures, and subsequently died at age 25 month (Table 1). DNA analysis revealed the homozygous *GNPTAB* mutation c.3503_3504delTC at exon 19, which is the most common ML II alpha/beta-causing mutation worldwide. Unfortunately, there was no parental DNA available to confirm the analysis.

The male and female progenitors were 31 and 36 years old, respectively, at the second conception. Prenatal obstetric ultrasound showed normal nuchal translucency, skeletal development and other fetal characteristics. At 16 weeks of pregnancy, amniocentesis was performed for biochemical and DNA testing, aiming at a very early performance of hematopoietic stem-cell transplantation, if the fetus was affected. The biochemical investigation (Table 1) showed inconclusive results, since the levels β-glucuronidase and α-mannosidase were within the normal range in the amniotic fluid (as expected for non-affected fetus), but in amniocytes only the level of β-glucuronidase was normal (as expected for non-affected fetus) while the levels of total hexosaminidase, β-galactosidase and α-iduronidase were below the normal range values (as expected for an affected fetus). DNA analysis showed the fetus was heterozygous for the c.3503_3504delTC mutation. The child was delivered at 39 weeks of pregnancy, weighing 3670 g, measuring 50 cm and normal physical examination. In the postnatal period, biochemical testing was conducted only in plasma and showed slight increases in α-L-iduronidase and α-mannosidase activities, as well as normal levels of total hexosaminidase. Placental GAG levels were 69 μg GAG/mg of protein, within the normal range (41–78 μg/mg). No further cells were available for additional analysis (Table 1).

Discussion/conclusions

To the best of our knowledge, this is the first reported prenatal investigation of ML II alpha/beta by a DNA-based approach and the first prenatal statement of ML II alpha/beta heterozygosity described in the literature as well. Reports of prenatal diagnosis of ML II alpha/beta are scarce and usually based on abnormal sonographic findings such as fetal hydrops, bone dysplasia and growth restriction (Heo et al., 2012; Chen et al., 2010; Yuksel et al., 2007; Saul et al., 2005; Carey et al., 1999; Ben-Yoseph et al., 1988). The case reported by Ben-Yoseph et al. (1988) was found to have heterozygote levels of GlcNAc-1-phosphotransferase in chorionic villi, but DNA analysis was not yet available at that time.

This case report corroborates the concept that biochemical diagnosis of ML II in the prenatal period can sometimes be inaccurate, as heterozygotes may exhibit intermediate activity of GlcNAc-1-phosphotransferase and, hence, milder abnormalities in lysosomal enzyme activities (Kornfeld and Sly, 2001; Leroy and DeMars,

1967; Cathey et al., 2010; Varki et al., 1982). However, as expected for "true" autosomal recessive disorders, this intermediate activity is not sufficient to cause storage of the substrates of the lysosomal hydrolases (e.g., GAGs) without clinical compromise.

In the present report, three out of the 4 enzymes evaluated in amniocytes showed low activities, but both the two enzymes evaluated in the amniotic fluid showed normal values, which suggests that intracellular levels of lysosomal enzymes can be more frequently abnormal than extracellular ones in fetuses heterozygous for ML II alpha/beta.

Prenatal diagnosis of autosomal recessive diseases is usually associated with some ethical dilemmas such as the possibility of identification of a heterozygous fetus that will never become symptomatic. However, if the fetus is affected, it could enable not only an early diagnosis but also an early treatment (e.g. hematopoietic stem-cell transplantation). Therefore, since this case demonstrates why biochemical analyses alone cannot be sufficient for prenatal diagnoses, we suggest DNA analysis should be performed whenever possible.

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5. DISCUSSÃO

As ML II e III são doenças raras com diversos aspectos a serem esclarecidos. Nesta tese, buscamos explorar aspectos desde o diagnóstico até características clínicas, passando por exploração de novas áreas como a pigmentação.

O diagnóstico bioquímico “indireto” (medida da atividade das enzimas lisossômicas em plasma e fibroblastos) não é o padrão-ouro; entretanto é o melhor disponível em nosso meio, Devido a questões práticas de assistência e falta de evidências na literatura (Brooks *et al,* 2007, Kornfeld & Sly,2010, Leroy & Cathey, 2012 ab), propusemos um protocolo diagnóstico baseado em revisão dos dados existentes em nossa coorte. Criamos um sistema de pontuação inédito visando a objetivar as alterações encontradas e facilitar a tomada de decisão, criando um protocolo efetivo e com menor custo possível. Com a evolução do diagnóstico molecular, alguns pontos poderão ser discutidos como, por exemplo, a necessidade de proceder com biópsia de pele e cultura de fibroblastos em todos os pacientes, visto que este procedimento é invasivo e o cultivo de fibroblastos ser delicado. Assim, neste momento e na realidade do nosso país, o protocolo bioquímico “indireto” é útil e facilita a assistência aos pacientes.

Embora existam relatos sobre as características clínicas das Mls, a maioria das evidência provém de relatos de casos ou séries com número pequeno de pacientes. A maior coorte foi de Cathey *et.al,* publicada em 2010, incluindo 61 probandos, proveniente de diversos países. O nosso estudo incluiu 27 pacientes provenientes de um único país, um número considerado razoável para uma doença tão rara. Conseguimos explorar diversos aspectos e sistemas afetados, mas, infelizmente, como parte da coleta de dados foi retrospectiva, muitas informações não estavam disponíveis. Nosso estudo foi

transversal, permitindo diversas inferências, entretanto a forma ideal de estudar estes pacientes seria através de uma coorte prospectiva, ideia que faz parte das nossas perspectivas futuras. Em nossa amostra houve predominância da ML II, o que difere um pouco da literatura, já que alguns autores sugerem frequência semelhante de ambos os tipos Um fator de confusão que pode existir é o fato da ML II ser clinicamente mais grave, aumentando a busca por diagnóstico e, consequentemente, o número de indivíduos diagnosticados. Outro ponto que exploramos foi a capacidade funcional e a restrição articular aferida por goniometria; como a ML II e III possuem estes fatores alterados, o uso de tais ferramentas pode intensificar a assistência através da mensuração objetiva das dificuldades e proporcionando auxílio focado para cada indivíduo.

Através das observações clínicas, pudemos estabelecer hipóteses como alterações na melanogênese refletindo possíveis alterações na pigmentação de olhos, pele e cabelos. Exploramos esta hipótese em um pequeno estudo clínico comparativo com controles e com genotipagem para polimorfismos reconhecidamente ligados à pigmentação em humanos. Observamos algumas alterações no estudo molecular, sugerindo que os pacientes como ML II e III realmente possuem alterações. Entretanto, idealmente deveríamos possuir controles pareados para amostra, visando a confirmar os achados que obtivemos. Outros polimorfismos também poderiam ser estudados, visto que a herança de pigmentação é algo complexo e que envolve diversas rotas.

O entendimento da ML II e III ainda não é completo e vem crescendo gradativamente, entretanto hoje ainda estamos distantes de tratamento específico para estes pacientes. Entretanto dados clínicos e o profundo entendimento da apresentação da doença são necessários para proporcionar assistência de qualidade a estes indivíduos. Ainda não existe consenso sobre o seguimento a longo prazo, como quais

exames e com qual regularidade deveriam ser realizados, quais tratamentos paliativos poderiam ser aplicados, além de formalizar recomendações como a sugestão de outros profissionais a se envolverem no processo (fonoaudiólogo, fisioterapeuta, terapeuta ocupacional). A aplicação prática dos nossos dados estende-se desde a assistência aos pacientes até aplicação em pesquisa, com várias lacunas a serem preenchidas.

6.CONCLUSÃO

As conclusões da presente tese estão abaixo relacionadas, de acordo com os objetivos inicialmente propostos.

Objetivos primário:

1. Caracterizar aspectos clínicos de uma amostra de pacientes brasileiros com ML II e III.

Objetivo secundário

2. Estudar as diferenças clínicas entre os subtipos de mucolipidose III (gama e alfa/beta);

Conclusões:

a) há maior frequência relativa dos pacientes ML II no Brasil;
b) os pacientes com ML II apresentam comprometimento somático e cognitivo mais grave e precoce que os pacientes com ML III;
c) os pacientes com ML III alfa/beta apresentam comprometimento somático maior que ML III gama e atraso do desenvolvimento apenas nos primeiros anos de vida;
d) o diagnóstico das ML II e III ocorre na infância, embora os sintomas surjam precocemente;
e) os pacientes ML II apresentam hiperparatireoidismo transitório no início da infância, e não existe evidência suficiente para o tratamento dessa complicação.
f)) o sequenciamento de nova geração (NGS) pode ser utilizado como método diagnóstico de ML II/III alfa/beta.

g) a genotipagem dos pacientes com ML II e III é importante para aconselhamento genético e diagnóstico pré-natal.

Objetivos primários:

2. **Caracterizar o fenótipo bioquímico de pacientes brasileiros com ML II e III, visando a elaboração de um protocolo laboratorial custo-efetivo para o diagnóstico das ML.**

   Conclusão: Nos pacientes com MLII/III da nossa amostra, houve aumento das enzimas lisossomais em plasma, com exceção da quitotriosidase; em fibroblastos apenas a atividade da beta-glicosidase foi normal, com atividade das demais enzimas reduzidas. Nossos dados sugerem diferenças no fenótipo bioquímico de ML II e III: apesar de dentro dos valores de referência, a atividade da alfa-N-acetilglicosaminidase em plasma foram mais elevados e da beta-glicosidase mais reduzidas em fibroblastos na ML II. As enzimas com maior diferença em relação à normalidade e com maior pontuação alfa-mannosidase (29 pontos), allfa–L-Iduronidase (28 pontos), beta-hexosaminidase total (27 pontoa), beta-glicuronidase (26 pontos), and α–N-acetilglicosaminidase (25 pontos). Sugerimos como painel de diagnóstico bioquímico indireto das ML II/III a pesquisa em plasma e fibroblastos de pelo menos três das enzimas supracitadas.

Objetivos secundários:

3. **Classificar os fototipos dos pacientes com ML e compará-los com seus pais e irmãos e com controles (indivíduos saudáveis), a fim de ser detectada possível tendência de hipomelanogênese;**
4. **Estudar a associação entre a cor dos olhos, cabelos e pele e os seguintes SNPs: [rs1126809 (*TYR* gene), rs16891982 (*SLC45A2* gene), rs1426654 (*SLC24A5* gene) and rs1129038 (*HERC2* gene)]**

Conclusão: A maioria dos pacientes apresentou fototipo I–III em taxa discrepante do fototipo de seus pais e de controles saudáveis comparados aos próprios pais. Observamos discrepância do genótipo-fenótipo, para rs1126809, rs16891982 e rs1426654 com características de olhos, cabelos e pele mais claro do que esperado e características mais escuras para rs16891982 (olhos, cabelos e pele), rs1426654 (cabelo e pele) e rs1129038 (olhos) . Nossos dados evidenciam que pode haver alteração da melanogênese em ML II/III, mas que estudos adicionais, com maior número de pacientes, são necessários para confirmar estes achados.

7. PERSPECTIVAS

A realização deste projeto permitiu compreender a importância do desenvolvimento, pelo nosso grupo de pesquisa, de novos projetos sobre ML II e III que tenham como objetivos os abaixo relacionados:

- Caracterização da pigmentação dos pacientes através de outros métodos.
- Comparação de frequência dos SNPs relacionados à pigmentação em controles pareados por idade e sexo. -Avaliar os melanossomos de pacientes com ML II e III por microscopia eletrônica.
- Avaliação da qualidade de vida em pacientes ML II e III e seus cuidadores através de instrumentos validados para mensuração objetiva da qualidade de vida.
- Acompanhamento da coorte de pacientes inclusa no presente estudo para maior entendimento da história natural da ML II e III, bem como as diferenças entre elas.

8. CONSIDERAÇÕES ÉTICAS

Este projeto é englobado por um estudo maior que foi encontra aprovado pela Comissão Científica e de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós Graduação do HCPA (projeto GPPG 07-244). Além disso o projeto complementar "Manifestações dermatológicas em pacientes com mucolipidose tipos II e III: um estudo controlado com ênfase no fototipo e nas alterações das hastes capilares" (projeto GPPG 12-0285) foi elaborado durante esta tese para estudos relacionados à pigmentação e igualmente aprovado pela Comissão Científica e de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós Graduação do HCPA. Em relação ao armazenamento de materiais ou uso de materiais armazenados em pesquisas anteriores, este estudo seguirá todas as normas pertinentes, sendo que a responsável pela guarda e pela autorização de uso de material será a Dra. Ida Vanessa D. Schwartz.

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ANEXO I

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: **ESTUDO ABRANGENTE SOBRE AS MUCOLIPIDOSES II E III NO BRASIL: UMA OPORTUNIDADE PARA A COMPREENSÃO DOS PROCESSOS GENÉTICOS QUE CONTROLAM O TRÁFEGO INTRACELULAR DE PROTEÍNAS**

Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Porto Alegre-RS. Tel: Tel: (51) 3308-6722

Paciente: _____

Prezado paciente ou responsável,

Esta pesquisa tem por objetivo a obtenção de informações relativas aos sintomas de pessoas com Mucolipidose II ou Mucolipidose III, às alterações presentes em suas enzimas e às alterações presentes no gene que ocasiona estas doenças. Estas informações serão obtidas por meio de entrevistas com você e/ou com os seus médicos, e mediante consulta a seu prontuário.

Caso a dosagem de todas as enzimas que podem estar alteradas na Mucolipidose II ou na Mucolipidose III não tenha sido ainda realizada em você, será necessário que você (paciente) seja submetido à coleta de sangue (10 mL para a análise das enzimas que podem estar alteradas em sangue) e realização de biópsia de pele (para a análise das enzimas que podem estar alteradas na pele). Caso a análise dos genes envolvidos na Mucolipidose II e na Mucolipidose III não tenha ainda sido realizada em você (paciente), também será necessária a coleta adicional de 05 mL de sangue. Você pode concordar ou não com a realização destes exames. Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). A biópsia de pele deverá ser realizada em condições higiênicas e sob anestesia local; o fragmento a ser retirado é pequeno (em torno de 0,3 cm) e superficial, não sendo, portanto, necessária a realização de pontos. O

desconforto e os riscos associados a estas avaliações serão minimizados pela realização da coleta por profissional treinado. Se você permitir, o material coletado, e que restar após a realização dos exames previstos neste estudo, será armazenado e utilizado em estudos futuros. Em relação a estas coletas, você declara que autorizou a coleta de (marcar com um X):

( ) 5 mL de sangue para a análise do gene envolvido nas Mucolipidoses II e III

( ) 10 mL de sangue para a análise das enzimas

( ) biópsia de pele para análise das enzimas

( ) eu não autorizei nenhuma das coletas acima relacionadas

Em relação ao armazenamento e utilização de algum material (sangue ou pele) que tenha restado após a realização dos exames previstos neste estudo, você declara que autorizou (marcar com um X):

( ) que este material poderá ser armazenado e poderá vir a ser utilizado em estudos futuros, desde que você revise e assine o termo de consentimento de tais estudos futuros.

( ) que este material não poderá ser armazenado e não poderá vir a ser utilizado em estudos futuros. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado.

Cabe salientar que as Mucolipidoses II e III não possuem, ainda, tratamento eficaz, e que esta pesquisa não tem como finalidade imediata a garantia de uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento destas doenças, o qual, no futuro, pode levar ao desenvolvimento de uma terapia mais efetiva. A identificação das mutações (alterações do DNA) presentes no gene que ocasiona estas doenças poderá ser importante para o aconselhamento genético da sua família e para o diagnóstico pré-natal. Não existe um prazo exato ou estipulado para que você receba os resultados dos exames realizados nesta pesquisa, mas estes lhes serão informados assim que estiverem

disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Departamento de Genética da Universidade Federal do Rio Grande do Sul.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

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RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

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Pelo presente termo, você declara que foi informado (a), de forma clara e detalhada, sobre a presente pesquisa, e que teve suas dúvidas esclarecidas por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira pela participação no estudo. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que recebeu cópia deste termo de consentimento.

Data: ___/___/_____

Paciente:_____

Responsável legal: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

Data: ____/_____/_____

Nome:_____

ANEXO II

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
Controles - crianças saudáveis

Projeto: **MANIFESTAÇÕES DERMATOLÓGICAS EM PACIENTES COM MUCOLIPIDOSE TIPOS II E III: UM ESTUDO CONTROLADO COM ÊNFASE NO FOTOTIPO E NAS ALTERAÇÕES DAS HASTES CAPILARES.**

Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Porto Alegre-RS. Tel: (51) 3308-6722

Nome do Voluntário: _____

Você está sendo convidado(a) a participar de um estudo clínico: MANIFESTAÇÕES DERMATOLÓGICAS EM PACIENTES COM MUCOLIPIDOSE TIPOS II E III: UM ESTUDO CONTROLADO COM ÊNFASE NO FOTOTIPO E NAS ALTERAÇÕES DAS HASTES CAPILARES. Esta pesquisa tem por objetivo a obtenção de informações sobre os sintomas de pessoas com Mucolipidose II ou Mucolipidose III e as alterações que causam estas doenças.

As mucolipidoses II e III são doenças genéticas raras e algumas das suas características ainda não foram bem estudadas, dentre as quais podemos citar as alterações de pele e de cabelo. Os dados dos pacientes com mucolipidose II e III serão comparados com o de pessoas de mesma idade e sexo sem a doença, como no seu caso. Além disso as características da pele serão comparadas à dos pais, tentando identificar diferenças que possam ser ocasionadas pela mucolipidose. Esses são o que chamamos de controles do estudo. Você está sendo convidado a participar desta pesquisa, porque gostaríamos de comparar o resultado do exame dos pacientes com mucolipidose aos pacientes com pessoas que não sejam portadores de uma doença genética, ou seja, ao seu exame ou do seu filho, para avaliarmos o que pode ser diferente das pessoas ditas saudáveis.

Sua participação envolverá uma anamnese e um exame clínico para avaliar a cor da pele e outras situações que possam vir a modificar tal coloração (como exposição solar, tabagismo e consumo de álcool). Durante o exame poderá ser requisitado que você mostre a parte interna do seu braço, uma vez que esta região está menos exposta ao sol. Além disso, se você concordar, cortaremos uma pequena mecha do seu cabelo, na parte posterior da cabeça.

Os procedimentos acima descritos não envolvem riscos de vida. Você pode sentir-se desconfortável ao ser examinado ou por responder perguntas em relação aos seus hábitos de vida (como tabagismo e consumo de álcool). Poderá haver pequeno desconforto durante

a coleta de cabelo, a alteração estética será minimizada coletando-se na parte posterior da cabeça,de modo que fique pouco visível. Você e sua família não terão nenhum benefício direto com a participação neste estudo como voluntário. O benefício que este estudo pode trazer é a contribuição para um melhor entendimento das mucolipidoses tipos II e III, o que, no futuro, pode levar ao desenvolvimento de uma terapia mais efetiva.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Departamento de Genética da Universidade Federal do Rio Grande do Sul (Tel: (51) 3308-6722).

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Data: ___/___/_____
Paciente:_____
Responsável legal: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

ANEXO III

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
Controles - crianças saudáveis

Projeto: **MANIFESTAÇÕES DERMATOLÓGICAS EM PACIENTES COM MUCOLIPIDOSE TIPOS II E III: UM ESTUDO CONTROLADO COM ÊNFASE NO FOTOTIPO E NAS ALTERAÇÕES DAS HASTES CAPILARES.**

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a coleta de cabelo, a alteração estética será minimizada coletando-se na parte posterior da cabeça,de modo que fique pouco visível. Você e sua família não terão nenhum benefício direto com a participação neste estudo como voluntário. O benefício que este estudo pode trazer é a contribuição para um melhor entendimento das mucolipidoses tipos II e III, o que, no futuro, pode levar ao desenvolvimento de uma terapia mais efetiva.

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Data: ___/___/_____
Paciente:_____
Responsável legal: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

ANEXO IV

FICHA DE AVALIAÇÃO CLÍNICA
Projeto ESTUDO ABRANGENTE SOBRE AS MUCOLIPIDOSES II E III NO BRASIL: UMA OPORTUNIDADE PARA A COMPREENSÃO DOS PROCESSOS GENÉTICOS QUE CONTROLAM O TRÁFEGO INTRACELULAR DE PROTEÍNAS

Ficha LEIM-HCPA número_____

Paciente: _____
Data de nascimento : _____/_____/_____

Sexo: ( ) masculino
( ) feminino

Naturalidade: _____

Médico assistente: _____
Endereço:_____
_____

Dados do nascimento:
-tipo de parto: _____
-peso: _____
-comprimento: _____
-perímetro cefálico: _____
-relato de manchas mongólicas extensas? ( ) sim ( )não
- complicações pré-natais: ( )sim ( ) não
_____
_____
- complicações durante o parto: ( )sim ( ) não
_____
_____
- complicações no período neonatal: ( )sim ( ) não
_____
_____

-outros:
_____
_____
_____
_____
_____

História familial (construir heredograma no verso):

-Consangüinidade parental: ( )sim ( ) não ( ) não informada

-Outros afetados na família?

( ) Sim. Número e grau parentesco:

_____
_____
_____ ( ) Não

( ) Não informado

Idade de início da sintomatologia: _____

Manifestações clínicas iniciais:

_____
_____
_____

Desenvolvimento neuropsicomotor:

	idade de aquisição	idade de perda
sustento cefálico		
sentar sem apoio		
caminhar sem apoio		
controle esfíncter vesical (diurno e noturno)		
controle esfíncter anal (diurno e noturno)		
palavras com no mínimo duas sílabas		
formar frases		

Escolaridade:
_____
_____

Internações hospitalares (data, motivo):
_____
_____
_____
_____
_____
_____

Procedimentos cirúrgicos (data, procedimento, intercorrências):
_____
_____
_____
_____
_____
_____

Tem síndrome da apnéia obstrutiva do sono? fez algum estudo específico para avaliar a presença desta complicação? (por exemplo, polissonografia)
_____
_____
_____
_____
_____

Tem ou teve crises convulsivas?
_____
Idade do primeiro episódio: _____

Fez avaliação oftalmológica? Resultados.
_____
_____
_____
_____
_____

Fez avaliação cardiológica? Resultados.

_____
_____
_____
_____
_____

Fez avaliação radiológica? Resultados.

_____
_____
_____
_____
_____
_____

Fez avaliação do metabolismo ósseo? Resultados.

_____
_____
_____
_____
_____

Fez avaliação para transplante de medula óssea? Resultados.

_____
_____
_____
_____
_____

Tem hipoacusia? Fez alguma avaliação específica para avaliar a presença desta complicação? Resultados.

_____
_____
_____

Faz uso de alguma medicação? Qual?

_____
_____
_____
_____
_____

Cpap/Bipap: ( ) sim ( ) não

Prótese auditiva: ( ) sim ( ) não

Último exame físico:
-data: _____
-idade: _____
-peso: _____
-comprimento: _____
-perímetro cefálico: _____
-opacificação de córnea? _____
-lesões papulares na pele? _____
-contraturas articulares? _____
-giba toracolombar? _____
-hepatomegalia? _____
-esplenomegalia? _____
-outros:
_____
_____
_____
_____
_____
_____

-Desenvolvimento neuropsicomotor:
_____
_____
_____
_____

Cognição:_____
_____
_____

Outras informações clínicas disponíveis/exames complementares:
_____
_____
_____
_____
_____
_____
_____
_____
___

Data de início da investigação bioquímica: _____

Data do diagnóstico laboratorial de ML II ou III: _____

Genótipo (gene *GNTPAB*): ( ) sim. Qual?_____
( ) não

Óbito: ( ) sim. Idade:_____
Causa:_____
( ) não

Investigação laboratorial realizada (plasma/leucócitos/fibroblastos):

Data	Atividade enzima	Tecido	Valor encontrado	Valor de referência
	Acetil-CoA glicosaminide N-acetiltransferase	Leucócitos		
	□-fucosidase	Leucócitos		
	□-galactosidase A	Plasma		
		Leucócitos		
	□-galactosidase B	Plasma		
		Leucócitos		
	□-glicosidase	Fibroblastos		
	□-L-iduronidase	Plasma		
		Leucócitos		
		Fibroblastos		
	□-manosidase	Plasma		
		Leucócitos		

		Fibroblastos		
	□-N-acetilglicosaminidase	Plasma		
	Arilsulfatase A	Plasma		
		Leucócitos		
		Fibroblastos		
	Arilsulfatase B	Leucócitos		
		Fibroblastos		
	□-galactosidase	Leucócitos		
		Fibroblastos		
	□-glicosidase	Leucócitos		
		Fibroblastos		
	□-glicuronidase	Plasma		
		Leucócitos		
		Fibroblastos		

	□-hexosaminidase A	Plasma		
		Leucócitos		
		Fibroblastos		
	□-hexosaminidase B	Plasma		
		Leucócitos		
		Fibroblastos		
	□-manosidase	Leucócitos		
		Fibroblastos		
	Esfingomielinase	Leucócitos		
		Fibroblastos		
	Galactocerebrosidase	Leucócitos		
		Fibroblastos		
	Galactose 6-sulfato-sulfatase	Leucócitos		
		Fibroblastos		

	GlcNAc-fosfotransferase	Fibroblastos		
	Heparan sulfamidase	Leucócitos		
		Fibroblastos		
	Iduronato-sulfatase	Plasma		
		Leucócitos		
	N-acetilglicosamina-6-sulfatase	Leucócitos		
		Leucócitos		
		Fibroblastos		
	Neuraminidase	Fibroblastos		
	Quitotriosidase	Plasma		

Exames realizados (urina) :

Data	Exame	Valor encontrado	Valor normal

	Dosagem glicosamimoglicanos		
	Cromatografia glicosaminoglicanos		
	Cromatografia de sialo-oligossacarídeos		

Exames realizados (papel-filtro) :

Data	Exame	Valor encontrado	Valor normal

ANEXO V

PROTOCOLO DE EXAME DERMATOLÓGICO – MUCOLIPIDOSE

1. Anamnese

Nome_____
DN:_____ Data do exame:_____ Idade ao exame:_____
Tipo de ML:_____ Mutação:_____
Local de Residência: _____
Local onde residiu por maior tempo: _____
Etnia: _____

Ascendência materna:_____
Ascendência paterna:_____

2. Fototipo (tabela anexa)

Se o exame for realizado <3 anos de idade, deve ser repetido após este período
Facilidade para queimadura solar: _____
Cor da pele:_____
Cor dos olhos e do cabelo:_____
Estação do ano em que foi realizado o exame:_____
Utiliza protetor solar? __ não __sim: FPS_____. Frequência: ____diariamente/ _____vezes por semana/ _____somente quando vai à praia ou piscina.
Exposição solar: costuma expor-se ao sol?
___não(ou até 20min por dia, antes das 11h ou depois 16h)
___ sim: tempo médio de exposição por dia: _____

2.1 Fototipo Pais e Irmãos:

2.1.1 Pai
Fototipo:_____
Tabagismo? ___não ___ sim: _____cigarros/dia
Consumo de álcool? ___não ___ sim: _____doses/dia (1 dose= 1 copo cerveja ou vinho)
2.1.2 Mãe
Fototipo:_____
Tabagismo? ___não ___ sim: _____cigarros/dia
Consumo de álcool? ___não ___ sim: _____doses/dia (1 dose= 1 copo cerveja ou vinho)
2.1.3 Irmãos
Fototipo Irmãos e idade:_____
_____
_____
_____
Tabagismo? ___não ___ sim: _____cigarros/dia
Consumo de álcool? ___não ___ sim: _____doses/dia (1 dose= 1 copo cerveja ou vinho)

3. Exame físico

3.1 Pele
Textura:_____
Espessura: _____
Elasticidade: _____
Mobilidade: _____
Turgor: _____

3.1 Manchas

Mongólicas: presentes ao nascimento? ___sim ___ não. Persistem?___sim ___ não.
Locais:_____
Outras (hipo ou hipercrômicas):_____

_____
_____
_____

3.2 Cabelo

Implantação:_____
_____
Distribuição:_____
_____
Área de perda de cabelo?(se sim, local)_____
_____
Quantidade: _____
_____
Cor: __preto__ castanho escuro__ castanho claro__louro __ruivo ___outro_____
Brilho: _____
_____
Espessura: _____
_____
Teste de tração: ___positivo ____negativo. Se positivo, coletar cabelo com bulbo

□Coleta de cabelo para tricoscopia
Resultado de exames complementares:_____
_____
_____
_____
_____
_____
_____
_____

3.3 Unhas

Forma e configuração, implantação, espessura, superfície, consistência, brilho e coloração.
_____
_____

3.4 Pêlos

Espessura, brilho, consistência, comprimento, distribuição, quantidade:_____
_____

4. Sudorese

Descrição subjetiva dos pais:_____

ANEXO VI

ENZIMAS LISOSSÔMICAS A SEREM PESQUISADAS PARA O DIAGNÓSTICO BIOQUÍMICO DAS MUCOLIPIDOSES TIPOS II E III NO LEIM-HCPA

Enzima lisossômica	Doença lisossômica associada	Tecido (valor de normalidade da atividade) - LEIM-HCPA	Referência
Alfa-fucosidase	alfa-fucosidose	Leucócitos: 40 - 140 nmoles/h/mg proteína Fibroblastos: 46 – 221 nmoles/h/mg proteína	LEIM-HCPA, SGM-HCPA
Alfa-galactosidase A	Doença de FAbry	Plasma: 8,9 – 39 nmoles/h/mL Leucócitos: 78 – 170 nmoles/h/mg proteína	DESNICK RJ *et al* (1973) MORGAN *et al* (1990)
Alfa--galactosidase B	Doença de Schindler	Plasma: 18 – 47 nmol/7h/mL	VAN DIGGELEN *et al*. (1988)
Alfa--glicosidase	Doença de Pompe	Fibroblastos: 21 – 139 nmoles/h/mg proteína	HERMANS *et al.* (1991)
Alfa-L-iduronidase	MPS I	Plasma: 6,8 – 13,7 nmoles/h/mL Leucócitos: 32 – 52 nmoles/h/mg proteína Fibroblastos: 74 – 148 nmoles/h/mg proteína	HOPWOOD *et al* (1979)
Alfa-manosidase	alfa-manosidose	Plasma: 17 – 56 nmol/h/mL Leucócitos: 60 – 400 nmoles/h/mg proteína Fibroblastos: 93 – 359 nmoles/h/mg proteína	PRENCE & NATOWICZ (1992)
Alfa-N-acetilglicosaminidase	MPS III-B	Plasma: 11-37 nmoles/h/mL	MARSH *et al* (1985)
Arilsulfatase A	Leucodistrofia metacromática	Plasma: negativo Leucócitos: 5 – 20 nmoles/h/mg proteína Fibroblastos: 20 – 50 nmoles/h/mg proteína	LEE-VAUPEL & CONZELMANN (1987)
Arilsulfatase B	MPS VI	Leucócitos: 72 – 176 nmoles/h/mg proteína Fibroblastos: 327 – 430 nmoles/h/mg proteína	KRESSE *et al* (1982)
Beta-galactosidase	Gangliosidose GM1, MPS IV-B, galactosialidose	Leucócitos: 78 – 280 nmoles/h/mg proteína Fibroblastos: 394 – 1440 nmoles/h/mg proteína	SUZUKI (1977)
Beta-glicuronidase	MPS VII	Plasma: 30-300 nmoles/h/mL Leucócitos: 23 – 151 nmoles/h/mg proteína Fibroblastos: 62 – 361 nmoles/h/mg proteína	BEAUDET *et al* (1975)
Beta-hexosaminidase A	Doença de Tay-Sachs, doença de Sandhoff	Plasma: 550 – 1675 nmoles/h/mL Leucócitos: 48 – 89% do total Fibroblastos: 46 – 81% do total	SINGER *et al.* (1973)
Beta-hexosaminidase B	Doença de Sandhoff	Plasma: 265 – 1219 nmoles/h/mL Leucócitos: 11 – 52% do total Fibroblastos: 19 – 54% do total	SINGER *et al.* (1973)
Esfingomielinase	Doença de Niemann-Pick (A ou B)	Leucócitos: 0,74 – 4,9 nmoles/h/mg proteína Fibroblastos: 49 – 72	LEIM-HCPA, SGM-HCPA
Galactocerebrosidase	Doença de Krabbe	Leucócitos: 15 – 84 nmoles/h/mg proteína Fibroblastos: 19 – 80 nmoles/h/mg proteína	WIEDERSCHAIN *et al,* (1992)
Galactose-6-sulfato-sulfatase	MPS IV-A	Leucócitos: 0,44 – 1,89 nmoles/h/mg proteína Fibroblastos: 0,53 – 1,03 nmoles/h/mg proteína	VAN DIGGELEN *et al* (1990)
Heparan sulfamidase	MPS III-A	Leucócitos: 5,5 – 24 nmoles/17h/mg proteína Fibroblastos: 26 – 84 nmoles/h/mg proteína	KARPOVA *et al* (1996)
Iduronato sulfatase	MPS II	Plasma: 122 – 463 nmoles/4h/mL Leucócitos:31 – 110 nmoles/h/mg proteína	VOZNYI *et al* (2001)
Neuraminidase	Sialidose	Fibroblastos: 30 – 38 nmoles/h/mg proteína	O'BRIEN & WARNER (1980)
Quitotriosidase	Aumentada em várias DL	Plasma: 8,85 – 132 nmoles/h/mL	HOLLAK *et al*. (1994)

MPS, mucopolissacaridose

APÊNDICES

APÊNDICE I

Mucolipidosis II and III alpha/beta in Brazil: Analysis of the *GNPTAB* gene

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Short communication

Mucolipidosis II and III alpha/beta in Brazil: Analysis of the *GNPTAB* gene

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ABSTRACT

Mucolipidosis II and III (MLII and MLIII) alpha/beta are rare autosomal recessive lysosomal storage diseases (LSDs) caused by pathogenic variations in the *GNPTAB* gene. *GNPTAB* gene codes for the α and β subunits of phosphotransferase, the enzyme responsible for synthesis of the mannose-6-phosphate (M_6P) marker that directs lysosomal enzymes to the lysosome.

Objectives: The objective of this study is to identify sequence variations of the *GNPTAB* gene in Brazilian patients with MLII and MLIII alpha/beta.

Method: Sequencing of the *GNPTAB* gene was performed in samples of gDNA extracted from the peripheral blood of patients with MLII/III diagnosed at a national reference center for LSDs.

Results: Twelve unrelated patients, from several regions of Brazil, were included in this study. Only one was born of consanguineous parents. All patients were found to carry at least one nonpathogenic variation. Nine causal sequence variations were found: c.242C>T (p.W81L); c.1123C>T (p.R375X); c.1196C>T (p.S399F); c.1208T>C (p.I403T); c.1514G>A (p.C505Y); c.1759C>T (p.R587X); c.2808A>G (p.Y937_M972del, novel mutation); c. 2269_2273delGAAAC (p.E757KfsX2, novel mutation); and c.3503_3504delTC (p.I1168QfsX5). Both pathogenic variations were identified in 8 of 12 patients; in four patients, only one pathogenic variation was identified. Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic variation found (n = 11/24 alleles). The deleterious effect of the c.2808A>C mutation on splicing was confirmed by cDNA analysis.

Discussion/conclusions: Our findings confirm that the *GNPTAB* gene presents broad allelic heterogeneity and suggests that, in Brazilian ML II and III patients, screening for mutations should begin at exon 19 of the *GNPTAB* gene. Further analyses will be conducted on patients in whom both pathogenic mutations have not been found in this study.

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Abbreviations: A, adenine; ARSA, arylsulfatase A; CAA, mRNA codon for amino acid glutamine; CAG, mRNA codon for amino acid glutamic acid; cDNA, DNA complementary to RNA; CNPq, Conselho Nacional de Pesquisa e Desenvolvimento; CUA, mRNA codon for amino acid leucine; CUG, mRNA codon for amino acid leucine; DBS, dried blood spot; dNTPs, deoxyribonucleoside triphosphate; EC, enzyme code; ELISA, enzyme-linked immunosorbent assay; FAPERGS, Fundação de Amparo à Pesquisa do estado do Rio Grande do Sul; FIPE, Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre; GAGs, glycosaminoglycans; gDNA, genomic deoxyribonucleic acid; GlcNAc-PT, UDP-N-acetylglucosamine:lysosomal hydrolase N-acetyl_l-phosphotransferase; *GNPTAB*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits"; *GNPTG*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, gamma subunit"; HCPA, Hospital de Clínicas de Porto Alegre; IDS, iduronate-sulfatase; IDUA, α-L-iduronidase; IEM, inborn errors of metabolism; kDa, kilodaltons; LC, liquid chromatography; MS, mass spectrometry; LREBM-HCPA, Laboratório de Erros Inatos do Metabolismo Hospital de Clínicas de Porto Alegre; LSD, lysosomal storage disease; $MgCl_2$, magnesium chloride; ML II, mucolipidosis type II; ML III, mucolipidosis type III; mM, millimolar; mRNA, messenger RNA; M_6P, mannose-6-phosphate; PCR, polymerase chain reaction; Pmol, picomol; PolyPhen, polymorphism phenotyping; PRONEX, Programa de Apoio a Núcleos de Excelência; RNA, ribonucleic acid; SIFT, sorting intolerant from tolerant; taq DNA polymerase, thermostable DNA polymerase.

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1. Introduction

Mucolipidosis type II alpha/beta (MLII; MIM#252500) and type III alpha/beta (MLIII; MIM#252600) are inherited autosomal recessive diseases caused by deficient activity of UDP-N-acetylglucosamine: lysosomal hydrolase N-acetyl-1-phosphotransferase (UDP-GlcNAc-phosphotransferase, GlcNAc-PT or phosphotransferase; EC 2.7.8.17) (Brooks et al., 2007; Tiede et al., 2005; Zarghooni and Dittakavi, 2009). Phosphotransferase plays a role in the synthesis of mannose-6-phosphate (M_6P), a marker molecule responsible for directing lysosomal hydrolases to the lysosome (Braulke et al., 2008; Cathey et al., 2008; Tiede et al., 2005). In the absence of M_6P residues, correct targeting of lysosomal hydrolases is impaired, which results in massive secretion of these enzymes in both the extracellular space and body fluids, as well as a decrease of their activity in cells such as fibroblasts (Braulke et al., 2008; Cathey et al., 2008; Encarnação et al., 2009). MLII is the most severe form of the disease, and is often apparent at birth; progression is rapid, leading to death as early as the first decade of life due to cardiorespiratory complications. Conversely, MLIII follows a slower clinical course, and patients have been known to survive until the eighth decade of life (Cathey et al., 2010; Encarnação et al., 2009; Kornfeld and Sly, 2001). MLII and III correspond to the extreme phenotypes associated with phosphotransferase, deficiency, and patients with intermediate clinical manifestations have also been described (Cathey et al., 2010).

Phosphotransferase is a hexameric protein composed of two α subunits, two β subunits, and two γ subunits (Bao et al., 1996; Tiede et al., 2005), where the α and β subunits are encoded by the *GNPTAB* gene, located in chromosome 12q23.3, and the γ subunits encoded by the *GNPTG* gene, located in chromosome 16p13.3 (Cathey et al., 2008; Encarnação et al., 2009; Kudo et al., 2005; Zarghooni and Dittakavi, 2009). Patients who are homozygous or compound heterozygous for pathogenic mutations in *GNPTAB* (ML alpha/beta) exhibit a phenotype consistent with MLII or MLIII, whereas patients who are homozygous or heterozygous for pathogenic mutations in *GNPTG* (ML gamma) exhibit a phenotype compatible with MLIII (Bargal et al., 2006; Cathey et al., 2010; Encarnação et al., 2009; Persichetti et al., 2009; Tappino et al., 2009).

The main objective of the present study was to conduct an analysis of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta.

2. Materials and methods

Twelve unrelated patients with a biochemical diagnosis of MLII or MLIII were included in the study. They were recruited from the cohort of patients (approximately 40,000) investigated for inborn errors of metabolism (IEM) at the Reference Laboratory for IEM at Hospital de Clínicas de Porto Alegre, Brazil (LREIM-HCPA), from 1983 to 2011. The LREIM-HCPA is a national reference laboratory for the diagnosis of lysosomal diseases, and its database of diagnoses probably includes most cases of MLII/III diagnosed in Brazil. Biochemical diagnosis of MLII/III at LREIM-HCPA includes analysis of arylsulfatase A (ARSA; EC 3.1.6.8) and several other lysosomal hydrolases, including α-L-iduronidase (IDUA; EC 3.2.1.76), iduronate-sulfatase (IDS; EC 3.1.6.12), β-glucuronidase (GUSB; EC 3.2.1.31), and β-hexosaminidase (EC 3.2.1.30), in plasma. Measurement of the activity of these enzymes in fibroblasts, as well as analysis of glycosaminoglycans (GAGs) and sialyloligosaccharides in urine, are also performed whenever samples are available. If only filter paper samples are available, the activity of IDS, GUSB, and hexosaminidases is analyzed.

Whole blood samples were collected for gDNA and RNA extraction. Determination of clinical severity (MLII or III) took into account the criteria usually reported in the literature, such as age at diagnosis, survival, and extent of skeletal involvement (and, consequently, patient height) (Cathey et al., 2010). The patients included in this study were under the care of different physicians, but a summary of their clinical records was always sent alongside each patient's blood sample, so that the classification assigned by the attending physician was always reviewed and confirmed by the investigators. Whenever possible, a sample of gDNA from the parents was also obtained for confirmation of the presence of the mutations found in the patient.

Genomic DNA was extracted from peripheral blood leukocytes with the DNeasy Blood and Tissue Kit (Qiagen, Germany). The 21 exons that comprise the *GNPTAB* gene, as well as the intron-exon boundaries and part of the 5′ and 3′ untranslated regions, were amplified from the specific sequences of oligonucleotides projected for this study. Amplification was performed by the polymerase chain reaction (PCR) using 50 ng of gDNA, 16 pmol of each oligonucleotide, 0.6 mM of dNTPs, 2.4 mM of $MgCl_2$, 1× reaction buffer, and 1 unit of *taq* DNA polymerase. The annealing temperatures and oligonucleotide sequences are available as supplementary data.

Sample sequencing was performed using the automatic ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The reference sequence of the *GNPTAB* gene was GenBank accession nº. NM_024312.3. Each mutation found was confirmed by sequencing performed with a new amplicon and the oligonucleotide inverse to that used in the first stage.

In silico analysis of the potential effect of missense mutations was done by means of PolyPhen2 (Polymorphism Phenotyping)

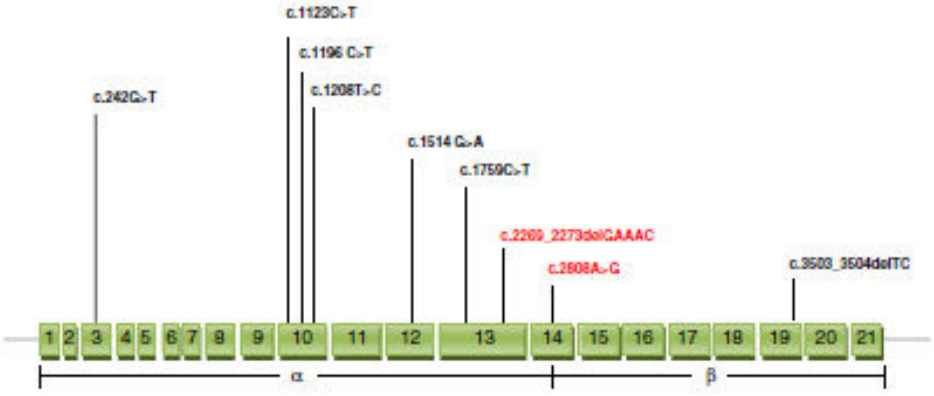

Fig. 1. *GNPTAB* gene: localization of pathogenic mutations found in the present study (modified from Tappino et al. (2009)). In red: mutations described for the first time in the present study. In black: mutations previously described in the literature. The α- and β-subunits are represented.

(http://genetics.bwh.harvard.edu.pph2/) and SIFT (Sorting Intolerant From Tolerant) (http://sift.jcvi.org/www.SIFT_seq_submit2.html). The MaxEntScan software was used to evaluate the effect of point mutations on generation or exclusion of splice sites (http://http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html). The frequency of synonymous codon usage was evaluated according to the database of codon usage in *Homo sapiens* (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). To evaluate the pathogenicity of the novel exonic point mutation found (c.2808A>C), a hundred control alleles were analyzed using 0.3 U of *Hyp 188I* restriction enzyme (New England Biolabs, USA), 2 μl of buffer 4 (supplied with the restriction enzyme) and 17.7 μl of amplicon; samples were incubated for 3 h at 37 °C. Total RNA extraction was performed on a whole blood sample from the patient presenting this mutation using the Paxgene blood RNA Kit (Qiagen, Germany), and conversion to cDNA was made using the Superscript II conversion kit (Invitrogen, USA), according to manufacturer instructions. cDNA was subsequently sequenced as described above.

This study was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Research Ethics Committee.

3. Results

Of the patients included, eight were classified as having MLD. Only one (patient 12) was born of consanguineous parents. Regarding biochemical investigation, fibroblast samples were unavailable for three patients (patients 6, 7 and 12); patient 6 was investigated only through a dried blood spot (DBS) sample. Urinary GAG measurements were normal in 10 of 10 patients, thin-layer chromatography of GAGs was abnormal in 4 of 10 patients (patient 4, dermatan/keratan sulfate; patients 5 and 7, dermatan sulfate; patient 9, keratan sulfate), while thin-layer chromatography of sialyloligosaccharides was abnormal in 1 of 3 patients (patient 10). Detailed data on the clinical and biochemical findings presented by the patients are available as supplementary material.

Nine causal sequence variations were found, two of which were novel: c.2808A>G (p.Y937_M972del) and c. 2269_2273delGAAAC (p.E757KfsX2) respectively (Fig. 1, Table 1). Both pathogenic mutations were identified in 8 of 12 patients, and only one pathogenic mutation was identified in four patients (Table 1). Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic mutation (n = 11/24 alleles; Table 1).

At least one nonpathogenic variant was found in each patient. The mutations c.365+145C>T in intron 4 (n = 17/24) and c.-41_-39delGGC in the 5′UTR (n = 16/24) were the most frequent nonpathogenic variants found, and the nonpathogenic mutation c.323+20delT (intron 3) has not been reported elsewhere. Additional information on nonpathogenic mutations is available as supplementary material.

3.1. The c.2808A>G mutation

As the c.2808A>G mutation (exon 14) was apparently not associated with an amino acid change, we decided to investigate its effect on splicing. According to MaxEntScan software (http://http://genes.mit.edu/burgellab/maxent/Xmaxentscan_scoreseq.html) results, this mutation would strongly interfere with *GNPTAB* mRNA splicing (Fig. 2). In fact, this mutation creates a similar sequence to the canonical donor splice site. cDNA analysis for this patient showed the presence of an abnormal transcript in which the final 108 bp of exon 14 is absent (Fig. 3). We did not perform expression studies, but this isoform is predicted to generate a truncated protein (p.Y937_M972del), with deletion of 36 amino acids of the phosphotransferase β-subunit. This mutation was not found in any of the 100 control alleles analyzed (Fig. 4).

Table 1
Pathogenic mutations found in the *GNPTAB* gene of 12 unrelated Brazilian patients with Mucolipidosis II and III alpha/beta.

Patients	Genotype (cDNA)	Genotype (protein)	Phenotype	In silico analysis of the effect in the protein[a]			Parental genotype**
				Polyphen2	SIFT	MaxEntScan	
1	c.[1123C>T]+[?]	p.[R375X]+[?]	ML II	N.A.	N.A.	NI	Mother: c.[1123C>T]+[N]
2	c.[1514G>A]+[7559C>T]	p.[G505R]+[R887X]	ML II	Probably deleterious	Intolerable	NI	Mother: c.[1514G>A]+[N] Father: c.[7559C>T]+[N]
3	c.[1308T>C]+[3503_3504delTC]	p.[=]+[L1168QfsX5]	ML II	Probably deleterious	Intolerable	NI	Mother: c.[1308T>C]+[N] Father: c.[3503_3504delTC]+[N]
4	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML II	N.A.	N.A.	N.A.	Mother: c.[N]+[N] Father: NA
5	c.[240G>T]+[?]	p.[W81L]+[?]	ML II	Probably deleterious	Intolerable	NI	NA
6	c.[2269_2273delGAAAC]+[2269_2273delGAAAC]	p.[E757KfsX2]+[E757KfsX2]	ML II	N.A.	N.A.	N.A.	Mother: c.[2269_2273delGAAAC]+[N]
7	c.[2808A>G]+[3503_3504delTC]	p.[Y937_M972del]+[L1168QfsX5]	ML II	N.A.	N.A.	Probably splice site interference (exon 14)	Mother: c.[3503_3504delTC]+[N]
8	c.[1196C>T]+[3503_3504delTC]	p.[S399F]+[L1168QfsX5]	ML III	Probably deleterious	Intolerable	NI	Father: (c.2808A>G)+[N] Mother: c.[1196C>T]+[N]
9	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML III	N.A.	N.A.	N.A.	Father: c.[3503_3504delTC]+[N] NA
10	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	N.A.	N.A.	N.A.	NA
11	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	N.A.	N.A.	N.A.	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]
12	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	N.A.	N.A.	N.A.	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]

?: Undescribed mutation; NA: not analyzed; NI: no interference; N: normal allele. Polyphen2 is available at http://genetics.bwh.harvard.edu/pph2/ and classifies variations as benign/possibly deleterious/probably deleterious. SIFT is available at http://sift.jcvi.org/www/SIFT_seq_submit2.html and classifies variation as tolerable or intolerable. MaxEntScan is available at http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html.

* Conducted only in case of missense mutations.

** Parents were analyzed only in relation to the mutations found in the proband.

4. Discussion

This is the first study to describe the pathogenic/nonpathogenic mutation spectrum of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta. As in other populations, this analysis confirmed that *GNPTAB* exhibits great allelic heterogeneity, that there are few recurrent mutations, and that c.3503_3504delTC is the most prevalent pathogenic mutation (Bargal et al., 2006; Cathey et al., 2010; Kudo et al., 2006; Paik et al., 2005; Tiede et al., 2005). Furthermore, three novel sequence variations (two pathogenic) are described herein.

The number of patients included in this study (n = 12) is significant, considering that all patients were Brazilian and that ML is quite rare and severe (http://www.geneclinics.org). Unfortunately, not all patients had undergone biochemical investigation in plasma and/or fibroblasts. It bears stressing, however, that all patients exhibited a clinical phenotype consistent with MLII or MLIII. Patient 6 was investigated using filter paper only, as described by Chamoles et al. (2001); as this patient was later confirmed as having MLII alpha/beta through DNA analysis, this finding corroborates the hypothesis that DBS samples can also be used for MLII/III screening. Interestingly, four patients exhibited an abnormal pattern on urinary thin-layer chromatography of GAGs, although their levels of total urinary GAGs were normal. Abnormal catabolism of GAGs is expected to occur in patients with MLII/III, as lysosomal enzymes involved in the catabolism of GAGs are deficient in these disorders. Levels of keratan sulfate (determined by ELISA), as well as dermatan sulfate and heparan sulfate (determined by LC/MS/MS), were found to be increased in plasma of some patients with MLII/II (Tomatsu et al., 2005, 2010). However, secretion of abnormal GAGs is usually too low in MLII/III patients to be detected by thin-layer chromatography.

The methodology used in the present study was able to identify 20 of 24 pathogenic alleles (roughly 83%). This rate is not very different from that reported in the literature (approximately 95%) (http://www.genedinics.org). The most frequently found pathogenic mutation was c.3503_3504delTC, which was present in homozygosity in three MLII patients (patients 10, 11 and 12) and in heterozygosity in another two MLII patients (patients 4 and 7) and three MLIII patients (patients 3, 8 and 9); the prevalence found for this mutation in our study (45%; 11 of 24 alleles) is similar to that described in the literature. Bargal et al. (2006) found this same mutation in 13 of 24 MLII patients, most of Arab-Muslim origin (11 homozygous; 2 compound heterozygous; 10 born to consanguineous couples). Encarnação et al. (2009) found this microdeletion in five patients with MLII (n = 9 of 18 alleles); four of these patients were homozygous for the mutation. Tappino et al. (2009) identified this mutation in 47 of 92 alleles; it was found in homozygosity in 14 patients with MLII, most of whom were Italian and born to non-consanguineous couples. Mutation c.3503_3504delTC was the only pathogenic mutation identified in the sample studied by Plante et al. (2008).

4.1. Effect on phenotype

Our data suggest that nonsense and frameshift mutations are associated with the severe phenotype (MLII alpha/beta), whereas missense mutations are associated with the attenuated phenotype (MLIII alpha/beta). These findings are in agreement with those previously described in the literature (Bargal et al., 2006; Encarnação et al., 2009; Tiede et al., 2005).

Thus far, c.3503_3504delTC, located at the region that codes for the β subunit of phosphotransferase, has been associated with the severe phenotype when found in homozygosity or when found in heterozygosity with nonsense mutations or frameshift mutations. Three of our patients with MLII alpha/beta presented this deletion in homozygosity (patients 10, 11 and 12). However, two patients with MLIII alpha/beta (patients 3 and 8, respectively) presented this deletion in heterozygosity with the missense mutations c.1208T>C (p.I403T) and c.1196C>T (p.S399F), both located at the region that codes for the α subunit of phosphotransferase. Other authors have also reported cases of compound heterozygosity for mutations in the α and β subunits (Cathey et al., 2010; Tappino et al., 2009), and Bargal et al. (2006) suggest that there is no intragenic complementation between these subunits. The genotype c.[1196C>T]+[3503_3504delTC] (patient 8) was also reported in a MLIII French patient by Bargal et al. (2006). In that same study, the authors reported that this patient exhibited a severe MLIII phenotype and that fibroblast testing showed cytoplasmic inclusions typical of patients with MLII. Our patient may also be considered a patient with severe MLIII alpha/beta, given the severity of his skeletal compromise, which is reflected by his present height. The c.[1208T>C]+[3503_3504delTC] genotype, on the other hand, is being described for the first time herein (patient 3, MLIII alpha/beta). Mutation c.1208T>C (p.I403T) has already been described by Tappino et al. (2009) in homozygosity in an Italian patient with MLII, as well as by Encarnação et al. (2009) in a Portuguese patient. Expression studies of the mutant p.I403T protein in COS cells conducted by Tappino et al. (2009) showed that this mutation had an expected molecular mass of 170 kDa, and the authors presumed that the resulting protein would be partially dysfunctional, which could explain the attenuated phenotype exhibited by these patients.

Genotype c.[1514G>A]+[1759C>T] was found in one patient with MLIII alpha/beta (patient 2). The first is a missense mutation (p.C505Y), while the second is a nonsense mutation (p.R587X). This

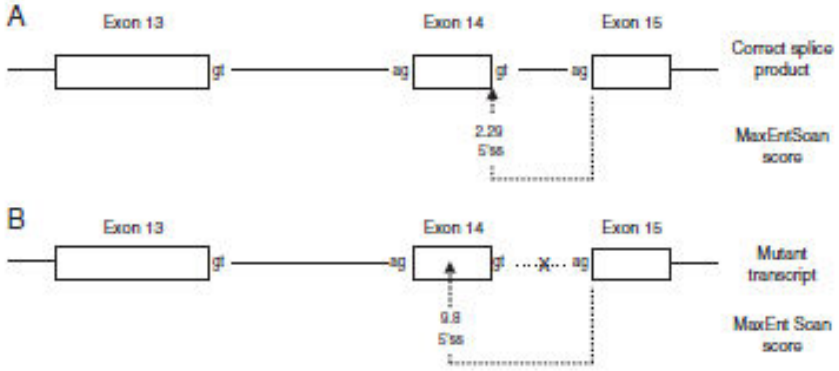

Fig. 2. Schematic representation of exons and introns 13, 14 and 15 of the *GNPTAB* gene and scores obtained with the MaxEntScan program for wild (A) and mutated (B) forms obtained in the presence of c.2808A>G. Dotted line: new splicing. X: splicing replaced.

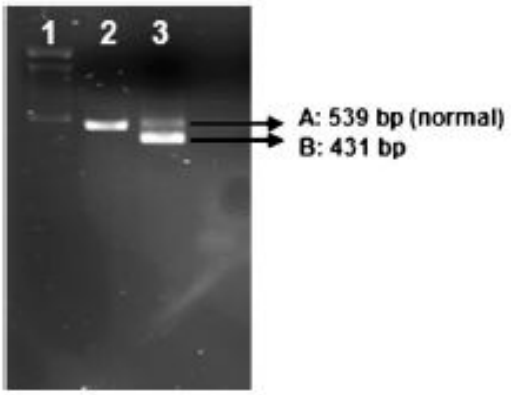

Fig 3. Electrophoresis of RT-PCR for the c.2808A>G mutation (patient 7) showing the normal transcript (A) and the alternative transcript (B). 1: Standard molecular weight; 2: negative control for c.2808A>G; 3: patient sample.

genotype is being reported here in for the first time, but the mutations have already been reported by Cathey et al. (2010), in compound heterozygosity, in a patient with MLII (p.R587X) and in one patient with MLIII (p.C505Y).

Genotype c.[242G>T]+[?] was found in a patient with ML II alpha/beta (patient 5). Encarnação et al. (2009) described this mutation, in homozygosity, in one of their Portuguese patients with MLIII. One hypothesis that might justify the phenotype of patient 5, with only one pathogenic mutation identified, would be the presence of two silent mutations in exon 1 of the second allele: c.18G>A and c.27G>A. The first induces a change from the sixth CUG codon (frequency of codon usage per thousand = 39.6) to CUA (frequency of codon usage per thousand = 7.2). The second mutation changes the ninth CAG codon (frequency of codon usage per thousand = 34.2) to CAA (frequency of codon usage per thousand = 12.3) (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). This could cause a greater change in the translation kinetics of the protein, generating a protein with an altered conformation (Komar, 2007; Sauna and Kimchi-Sarfaty, 2011). Unfortunately, parental DNA and patient fibroblasts were unavailable, and, therefore, we are not able to confirm whether these mutations are in *cis* or in *trans*. Additional studies will be conducted to confirm this hypothesis.

5. Conclusions

This was the first DNA analysis-based study conducted in Brazilian patients with ML alpha/beta. Its findings suggest that analysis of *GNPTAB* in these patients should begin by exon 19, thus optimizing the investigation and reducing costs. The results obtained herein emphasize the need for further studies, such as application of other techniques that will enable completion of genotyping of the patients whose genotype has not been fully characterized (e.g. sequencing of promoter and intronic regions, exclusion of large deletions/rearrangements), and for determination of the frequency of recurrent variants in healthy Brazilian individuals.

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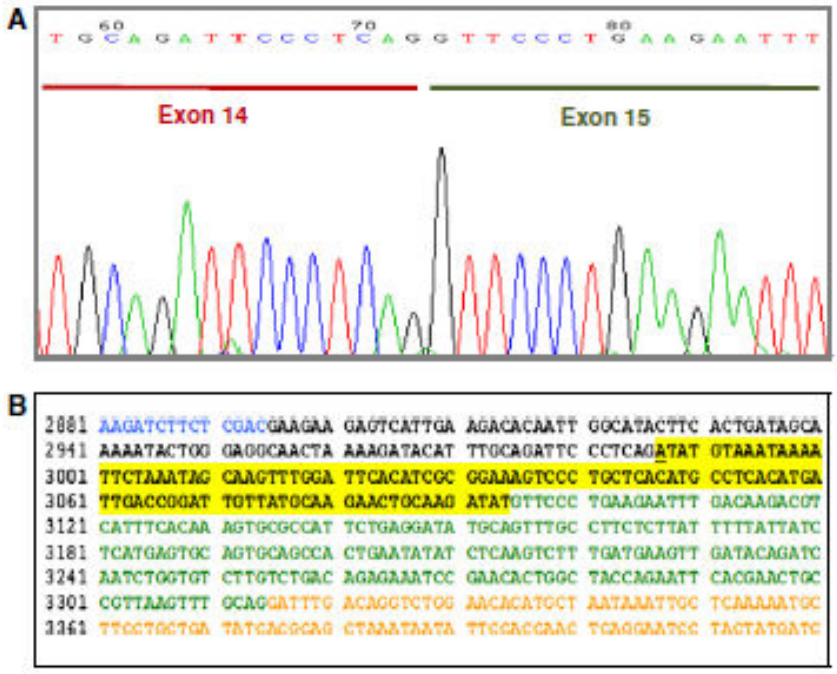

Fig 4. A) Patient 7 (genotype: c.2808A>G; p.V937_M972del): Sequencing of the abnormal fragment amplified by RT-PCR. B) Normal cDNA sequence of the *GNPTAB* gene. In blue, part of exon 13; in black, exon 14; in green, exon 15; in orange, exon 16; underlined, mutation site; in yellow, 108 bp lost due to the c.2808A>G mutation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.03.105.

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APÊNDICE II

A *de novo* or germline mutation in a family with Mucolipidosis III gamma: Implications for molecular diagnosis and genetic counseling

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Short Communication

A *de novo* or germline mutation in a family with Mucolipidosis III gamma: Implications for molecular diagnosis and genetic counseling

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ABSTRACT

Mucolipidosis III (ML III) gamma is a very rare autosomal-recessive disorder characterized by the abnormal trafficking and subcellular localization of lysosomal enzymes due to mutations in the *GNPTG* gene. The present study consists of a report of a Brazilian compound heterozygote patient with ML III gamma resulting from one mutant paternal allele and one allele that had most likely undergone a *de novo* or maternal germline mutation. This is the first report of a *de novo* mutation in ML III gamma. This finding has significant implications for genetic counseling.

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1. Introduction

Mucolipidosis II (ML II disease, inclusion cell disease or I-cell disease) and III (ML III, pseudo-Hurler polydystrophy) are autosomal recessive disorders caused by defects in the GlcNAc-1-phosphotransferase (EC 2.7.8.17) complex, which is composed by three subunits: α, β, and γ. Mutations in the gene encoding the α- and β-subunits (*GNPTAB*) lead to ML II alpha/beta (OMIM #252500), or to the less clinically severe

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condition, ML III alpha/beta (OMIM #252600). ML III gamma (OMIM #252605) is caused by mutations in the gene encoding the γ-subunit of GlcNAc-1-phosphotransferase [1,2], and is thought to be the mildest form of the disease. Very few cases of ML III gamma are reported in the literature, maybe because the disease is underdiagnosed due to its relatively mild and unspecific clinical findings, which is suggested by a recent report of ML III gamma patients diagnosed through next generation sequencing [3]. To date, approximately 28 mutations have been reported in the *GNPTG* gene. A large number of these mutations are unique or rare [4].

De novo mutations are not rare events and the perception they are potentially important in genetic diseases, even in autosomal recessive conditions, have major implications for genetic counseling [4,5]. The present study consists of a report of a Brazilian compound heterozygote patient with ML III gamma resulting from the inheritance of one mutant paternal allele and one maternal allele that had most likely undergone a *de novo* or germline mutation.

2. Materials and methods

2.1. Case report

The proband, a female born at term to young (maternal and paternal age at conception was 27 years old) and non-consanguineous parents, has been described previously [6]. She was referred for clinical genetic evaluation due to large joint contractures. On physical examination, the patient presented contractures and restrictions of movement, especially in the hands, feet and shoulders, and heart systolic murmur, audible mainly at the left sternal border. The two-dimensional color Doppler echocardiography revealed the presence of mild thickening of aortic valve leaflets with mild regurgitation. All other clinical parameters were within normal limits for the patient's age. The exam was performed through the subcostal window, as thoracic deformity prevented the use of standard echocardiographic measures of pulmonary artery systolic pressure. Electroneuromyography of the upper limb was normal, and showed no electrophysiological evidence of peripheral neuropathy. The somatosensory evoked potential of the upper and lower limbs was also normal. The patient was diagnosed as having ML III when she was 8 years old (Table 1), and is currently stable. At the time of the study, she was 16 years old and attended regular school. Previous *GNPTAB* sequencing showed no alterations.

2.2. GNPTG analysis and maternity testing

Genomic DNA was extracted from leukocytes (patient and both parents), buccal cells (patient and mother) and fibroblasts (patient) after informed consent was given.

GNPTG was amplified in five fragments containing exons 1 to 2, 3, 4 to 7, 8 to 9 and 10 to 11 as described by Persichetti et al. [7] with modifications. The fragment which comprises exons 4 to 7, where the mutation c.244_247dupGAGT is located, was also amplified using a second pair of primers [8]. Samples were submitted to DNA sequencing, performed on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). All samples were amplified and sequenced twice. The sequence of the *GNPTG* gene used as a template was GenBank accession no. NG_016985.1.

Table 1
Biochemical characterization of a patient with Mucolipidosis III gamma.

Enzymes	Sample	Patient	References values
Arylsulfatase A (EC 3.1.6.8)	Plasma	+	Negative
α-L-Iduronidase (EC 3.2.1.76)	Plasma	176	32–52 nmol/h/ml
β-Glucuronidase (EC 3.2.1.31)	Plasma	475	30–300 nmol/h/ml
α-Mannosidase (EC 3.2.1.24)	Plasma	1,548	17–56 nmol/h/ml
Iduronate-sulfatase (EC 3.1.6.12)	Plasma	1894	122–463 nmol/h/ml
Total β-hexosaminidases (EC 3.2.1.52)	Plasma	12,675	1000–2857 nmol/h/ml
α-Mannosidase (EC 3.2.1.24)	Fibroblasts	16	60–400 nmol/h/mg
β-Galactosidase (EC 3.2.1.23)	Fibroblasts	132	394–1440 nmol/h/mg
β-Glucuronidase (EC 3.2.1.31)	Fibroblasts	8.6	62–361 nmol/h/mg
α-Fucosidase (EC 3.2.1.51)	Fibroblasts	2.1	46–221 nmol/h/mg

Total RNA extraction was performed on a whole blood sample of the patient and her relatives, as well as three controls using the Paxgene blood RNA Kit (Qiagen, Germany), and conversion to cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. The *GNPTG* mRNA levels were determined by qRT-PCR using 2× SYBR Green PCR Master Mix (Applied Biosystem) with the Mx3000P (Stratagene, Amsterdam, NL). *GAPDH* was chosen as housekeeping gene. Primers and conditions were performed as described by Ho et al. [9] with modifications. The relative quantification of the RNA was normalized to the level of *GAPDH* mRNA in the same cDNA using the comparative CT method ($2^{-\Delta\Delta CT}$).

For maternity testing, DNA from each sample was analyzed by PCR multiplex reaction using the AmpFlSTR® Identifiler® PCR Amplification kit (Applied Biosystems®), which enables the analysis of the 15 STR markers (STRs) using fluorescent primers, according to the manufacturer's instructions. PCR products were then resolved by capillary electrophoresis in an ABI3130xl genetic analyzer (Applied Biosystems®) using GeneScan™ 500 LIZ® as an internal marker and alleles were identified through GeneMapper® Software® v1.2 (Applied Biosystems®).

2.3. Statistical analysis

Values obtained for the relative quantification of *GNPTG* mRNA in patient, parents and control samples were compared using Student's test (IBM SPSS Statistics version 20). P values lower than 0.05 were considered statistically significant.

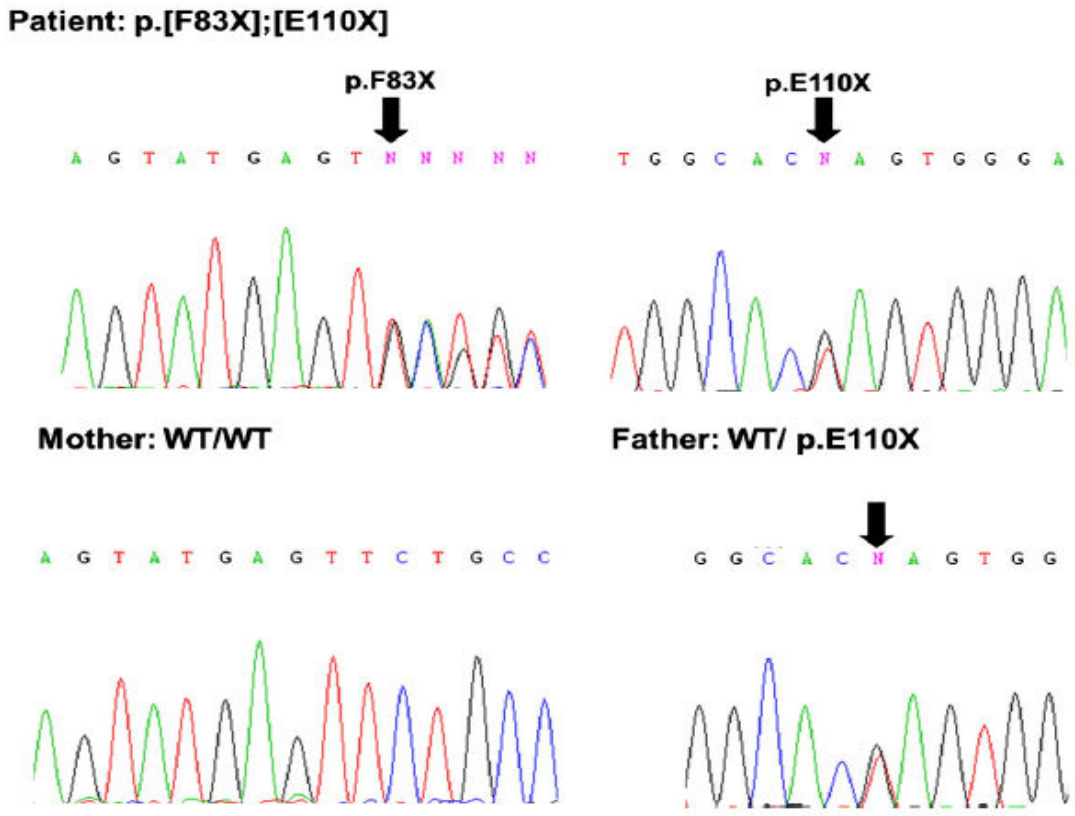

Fig. 1. *GNPTG* gene sequencing of the patient, her mother and father (leukocytes). WT = wild type.