Universidade Federal do Rio Grande do Sul

Programa de Pós-graduação em Genética e Biologia Molecular

Aspectos moleculares relacionados ao diagnóstico e progressão da Doença de Fabry

Gabriela Pasqualim

Orientadora: Dra Ursula da Silveira Matte

Coorientador: Dr Filippo Vairo

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Lista de abreviaturas, símbolos e unidades

3' UTR	3' *Untranslated Region* - Região 3' não traduzida
5' UTR	5' *Untranslated Region* - Região 5' não traduzida
cDNA	DNA codificante
del	deleção
DF	Doença de Fabry
DL	doença lisossômica
EIMs	erros inatos do metabolismo
ELs	Esfingolipídios
ERT	*Enzyme replacement therapy*
Gb3	globotriaosilceramida
GLA	Gene que codifica a enzima α-Galactosidase A
HGMD	Human Genome Mutation Database
HRM	*High resolution melting*
IC	Intervalo de Confiança
Kb	*Kilo base* - quilobase
kDa	quilodalton
liso-Gb3	globotriaosilesfingosina
M6P	manose-6-fosfato
MLPA	Amplificação Multiplex de Sondas Dependente de Ligação
MPS	Mucopolissacaridose
pb	Pares de base
PCR	Reação em cadeia da polimerase
qRT-PCR	*quantitative real-time PCR*
S1P	esfingosina-1-fosfato
SAP	saposina
TRE	Terapia de reposição enzimática
VPN	Valor Preditivo Negativo
VPP	Valor Preditivo Positivo
VUS	*variant of uncertain significance*
α-GAL / α-Gal A	Enzima α-galactosidase A

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Resumo

A Doença de Fabry (DF) é uma doença rara com herança ligada ao X causada pela deficiência de atividade da hidrolase lisossomal α-galactosidase A (α-GAL), codificada pelo gene *GLA*. Este defeito enzimático leva ao acúmulo de glicoesfingolipídios, principalmente globotriaosilceramida (Gb3) pelo corpo. Pacientes com a forma clássica apresentam baixa atividade residual da enzima com início dos sintomas na infância e desenvolvimento de sintomas cardíacos, cerebrovasculares e/ou renais. Pacientes com apresentações não clássicas tem atividade enzimática residual maior, início tardio dos sintomas e podem ser mono ou oligo-sintomáticos. Características marcantes da DF são alta variabilidade alélica e fenotípica. Dessa forma, o objetivo desta tese foi investigar aspectos moleculares relacionados ao diagnóstico e progressão da Doença de Fabry na população brasileira. Para triagem bioquímica de pacientes do sexo feminino em populações de risco, foi proposta uma metodologia de uso combinado de atividade de α-GAL em leucócitos e plasma. Com o uso de valores de referência gênero-específicos, esta metodologia amplamente acessível pode reduzir o número de amostras que necessitam de triagem genética em 35%. Com relação à triagem molecular, foi descrito um protocolo com a técnica de HRM (*high resolution melting*) para amostras de sangue coletadas em EDTA. Todas as variantes analisadas neste estudo resultaram em perfis distintos dos controles normais. Além disso, com exceção das variantes localizadas no éxon 7, também foi possível a identificação de todos os genótipos diferentes analisados. A análise molecular do gene *GLA* por sequenciamento de Sanger foi realizada em 408 pacientes brasileiros, provenientes de 213 famílias, com histórico familiar ou suspeita clínica de DF. Conforme o esperado para uma doença com mutações privadas, a maioria destas variantes foi encontrada em famílias únicas. Das 26 variantes identificadas em regiões codificantes, cerca de 80% eram do tipo *missense,* o que está de acordo com a literatura. Em seguida, a existência de genes modificadores do fenótipo cardíaco foi avaliada por análise de exoma de pacientes com ou sem doença cardíaca. A frequência da variante rs3188055 gene inositol polifosfato 5-fosfatase (*INPP5F*) foi significativamente maior no grupo com doença cardíaca. Portanto; *INPP5F,* envolvido na maturação e reciclagem dos endossomos e regulação negativa da

hipertrofia cardíaca, é um possível modulador da doença cardíaca em DF. Finalmente, a variabilidade fenotípica vista em mulheres com DF foi analisada com o uso de transcriptomas de células sanguíneas. Foram obtidos perfis de expressão distintivos para pacientes com diferentes manifestações: exclusivamente cardíacas, exclusivamente renais ou ambas. Os genes com expressão diferencial validados (*DEFA1, A1B, A3; CEACAM8, PRKXP1, IFI44, IFI44L, IFIT1, HERC5, EIF2AK2* e *RSAD2*), representam possíveis vias especificas das manifestações analisadas, envolvidas com a variabilidade vista em DF, que podem auxiliar no manejo clínico dos pacientes.

Abstract

Fabry disease (FD) is a rare X-linked disorder resulting from deficient activity of the lysosomal hydrolase α-galactosidase A (α-GAL), encoded by the *GLA* gene. This deficiency leads to the accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3), throughout the body. Patients with the classical form of the disease have low residual enzymatic activity with childhood onset of symptoms and development of cardiac, cerebrovascular and/or renal complications. Patients with non-classical phenotypes have higher residual activity, later onset of symptoms and are mono or oligo-symptomatic. Striking features of FD are high allelic and phenotypic variability. Thus, the aim of this thesis was to investigate molecular aspects related to the diagnosis and progression of Fabry disease in the Brazilian population. For biochemical screening of female patients in risk populations, a methodology of combined use of α-GAL activity in leukocytes and plasma was proposed. With the use of gender-specific reference values, this widely accessible methodology can reduce the number of samples that require genetic screening by 35%. Regarding molecular screening, a protocol with high resolution melting (HRM) technique for blood samples collected in EDTA was described. All the variants analyzed in this study displayed different profiles from normal controls. In addition, with the exception of variants located in exon 7, it was also possible to identify all the different genotypes analyzed. The molecular analysis of the *GLA* gene by Sanger sequencing was performed in 408 Brazilian patients, from 213 families, with family history or clinical suspicion of FD. As expected for a disease with private mutations, most of these variants were found in single families. Of the 26 variants identified in coding regions, about 80% were missense, which is in agreement with the literature. Then, the existence of cardiac phenotype modifier genes was evaluated by exome analysis of patients with and without heart disease. The frequency of variant rs3188055 variant in Inositol Polyphosphate-5-Phosphatase F gene (*INPP5F*) was significantly higher in the group with heart disease. Therefore, *INPP5F*, which is involved in the maturation and recycling of endosomes and negative regulation of cardiac hypertrophy, is a possible modulator of cardiac disease in FD. Finally, the phenotypic variability seen in women with FD was analyzed with the use of transcriptomes from whole blood. Distinctive expression profiles were obtained for patients with different

manifestations: exclusively cardiac, exclusively renal or both. The validated genes with differential expression (*DEFA1, A1B, A3, CEACAM8, PRKXP1, IFI44, IFI44L, IFIT1, HERC5, EIF2AK2* and *RSAD2*) indicate possible disease specific pathways involved in phenotypic variability seen in FD, which might aid in patient management.

1. Introdução

1.1. Lisossomos

Os lisossomos são organelas delimitadas por bicamadas lipídicas compostas por proteínas específicas, principalmente proteínas associadas à membrana lisossômica tipos 1 e 2 (LAMP 1 e 2) e proteínas integrais de membrana lisossômica tipos 1 e 2 (LIMP 1 e 2) (Saftig and Klumperman 2009). Em seu interior, diversas hidrolases lisossomais atuam na degradação de compostos. O endereçamento destas enzimas se dá principalmente pela via de receptores de manose-6-fosfato (M6P). Após a síntese no reticulo endoplasmático, os precursores das enzimas lisossomais são transferidos para o complexo de Golgi, onde ocorre a adição de resíduos de M6P. Então, após a ligação a receptores de M6P, os precursores são liberados e diretamente transportados aos endossomos. Parte das enzimas produzidas é exocitada, mas pode ser recaptada via ligação aos mesmos receptores de M6P presentes na membrana plasmática. No pH ácido do lisossomo, as enzimas se desassociam dos receptores, que são reciclados, e adquirem sua forma ativa após processamento (Braulke and Bonifacino 2009).

Proteínas associadas também são necessárias para o funcionamento dos lisossomos. A manutenção do pH lisossomal, por exemplo, é feita pela ação de bombas de prótons como o complexo H+ ATPase tipo V. Além disso, as proteínas de membrana associadas a vesículas (VAMPs) modulam a capacidade dos lisossomos de se fusionar com outras vesículas, outro fator fundamental para ação destas organelas (Saftig and Klumperman 2009).

Classicamente o lisossomo é visto apenas como uma organela com função limitada a degradação e reciclagem de biomoléculas. Entretanto, possui funções diversas dentre as quais pode-se citar: transdução de sinal, apresentação de antígenos e resposta imune, autofagia, morte celular, reparo de membranas, degradação de DNA mediada por DNAse, homeostase do colesterol, remodelamento ósseo e tecidual, invasão tumoral e metástase e resistência tumoral à fármacos (Saftig and Klumperman 2009; Matte and Pasqualim 2016). Mais detalhes sobre a biogênese e funções dos lisossomos estão descritas

no artigo “Lysosome: The Story Beyond the Storage” (Matte and Pasqualim 2016), incluído na seção de anexos desta tese (anexo I).

1.2. Doenças lisossômicas

A deficiência de proteínas lisossomais resulta no desenvolvimento de doenças lisossômicas (DLs). Estes erros inatos do metabolismo (EIMs) são mais comumente causados por deficiências de enzimas lisossomais. Com menor frequência, também são ocasionados por defeitos em proteínas lisossômicas não enzimáticas ou proteínas não lisossomais envolvidas na biogênese do lisossomo (Filocamo and Morrone 2011).

Apesar de serem genética e fenotipicamente distintas, partilham características em comum como o acúmulo de metabólitos não degradados ou parcialmente degradados no sistema endossomal-autofágico-lisossomal (Platt et al. 2012). Além disso, a maioria das proteínas lisossomais é codificada por genes constitutivos e agem em diferentes substratos que possuem o mesmo resíduo terminal. Consequentemente, as DLs são multissistêmicas e apresentam grande amplitude fenotípica (Neufeld and Muenzer 2001; Wraith 2002; Wilcox 2004; Filocamo and Morrone 2011). Outro aspecto importante é o caráter progressivo dessas enfermidades, com acúmulo detectado já na placenta em alguns casos (Baldo et al. 2011). O modo de herança também é partilhado pela maioria destas doenças, que são autossômicas recessivas. As exceções são Doença de Fabry, Mucopolissacaridose do tipo II (Doença de Hunter) e Doença de Danon, todas com herança ligada ao X.

No Brasil, foram detectados 1 caso de DL por 19.942 nascidos vivos no período de 2000 a 2013, sendo o grupo de EIM mais frequentemente detectado (Giugliani et al. 2017). Essa estimativa se baseia no diagnóstico bioquímico de populações de risco em feito pelo Serviço de Genética do Hospital de Clínicas de Porto Alegre (SGM/HCPA), um dos mais completos centros para diagnóstico de DLs do país, sendo reconhecido como um centro de referência. Porém, este dado pode ser uma subestimativa, já que inclui dados de apenas um centro, e está abaixo de estimativas mundiais de prevalência de 1:9.000 nascidos vivos

(Meikle et al. 1999; Fuller et al. 2006). Dessa forma, apesar de individualmente raras, as DLs são frequentes quando avaliadas em conjunto.

Atualmente mais de 50 DLs foram descritas. Existem diferentes formas de classificação deste grupo de doenças. Historicamente esta divisão é feita tendo como base o tipo de substrato acumulado: mucopolissacaridoses (glicosaminoglicanos), glicogenoses (glicogênio), oligossacaridoses (oligossacarídeos) e lipidoses, onde se incluem as esfingolipidoses (lipídios e esfingolipídios, respectivamente) (Wraith 2002). Esta classificação não é adequada a todas as DLs. A Mucolipidose do tipo II, por exemplo, é ocasionada por um defeito genético primário que acarreta em deficiências enzimáticas múltiplas e consequente acúmulo de múltiplos tipos de substratos. Dessa forma, o tipo de defeito molecular apresentado também é usado para divisão em categorias como: deficiências de enzimas lisossomais, defeitos de tráfego de enzimas lisossomais, defeitos em proteínas lisossomais solúveis não enzimáticas e defeitos de proteínas de membrana lisossomal (Filocamo and Morrone, 2011; Platt, Boland and van der Spoel, 2012).

1.3. Esfingolipidoses

As DLs caracterizadas pelo acúmulo de esfingolipídios são denominadas esfingolipidoses. Esfingolipídios (ELs), juntamente com colesterol e glicerofosfolipídios, são componentes da membrana plasmática eucariótica e das membranas de algumas organelas, com o complexo de Golgi e os lisossomos. Além disso, os ELs são biomoléculas ativas envolvidas na sinalização celular e regulação do crescimento celular, diferenciação, senescência e apoptose (Bartke and Hannun 2009).

Com relação a sua estrutura, o elemento básico de um EL é uma base esfingóide de cadeia longa, como a esfingosina ou esfinganina (Figura 1A e 1B). A ligação de um ácido graxo a esta base forma uma ceramida (Figura 1C), composto intermediário para a biossíntese de todos os compostos mais complexos.

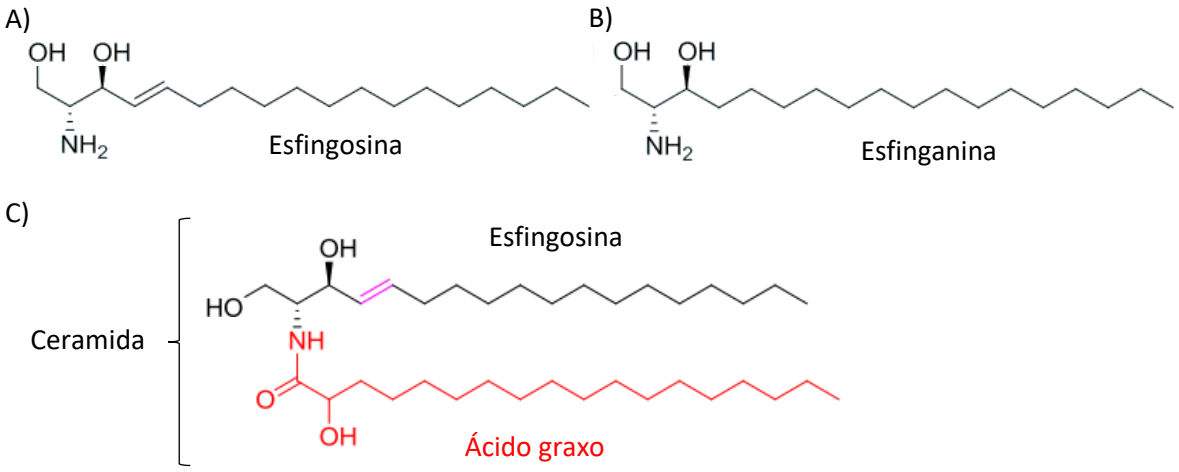


Figura 1: Estruturas mais comuns para bases esfingóides (A e B) e exemplo de ceramida (C). Adaptado de Fredi and Tinoco (2015).

Finalmente, a adição de fosofocolina ou um ou mais sacarídeos às ceramidas dá origem a esfingomielina e aos glicoesfingolipídios, respectivamente (Futerman and Riezman 2005; Fredi and Tinoco 2015). Os glicoesfingolipídios (Figura 2) podem ser classificados em 4 classes principais de acordo com os carboidratos adicionados em:

- Cerebrosídeos: contém um monossacarídeo ligado a ceramida. Comumente possuem galactose (galactocerebrosídeo), ou, em menor frequência, glicose (glicocerebrosídeo).

- Sulfatídeos: são cerebrosídeos com resíduos de galactosil sulfatados (galactocerebrosídeos sulfatados).

-Globosídeos: são cerebrosídeos com sacarídeos adicionais, predominantemente galactose, glicose ou N-acetilgalactosamina. A globotriaosilceramida está incluída nesta classe e é composta de dois resíduos de galactose ligados a uma glicosilceramida.

- Gangliosídeos: são similares aos globosídeos, mas contém uma ou mais moléculas de ácido N-acetilneuramínico (ácido siálico).

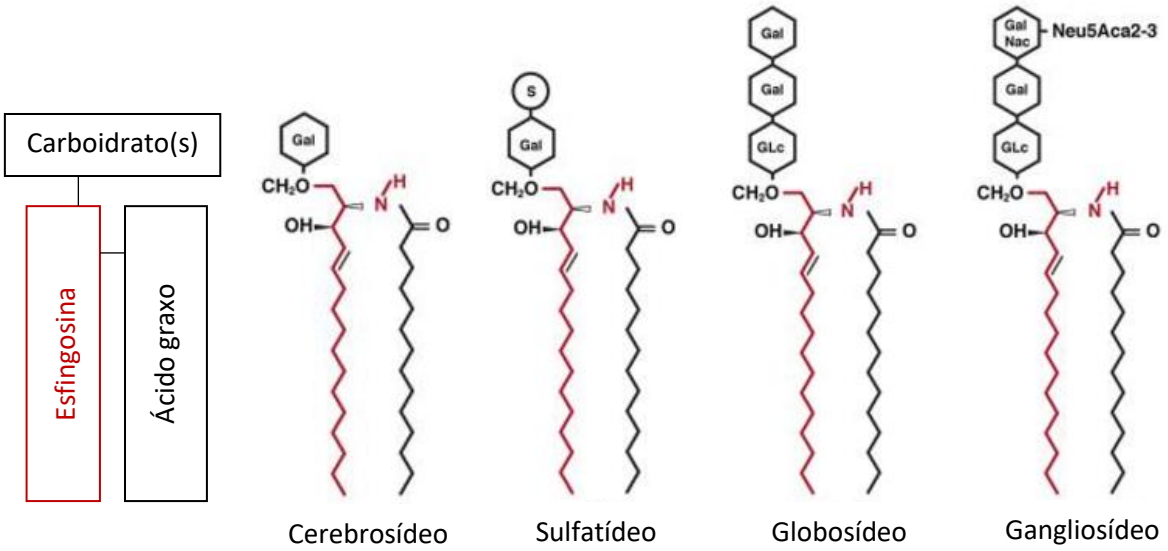


Figura 2: Estrutura dos glicoesfingolipídios. Gal: galactose; S: grupo sulfato; GLc: glicose; GalNac: N-acetilgalactosamina; Neu5Aca2-3: ácido siálico. Adaptado de Mencarelli and Martinez-Martinez (2013)

Os glicoesfingolipídios ainda podem ser subclassificados como neutros (sem sacarídeos carregados ou grupos iônicos diretamente ligados à ceramida), ácidos (com uma ou mais moléculas de ácido siálico) ou sulfatados.

Existe um *turnover* contínuo dos ELs e tanto a biossíntese quanto o catabolismo destas moléculas requerem a ação sequencial de uma série de enzimas e proteínas específicas; sendo o produto da reação anterior, o substrato da reação seguinte. As etapas de síntese dos ELs iniciam com a formação de ceramidas no lado citoplasmático do retículo endoplasmático pela ação de ceramidas sintases. As ceramidas são então transferidas para a membrana do complexo de Golgi onde a glicosilceramida é formada e convertida em lactosilceramida. Finalmente, a adição de carboidratos complexos por glicosilases dá origem aos glicoesfingolipídios e gangliosídeos, que atingem a membrana plasmática com o fluxo vesicular exocítico (Schulze and Sandhoff 2014).

O catabolismo dos ELs ocorre dentro dos lisossomos, acompanhando a degradação de membranas provenientes de processos autofágicos, fagocíticos ou endocíticos. Este processo requer a ação de hidrolases lisossomais específicas e é estimulado pelo lipídio aniônico bis(monoacilglicero)fosfato (BMP), presente nas membranas intraendossomais

(Sandhoff 2013). Além disso, por se tratarem de moléculas anfipáticas e enzimas hidrofílicas, é necessária a ação das proteínas ativadoras de esfingolipídios ou saposinas (SAP). Essas glicoproteínas podem agir como cofatores ligando-se diretamente às hidrolases (SAP A, C, D) ou como detergentes naturais, ligando-se aos substratos lipídicos (SAP B) (Munford et al. 1995). A deficiência de alguma dessas enzimas ou proteínas leva ao acúmulo de ELs não degradados ou parcialmente degradados nos lisossomos e ao estabelecimento das esfingolipidoses. A Figura 3 ilustra as vias de degradação dos ELs nos lisossomos e algumas das esfingolipidoses relacionadas. Mais detalhes sobre estas doenças estão descritos na Tabela 1.

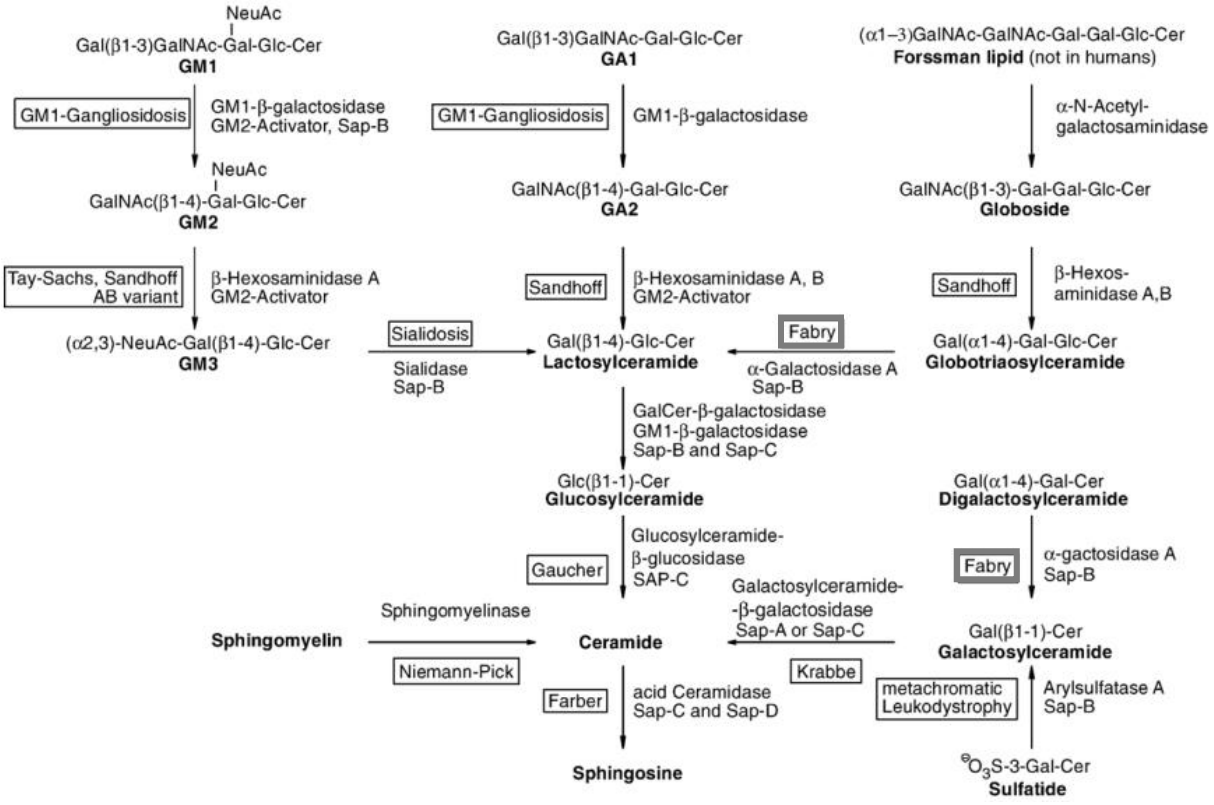


Figura 3: Vias de degradação de alguns esfingolipídios selecionados nos lisossomos. Os nomes das doenças relacionadas estão em retângulos e os das enzimas afetadas em negrito. Sap: saposina. Adaptado de Kolter and Sandhoff (2006).

Tabela 1: Classificação das esfingolipidoses e defeitos de ativação de esfingolipídios. Adaptado de Winchester (2012).

Doença (# fenótipo OMIM)	**Proteína deficiente**	**Material acumulado**	**Gene**
Deficiência de prosaposina (611721)	Prosaposina	Glicolipídios não-neuronais, Material ubiquitinado em neurolisossomos	*PSAP*
Doença de Fabry (301500)	α-galactosidase A	Glicosídeos galactosilados	*GLA*
Doença de Farber (228000)	Ceramidase ácida	Ceramida	*ASAH1*
Doença de Gaucher tipos I, II & III (230800, 230900, 231000)	β-glicosidase	Glicosilceramida	*GBA*
Doença de Gaucher atípica (610539)	Saposina C	Glicosilceramida	*PSAP*
Gangliosidose GM1 tipos I, II & III (230500, 230600, 230650)	β-galactosidase	Gangliosídeo GM1, queratan sufato, oligossacarídeos, glicolipídios	*GLB1*
Gangliosidose GM2			
Tay–Sachs (272800)	β-hexosaminidase A	Gangliosídeo GM2, oligossacarídeos globosídeo, glicolipídios	*HEXA*
Sandhoff (268800)	β-hexosaminidase A + B	Gangliosídeo GM2, oligossacarídeos	*HEXB*
Defeito do ativador de GM2 variante AB (272750)	Proteína ativadora de GM2	Gangliosídeo GM2, oligossacarídeos	*GM2A*
Leucodistrofia metacromática (250100)	Arilsulfatase A	Sulfatídeos	*ARSA*
Leucodistrofia metacromática (249900)	Saposina B	Sulfatídeos	*PSAP*
Leucodistrofia de células globosas / Doença de Krabbe (245200)	β-galactocerebrosidase	Galactosilceramida	*GALC*
Krabbe atípica pela deficiência de saposina A (611722)	Saposina A	Galactosilceramida	*PSAP*
Niemann–Pick A & B (257200 & 607616)	Esfingomielinase	Esfingomielina	*SMPD1*

As manifestações fenotípicas das esfingolipidoses apresentam grande variação entre doenças diferentes e entre pacientes com a mesma doença. Isso se deve a diferentes fatores. Um deles é o padrão de distribuição tecido-específica dos ELs. Gangliosídeos complexos são principalmente expressos nas membranas de neurônios enquanto ceramidas são essenciais para formação da barreira impermeável na pele. Portanto, defeitos no catabolismo desses compostos levam a danos no sistema nervoso central e a fenótipos cutâneos severos, respectivamente. Galactosilceramidas e sulfatídeos são componentes característicos da bainha de mielina e também estão presentes nos rins e os globosídeos são expressos em órgãos viscerais (Kolter and Sandhoff 2006; Schulze and Sandhoff 2014). Além disso, a atividade residual das enzimas afetadas também está inversamente correlacionada com a gravidade do fenótipo. Entretanto, em algumas DLs, como a Doença de Fabry, indivíduos que partilham o mesmo genótipo, e a mesma atividade enzimática residual, também podem apresentar variações fenotípicas.

1.4. Doença de Fabry

A Doença de Fabry (DF; OMIM # 301500) é uma esfingolipidose causada pela deficiência de atividade da hidrolase lisossomal α-galactosidase A (GLA, EC 3.2.1.22) com herança ligada ao X. Este defeito enzimático leva ao acúmulo de glicoesfingolipídios, principalmente globotriaosilceramida (Gb3), nos tecidos e fluidos corporais. Sinais típicos incluem acroparestesias, angioqueratomas, córnea *verticillata*, sintomas gastrointestinais e anormalidades de transpiração. Pacientes com a forma clássica apresentam baixa atividade residual da enzima com início dos sintomas na infância e desenvolvimento de sintomas cardíacos, cerebrovasculares e/ou renais. Pacientes com apresentações não clássicas tem atividade enzimática residual maior, início tardio dos sintomas e podem ser mono ou oligo-sintomáticos (Schiffmann et al. 2016).

1.4.1. Frequência

A DF é uma das doenças lisossômicas mais frequentes, sendo encontrada em todos os grupos étnicos. Dados de triagens neonatais estimaram a prevalência de DF em homens em cerca de 1:3.500 na Europa (Spada et al. 2006; Mechtler et al. 2012) e 1:1.250 na Ásia (Hwu et al. 2009). Entretanto, tendo em vista que esta frequência não é observada em adultos e que algumas das variantes consideradas patogênicas nestes estudas tiveram seu significado clínico revisto, é possível que estes dados sejam superestimativas. Mulheres com DF podem ser tão gravemente afetadas quanto homens. Contudo, apresentam níveis enzimáticos que se sobrepõe aos normais. Isso dificulta sua detecção nestas triagens que geralmente usam a atividade enzimática como o primeiro nível da análise. No Brasil, a frequência mínima de DF diagnosticada por ensaios bioquímicos foi de aproximadamente 0,22:100.000 nascidos vivos no período de 2000 a 2013 (Giugliani et al. 2017). Porém, este dado pode ser uma subestimativa, pois inclui dados de apenas um centro de diagnóstico.

1.4.2. Histórico

A DF foi descrita pela primeira vez em pacientes do sexo masculino com angioqueratomas por dois dermatologistas em 1898: Anderson na Inglaterra e Fabry na Alemanha (Anderson 1898; Fabry 1898). Em uma análise mais compreensiva de 8 famílias, foi sugerido o modo de herança ligado ao sexo e descritas outras manifestações da doença como: doença cerebrovascular, doença arterial coronariana, falência renal e dores nas extremidades. O desenvolvimento desses sintomas foi relacionado à deposição de "fosfatídeos" nos glomérulos renais, músculo liso e miocárdio (Wise et al. 1962). Em seguida, dois glicosfingolipídios neutros (globotriaosilceramida e digalactosilceramida) acumulados no rim de um paciente do sexo masculino foram isolados e caracterizados. Isso levou a classificação de DF como uma esfingolipidose (Sweeley and Klionsky 1963). A enzima envolvida (α-galactosidase A) foi identificada como ceramida trihexosidase, tendo sua especificidade anomérica descrita posteriormente (Brady et al. 1967; Kint 1970). Finalmente, o modo de herança ligada ao X foi confirmado experimentalmente e o gene da

α-galactosidase A foi clonado e caracterizado (Opitz et al. 1965; Bishop et al. 1988; Kornreich et al. 1989).

1.4.3. Aspectos bioquímicos

A α-galactosidase A (α-GAL ou α-Gal A, EC 3.2.1.22) é uma glicoproteína homodimérica e cada monômero é composto por dois domínios (Figura 4). O domínio 1, N-terminal, tem estrutura barril $(\beta/\alpha)_8$ e contém o sítio ativo formado pelas extremidades C-terminais das fitas β1 a β7, próximo ao centro do barril. O domínio 2, C-terminal, apresenta oito fitas β antiparalelas organizadas em duas folhas β. Na enzima ativa, após clivagem do peptídeo sinal de 31 resíduos, o domínio 1 se estende dos resíduos 32 a 300 e o domínio 2, do resíduo 331 ao 429. Além disso, a enzima tem sua superfície glicosilada, longe do sítio ativo, em 3 resíduos de asparagina: Asn139, Asn192 e Asn215. O resíduo Asn139 se liga a carboidratos complexos, enquanto Asn192 e Asn215 ligam-se a oligossacarídeos ricos em manose. Portanto, Asn192 e Asn215 estão envolvidos no endereçamento lisossomal pela via dos receptores de manose-6-fostato (Bekri 2006).

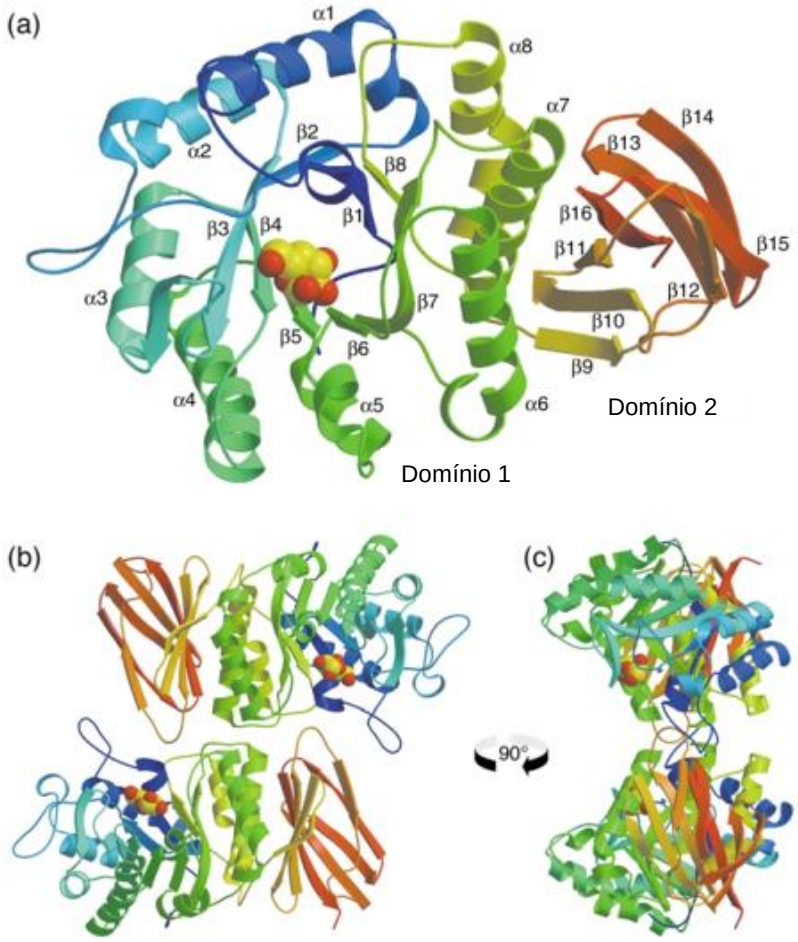


Figura 4: Estrutura da α-Galactosidase A. (a) Estrutura do monômero. A extremidade N-terminal está colorida de azul e a C-terminal, de vermelho. A enzima possui 2 domínios. O domínio 1 contém o sítio ativo no centro de das folhas β no barril $(\beta/\alpha)_8$. O domínio 2 possui oito filhas β antiparalelas organizadas em duas folhas β. O ligante galactose é representado em amarelo e vermelho. (b) e (c) Duas visões do dímero de α-Galactosidase A, forma na qual a enzima é ativa. Adaptado de Garman and Garboczi (2004).

O sítio ativo é uma região muito conservada nessa família de hidrolases. Em α-GAL (Figura 5), ele é formado pelas cadeias laterais de 15 resíduos: Trp47, Asp92, Asp93, Tyr134, Cys142, Lys168, Asp170, Cys172, Glu203, Leu206, Tyr207, Arg227, Asp231, Asp266 e Met267. As duas cisteínas, Cys142 e Cys172, formam uma ponte dissulfeto entre si (Garman and Garboczi 2004).

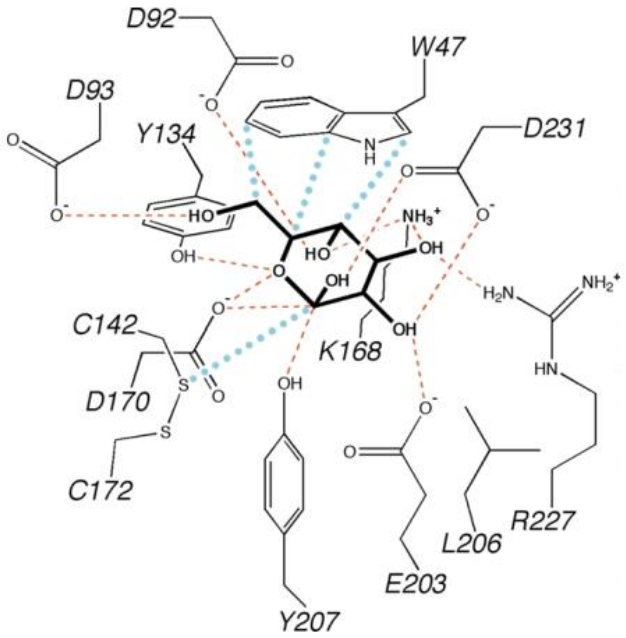


Figura 5: Sítio ativo da α-Galactosidase A. O ligante α-galactose é mostrado em negrito e as cadeias laterais dos aminoácidos com os quais interage estão identificadas. Pontes de hidrogênio e interações polares estão indicadas em vermelho; ligações de van der Waals em azul. (Garman and Garboczi 2004)

A α-GAL é sintetizada como um precursor glicosilado de 429 aminoácidos e 52 kDa no retículo endoplasmático. Como ocorre nas demais enzimas lisossômicas, o precursor recebe resíduos de M6P no complexo de Golgi e, após transporte aos lisossomos pela via de M6P, é processado para a forma madura de 398 aminoácidos e 46 kDa com a clivagem do peptídeo sinal (Lemansky et al. 1987). Cerca de 5 a 20% da enzima produzida é secretada antes de chagar aos lisossomos (Kornfeld 1986). A reabsorção pode ser feita pela mesma célula ou células vizinhas e envolve principalmente os receptores de M6P da membrana plasmática e também os receptores sortilina e megalina (Prabakaran et al. 2011).

A função biológica da α-GAL é catalisar a remoção de resíduos terminais de α-D-galactose (Figura 6) de glicoesfingolipídios neutros, glicoproteínas e oligossacarídos, com especificidade anomérica para a ligação glicosídica α1,4. Esta reação ocorre durante o catabolismo de macromoléculas com o auxílio da SAP-B (Garman and Garboczi 2004). Na DF, a deficiência dessa enzima leva ao acúmulo de glicoesfingolipídios neutros,

principalmente globotriaosilceramida (Gb3) e seu derivado deacilado (sem a cadeia de ácidos graxos) globotriaosilesfingosina (liso-Gb3) (Aerts et al. 2008). Em menor escala, acumulam-se também digalactosilceramida (galabiosilceramida) e antígenos do grupo sanguíneo B. Em situações normais, as maiores concentrações Gb3 são encontradas nos rins, aorta, baço e fígado. Em indivíduos com DF, há acúmulo desse substratos em todos os tecidos; com maior concentração nos rins, coração e nódulos linfáticos (Desnick et al. 2001). O acúmulo de digalactosilceramida em DF ocorre nos rins, pâncreas, coração, pulmões, sedimento urinário e gânglios espinhais e simpáticos do sistema nervoso.

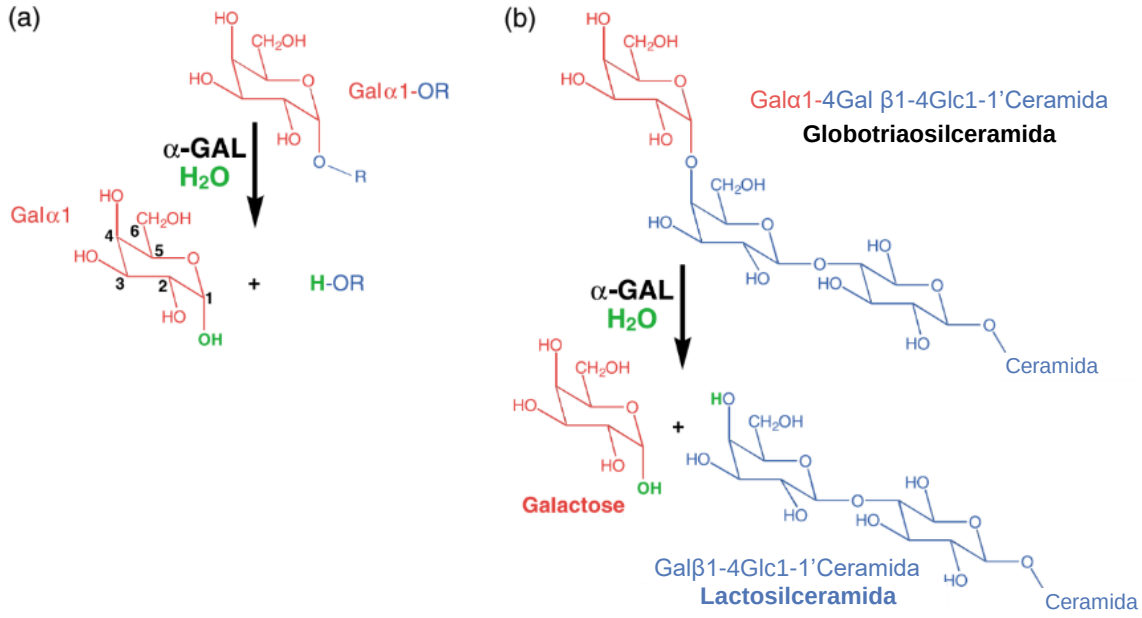


Figura 6: Reação catalisada pela enzima α-Galactosidase A (α-GAL). (a) Reação geral. Uma galactose terminal é clivada de um oligossacarídeo, glicoproteína ou glicolipídio, produzindo α-galactose (Galα-1) e um álcool (H-OR). (b) α-GAL e Doença de Fabry. Normalmente, o substrato globotriaosilceramida é clivado pela α-GAL para gerar lactosilceramida. Com a deficiência da enzima funcional, a globotriaosilceramida se acumula nos tecidos. Gal: galactose; Glc: glicose. Adaptado de Garman and Garboczi (2004).

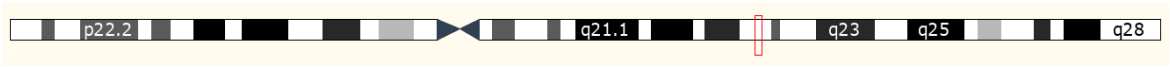
Na década de 1970, α-GAL foi descrita com parte de um par de isoenzimas, juntamente com a enzima então chamada α-galactosidase B (Schram et al. 1977; Dean et

al. 1977). Os genes codificantes dessas enzimas são homólogos e ambas são muito similares estruturalmente, com cerca de 50% de identidade na sequência de aminoácidos (Wang et al. 1990). Existe uma sobreposição da ação catalítica de ambas. A α-GAL tem afinidade por macromoléculas com resíduos de α-galactose terminais e a "α-galactosidase B" realiza também a clivagem de N-acetilgalactosaminas terminais. Entretanto, a clivagem de α-galactoses terminais por "α-galactosidase B" ocorre com uma eficiência muito menor. Atualmente, esta enzima é denominada α-N-acetilgalactosaminidase (α-NAGAL, E.C. 3.2.1.49). Sua deficiência está relacionada a Doença de Schindler / Kanzaki, onde ocorre o acúmulo de substratos contendo sacarídeos com ácido-siálico e galactose terminais, similar ao que ocorre nas doenças sialidose e galactosialidose. Apesar da sobreposição na especificidade das enzimas, a deficiência de α-GAL na Doença de Fabry não é compensada pela atividade em níveis normais de α-NAGAL (Garman and Garboczi 2002; Garman and Garboczi 2004; Clark and Garman 2009).

1.4.4. Aspectos genéticos

A enzima α-galactosidase A é codificada pelo gene *housepkeeping GLA* (MIM #300644) localizado no braço longo do cromossomo X, na região Xq22.1 (Desnick et al. 2001, Ensembl #ENSG00000102393, Figura 7A). *GLA* está na fita reversa e, na fita oposta, é flanqueado pelos genes *RPL36A* (proteína ribossomal L36a) e *HNRNPH2* (Ribonucleoproteína nuclear heterogênea H2) (Figura 7B). Este locus apresenta um processo de *read-through* transcricional natural entre ambos genes que dá origem a proteína RPL36A-HNRNPH2, similar a proteína ribossomal L36a (GeneCards, www.genecards.com).

A)



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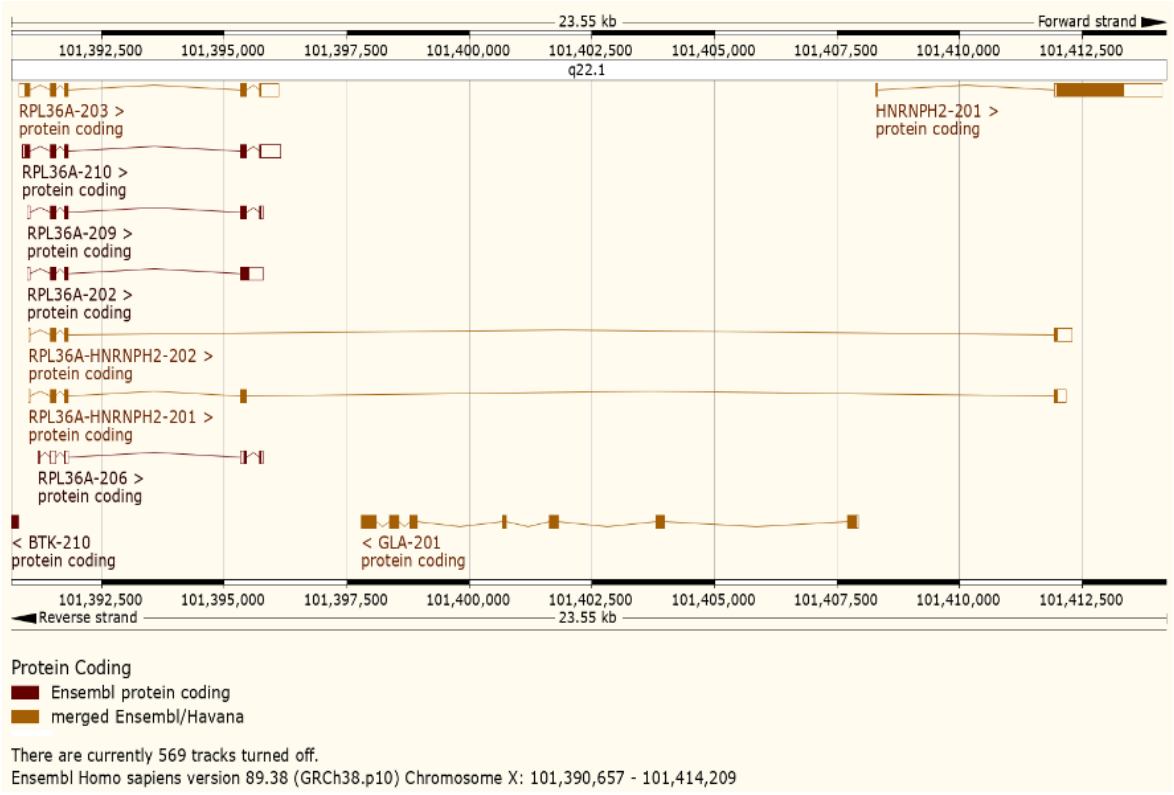

Figura 7: Localização cromossômica do gene *GLA*. A) Mapa citogenético do cromossomo X, com destaque em vermelho para região Xq22.1. B) Ampliação da região Xq22.1. *GLA:* α-galactosidase; *RPL36A:* proteína ribossomal L36a; *HNRNPH2*: Ribonucleoproteína nuclear heterogênea H2, RPL36A-HNRNPH2: *read-through* dos genes *RPL36A e HNRNPH2*. Fonte: Ensembl, transcrito ENSG00000102393.

O gene *GLA* é composto por 12.436 pb divididos em sete éxons que variam entre 92 a 309 pb e seis íntrons que variam de 218 a 3.725 pb (Bishop et al. 1988; Kornreich et al. 1989, GeneBank: NG_007119.1, Figura 8A). O transcrito principal deste gene tem 1.318 pb e dá origem a enzima de 429 aminoácidos. Através de *splicing* alternativo, um transcrito secundário é produzido (Figura 8B) em quantidades muito reduzidas em relação ao normal. Neste transcrito, há ativação de um éxon críptico, dentro do íntron 4. Porém, devido a criação de um códon de terminação prematuro, a proteína resultante tem apenas 222

aminoácidos. Além disso, o mRNA resultante é rapidamente degrado pela via de NMD (*nonsense-mediated mRNA decay*) (Ishii et al. 2002; Chiang et al. 2017).

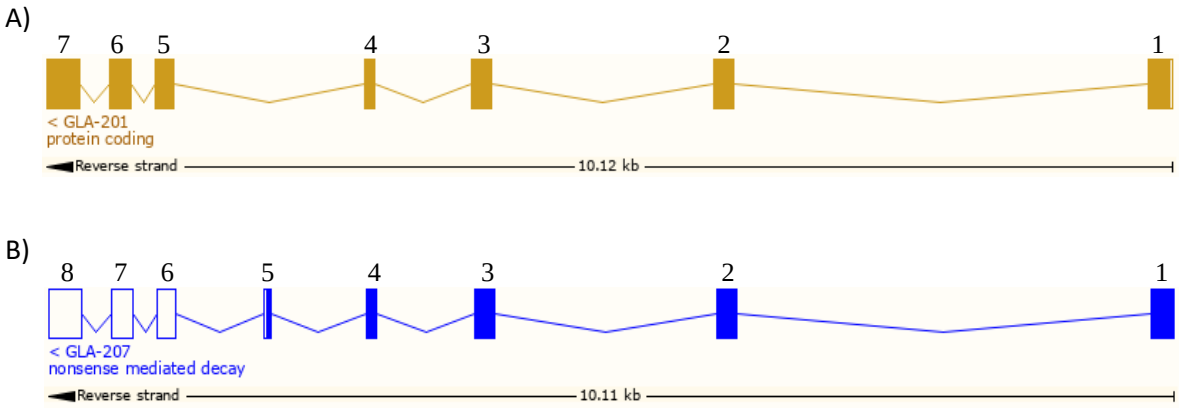

Figura 8: Estrutura do gene *GLA* na fita reversa do cromossomo X. A numeração dos éxons está indicada acima dos mesmos. A) Transcrito normal com 7 éxons e 429 aminoácidos. B) Transcrito alternativo, com 8 éxons e 222 aminoácidos. Quadros vazados representam regiões exônicas não traduzidas. Fonte: Ensembl, transcritos ENST00000218516.3 e ENST00000493905.6.

Outra característica estrutural deste gene é o enriquecimento de elementos repetitivos, que representam cerca de 30% da sequência (Figura 9). No total, 12 elementos *Alu* estão distribuídos pelos 6 íntrons e região 3' UTR (Kornreich et al. 1990). Estas sequências estão associadas a eventos recombinatórios. Porém, apesar de sua alta densidade, sequências *Alu* não estão frequentemente envolvidas em rearranjos gênicos em *GLA* (Kornreich et al. 1990). Além disso, rearranjos não são causas frequentes de DF.

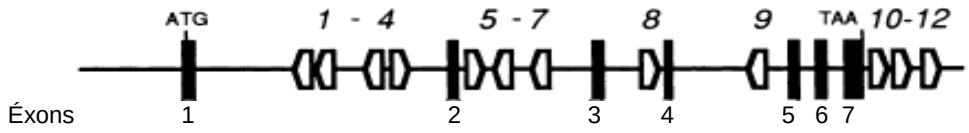

Figura 9: Elementos repetitivos *Alu* (formas pentagonais) no gene *GLA*. ATG: códon de iniciação; TAA: códon de parada. Adaptado de Desnick et al. (2001).

Um ponto importante e distintivo das demais esfingolipidoses, e doenças lisossômicas, é a presença de variantes privadas, observadas em uma ou poucas famílias, com consequente ausência de variantes recorrentes, em geral. Exceções incluem a variante c.936+919G>A, relacionada ao fenótipo não-clássico e muito frequente na população asiática; e algumas mutações em dinucleotídeos CpG, conhecidos por serem *hot-spots* mutacionais, como: Arg118, Asn215, Arg227, Arg342 e Arg356. Isso se traduz em um alto nível de heterogeneidade alélica nesta doença quando comparada às demais esfingolipidoses e defeitos de ativação de esfingolipídios, que tem em média 173 variantes descritas (Tabela 2).

Tabela 2: Número de variantes descritas em genes relacionados às esfingolipidoses e defeitos de ativação de esfingolipídios.

Gene	**Doenças(s) relacionadas**	**Nº de variantes***
GLA	Doença de Fabry	911
GBA	Doença de Gaucher tipos I, II & III	460
SMPD1	Niemann–Pick A & B	237
GALC	Doença de Krabbe	230
ARSA	Leucodistrofia metacromática	217
GLB1	Gangliosidose GM1 tipos I, II & III	213
HEXA	Tay–Sachs	182
HEXB	Sandhoff	108
ASAH1	Doença de Farber	55
PSAP	Krabbe atípica pela deficiência de saposina A, Leucodistrofia metacromática, Doença de Gaucher atípica	24
GM2A	Defeito do ativador de GM2 variante AB	9

*Variantes depositadas no Human Genome Mutation Database em outubro de 2017 (HGMD Professional v2017.2)

O *Human Mutation Database* (Stenson et al. 2009), um banco de dados curado de mutações humanas, conta com 911 variantes no gene *GLA* em sua versão profissional

2017.2 (Tabela 3). Em agosto de 2016 (versão 2016.2), este número era de 845 variantes. Mais da metade das mutações descritas são de sentido trocado ou sem sentido (69.8%). Pequenas alterações dentro da região codificante representam 89,2% das mutações. Por outro lado, grandes rearranjos correspondem a apenas 5,5% do total de variantes descritas. Essas características fazem com que o sequenciamento convencional por Sanger seja um método apropriado para diagnóstico de DF.

Tabela 3: Variantes do gene *GLA* depositadas no *Human Genome Mutation Database* (HGMD).

Tipo	**N**	**% total**
Sentido trocado / Sem sentido	636	69.8%
Pequenas deleções	124	13.6%
Pequenas inserções	40	4.4%
Pequenas indels	13	1.4%
Splicing	42	4.7%
Regulatórias	5	0.5%
Grandes deleções	37	4.1%
Grandes inserções	6	0.7%
Rearranjos complexos	7	0.8%
Total	**845**	**100%**

Fonte: HGMD Professional versão 2017.2

1.4.5. Diagnóstico

O diagnóstico de DF é feito em geral pelo menos 3 anos depois do aparecimento dos sinais e/ou sintomas em ambos os sexos, ainda que, em alguns casos, possa ser feito em até 20 anos (Mehta et al. 2004; Ramaswami et al. 2006). Razões para isso incluem a raridade da doença e o consequente desconhecimento por parte de muitos médicos e a diversidade de sintomas não específicos que se assemelham aos de doenças mais comuns (Mehta et al. 2010).

Em hemizigotos, o diagnóstico pode ser feito na maioria das vezes por medida da atividade enzimática de α-GAL em leucócitos. Dosagens em plasma ou papel filtro também podem realizadas, mas são menos confiáveis por apresentarem níveis maiores de falsos positivos por degradação da amostra e/ou devido a existência da variante p.Asn313Tyr, relacionada a pseudodeficiência em DF. Pacientes com forma clássica doença apresentam níveis muito reduzidos de atividade de α-GAL. Por outro lado, pacientes com a forma não-clássica podem apresentar atividade residual maior. Geralmente, níveis abaixo de 25% do normal podem ser considerados diagnósticos e níveis entre 25% a 35% devem ser confirmados por outros métodos (Desnick et al. 2001).

Heterozigotas podem apresentar níveis enzimáticos muito variáveis, com sobreposição aos níveis normais. Dessa forma, a medida isolada da atividade enzimática em um único tipo de amostra não é recomendada para diagnóstico de mulheres com DF. A análise do gene *GLA* completo ou da região afetada pela variante familiar é crucial para o diagnóstico de mulheres com suspeita ou história familiar de DF.

Vale ressaltar que os ensaios bioquímicos devem sempre incluir a adição de α-N-acetilgalactosamina, um inibidor específico da enzima α-N-acetilgalactosaminidase que possui especificidade cruzada com α-GAL. Além disso, outra enzima lisossomal deve ser usada como referência e medida concomitantemente para garantir a qualidade da amostra e do ensaio (Gal et al. 2011).

A dosagem de Gb3 e liso-Gb3 plasmáticos, urinários ou teciduais já foi sugerida como método diagnóstico em ambos os sexos. Porém, Gb3 também se encontra elevado em pacientes com doença cardíaca sem DF (Schiffmann et al. 2014). Além disso, apesar da alta especificidade, o uso de liso-Gb3 urinário pode acarretar em falsos-negativos, principalmente em variantes atípicas (Auray-Blais et al. 2010). Atualmente, Gb3, liso-Gb3 e seus análogos são comumente usados como biomarcadores para acompanhamento da progressão da doença e eficácia dos tratamentos (Ferreira et al. 2015a; Arends et al. 2016). Porém, a aplicação no monitoramento da eficácia do tratamento por TRE apresenta deficiências, já que esses marcadores atingem um *plateau* após 3 meses de tratamento em pacientes que utilizam qualquer uma das enzimas disponíveis (van Breemen et al. 2011).

Para casos de diagnóstico pré-natal, recomenda-se a análise da variante familiar em cultivos de amniócitos ou vilosidades coriônicas. O diagnóstico bioquímico também pode ser realizado em fetos do sexo masculino pela medida de α-GAL em vilosidades coriônicas (cultivadas ou não) ou em amniócitos cultivados coletados na 10ª e 14ª semanas de gestação, respectivamente (Germain 2010).

Devido ao tamanho reduzido do gene e do perfil das mutações descritas, que engloba menos de 5% de grandes rearranjos gênicos, o diagnóstico genético de DF pode ser feito por sequenciamento de Sanger na maior parte dos pacientes. Caso a variante patogênica não seja identificada por Sanger em pacientes com diagnóstico bioquímico confirmado, outros métodos de diagnóstico genético devem ser aplicados. Entre as opções disponíveis, pode-se citar o MLPA (*Multiplex Ligation-dependent Probe Amplification*). Esta é uma técnica de análise de alterações no número de cópias (deleções e duplicações) de regiões como éxons ou genes inteiros. É baseada na hibridização simultânea de sondas em genes alvo e de referência e posterior amplificação e análise de fragmentos. O sequenciamento de cDNA também pode ser utilizado para análise de grandes inserções, deleções, indels, e inversões, sendo útil também para confirmação de possíveis alterações de *splicing*.

Além disso, técnicas de citogenética molecular podem ser úteis para a detecção de outras alterações cromossômicas concomitantes com a variante causal de DF que agravam o fenótipo. Apesar de raros, estes eventos já foram descritos na literatura (Hossain et al. 2017). Na hibridação genômica comparativa baseada em microarranjos de oligonucleotídeos (aCGH) ocorre a hibridação de amostras de DNA marcadas fluorescentemente do paciente e de um controle em sondas específicas. Por comparação da intensidade da fluorescência é possível a detecção de deleções e duplicações de grandes segmentos gênicos. Os microarranjos de SNP são uma variação desta técnica e permitem também a detecção de perda de heterozigosidade e dissomia uniparental. Uma limitação de ambas metodologias é a necessidade de confirmação dos pontos de quebra exatos dos rearranjos por técnicas complementares, como sequenciamento de Sanger. Atualmente, o uso do sequenciamento massivo paralelo (“sequenciamento de nova geração”), tem se tornado cada vez mais acessível e utilizado no diagnóstico de DF e outras doenças,

permitindo a detecção de mutações de ponto a grandes rearranjos. Esta técnica inclui o sequenciamento de genomas inteiros, de exomas e o uso de painéis com genes específicos, que tem o benefício de diminuir implicações éticas relacionadas a achados incidentais. Mais detalhes sobre as técnicas utilizadas frequentemente no diagnóstico genético de DF e de outras doenças lisossômicas estão descritos no artigo "*Current molecular genetics strategies for the diagnosis of lysosomal storage disorders*" (Giugliani et al. 2016a), incluído na seção de anexos desta tese (anexo II).

Devido à natureza não especifica dos sintomas, muitos estudos na literatura relatam triagem para DF em grupos de risco como pacientes submetidos a transplante renal, com acidente vascular cerebral ou cardiomiopatia hipertrófica (Linthorst et al. 2010; Goeggel Simonetti et al. 2015; Coutinho et al. 2017; Kubo et al. 2017; Yılmaz et al. 2017). Além disso, estudos de triagem neonatal para DF e outras doenças lisossômicas vem se tornando cada vez mais frequentes e com maior abrangência (Colon et al. 2017; Liao et al. 2017); inclusive no Brasil, com pode ser observado no anexo III desta tese (Bravo et al. 2017). Os métodos de detecção envolvidos nestes projetos geralmente envolvem dosagem enzimática em amostras de sangue em papel filtro como primeira etapa da triagem, o que leva a uma subestimativa do número de mulheres heterozigotas. Alternativamente, técnicas de *screening* de mutações, como HRM (*high resolution melting*), já foram utilizadas (Bono et al. 2011; Tai et al. 2012). Essa técnica se baseia na diferença da curva de dissociação de amplicons na presença de corantes fluorescentes saturantes e não requer manipulação da amostra após a amplificação gênica. A genotipagem por espectrometria de massas também já foi descrita em uma população taiwanesa (Lee et al. 2014). Apesar da possibilidade de avalição de um alto número de amostras simultâneas, essa metodologia é limitada a análise de alelos específicos definidos previamente pelo usuário.

1.4.6. Mecanismos patológicos

A DF é caracterizada pela deficiência de α-GAL e consequente acúmulo de glicoesfingolipídios, principalmente Gb3, que começa já no período gestacional (Vedder et al. 2006). Estes substratos se acumulam principalmente nas células endoteliais; pericitos e

músculo liso de capilares cutâneos, vênulas e arteríolas (Desnick et al. 2001). Apesar do início precoce da deposição de substratos, a maioria dos pacientes se mantem assintomática durante os primeiros anos de vida (Germain 2010).

O padrão de depósito de Gb3 se correlaciona com os principais achados clínicos de DF e de forma geral os níveis de Gb3 são elevados na urina e no plasma de homens com a forma clássica. Porém, heterozigotas e pacientes com formas atípicas podem apresentar valores normais. Além disso, os níveis de Gb3 não se correlacionam com a gravidade da doença e apesar da normalização de Gb3 com a terapia de reposição enzimática pode haver progressão do quadro clínico (Young et al. 2005; Rombach et al. 2010). Dessa forma, o acúmulo primário Gb3 não é suficiente para explicar a toda a patofisiologia da doença.

Porém, Gb3 induz a produção de espécies reativas de oxigênio e consequente dano oxidativo, estimulando a inflamação, e aumenta a transcrição de moléculas de adesão envolvidas na vasculopatia da DF (Shen et al. 2008). Além disso, a deposição de Gb3 está correlacionada com as manifestações oftalmológicas (Sodi et al. 2007). Do mesmo modo, existe associação entre Gb3 e manifestações em células renais, que são mais resistentes à normalização com terapia de reposição enzimática. A deposição de Gb3 nos rins pode ser vista em todos os tipos celulares por microscopia eletrônica, independente da aparência histológica normal (Valbuena et al. 2008). Este acúmulo é progressivo com a idade e está associado a alterações na morfologia e danos aos podócitos. Os podócitos são células com capacidade regenerativa limitada, que mantem inclusões maiores de Gb3 e por mais tempo que células renovadas com frequência. Dessa forma, danos a estas células não são compensados por replicação das células restantes, levando a diminuição de seu número total e consequente glomeruloesclerose, um achado comum na nefropatia avançada de DF (Fall et al. 2016; Trimarchi et al. 2016). *In vitro*, o acúmulo de Gb3 em linhagens de podócitos também está associado com desregulação da autofagia, com aumento do número de autofagossomos e diminuição do seu regulador negativo mTOR (Liebau et al. 2013)

Além de Gb3, também há acúmulo significativo de sua forma deacilada, liso-Gb3. Vale ressaltar que na maioria dos estudos de associação entre Gb3 e manifestações da

doença, não se faz uma caracterização completa dos glicoesfingolipidios acumulados, de forma que os resultados podem ser devidos ao acúmulo concomitante de Gb3 e liso-Gb3. Os níveis plasmáticos deste composto em hemizigotos com a forma clássica da doença são 100 vezes maiores que os níveis normais. Porém, em pacientes com a forma não clássica e alta atividade residual de α-GAL, seus níveis são muito baixos (Aerts et al. 2008; Arends et al. 2016). Em heterozigotas pré-sintomáticas os níveis podem estar dentro do padrão normal (Rombach et al. 2010; Smid et al. 2015). A síntese de liso-Gb3 pode ocorrer por glicosilação sequencial de bases esfingóides ou por deacilação do Gb3 acumulado pela enzima ceramidase ácida. É uma molécula bioativa importante na patofisiologia da doença. Ele inibe a atividade da α-GAL e, por consequência, aumenta o acúmulo progressivo de Gb3 (Aerts et al. 2008). O tempo de exposição a esta molécula foi correlacionado com a gravidade da doença (Rombach et al. 2010).

No modelo animal de DF há uma relação direta entre os níveis de liso-Gb3 e dor, uma manifestação frequente e debilitante nos pacientes com DF. O aumento de liso-Gb3 sensibiliza os neurônios sensoriais, aumentando o influxo de cálcio e aumentando as correntes dependentes de cálcio nos neurônios dos gânglios da raiz dorsal, o que resulta na hipersensibilidade a dor (Choi et al. 2015). Experimentos *in vitro* com podócitos cultivados indicam que o liso-Gb3 pode estar envolvido com dano glomerular através do aumento da expressão do fator de crescimento transformador β1 (TGF-β1) e do receptor para fator de inibição de migração de macrófagos (CD74), ambos envolvidos com a nefropatia diabética. TGF-β1 é um mediador da expressão de matriz extracelular, fibrose e dano aos podócitos. CD74 é um receptor da citocina MIF (*Macrophage migration inhibitory factor*), e está associado à glomerulonefrite (Sanchez-Niño et al. 2011). Além disso, a ativação da via de sinalização Notch1 por liso-Gb3 também contribui para o dano aos podócitos e fibrose renal através da superexpressão de proteínas da matriz extracelular e ativação de vias inflamatórias (Sanchez-Niño et al. 2015).

Na doença cardíaca, há depósito de glicoesfingolipídios em todos os componentes celulares do coração, incluindo cardiomiócitos, células do sistema de condução, fibroblastos valvulares, células endoteliais e células da musculatura lisa vascular (Zarate and Hopkin 2008). Entretanto, o acúmulo de substratos representa menos de 5% da massa

cardíaca total (Elleder et al. 1990), o que indica que outros mecanismos patológicos também estão envolvidos. A indução de estresse oxidativo pelos glicoesfingolipídios também afeta o sistema cardíaco, levando a danos ao DNA, mitocôndrias e proteínas em cardiomiócitos. Isso leva à degradação de elementos contráteis, disfunção contrátil e rigidez do miocárdio, redução da síntese de ATP necessária à contração e morte celular (Chimenti et al. 2015; Frustaci et al. 2017). Os níveis plasmáticos de liso-Gb3 foram relacionados com a massa do ventrículo esquerdo (Rombach et al. 2010). A hipertrofia desta região é um dos achados clínicos mais comuns em DF. Além disso, a hipertrofia dos cardiomiócitos em geral é uma etapa inicial da disfunção cardíaca, com subsequente aparecimento de anormalidades do interstício e fibrose (Linhart and Elliott 2007). Finalmente, a indução da proliferação de células musculares lisas por liso-Gb3 pode estar envolvida com o espessamento visto na camada íntima-média nas carótidas (Aerts et al. 2008).

As manifestações cerebrovasculares, como acidentes vasculares cerebrais, podem ser consequências diretas de embolismo cardiogênico. Alternativamente, podem ser devido às mudanças nas paredes dos vasos, secundárias ao acúmulo dos glicoesfingolipídios, com alteração das vias de sinalização e ativação da cascata de coagulação que levam ao estreitamento do lúmen e aumento da fragilidade (Zarate and Hopkin 2008).

A esfingosina-1-fosfato (S1P) é outra molécula bioativa com níveis elevados no plasma pacientes com DF. Em hemizigotos, há um aumento de 30% a 40% em relação a controles normais. Em mulheres, os níveis podem ser iguais aos masculinos, mas devido à grande variação dos níveis, não há diferença significativa com controles. Este esfingolipídio é gerado pela degradação lisossômica de esfingomielina e glicoesfingolipídios, que dá origem a esfingosina que; por sua vez, é fosforilada por esfingosina quinases no citoplasma. A S1P é capaz de estimular a proliferação de células musculares lisas vasculares de forma dose-dependente *in vitro.* No modelo animal de DF, foram detectados níveis de 2 a 3 vezes maiores que o normal nos rins, e a administração de S1P levou a indução de hipertrofia cardíaca. Em pacientes com DF, está correlacionada com o aumento da espessura da

camada íntima-média da parede da carótida e hipertrofia do ventrículo esquerdo, sem aumento da pressão arterial (Brakch et al. 2010; Mirzaian et al. 2016).

1.4.7. Aspectos clínicos

A DF possui herança ligada ao X e as manifestações clássicas iniciam na infância ou adolescência e incluem crises periódicas de dor e parestesias das extremidades (acroparestesias), lesões cutâneas vasculares (angioqueratomas), alterações na produção de suor, lesões oculares características (opacidade de córnea e cristalino) e sintomas gastrointestinais (dor abdominal e diarreia) (Laney et al. 2014). Na idade adulta, as manifestações progridem para graves complicações renais, cardíacas e cardiovasculares que levam a morte precoce. A expectativa de vida de homens e mulheres com FD é reduzida em 5 e 16 anos, respectivamente, quando comparada à média populacional (Waldek et al. 2009).

Pacientes com até 25% a 30% de atividade residual da enzima tem apresentação clinica não clássica, com início tardio da doença e fenótipo mais brando. Estes pacientes são essencialmente assintomáticos na idade em que hemizigotos clássicos já apresentam o quadro completo da doença. Podem apresentar sintomas restritos a um ou poucos órgãos, mais frequentemente manifestações cardíacas (variante cardíaca) ou renais (variante renal) (Schiffmann and Ries 2016).

Heterozigotas tem amplitude fenotípica muito ampla. Podem ser assintomáticas ou apresentarem todas as manifestações clínicas clássicas, geralmente com gravidade menor do que visto em homens. Porém, algumas heterozigotas podem apresentar manifestações clínicas e evolução tão grave quanto aquelas em hemizigotos (Schiffmann and Ries 2016).

As principais manifestações clínicas da DF estão descritas a seguir:

- Angioqueratomas ou angiectasias (Figura 10): são as manifestações precoces mais visíveis. Podem aparecer na infância, mas são mais frequentes encontradas a partir dos 14-16 anos. São lesões de pele roxo-avermelhadas que tendem a aumentar em número e

tamanho com a idade, se concentrando nas regiões abdominal, genital e extremidades inferiores (Zarate and Hopkin 2008).

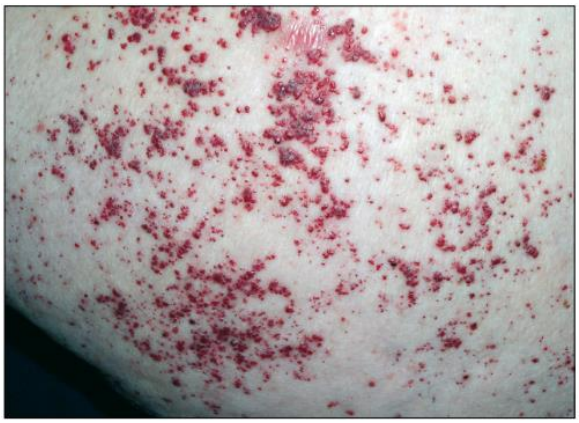

Figura 10: Exemplo de angioqueratomas típicos em um paciente com Doença de Fabry (Zarate and Hopkin 2008).

- Dor neuropática (acroparestesias): existem dois tipos de dor descritos em DF, a dor crônica caracterizada por sensação de formigamento e queimação nas extremidades (acroparestesias) e as crises episódicas de dor. As crises são descritas como uma sensação excruciante de ardência e/ou dor em pontada nas extremidades (palmas das mãos e solas dos pés) com irradiação proximal (Desnick et al. 2001). As crises de dor também podem incluir dor abdominal e são desencadeadas por estresse, febre, exercício e rápidas mudanças na temperatura ambiente. São causadas por neuropatia de fibras pequenas e tendem a se tornar menos frequentes e graves com a idade pela deterioração da função sensorial. Esse sintoma é um dos mais precoces e generalizados, inicia na infância ou adolescência e atinge de 60 a 80% dos pacientes com a forma clássica (Schiffmann and Ries 2016).

- Manifestações oculares: as alterações oculares são características e envolvem a córnea, cristalino, conjuntiva e retina. Córnea *verticillata* é a manifestação mais frequente, presente em mais de 70% dos pacientes, geralmente sem outros sintomas oculares concomitantes. Aparece inicialmente como opacificação difusa e depois como faixas fusiformes. Pode ser detectada por exame com lâmpada de fenda, juntamente com tortuosidade dos vasos da retina e conjuntivo (Figura 11) (Sodi et al. 2007).

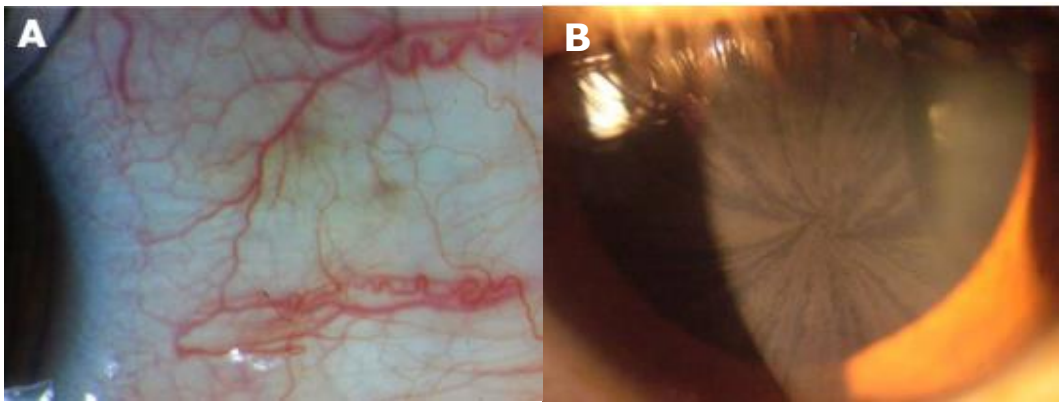

Figura 11: Manifestações oculares da Doença de Fabry. A) Tortuosidade dos vasos conjuntivos (Germain 2010); B) Córnea *verticillata* (Sodi et al. 2007).

- Manifestações renais: os sintomas renais são progressivos e avançam com a idade. Podocitúria (aparecimento de podócitos na urina) é o primeiro sinal de comprometimento renal, presente em indivíduos pré-sintomáticos (Fall et al. 2016; Trimarchi et al. 2016). Durante a segunda a terceira décadas de vida geralmente há microalbuminúria e proteinúria, que contribuem diretamente para progressão da nefropatia. Em seguida, ocorrem anormalidades da função tubular e declínio da função renal global. Finalmente, os pacientes apresentam uremia/azotemia (aumento de ureia na urina/compostos nitrogenados no sangue) em torno da terceira ou quarta década de vida levando à insuficiência renal e à necessidade de diálise e transplante renal (Germain 2010; Waldek and Feriozzi 2014).

- Manifestações cardíacas: pacientes com DF podem ter virtualmente qualquer manifestação cardíaca. Todas as estruturas cardíacas, incluindo o miocárdio, sistema de condução e válvulas podem ser afetados (Mehta et al. 2010). As manifestações mais frequentes incluem hipertrofia de ventrículo esquerdo, doença valvular (especialmente insuficiência mitral), anormalidades de condução miocárdica, arritmias, angina e dispneia (Linhart et al. 2007; Kampmann et al. 2008). Dados do *Fabry Outcome Survey*, um registro mundial observacional, indicam que na consulta inicial, a frequência de qualquer sintomas cardíaco varia de 39% em mulheres não tratadas a 66% em homens tratados com terapia

de reposição enzimática (Linhart et al. 2007). Além disso, a doença cardíaca é a causa mais frequente de mortalidade em pacientes de ambos os sexos (Linhart et al. 2007; Mehta et al. 2009; Waldek et al. 2009).

- Manifestações cerebrovasculares: no sistema nervoso central a DF se manifesta predominantemente através de doença vascular, de natureza primariamente multifocal e de pequenos vasos (Germain 2010). O ataque isquêmico transitório (AIT) e o acidente vascular cerebral (AVC) são os sintomas mais frequentes e recorrentes, com frequência de 27% em mulheres e 12% em homens. A média da idade de aparecimento desses sintomas é de 28 anos em homens e 43 em mulheres. Porém, AIT também foi descrito em crianças (Mehta et al. 2004). Dos pacientes que apresentam AVCs, de 40 a 50% tem seu primeiro evento antes de receberem o diagnóstico de DF e mais de 70% não tem manifestações renais ou cardíacas associadas (Sims et al. 2009).

1.4.8. Tratamento

Tendo em vista que a DF apresenta manifestações de natureza não específica e que são indistinguíveis de complicações similares presentes na população em geral, ela é responsiva a tratamento clínico e cirúrgico padrão (Schiffmann and Ries 2016). Dessa forma, intervenções complementares convencionais podem ser indicadas de acordo com o quadro clínico dos pacientes (Tabela 4).

Tabela 4: Tratamentos não específicos para Doença de Fabry

Manifestação	**Terapia**
Acroparestesia	Anticonvulsivantes (p. ex. carbamazepima, gabapentina, fenitoína)
Acidente vascular cerebral (prevenção)	Anticoagulantes e antiplaquetários (p. ex clopidogrel, ácido acetilsalicílico)
Manifestações cardiovasculares	Diuréticos, inibidores da enzima de conversão da angiotensina, bloqueadores dos receptores de angiotensina, beta-bloqueadores, implante de marca-passo
Manifestações renais	Anti-hipertensivos, inibidores da enzima de conversão da angiotensina, bloqueadores dos receptores de angiotensina, diálise, transplante renal.
Anidrose	escolha de um ambiente de trabalho frio e redução do esforço físico

Adaptado de Schiffmann and Ries (2016)

O tratamento específico para DF é a administração da enzima deficiente através da terapia de reposição enzimática. Além disso, o tratamento com chaperonas farmacológicas já foi aprovado para uso clínico na Europa e produtos para terapia redução de substrato estão em desenvolvimento. Essas três abordagens terapêuticas estão descritas abaixo. De forma complementar, a aplicação destas e outras terapias para em outro grupo de DLs, as mucopolissacaridoses, estão descritas nos anexos IV e V desta tese.

1.4.8.1. Terapia de reposição enzimática

A terapia de reposição enzimática (TRE) consiste na administração de enzimas recombinantes glicosiladas e se baseia na via de endereçamento lisossomal pelos receptores de manose-6-fostato. Atualmente existem dois medicamentos disponíveis aprovados para TRE: agalsidase alfa (Replagal, Shire Human Genetic Therapies Inc.) e

agalsidase beta (Fabrazyme, Genzyme Corp). A agalsidase alfa é produzida em uma linhagem de fibroblastos humanos, administrada na dosagem de 0,2 mg/kg durante aproximadamente 40 minutos, geralmente sem pré-medicação. A agalsidade beta é produzida em células CHO (ovário de hamster chinês) e administrada em infusão intravenosa na dose de 1mg/kg, durante cerca de 3 horas, com pré-medicação utilizada rotineiramente. Ambas possuem propriedades bioquímicas e estruturais semelhantes e são administradas de forma intravenosa a cada 15 dias (Clarke 2007). Porém, devido à diferença nos sistemas de produção as estruturas das cadeias de oligossacarídeos são distintas e resultam em variações na distribuição tecidual e na resposta à dosagem (Garman and Garboczi 2004). A agalsidase beta contém uma proporção maior de resíduos de manose-6-fostato, o que aumenta sua ligação ao receptor de manose e sua captação celular (Lee 2003).

A Pegunigalsidase alfa (Protalix Biotherapeutics) atualmente está sendo avaliada em estudos clínicos de fase III e poderá ser uma terceira opção terapêutica para TRE. Esta enzima é produzida em células BY2 de tabaco. Além disso, é modificada quimicamente com a adição de cadeias de polietilenoglicol 2000 (PEG2000) que ligam de forma covalente as duas subunidades da enzima. A proteína resultante é mais estável e ativa do que ambas agalsidades, além de possuir meia-vida maior (Kizhner et al. 2015).

De uma forma geral a TRE normaliza os níveis de Gb3 e liso-Gb3. Ela reduz o declínio da função glomerular renal, principalmente se iniciada antes da taxa de filtração glomerular estar muito reduzida. A dor neuropática tende a se reduzir com o passar do tempo em adultos e crianças, mas não é curada. Não há redução do risco de acidente vascular cerebral e há poucas evidências que suportem grandes melhoras nos sintomas cardiovasculares (Schiffmann and Ries 2016). Porém, apesar de não reduzir a hipertrofia do ventrículo esquerdo, manifestação cardíaca mais frequente, a TRE é capaz de prevenir a formação de fibrose se iniciada antes de seu estabelecimento (Nagano et al. 2016). Dessa forma, é possível que a TRE em DF não seja capaz de reverter sintomas já estabelecidos em diversos sistemas, assim como visto em outras doenças lisossômicas (Pasqualim et al. 2015a).

1.4.8.2. Terapia com chaperonas farmacológicas

Chaperonas farmacológicas são pequenas moléculas que podem ser administradas oralmente. Elas atuam através da ligação seletiva a enzimas com alterações na estrutura causadas por mutações, promovendo o dobramento correto, estabilização e tráfego lisossomal. Uma limitação importante desse tratamento é a eficácia restrita a determinadas mutações que não alterem a fase de leitura, como mutações de ponto e, excepcionalmente, pequenas duplicações. Portanto, não é indicada para pacientes com mutações sem sentido, alterações de *splicing*, deleções e/ou inserções que alterem a fase de leitura ou grandes rearranjos. De qualquer forma, é necessária a avaliação experimental prévia da ação na variante específica do paciente.

O cloridrato de 1-deoxigalactojirimicina ou migalastat (Galafold, *Amicus Therapeutics*) é uma chaperona que se liga de forma reversível ao sitio ativo da α-galactosidase A. Após 6 meses de tratamento com migalastat em pacientes com mutações suscetíveis ou não, não houve diferença de resposta entre os grupos tratados e o grupo placebo (Germain et al. 2016). Entretanto, após 18 meses de tratamento apenas em pacientes com mutações suscetíveis, migalastat foi mais eficaz do que TRE redução da massa do ventrículo esquerdo, e equivalente em relação à função renal e redução de Gb3 e liso-Gb3 (Hughes et al. 2017).

1.4.8.3. Terapia de redução de substrato

O racional da terapia de redução de substrato é a diminuição da síntese de precursores dos glicoesfingolipídios através da inibição da enzima glicosilceramida sintase (GCS). Ela pode ser administrada como uma terapia adjuvante ou alternativa em pacientes que não tolerem a TRE. Atualmente esta já é uma terapia aprovada para outra esfingolipidose, a Doença de Gaucher, através da administração de miglustat (Zavesca, Actelion Pharmaceuticals do Brasil). Em Fabry, estudos pré-clinicos mostram que a administração de outro antagonista da GSC (Genz-682452) diminui o acúmulo de Gb3 e liso-Gb3 em diversos tecido em camundongos. Benefícios adicionais dessa terapia incluem a administração oral e a capacidade da molécula de cruzar a barreira hemato-encefálica e

agir no sistema nervoso (Ashe et al. 2015). Recentemente, um ensaio clínico de fase I demonstrou a ausência de efeitos adversos graves decorrentes do uso de um novo antagonista, Lucerastat (Guérard et al. 2017).

1.4.9. Doença de Fabry em mulheres

Historicamente, a DF era considerada uma doença com herança ligada ao X recessiva e, portanto, as mulheres heterozigotas eram consideradas portadoras assintomáticas. Este mesmo raciocínio é aplicado a outra DL causada por deficiência de uma hidrolase com herança ligada ao X, a Mucopolissacaridose do tipo II (MPS II). Raramente portadoras desta doença manifestam sintomas, com menos de 20 casos descritos na literatura. Com frequência, estas mulheres afetadas têm o fenótipo grave. Na maioria dos casos, a inativação preferencial do alelo normal foi apontada como motivo para expressão do fenótipo (Tuschl et al. 2005; Piña-Aguilar et al. 2013). Além disso, alterações cromossômicas numéricas ou estruturais também estão relacionadas (Burton and Giugliani 2012).

Apesar de serem razoáveis para MPS II, as definições “recessiva” e “portadora” não são adequadas para DF e outras doenças com herança ligada ao X, já que não se aplicam ao amplo espectro de penetrância e expressividade observado (Dobyns et al. 2004). As classificações “dominante” e “recessiva” foram primeiramente aplicadas a autossomos e posteriormente a cromossomos sexuais. Originalmente foram criadas para descrever o mecanismo de compensação de dose visto em *Drosophila*. Teoricamente, as doenças dominantes teriam fenótipo similar em ambos os gêneros e um excesso de mulheres afetadas, enquanto as recessivas raras teriam apenas homens afetados. No entanto, os estudos de registro internacional de doença de Fabry (FOS – *Fabry Outcome Registry*), por exemplo, indicam uma proporção similar entre homens e mulheres afetados, o que não está de acordo com nenhuma das definições clássicas (Ries and Gal 2006). Dessa forma, a classificação utilizada atualmente para o tipo de herança visto em Fabry é apenas “ligada ao X”.

O termo “portadora” (comumente usado em MPS II e outras doenças) descreve o status genético de heterozigose e, consequentemente, a chance de transmitir a doença para seus descendentes. No entanto, não fornece informação alguma sobre o fenótipo apresentado pela mulher em doenças ligadas ao X (Ries and Gal 2006). Novamente, o quadro visto em mulheres com DF é muito diferente de MPS II. A maioria das heterozigotas com DF é sintomática (Deegan et al. 2006). Um estudo clássico estimou que DF tenha penetrância quase total em homens e intermediária (70%) em mulheres, o que significa que 70 a cada 100 mulheres desenvolvam sintomas (Dobyns et al. 2004). Porém estudos elaborados com dados do FOS indicam que a penetrância em mulheres é ainda maior (Beck et al. 2015; Giugliani et al. 2016b). Os sintomas mais comuns em heterozigotas são os neurológicos, com 77% de frequência, seguidos de sintomas cardíacos (59-62%) e renais (40%) (Deegan et al. 2006; Linhart et al. 2007).

A expressividade vista em DF também é muito variável, tanto em mulheres apresentam quanto em homens. Há desde casos assintomáticos até casos com necessidade de transplante renal. Porém, heterozigotas geralmente tem início mais tardio dos sintomas, que tendem a ser mais leves do que homens com mutações similares no gene *GLA* (Schiffmann and Ries 2016).

Alguns autores consideram que o principal motivo para variabilidade de expressão em mulheres seja uma inativação enviesada do cromossomo X. De acordo com a hipótese postulada por Lyon, um dos cromossomos X das mulheres é inativado aleatoriamente em estágios precoces do desenvolvimento embrionário. Depois de estabelecida, a inativação é transmitida a todas as células-filhas de forma relativamente estável, permanente e irreversível (Lyon 1961). Contudo, os níveis de enviesamento tendem a aumentar com a idade (Amos-Landgraf et al. 2006). Como resultado, heterozigotas são mosaicos a nível celular, apresentando uma população de células com o alelo normal ativo e outra com o alelo mutante. Níveis de inativação a partir de 25%:75%, ou 20%:80% de acordo com alguns autores, são considerados patológicos (Busque et al. 2009). Assim, heterozigotas sintomáticas de DF apresentariam inativação preferencial superior a 75%-80% do alelo normal e preponderância da atividade do alelo mutante em tecidos-alvo (Maier et al. 2006).

No entanto, os estudos têm apresentado resultados conflitantes sobre este tema, que vão desde a associação entre altos níveis de inativação preferencial (acima de 95%) e o fenótipo clínico em fibroblastos de gêmeas monozigóticas com fenótipos discordantes (Redonnet-Vernhet et al. 1996) até relatos que mostram ausência de correlação entre inativação preferencial e fenótipo (Maier et al. 2006; Elstein et al. 2012; Juchniewicz et al. 2017).

Em decorrência dessa discrepância de achados, outras teorias surgem para explicar os sintomas apresentados pelas mulheres com doença de Fabry. Wang et al. (2007) propuseram que a níveis variados de inativação do X com limiares diferentes de atividade considerada normal da enzima em tecidos diferentes são responsáveis pela heterogeneidade clínica. Outra hipótese para explicar o fenótipo de heterozigotas é a indução cruzada, o oposto da correção cruzada. Neste caso, o produto da deficiência de uma célula induz o fenótipo deficiente em outra célula (Pinto et al. 2010). Essa teoria se baseia no fato de liso-Gb3 ter níveis elevados no plasma e urina de heterozigotas sintomáticas e estar associado a gravidade do quadro clínico. Além disso, é uma molécula altamente difusível e capaz de inibir a atividade de α-GAL induzir o acúmulo de Gb3 (Aerts et al. 2008; Niemann et al. 2014).

Recentemente se propôs que, independentemente do nível de inativação do X, a correção cruzada, onde o produto de células normais corrigem células deficientes, é um processo ineficiente em DF. Isso pode ser causado pela quantidade de enzima secretada pelas células normais ser insuficiente para corrigir a deficiência das células mutadas e/ou a enzima secretada não ser adequadamente internalizada (Fuller et al. 2015).

Finalmente, existe a possibilidade de genes modificadores atuarem sobre o fenótipo. Relatou-se que Gb3 tem uma ação pró-inflamatória mediada por TRL4 (De Francesco et al. 2013) ou estresse oxidativo (Shen et al. 2008; Biancini et al. 2012). Dessa forma mutações em genes envolvidos em inflamação poderiam alterar o desenvolvimento da doença. A associação entre genótipos de genes de resposta inflamatória (TNF-α), da família das interleucinas (IL-10, IL-1β e IL-1 α), fator V de Leinden e da proteína Z com as manifestações cerebrovasculares da doença também já foi descrita (Altarescu et al. 2005;

Safyan et al. 2006). A presença do polimorfismo -174G>C (NM_000600.4:c.-237C>G, rs1800795) na região promotora do gene da IL-6 chegou a ser proposto como marcador do prognóstico da doença em heterozigotas (Altarescu et al. 2008). O polimorfismo Q192R (NP_000437.3: p.Gln192Arg, rs662) da enzima paraoxonase (PON1) também já foi descrito em alta frequência em pacientes com Fabry com manifestações cardiovasculares. Essa enzima tem ligação ao perfil lipídico já que está envolvida na oxidação de lipoproteínas e tem efeito protetor contra doenças cardíacas (Barris-Oliveira et al. 2012). Da mesma forma, polimorfismos do gene da enzima óxido nítrico sintase endotelial (NOS3) também estão associados a cardiomiopatia hipertrófica vista em DF (Rohard et al. 2008).

2. Objetivos

2.1. Objetivo Geral

2.1.1. Investigar, de forma abrangente, aspectos moleculares relacionados ao diagnóstico e progressão da Doença de Fabry na população brasileira.

2.2. Objetivos Específicos

2.2.1. Propor um método mais preciso para o diagnóstico enzimático de mulheres com Doença de Fabry.

2.2.2. Propor um método simples para triagem de mutações em indivíduos com suspeita de Doença de Fabry.

2.2.3. Estabelecer o perfil mutacional de pacientes brasileiros com Doença de Fabry.

2.2.4. Investigar possíveis genes modificadores de fenótipo em pacientes brasileiros com Doença de Fabry.

2.2.5. Avaliar perfis de expressão gênica específicos da doença cardíaca e renal em heterozigotas com Doença de Fabry.

3. Resultados

Os resultados serão descritos em 5 capítulos conforme descrito abaixo:

#	Nome	Situação
I	*Fabry disease: a new approach for the screening of females in high-risk groups*	Artigo publicado no periódico *Clinical biochemistry*
II	*Simple and efficient screening of patients with Fabry disease with high resolution melting*	Artigo sob revisão no periódico *Clinical biochemistry*
III	*Mutational profile of Brazilian patients with suspicion of Fabry disease*	Artigo submetido ao periódico *Molecular Genetics and Metabolism*
IV	*The cardiac phenotype in Fabry Disease: a search for modifier genes*	Artigo a ser submetido ao periódico *American Journal of Human Genetics*
V	*Gene expression signatures for specific disease manifestations revealed by transcriptome analysis of women with Fabry Disease*	Artigo a ser submetido ao periódico *Human Genetics*

3.1. Artigo I

Pasqualim G, Simon L, Sperb-Ludwig F, Burin MG, Michelin-Tirelli K, Giugliani R and Matte U (2014) **Fabry disease: a new approach for the screening of females in high-risk groups**. Clinical biochemistry 47:657–62. doi: 10.1016/j.clinbiochem.2014.02.014

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Fabry disease: A new approach for the screening of females in high-risk groups

Gabriela Pasqualim [a,b], Laura Simon [a,b], Fernanda Sperb-Ludwig [b], Maira Graef Burin [c], Kristiane Michelin-Tirelli [c], Roberto Giugliani [a,b,c,d,e,*], Ursula Matte [a,b]

[a] Post-Graduation Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, Brazil
[b] Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[c] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[d] Department of Genetics, UFRGS, Porto Alegre, RS, Brazil
[e] INAGEMP, Porto Alegre, RS, Brazil

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ABSTRACT

Objective: Fabry disease (FD) is a rare X-linked inborn error of metabolism caused by deficient activity of lysosomal α-galactosidase A (α-GAL). Due to random X inactivation, α-GAL activity in heterozygous females ranges from very low to overlapping normal values. Determining this specific range and altering assays cutoffs could become a valuable tool for minimizing the need in DNA sequencing for screening of all potential carriers. Therefore, the aim of this study was to establish the range of enzyme in dried blood spots (DBS), plasma and leukocytes that suggests carrier status for FD.

Design and methods: α-GAL gene was sequenced in 453 women with clinical suspicion and/or positive family history of FD. This data was compared to the α-GAL activity measured in DBS (dried blood spots) and/or plasma and/or leukocytes.

Results: About 12% of the samples had pathogenic mutations (c.30_32delG, c.718_719delAA, p.R118C, p.S126G, p.Y152X, p.A156D, p.C202Y, p.N215S, p.P259R, p.D264Y, p.V269M, p.R342Q and p.R356W). When compared to genotype, DBS was the least reliable biochemical test for screening, with very low specificity. Plasma and leukocyte activities presented high AUC in ROC curve analysis, both over 84%. When cutoffs were altered to identify all carriers, leukocyte specificity was higher than that of plasma (35.2% and 27.6%, respectively). Moderated correlation and agreement coefficients were found between them, which reinforces the need for using both data combined.

Conclusion: A combined approach involving plasma and leukocyte α-GAL activities, with distinct cutoffs for men and women, could represent a more accurate, faster and less expensive tool to screen women for FD in high-risk groups in middle- and low-income countries.

Introduction

Fabry disease (FD; OMIM 301500) is a rare X-linked inborn error of glycosphingolipid catabolism caused by deficient activity of lysosomal α-galactosidase A (α-GAL or α-Gal A; EC3.2.1.22) [1]. The gene encoding α-GAL (*GLA*, OMIM 300644, RefSeq X14448) is located on the X-chromosome (Xq22.1) [1] and it is composed of seven exons, spanning 12 kb [2,3]. The cDNA encodes a precursor polypeptide of 429 amino acids, including a 31-residue leader sequence, which is processed into a 370 amino acid glycoprotein that is functional as a homodimer [4]. To date (November 2013), there are 675 mutations in the *GLA* gene described in the Human Gene Mutation Database [5].

α-GAL deficiency results mainly in progressive accumulation of globotriaosylceramide (Gb3 or GL-3; also known as ceramide trihexoside or CTH) in the lysosomes [6]. In patients with the classic form of the disease, this accumulation of Gb3 and related glycosphingolipids in vascular endothelial cells of the kidneys, heart, skin, and brain leads to the main disease manifestations [7]. Clinical manifestations may also include hypohidrosis, angiokeratomas, acroparesthesias and corneal opacity [8].

Diagnosis can be confirmed in male patients by α-GAL activity assay preferably in peripheral leukocytes, since they have very low enzyme activity. If leukocyte analysis is unavailable, plasma may be used [9]. The assay of α-GAL activity in dried blood spots (DBS) may also be a useful tool for the screening of men [6,10–17]. Female carriers who can also develop mild to severe clinical manifestations [1]

* Corresponding author at: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, RS. 90035-903, Brazil. Fax: +55 51 3359 8010.
E-mail address: rgiugliani@hcpa.ufrgs.br (R. Giugliani).

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present α-GAL activities which range from very low levels to normal values, due to random X inactivation [18].

Although measurement of α-GAL in women is not accurate for the diagnosis of FD, determining the range of the enzyme activity presented by heterozygous females could become a valuable tool for minimizing the burden of screening by DNA sequencing all potential carriers. This strategy would diminish the total number of sequencing reactions and, by consequence, greatly reduce the costs and the time needed for diagnosis, especially in those centers without high-throughput technologies implemented. Therefore, the aim of this study was to establish the range of enzyme in DBS, plasma and leukocytes that suggests carrier status for FD.

Materials and methods

Samples

The Medical Genetics Service of the Hospital de Clínicas de Porto Alegre is a reference center for inborn errors of metabolism and as such receives a large amount of samples from all over Brazil and other Latin America countries. All samples included in this study are from Brazilian patients. The inclusion criteria for this study was the suspicion of the attending physician that the patient had Fabry disease, based in either clinical observations and/or positive family history.

All samples were shipped within 24 h of collection. DBS and FTA samples were sent at room temperature. Leukocytes, plasma and EDTA tubes were sent in ice-filled containers and were stored at 4 °C until diagnosis was completed. In addition, samples were processed within 24 h of arrival.

In total, samples from 453 female patients with clinical suspicion of Fabry disease were analyzed. The study was approved by the Ethics Research Committee of our institution and the patients gave written informed consent.

Genotyping

Whole blood genomic DNA was extracted either from EDTA tubes with EasyDNA Kit (Invitrogen, USA) or from FTA Elute Cards (Whatman, USA), both according to the manufacturer's instructions. PCR of the seven *GLA* exons was performed separately with 20 ng of genomic DNA, 1× PCR buffer, 0.1 mM of dNTPs, 4 mM of $MgCl_2$, 0.5 pM of primers, 0.5 U of Taq DNA polymerase (Life Technologies, USA) and distilled water qsp 20 μl. Primers pair sequences were described by Shabbeer et al. [19]. All reactions were made in a Veriti Thermal Cycler (Applied Biosystems, USA) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s and 1 cycle at 72 °C for 7 min. Following purification with EXO-SAP and quantification with Low Mass Reader (Invitrogen, USA), sequencing was done in ABI3500 genetic analyzer using BigDye Terminator v3.1 (Applied Biosystems, USA). Sequences were analyzed by comparison to genomic reference sequence (GenBank accession number X14448.1) and all alterations were confirmed by reverse strand sequencing.

Biochemical data

Results of α-GAL activities in DBS (n = 377) and/or plasma (n = 93) and/or leukocytes (n = 88) were obtained from patients' laboratory records. All biochemical analyses were performed in the Medical Genetics Service of the Hospital de Clínicas de Porto Alegre using fluorogenic methods and reference values previously determined by this service [10,20,21]. Briefly, for measuring DBS α-GAL activity, one 3 mm punch was added to 40 μL of 0.25 M N-acetyl-D-galactosamine and 100 μL of 5 mM 4-metilumbeliferil-α-D-galactoside diluted in 1 mL of 0.15 M phosphate–citrate buffer pH 4.8. After gentle mixing, samples were incubated at 37 °C for 20 h in a water bath with agitation. Reaction was stopped by adding 600 μL of 0.1 M ethylenediamine pH 11.4. For leukocytes, 50 μL of 5 mM 4-metilumbeliferil-α-D-galactoside in 50 mM phosphate–citrate buffer pH 4.8 and 20 μL of 0.25 M N-acetyl-D-galactosamine were added to a 20 μL of cell suspension. The reaction was incubated for 2 h at 37 °C and stopped by adding 1.5 mL of 0.5 M glycine–sodium hydroxide buffer pH 10.3. For plasma, 100 μL of sample was added to 100 μL of substrate–inhibitor mix (10 mM 4-metilumbeliferil-α-D-galactoside, 0.2 M N-acetyl-D-galactosamine in 1 mL of 0.5 M sodium acetate buffer pH 4.8). The reaction was incubated for 2 h at 30 °C and stopped by adding 1.5 mL of 0.5 M glycine–sodium hydroxide buffer pH 10.3. For all types of samples, the activity of a reference enzyme, usually β-galactosidase, was always measured to assure sample quality. In addition, analysis always included samples from normal and affected controls to guarantee test quality.

Statistical analyses

ROC (Receiver Operating Characteristic) curve analyses of the genetic and biochemical data, Pearson's correlation coefficient and Cohen's kappa coefficient between plasma and leukocyte activities were performed with the software IBM SPSS Statistics v.20 (IBM Company, USA). In order to describe the biochemical diagnosis performance, positive predictive value (PPV) and negative predictive value (NPP) were calculated. They represent true positives and true negatives, respectively, and were calculated with Microsoft Excel 2010 (Microsoft Company, USA), considering molecular results as gold standard.

Table 1
Mutations in the *GLA* gene found in genomic DNA from 453 patients with either clinical suspicion or positive family history of Fabry disease.

Mutation			Number of patients	Reference
rs id (db SNP)	Effect on cDNA	Effect on protein		
NA	c.30_32delG	p.L120X	3	[22]
NA	c.718_719delAA	p.S248X	1	[23]
rs148158093	c.352C>T	p.R118C	2	[24]
rs149391489	c.376A>G	p.S126G	1	[25]
NA	c.456C>A	p.Y152X	2	[26]
NA	c.467C>A	p.A156D	13	[27]
NA	c.605G>A	p.C202Y	1	[28]
rs28935197	c.644A>G	p.N215S	3	[29]
NA	c.455C>G	p.P259R	2	[30]
rs190347120	c.790G>T	p.D264Y	10	[19]
NA	c.805G>A	p.V269M	8	[31]
rs28935493	c.1025G>A	p.R342Q	6	[32]
rs104894827	c.1066C>T	p.R356W	2	[33]
			54	

NA: not available.

Results

Genotyping

From the 453 samples sequenced, 54 were heterozygous for pathogenic *GLA* mutations. Thirteen previously described pathogenic mutations were found; most of them, in more than one patient (Table 1). All patients carrying pathogenic mutations are herein called "heterozygotes" and those who do not were named as "controls".

α-GAL activity data

The median α-GAL activity in DBS (Fig. 1A) was 3.7 ± 3.2 nmol/h/mL in heterozygotes and 6.2 ± 3.5 nmol/h/mL in controls. In leukocytes (Fig. 1B) and plasma (Fig. 1C) heterozygote means were 23.9 ± 11.6 nmol/h/mg prot and 4.5 ± 2.5 nmol/h/mL, and control values were 41.6 ± 13.1 nmol/h/mg prot and 11.1 ± 5.4 nmol/h/mL, respectively.

ROC curve

ROC curve analysis between genotype and α-GAL activity measured in DBS (Fig. 2) gave AUC (area under curve) of 0.768 ± 0.059 ($p < 0.001$). Currently used reference values (RV) for this method range from 2.9 to 14.4 nmol/h/mL. The lowest RV had a sensitivity of 42.3% and specificity of 90.9%. To achieve 100% sensitivity (no false negatives) it would be necessary to change the lowest reference value to 13.95 nmol/h/mL, which would represent a drop in specificity to 2.6%.

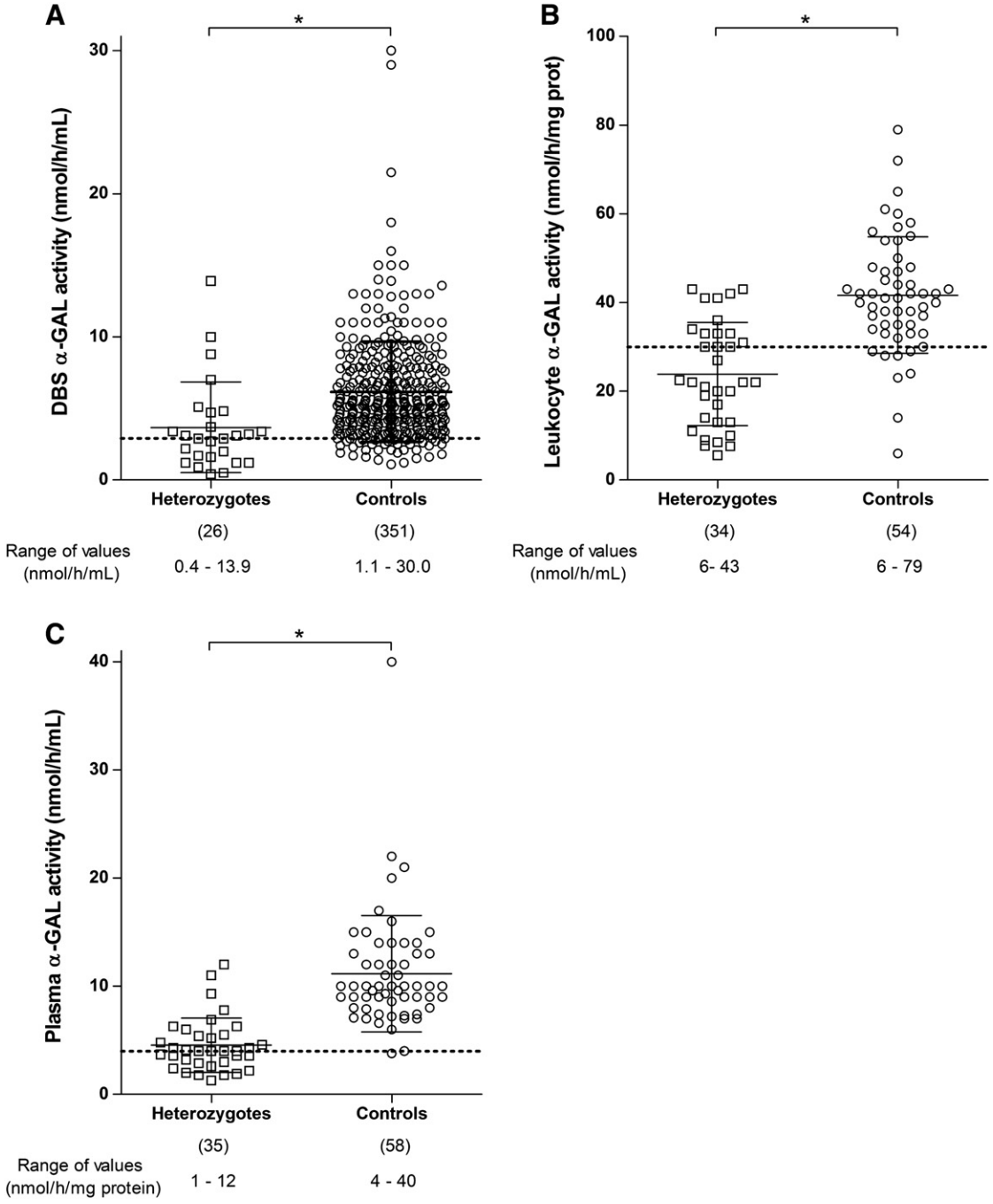

Fig. 1. Levels of α-GAL activity measured in women with clinical suspicion and/or family history of Fabry disease with or without pathogenic mutations. Numbers in parenthesis represent n for each group. A) Dried blood spot. B) Leukocyte and C) plasma activities. Bars represent mean ± SD. Independent samples *t*-test, $p < 0.001$.

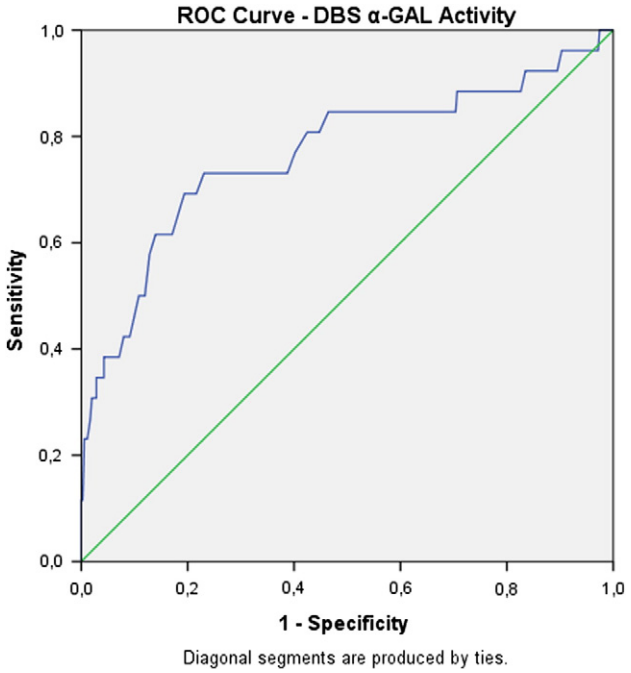

Fig. 2. ROC curve analysis between genotype and α-GAL activity measured in Dried Blood Spots of 377 women with clinical suspicion and/or familial history of Fabry disease. AUC = 0.768 ± 0.059 (p < 0.001).

ROC curve analysis for leukocyte activity (Fig. 3) resulted in an AUC of 0.846 ± 0.042 (p < 0.001). Current reference values for this method range between 30 and 63 nmol/h/mg prot. The lowest RV presented sensitivity and specificity of 58.8% and 85.2%, respectively. In order to identify all heterozygotes, this value should be altered to 43.5 nmol/h/mg prot. However, specificity would be reduced to 35.2%.

In plasma (Fig. 4), AUC was 0.927 ± 0.031 (p < 0.001). Current reference values range from 4 to 22 nmol/h/mL. The lowest RV resulted in sensitivity of 42.9% and specificity of 96.6%. Maximum sensitivity

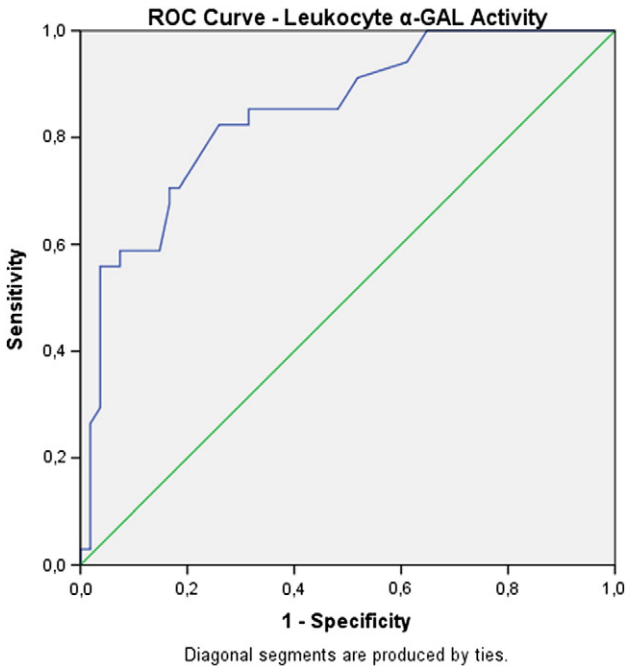

Fig. 3. ROC Curve analysis between genotype and α-GAL activity measured in leukocyte of 88 women with clinical suspicion and/or familial history of Fabry disease. AUC: 0.846 ± 0.042 (p < 0.001).

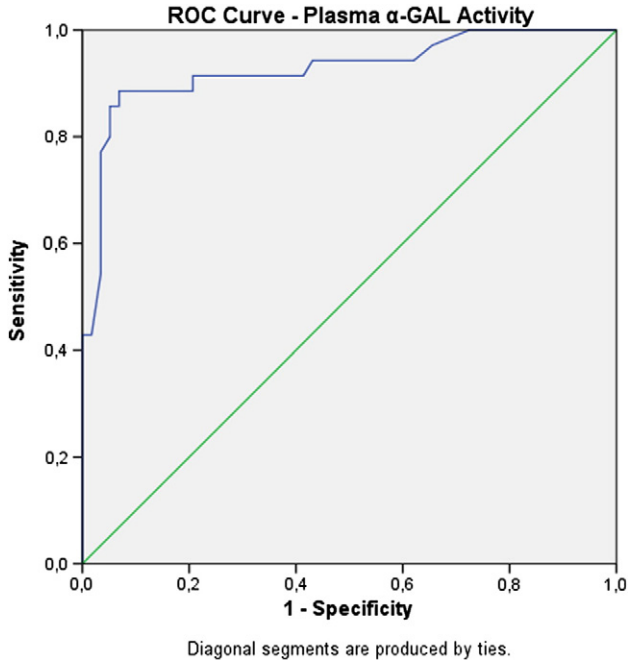

Fig. 4. ROC Curve analysis between genotype and α-GAL activity measured in plasma of 93 women with clinical suspicion and/or familial history of Fabry disease. AUC: 0.927 ± 0.031 (p < 0.001).

and NPV could be achieved by changing this value to 12.5 nmol/h/mL and reducing specificity to 27.6%.

Predictive values

Positive and negative predictive values for α-GAL activity in DBS, leukocytes and plasma with currently used RV and the new values suggested by this study (which aim to maximize sensitivity) are listed in Table 2.

Correlation and agreement coefficients

The aim of screening methods is to identify all affected individuals, in this case, heterozygotes, resulting in the fewer number of false positives as possible. Therefore, α-GAL activity in DBS was considered unfitted for Fabry disease screening in women due to its very low specificity and was removed from posterior analyses. In the 84 individuals for whom leukocytes and plasma were available, Pearson's correlation coefficient between α-GAL activities (Supplementary Fig. 1) was 0.508 (p < 0.001) and Cohen's kappa agreement coefficient was 0.472 (p < 0.001).

Discussion

Several authors reported screening tests for Fabry disease in newborns, hemodialysis patients and/or patients with heart conditions such as hypertrophic cardiomyopathy and left ventricular hypertrophy

Table 2
Predictive values for female α-GAL activity in different sample types.

	DBS (n = 377)		Leukocyte (n = 88)		Plasma (n = 93)	
	RV	NV	RV	NV	RV	NV
PPV	0.25	0.07	0.71	0.49	0.94	0.45
NPV	0.96	1.00	0.77	1.00	0.74	1.00

RV: currently used reference values. SV: new values suggested by this study based on achieving maximal sensitivity.

[24,34,35]. Most studies, particularly the ones aiming newborn screening, focused on male patients and verified α-galactosidase levels in DBS and/or plasma, confirming results with leukocytes and/or DNA sequencing. Due to random X chromosome inactivation, female heterozygotes present variable enzyme activity levels, overlapping normal range [18]. Therefore, gene sequencing became the "gold standard" for diagnosis [9]. However, screening by enzyme activity level with proper cutoff points that are able to identify most heterozygotes could help to lower the time and cost for the diagnosis of females, reducing dramatically the number of patients to be sequenced. In this study, in order to determine appropriated cutoff values for screening of females in high-risk populations, the *GLA* gene of 453 females with clinical suspicion or family history of Fabry disease was sequenced and compared to activity levels on DBS, leukocytes and plasma. This was the first time that an analysis of this kind was done in such a large number of women with molecular diagnosis for Fabry disease, which allowed the determination of specific cutoff points for this population.

Given that the primary focus of this study was sensitivity, aiming to identify all heterozygotes, the new cutoff values were calculated with ROC curve analysis at a point of 100% sensitivity. Another statistical approach, the Youden statistics, which takes into account both sensitivity and specificity for calculating cutoffs, was also performed, resulting in 90% and 83.3% sensitivity for plasma and leukocytes, respectively, with cutoffs of 6.95 and 34.5 (data not shown). Due to the large sample analyzed, we believe that it is unlikely that the addition of new patients would substantially change the parameters found, therefore we chose using the ROC curve cutoffs that will be discussed below.

Out of 453 samples analyzed, 12% presented pathogenic alterations. Patients with the mutations p.V269M, p.P259R or p.N215S had higher enzymatic levels in both plasma (over 7.8 nmol/h/mL) and leukocyte (over 40 nmol/h/mg prot) (data not shown). Unfortunately, no data on DBS were available for these patients. These data is similar to what was reported by Winchester and Young [36] about p.N215S in men. Individuals carrying this mutation showed higher levels both in plasma and in leukocytes of residual activity when compared to other affected male individuals. The repercussion of this increased residual activity on patient's phenotype was beyond the scope of this retrospective study.

Since DBS is the preferred method for sending samples between referral centers, it was not a surprise that it corresponded to the largest number of samples analyzed in this study, 377 patients, including 26 heterozygotes. As shown by the ROC curve analyses, it was the least accurate of the three biochemical methods, with the lowest AUC (0.768 ± 0.059), although it was still in an acceptable range for diagnosis. When current reference values were used, it also presented the lowest sensitivity and specificity. The large overlapping region with control levels, which resulted in such low parameters, is in accordance with previous reports in the literature [11–14]. If the cutoff value was altered to achieve maximum sensitivity, it would still maintain the lowest specificity. That would result in sequencing almost all patients (over 97%), rendering it completely unsuitable as a screening method before genotyping. Therefore, our data suggest that the measurement of enzyme activity in DBS is not a reliable method to screen for FD in females. Therefore, enzyme results in DBS should be analyzed carefully before excluding patients from next steps of screening.

Leukocyte and plasma enzymatic levels showed very similar results. Both have shown high accuracy with AUCs of over 0.845 and a difference of only 8% between them. Moreover, when cutoffs were elevated to ensure identification of all heterozygotes both had PPV of approximately 50%. However, in this scenario, leukocyte activity presented higher specificity than plasma, 35.2% and 27.6% respectively, which would result in lower false-positives. Interestingly, even though the new cutoffs were used, enzyme activity in plasma and leukocytes had only moderated correlation and agreement coefficients, which reinforces the need for using both data combined.

Taken together, these data suggest that a combined approach involving measurement of enzyme activity in plasma and leukocytes could be used to select the samples to be genotyped in a screening program. According to our data, if only plasma activities were evaluated with new cutoff values, there would be a reduction of 17% in the number of samples that would have to be sequenced to reach the diagnosis, as reference values would be higher than enzymatic levels found in all heterozygotes. If only leukocytes were used, this reduction would be of 21%. However, if results of both types of samples were combined, more than 35% of the patients would be discarded as suspect of FD by having enzyme activity within reference levels in both plasma and leukocytes (Supplementary Fig. 1, samples inside full line rectangle). Thus, the use of specific cutoffs with 100% sensitivity for women minimizes time and costs for the screening of female carriers for FD.

It is important to point out that although we included groups of patients with positive family history and with clinical suspicion in the analysis, our conclusion refers only to screening of females in high-risk populations, such as patients in hemodialysis or those who present hypertrophic cardiomyopathy. In the clinical management, patients are suspected of Fabry disease based on their clinical phenotypes and then referred to molecular diagnosis. However, in the screening of high-risk populations the identification of women by direct sequencing is still very expensive, not widely available and time consuming (at least in some countries). Therefore, an enzyme-based method that could reduce the number of women being analyzed by sequencing would be cost-effective. It is also worth noticing that, since analytical methods differ among distinct laboratories, cutoff values should be specifically determined by each one. For females with positive family history identified as obligate carriers by pedigree analysis, particularly if the family mutation was already identified, performing molecular analysis is mandatory.

In addition, it is important to reinforce that cutoff values shown here were not artificially defined, but were calculated based on a large and representative sample of the Brazilian population with clinical symptoms of Fabry disease. The determination of specific cutoff points for specific populations could represent a significant cost reduction for middle- and low-income countries, where facilities for genetic analyses are scarce and where the costs of molecular tests are usually not covered by health insurance. However, the main weakness of this work is the lack of clinical data and other markers of Fabry disease, such as GB3 analysis.

In conclusion, a combined approach involving measurement of α-GAL in plasma and leukocytes, with distinct cutoffs for men and women, should be strongly considered to screen for FD in females from high-risk groups in middle- and low-income countries.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2014.02.014.

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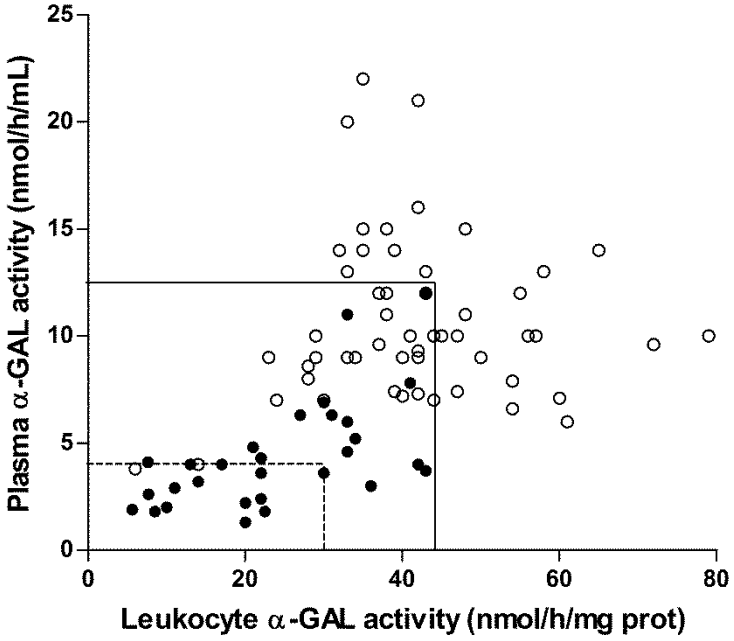

Supplementary figure 1: Correlation between α-GAL activities measured in leukocytes and plasma of 71 women with clinical suspicion and/or familial history of Fabry Disease with (filled circles) or without (empty squares) pathogenic mutations. r= 0.493, p<0.001. Dotted lines represent current lowest reference values. Full lines represent lowest reference value suggested by this paper

3.2. Artigo II

Pasqualim G, Santos B, Giugliani R and Matte U (2017) **Simple and efficient screening of patients with Fabry disease with high resolution melting**.

Artigo sob revisão no periódico *Clinical biochemistry*.

Simple and efficient screening of patients with Fabry disease with high resolution melting

Gabriela Pasqualim[1,2], Bruna Almeida dos Santos[2], Roberto Giugliani[1,2,3,4,5], Ursula Matte[1,2,4]

[1]: Post-Graduation Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[2]: Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[3]: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[4]: Department of Genetics, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[5]: INAGEMP, Porto Alegre, RS, 90035-903, Brazil

Corresponding author: Dr Ursula Matte. Address: Gene Therapy Center, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, RS. 90035-903, Brazil. Phone: +55 51 3359 8838, Fax: +55 51 3359 8010. E-mail address: umatte@hcpa.edu.br

ABSTRACT

Fabry disease (FD [MIM: 301500]) is a disorder caused by mutations in the alpha-galactosidase gene (*GLA*). Although it displays X-linked inheritance, women are often symptomatic and can be as severely affected as male individuals. We performed high resolution melting (HRM) analysis in one hundred and three individuals, 79 females and 24 males, with a total of 27 different variants in 30 different genotypes. We standardized a protocol using EvaGreen, a release-on-demand dye specific for HRM, added to the PCR reaction. Amplification was performed in a conventional real-time system with HRM capability. All genotypes in all amplicons were distinguishable from wild type. In most amplicons it was even possible to differentiate each genotype from the others. In conclusion, we developed a simple, fast and highly sensitive HRM based protocol that may facilitate genetic screening of FD.

Keywords: Fabry disease, genetic screening, high resolution melting analysis.

Highlights

1. The described HRM protocol is a simple screening method suitable for FD patients.

2. All genotypes in all amplicons were distinguishable from wild type.

3. This is the largest cohort of FD patients analyzed by HRM, covering the whole coding region.

1. Introduction

Fabry disease (FD [MIM: 301500]) is a genetic disorder caused by mutations in the alpha-galactosidase gene (*GLA*), that encodes for the lysosomal hydrolase α-galactosidase A (GLA, EC 3.2.1.22). Its deficiency leads to accumulation of globotriaosylceramide (Gb_3) and other glycosphingolipids [1]. Variants resulting in low residual enzymatic activity are associated with the classical form of the disease, with childhood onset of signs and symptoms and development of cardiac, cerebrovascular and/or renal manifestations. Variants resulting in high residual activity are associated with non-classical mono or oligo-symptomatic phenotypes and later disease onset [2].

Although FD displays X-linked inheritance, women are often symptomatic and can be as severely affected as male individuals. However, diagnosis of female patients must be performed by molecular analysis as enzyme levels can be within normal range. Men, on the other hand, can be reliably diagnosed by low enzymatic activity [1]. Genetic analysis also helps in genotype-phenotype correlation, genetic counselling and screening of family members.

GLA (GeneBank Accession Number: NG_007119.1) is a small gene located at Xq22.1 that spans 12 kb and that is divided in seven exons ranging from 92 to 309 bp [3]. Despite the existence of a few mutational hot-spots, FD is characterized by the presence of private mutations that result in great allelic heterogeneity. Currently, about 1,000 *GLA* mutations have been described in the Human Mutation Database [4] and over 1,500 entries are listed in the Fabry database [5]. The majority of these variants are missense or affect one or a few bases, which makes Sanger sequencing an appropriated method for molecular diagnosis. Since there are no recurrent pathogenic mutations and variants are mostly homogeneously distributed, the whole gene must be analyzed. Therefore, the development of fast screening methods may reduce costs and length of diagnosis, being particularly important for screening programs of high risk female patients.

High resolution melting (HRM) analysis is a closed-tube assay with no post-PCR processing that evaluates differences in the dissociation (melting) profiles of amplicons mixed with saturating fluorescent dyes. The position and shape of melt curves are affected

by GC content and distribution and by fragment length which are altered by mutations [6]. Due to its low cost, high-throughput capability and sensitivity, HRM has been increasingly applied for mutation specific genotyping or mutation screening. However, no protocols were validated for FD HRM screening in the Brazilian population. Therefore, the purpose of this study was to develop a pre-sequencing genetic screening method based on HRM analysis suitable for the mutation profile seen on FD patients.

2. Materials and methods

2.1. Samples

One hundred and three individuals (79 females and 24 males) with FD and previous molecular analysis of the *GLA* gene performed by Sanger sequencing were analyzed. In total 27 different variants were studied (table 1). This study was approved by the Ethics Research Committee of our institution (#03-441 and #15-0196) and the patients gave written informed consent.

Stored DNA from whole blood samples were quantified with Nanodrop spectrophotometer (Thermo Fischer Scientific, USA) and diluted with TE buffer. To facilitate screening of males, artificial heterozygotes were created by mixing samples with male sequenced controls in a 1:1 ratio.

Table 1: *GLA* variants for which HRM protocol was validated.

#	Location	cDNA effect[1]	Protein effect[2]	rsID
1	5' UTR	c.-30G>A	-	rs3027584
2	5' UTR	c.-12G>A	-	rs3027585
3	5' UTR	c.-10C>T	-	rs2071225
4	Exon 1	c.4C>T	p.Gln2Ter	rs869312313
5	Exon 1	c.32delG	p.Gly11Alafs	rs869312278
6	Exon 1	c.44C>A	p.Ala15Glu	rs869312304
7	Exon 1	c.167G>A	p.Cys56Tyr	rs869312258
8	Exon 2	c.195T>C	p.Ser65Ser	rs782803696
9	Exon 2	c.334C>T	p.Arg112Cys	rs104894834
10	Exon 2	c.352C>T	p.Arg118Cys	rs148158093
11	Exon 3	c.398T>A	p.Ile133Asn	N/A
12	Exon 3	c.456C>A	p.Tyr152Ter	N/A
13	Exon 4	c.560T>C	p.Met187Thr	rs869312342
14	Exon 4	c.605G>A	p.Cys202Tyr	rs869312344
15	Exon 4	c.612G>A	p.Trp204Ter	N/A
16	Intron 4	c.640-16A>G	-	rs2071397
17	Exon 5	c.644A>G	p.Asn215Ser	rs28935197
18	Exon 5	c.776C>G	p.Pro259Arg	rs869312399
19	Exon 5	c.790G>T	p.Asp264Tyr	rs190347120
20	Exon 6	c.805G>A	p.Val269Met	rs869312427
21	Exon 6	c.812G>C	p.Gly271Ala	rs869312429
22	Exon 6	c.870G>A	p.Met290Ile	rs869312438
23	Exon 6	c.982G>A	p.Gly328Arg	rs104894832
24	Exon 7	c.1025G>A	p.Arg342Gln	rs28935493
25	Exon 7	c.1033_1034delTC	p.Ser345Argfs	rs398123198
26	Exon 7	c.1066C>T	p.Arg356Trp	rs104894827
27	Exon 7	c.1102G>A	p.Ala368Thr	rs144994244

[1]: GenBank NM_000169.2; [2]:GenBank NP_000160.1.

2.2. PCR and HRM

Amplification of the seven *GLA* exons was performed in 8 separate PCR reactions (exon 7 was amplified in two overlapping fragments due to its size). Reactions contained 20 ng of genomic DNA, 1× PCR buffer, 1x EvaGreen (Biotium, USA), 0.1 mM of dNTPs, 0.1 µM of each primer and 1 U of Platinum Taq DNA polymerase (Thermo Fischer Scientific, USA) in a final volume of 20 µL. Amplicon length ranged from 214 to 280 bp and Magnesium Chloride ($MgCl_2$) concentration for each fragment was empirically determined to provide

the best resolution of melt curves. Detailed information on primer sequences, amplicons sizes, $MgCl_2$ and melt temperature for each amplicon are described in supplementary table S1. All reactions were made at least in duplicates in a StepOne Real-Time PCR instrument (Thermo Fischer Scientific, USA). PCR cycling conditions were 1 cycle of 95°C for 5 min followed by 45 cycles at 95°C for 15 s, Tm for 30 s and 72°C for 30 s.

Immediately after PCR cycling, HRM was performed in the same instrument according to manufacturer's recommendations. Amplicons were heated to 95°C for 10 s, cooled to 60°C for 1 min, followed by melt curve generation by heating until 95°C with 0.3% continuous ramp rate. Melt curves were analyzed with High Resolution Melt Software v3.0.1 (Thermo Fischer Scientific, USA). For each amplicon, from one to two samples from each genotype were identified as controls and software automatically classified the remaining.

3. Results and discussion

At least 2 variants were tested for each exon and all presented distinct melt curves from wild type controls (figure S1). However, allele discrimination is easier using normalized difference plots, shown in figure 1. For all amplicons and all of the tested variants it was possible to discriminate between wild type and heterozygotes, either in female or artificial male subjects. The highest allelic heterogeneity was seen in the amplicon including exon 1 and part of 5'UTR (figure 1A). We analyzed samples with eight different variants and nine different genotypes, since variants c.-10C>T and c.-12G>A were detected together in three patients. All genotypes were distinguishable from wild type. However, with increase number of concomitant genotypes per analysis, differences between mutated alleles were less evident. Exon 7, on the other hand, despite a reduced number of variants, all located within the first amplicon, did not yield such a clear discrimination plot (figure 1G). Even though all four variant genotypes showed different curves from wild type, it was also not possible to differentiate clearly between different genotypes. A similar result for this region was described in a study that utilized LightCycler

reagents and instrument [7]. This indicates the observed result is related to the sequence characteristics and not a specific limitation of the system used.

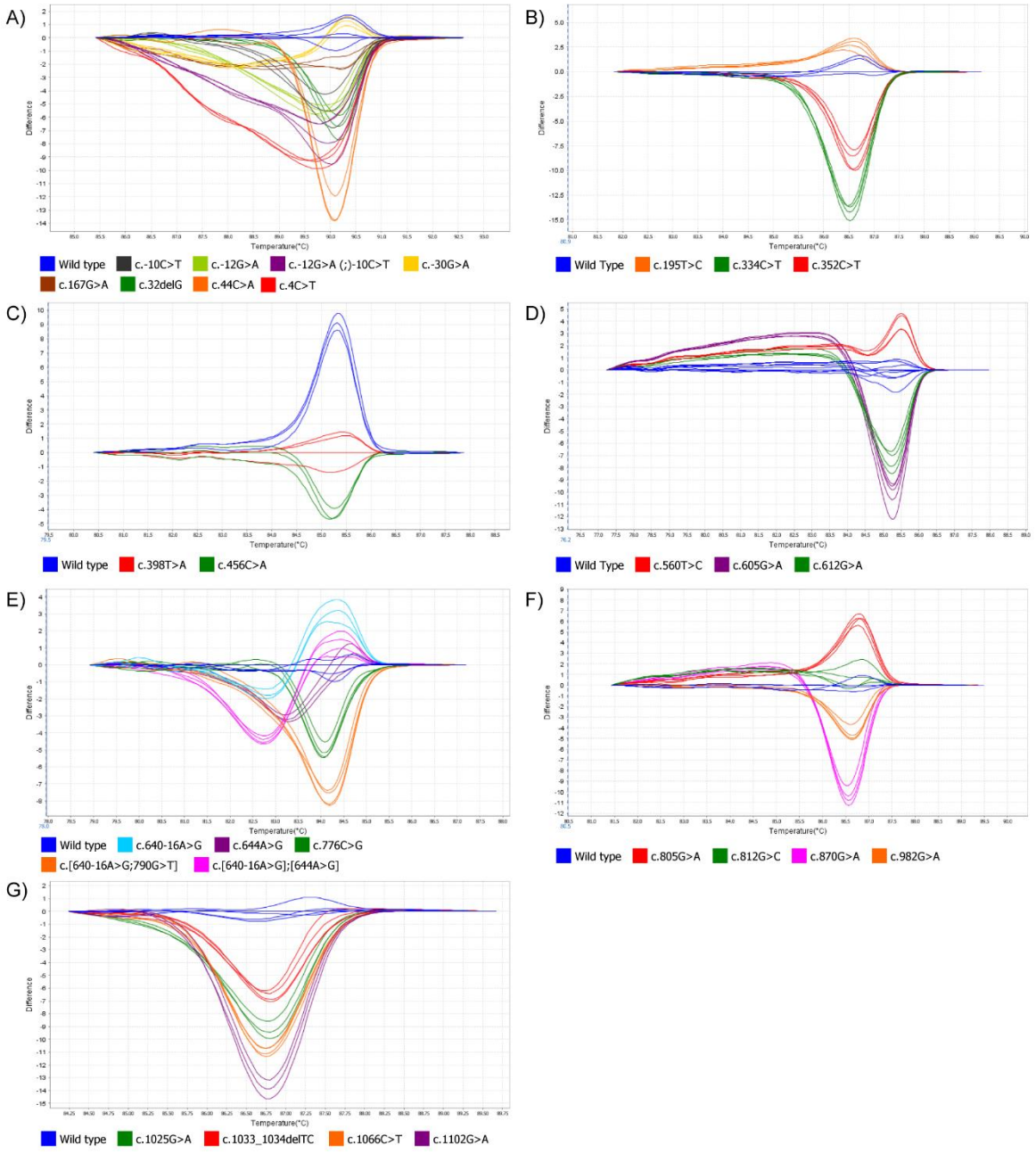

Figure 1: HRM analysis of GLA gene. Normalized difference plots from each amplicon from samples of females and artificial heterozygous males. Wild type curves are shown in dark blue for all exons. A-G: Exons 1 to 7 with respective variants.

We used a conventional PCR reaction together with a “release-on-demand” fluorescent dye instead of more expensive master-mixes. This represents an increase of less than USD 0.05 in reagents per standard PCR reaction to perform a high sensitivity assay capable of, in most cases, genotype specific mutations. Moreover, it leads to a significant reduction in sequencing costs, since only amplicons with altered patterns of melting would have to be further analyzed. Additionally, the equipment utilized was an affordable regular peltier block based real-time system with HRM capability but not specifically design for HRM analysis. Other specialized equipment such as LightScanner (BioFire Defense, USA) and LightCycler (Roche Molecular Systems, USA) have increased number of data points collection per degree and higher melting rates, which drastically decreases melting time [8]. Therefore, they can increase throughput but demand higher initial investment.

In addition to intrinsic sequence features as GC length and content, other factors such as DNA quality/purity and ionic strength are known to affect melting profiles [9]. Therefore, it is recommended that all samples undergo the same extraction protocol. In this study, we used DNA from peripheral blood collected in EDTA tubes. Although DNA from these samples was extracted with three different protocols (standard in house salting-out, commercial kits with manual or automatic extraction), after dilution with the same buffer the differences in DNA extraction methods did not influence results. On the other hand, we were unable to use samples from stored filter paper, as they resulted in high degree of variation in melt curves when compared to EDTA samples. Therefore, these samples had to be excluded from analysis and are not accounted for in any section of this report. However, samples included represent 82% of the variants already identified by our group in Brazilian patients with FD [10,11].

Finally, our protocol allowed differentiating each genotype from the others and consequently genotyping multiple variants simultaneously. However, it is not possible to exclude the possibility that a different variant might present a similar melt profile to the ones analyzed. Therefore, HRM might only be considered a genotyping method in family studies, where only one mutation at time is evaluated. For screening programs, HRM should be followed by sequencing to confirm findings.

Diverse screening methods such as single-strand conformation polymorphism (SSCP) and denaturing high-performance liquid chromatography (DHPLC) have been employed for diagnosis of FD [12]. These techniques are time consuming and laborious, requiring extensive post-PCR manipulation. On the other hand, HRM is a closed-tube technique that requires no post-PCR processing. Besides, it is a non-destructive method and altered samples may be directly sequenced afterwards. Thus, it reduces contamination and errors from multiple processing of samples, length of diagnosis and increases throughput.

Previous studies with HRM in FD included a small number variants analyzed, and/or did not evaluated variants in all exons of GLA [7,13,14]. Our study is the most comprehensive so far, with the highest total amount and average amount of variants per exon analyzed. In conclusion, we developed a fast and highly sensitive HRM based protocol that may facilitate genetic screening of FD.

4. Acknowledgements

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Supplemental data

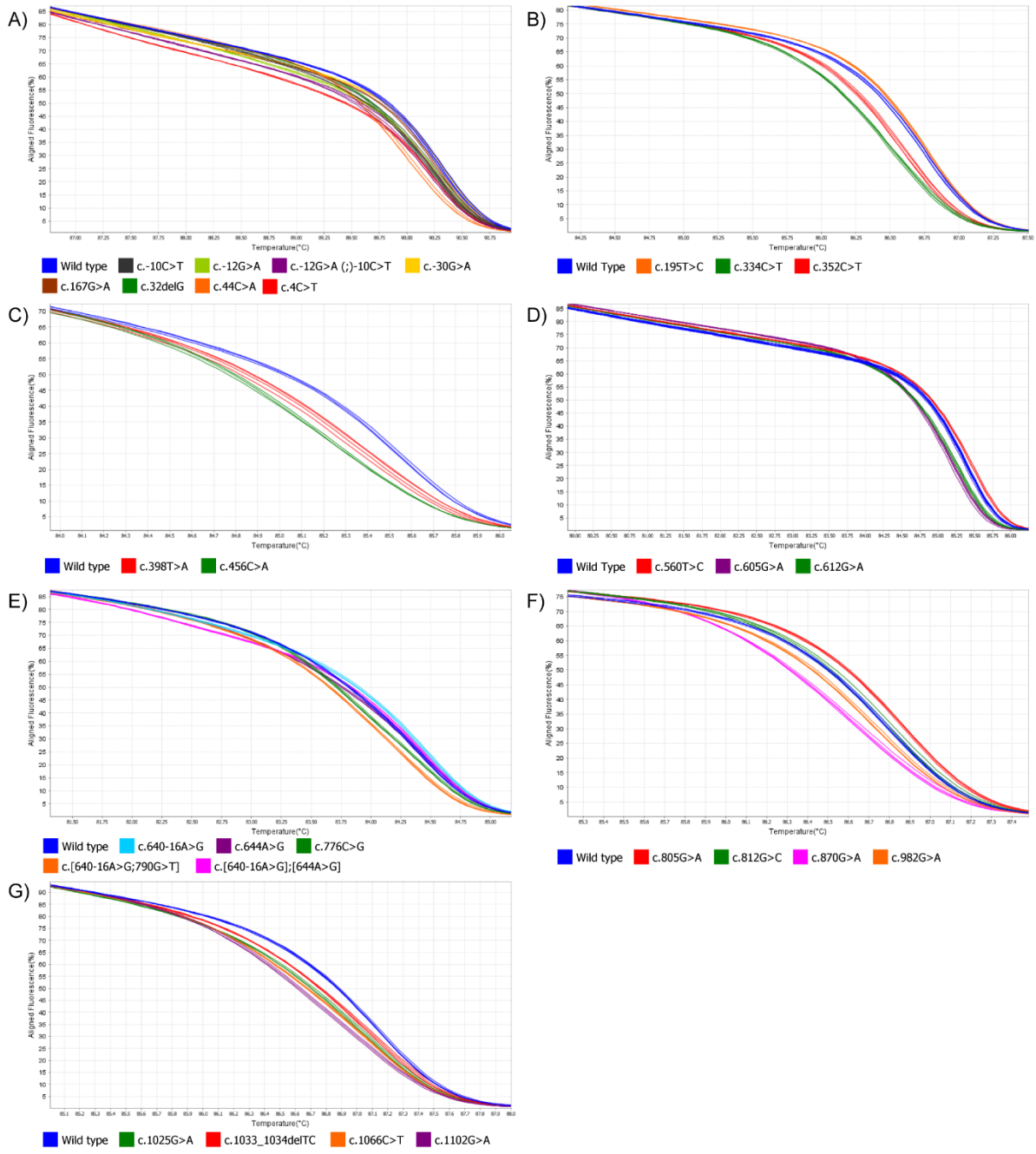

Figure S1: HRM analysis of *GLA* gene. Align melt curves from each amplicon from samples of females and artificial heterozygous males. Wild type curves are shown in dark blue for all exons. A-G: Exons 1 to 7 with respective variants.

Table S1: Primer sequences, amplicon sizes and PCR conditions used for *GLA* amplification.

Exon	Primer Forward (5'>3')	Primer Reverse (5'>3')	Amplicon size (pb)	$MgCl_2$ conc. (mM)	Tm (°C)
1[a]	TTAAAAGCCCAGGTTACCCG	AAAGCAAAGGGAAGGGAG	280	4	62
2	ATTGTAATGATTATTGGAATTTCTCT	CTGAATGAACAAGAACATTATCTAT	239	2.5	60
3[a]	TCTCTTTCTGCTACCTCACG	TCTTTCCTTTGTGGCTAAATC	282	4	60
4[b]	**GCCGCCCCCGCCGC**CAGCTGGAAATTCATTTCTTT	**CCCCCGCCCG**CCTTGGTTTCCTTTGTTGTCA	239	4	62
5[a]	GAAGGCTACAAGTGCCTCCT	AGCCTACCGCAGGGTCTT	293	4	64
6	CTCTCTTGTTTGAATTATTTCATTCT	ATAAAGCCATCTTAAAATATATACTCTTA	267	3	60
7.1	GCTAAGCAACCACACTTTCTTGG	TCCATTCATAGAACCCTAGCTTCC	230	4	64
7.2	CAGCTCCTCCCTGTGAAA	AGCCACCTAGCCTTGAGC	214	4	60

[a]: Tai et al., 2012 [b]: Shabbeer, Robinson, & Desnick, 2005; Nucleotides in bold are GC clamps.

Table S1 references:

C.-L. Tai, M.-Y. Liu, H.-C. Yu, C.-C. Chiang, H. Chiang, J.-H. Suen, S.-M. Kao, Y.-H. Huang, T.J.-T. Wu, C.-F. Yang, F.-C. Tsai, C.-Y. Lin, J.-G. Chang, H.-D. Chen, D.-M. Niu, The use of high resolution melting analysis to detect Fabry mutations in heterozygous females via dry bloodspots., Clinica Chimica Acta. 413 (2012) 422–7. doi:10.1016/j.cca.2011.10.023.

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3.3. Artigo III

Pasqualim G, Pereira FS, Simon L, Mayer FQ, Burin MG, Michelin-Tirelli K, Vargas CR, Zamproni L, Gonçalves M, Guimarães I, Vairo F, Giugliani R and Matte U (2017) **Mutational profile of Brazilian patients with suspicion of Fabry disease.**

Artigo submetido ao periódico *Molecular Genetics and Metabolism*.

Mutational profile of Brazilian patients with suspicion of Fabry disease

Gabriela Pasqualim[1,2], Fernanda dos Santos Pereira[2], Laura Simon[2], Fabiana Quoos Mayer[2], Kristiane Michelin-Tirelli[3], Maira Graeff Burin[3], Carmen Regla Vargas[3], Filippo Vairo[3], Roberto Giugliani[1,2,3,4,5], Ursula Matte[1,2,4]

[1]: Post-Graduation Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[2]: Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[3]: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[4]: Department of Genetics, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[5]: INAGEMP, Porto Alegre, RS, 90035-903, Brazil

Corresponding author: Dr Ursula Matte.

Address: Gene Therapy Center, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, RS. 90035-903, Brazil. Phone: +55 51 3359 8838, Fax: +55 51 3359 8010.

E-mail address: umatte@hcpa.edu.br

Abstract

Fabry disease (FD) is a lysosomal disorder resulting from deficient α-galactosidase A (GLA) activity that leads to the accumulation of glycosphingolipids. Due to its X-linked inheritance, genetic analysis is crucial for the identification of women with FD. In this study, we report the analysis through direct sequencing of *GLA* of 408 Brazilian individuals with family history (n=226) or clinical suspicion of FD (n=182) belonging to 213 families. In non-coding regions, variants c.-12G>A, c.-10C>T, c.640-16A>G, and c.1000-22C>T were detected with frequencies of 0.10, 0.11, 0.14 and 0.36, respectively. In coding regions, 26 variants were identified in 135 individuals (56 males, 79 females) of 31 families. Most were missense mutations identified in single families. Two previously unreported variants were found in females: p.Ser65Ser (located in an exon-intron junction) and p.Pro323Thr. *In silico* analysis of p.Ser65Ser indicated possible effect in splicing. However, despite low GLA plasma activity, no splice defects were identified by cDNA sequencing. p.Pro323Thr was identified in a female individual with stroke. Predicting tools resulted in 70% agreement on pathogenicity. This study reports a comprehensive analysis of *GLA* variants in the Brazilian population that might aid in management and genetic counseling of families with suspected or confirmed cases of FD.

Key words

Fabry disease, α-galactosidase, genetic diagnosis, lysosomal storage disease.

1. Introduction

Fabry disease (FD; OMIM #301500) is an X-linked disorder resulting from deficient activity of the lysosomal hydrolase α-galactosidase A (GLA, EC 3.2.1.22). This enzymatic defect leads to the accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3), throughout the body. Typical symptoms include acroparesthesia, angiokeratomas, corneal dystrophy, gastrointestinal symptoms, and sweating abnormalities [1]. Patients with the classical form have low residual enzymatic activity with childhood onset of symptoms and development of cardiac, cerebrovascular and/or renal complications [2]. Patients with non-classical phenotypes have higher residual activity, later onset of symptoms and are mono or oligo-symptomatic [3]. Data from newborn screenings estimated the prevalence of FD in males to be about 1:3,500 in Europe [4,5] and as high as 1:1,250 in Asia [6]. Female individuals with FD may be as severely affected as men. However, they usually present enzyme activity levels overlapping normal range, which hampers their detection through enzyme analysis (a common first tier of newborn screenings). Therefore, whenever the family history is non informative about carrier status, genetic analysis is crucial for identification of women with FD.

GLA is a homodimeric protein, formed by a polypeptide of 429 amino acids including a 31-residue signal sequence [7]. Its coding gene, *GLA* (OMIM #300644), is mapped to Xq22.1, and spans 12.436 bp divided in seven exons of 90 to 300 bp [8]. FD is characterized by private mutations in *GLA* and presents great allelic heterogeneity. To date, more than 900 mutations in the GLA gene have been described in the Human Mutation Database [9]. Over 60% of mutations described are missense/nonsense and more than 90% of all variants are point mutations or small insertions/deletions, therefore standard Sanger sequencing is an effective diagnostic method. Some missense mutations are responsive to pharmacological chaperones, expanding treatment options beyond enzyme replacement therapy for part of FD patients [10]. Therefore, identification of *GLA* variation is essential not only to detection of female patients and genetic counseling, but also for guidance about treatment options. In this study, we report the analysis through direct sequencing of 408 *GLA* Brazilian patients with family history or clinical suspicion of Fabry disease.

2. Materials and methods

2.1. Patients and Samples

The Medical Genetics Service at Hospital de Clínicas de Porto Alegre (MGS/HCPA) is a reference center for inborn errors of metabolism and as such investigates samples referred from all over Brazil and from other Latin American countries. Samples analyzed in this study are from Brazilian individuals with positive family history or clinical suspicion of FD. All male patients included had confirmed biochemical diagnosis with low enzyme activity in leukocytes and/or plasma. Whole blood samples were collected in FTA cards (Whatman, USA), EDTA or PAXgene (Qiagen, Germany) tubes directly at HCPA or elsewhere and shipped within 24 h of collection. Shipping was made in ice-filled containers (EDTA tubes) or at room temperature (FTA cards and PAXgene tubes). Processing was performed within 24 h of arrival. Data regarding Gb3 measurements were collected from patients charts. This study was approved by the Ethics Research Committee of our institution (#03-441 and #15-0196) and the patients gave written informed consent.

2.2. Biochemical and molecular analysis

Analysis of GLA activity in leukocytes and/or plasma was performed using fluorogenic method with gender specific reference values previously determined [11–13]. Quality control measures involved inclusion of a reference enzyme to assure sample quality and control samples to assure test quality. Urinary Gb3 analysis was also performed, in selected cases. Samples were analyzed by LC-MS/MS (liquid chromatography–tandem mass spectrometry) as previously reported [14]

Genomic DNA was purified from FTA Classic cards with FTA purification reagent (Whatman, USA) and extracted from FTA Elute cards with water. Extraction from peripheral blood samples in EDTA was made with either EasyDNA Kit (Invitrogen, USA), Wizard Genomic DNA Purification Kit (Promega, USA) or standard salting-out protocol. PCR reactions, purification and sequencing were performed as previously described [13]. Electropherograms were compared to the genomic reference sequence (GenBank: NG_007119.1) and all alterations were confirmed by sequencing of the opposite strand. In

patients with family history of FD and known causative mutation, only the affected exon and its flanking regions were analyzed.

For patients bearing possible splice site mutations, total RNA was extracted from PAXgene tubes with PAXgene Blood RNA kit (Qiagen, Germany) and converted to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to manufactures' recommendations. Primers 5'- GGGCTAGAGCACTGGACAAT -3' and 5'- TCTGGGCATCAATGTCGTAG-3' were used to amplify a fragment ranging from exon 1 to 3. Reactions contained 35ng of cDNA, 1× PCR buffer, 0.2 mM of dNTPs, 3 mM of MgCl2, 0.8 µM of primers and 2U of Taq Platinum DNA polymerase (Thermo Fisher Scientific, USA). Cycling conditions were : 95 °C for 5 min, followed by 35 cycles at 95 °C for 30s, 60°C for 30s and 72 °C for 30s and 1 cycle at 72 °C for 10 min. After purification with EXO-SAP (GE Healthcare Life Sciences, USA) and agarose gel electrophoresis quantification with Low DNA Mass Ladder (Thermo Fisher Scientific, USA), samples were sequenced in an ABI3500 genetic analyzer using BigDye Terminator v3.1 (Thermo Fisher Scientific, USA) and compared to GenBank: NM_000169.2. Altered sequencing results were confirmed by reverse strand sequencing. Comparisons with the general population were made with data from ABraOM, a repository with genomic variants formed exclusively by 609 elderly Brazilian individuals [15]

2.3. *In silico* analysis

Prediction of pathogenicity was made with: Polyphen2 [16], SIFT [17], Provean [18], Mutpred2 [19], SNP&GO [20], Phanter [21] and M-CAP [22]. Analysis of exonic splicing enhancers (ESE) and splicing alterations was made with RESCUE-ESE [23], PESX [24,25], MutationTaster2 [26], MutPred Splice [27] and Human Splice Finder (HSF) 3.0 [28]. Responsiveness to pharmacological chaperones was tested with Fabry_CEP [29].

2.4. Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics version 22.0 software (SPSS Inc., USA). Differences in allele frequencies were assessed with Fischer's Exact Test or Monte Carlo method, as indicated, both followed by Bonferroni correction.

3. Results

In total, 408 individuals (56 males and 352 females) with family history (n=226) or clinical suspicion (n=182) of FD from 213 families were genotyped at HCPA. Four common variants were detected in non-coding regions, two in 5'UTR, one in intron 4 and one in intron 6 (table 1). The highest frequency was observed for c.1000-22C>T, which was found in 113 patients including 13 females and 10 males who also harbored exonic mutations. Its frequency was also increased in comparison to the frequency found in the Brazilian population (P value = 0.027). Variant c.640-16A>G was found in 45 individuals, including all carriers of p.Asp264Tyr (3 males and 3 females) and p.Met290Ile (3 females). Of note, p.Met290Ile was found with the haplotype c.-10C>T/ c.640-16A>G/ c.1000-22C>T in all carriers. Variant c.-10C>T was identified exclusively in females (n=46). Out of the 40 carriers of variant c.-12G>A, only 1 was male.

Table 1: Frequency of variants identified in non-coding regions of *GLA.*

Location	cDNA[1]	rsID	Frequency in this study	Frequency in Brazil[2]	P value[3]
5' UTR	c.-12G>A	rs3027585	0.10	0.09	0.407
5' UTR	c.-10C>T	rs2071225	0.11	0.06	0.003
Intron 4	c.640-16A>G	rs2071397	0.14	0.12	0.449
Intron 6	c.1000-22C>T	rs2071228	0.36	0.30	0.027*

[1]GenBank: NM_000169.2, [2]Data from AbraOM, a database for Brazilian genomic variants.
[3] Fischer exact test. *Not significant after Bonferroni correction.

In coding regions, 26 variants were identified in 135 individuals (56 males and 79 females) of 31 families (Table 2).

Table 2: Frequency of variants identified in coding regions of *GLA.*

#	Location	cDNA effect[1]	Protein effect[2]	rsID	Frequency (%)[3]	Families	Hemizygotes	Heterozygotes	Ref
1	Exon 1	c.4C>T	p.Gln2Ter	rs869312313	3.2%	1	1	0	[30]
2	Exon 1	c.32delG	p.Gly11Alafs	rs869312278	3.2%	1	10	12	[31]
3	Exon 1	c.44C>A	p.Ala15Glu	rs869312304	3.2%	1	2	3	[32]
4	Exon 1	c.167G>A	p.Cys56Tyr	rs869312258	3.2%	1	2	1	[33]
5	Exon 2	c.195T>C	p.Ser65Ser	rs782803696	3.2%	1	0	6	This study
6	Exon 2	c.334C>T	p.Arg112Cys	rs104894834	3.2%	1	3	6	[34]
7	Exon 2	c.352C>T	p.Arg118Cys	rs148158093	9.7%	3	0	6	[4]
8	Exon 3	c.398T>A	p.Ile133Asn	N/A	3.2%	1	1	1	[14]
9	Exon 3	c.456C>A	p.Tyr152Ter	N/A	3.2%	1	3	0	[35]
10	Exon 3	c.467C>A	p.Ala156Asp	rs869312307	6.5%	2	1	1	[36]
11	Exon 4	c.560T>C	p.Met187Thr	rs869312342	3.2%	1	1	3	[37]
12	Exon 4	c.605G>A	p.Cys202Tyr	rs869312344	3.2%	1	1	1	[38]
13	Exon 4	c.612G>A	p.Trp204Ter	N/A	3.2%	1	1	0	[39]
14	Exon 5	c.644A>G	p.Asn215Ser	rs28935197	3.2%	1	1	1	[40]
15	Exon 5	c.776C>G	p.Pro259Arg	rs869312399	3.2%	1	2	0	[41]
16	Exon 5	c.790G>T	p.Asp264Tyr	rs190347120	3.2%	1	3	3	[37]
17	Exon 6	c.805G>A	p.Val269Met	rs869312427	6.5%	2	12	20	[37]
18	Exon 6	c.812G>C	p.Gly271Ala	rs869312429	3.2%	1	0	1	[42]
19	Exon 6	c.870G>A	p.Met290Ile	rs869312438	3.2%	1	0	3	[37]
20	Exon 6	c.937G>T	p.Asp313Tyr	rs28935490	6.5%	2	0	2	[43]
21	Exon 6	c.967C>A	p.Pro323Thr	rs147737890	3.2%	1	0	1	This study
22	Exon 6	c.982G>A	p.Gly328Arg	rs104894832	3.2%	1	3	2	[34]
23	Exon 7	c.1025G>A	p.Arg342Gln	rs28935493	3.2%	1	5	0	[44]
24	Exon 7	c.1033_1034delTC	p.Ser345Argfs	rs398123198	3.2%	1	1	2	[45]
25	Exon 7	c.1066C>T	p.Arg356Trp	rs104894827	3.2%	1	3	3	[46]
26	Exon 7	c.1102G>A	p.Ala368Thr	rs144994244	3.2%	1	0	1	[47]

[1]: GenBank NM_000169.2; [2]:GenBank NP_000160.1; [3]: Based on number of carrier families. N/A: Not available.

Exon 6 had the highest frequency of variants (23.1%) followed by a more even distribution in the remaining coding regions: 15.4% in exons 1 and 7 and 11.5% in exons 2 to 5. Missense mutations were significantly more common than any other type, representing 80.8% of total. Both the increased number of variants identified in exon 6 and the higher rate of missense variants are in accordance with data from the HGMD database (figure 1 and table S1). Small deletions, nonsense and synonymous mutations were also found but at lower rate.

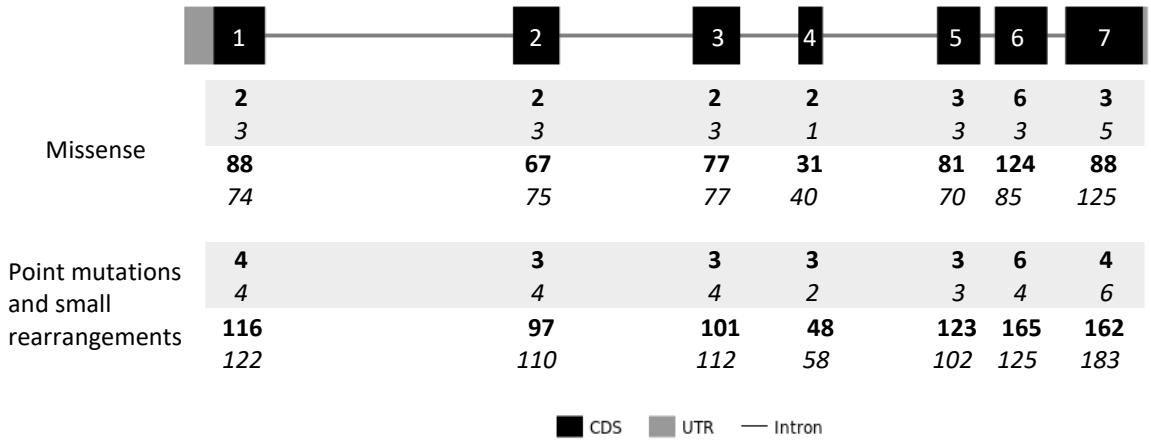

Figure 1: Distribution of coding *GLA* variants. Data from this study (grey lines) or the HGMD version 2016.2. Frequencies of observed (bold) and expected (italic) number of variants (if equal distribution based on coding region size). In HGMD, both classes of variants show statistically significant differences in distribution [Monte Carlo P value: ≤0.001 (0.000-0.001)] Point mutations: missense and nonsense variants; Small rearrangements: deletions, insertions and indels involving up to 20 bp. Diagram based on GenBank: NG_007119.1 with introns rescaled to 25% of original size.

The majority of variants was found in in single families, with exception of p.Arg118Cys, found in 3 families; and p.Ala156Asp, p.Val269Met and p.Asp313Tyr present in 2 families each. Most variants have also been previously described in FD patients with classical phenotype and in males with low enzymatic activity and do not require further explanation. However, seven variants were found only in female individuals and are not unequivocally associated with FD: p.Arg118Cys (n=6), p.Gly271Ala (n=1), p.Met290Ile (n=3), p.Asp313Tyr (n=2), p.Ala368Thr (n=1), c.195T>C (n=6), and p.Pro323Thr (n=1).

Patients with p.Arg118Cys presented urinary Gb3 ranging from 5.9 to 21.73 µg Gb3/mg creatinine [average 14.8, and reference value (RV) ≤ 15 µg Gb3 / mg creatinine]. The only female subject with p.Gly271Ala presented very low enzyme activity in leucocytes: 2.3 nmol/h/mg protein (RV: 43 to 63 nmol/h/mg protein). The range of leukocyte activity in individuals with p.Met290Ile was 16 to 26 nmol/h/mg protein and urinary Gb3 was normal. The individual with p.Ala368Thr presented normal enzyme activity in both plasma (13 nmol/h/mL, RV: 12 – 22 nmol/h/mL) and leukocytes (40 nmol/h/mg protein)

Variants c.195T>C (p.Ser65Ser) and c.967C>A (p.Pro323Thr) were not previously described in the literature in individuals with FD, despite being deposited in dbSNP. c.195T>C was deposited in dbSNP by the ExAc Consortia but had a low coverage on this database. Its frequency on the Genome Aggregation Database (gnomAD), which includes data from ExAC, is 0.00002250 [48]. In this study, it was identified in 6 female individuals with 40 to 56 years of age with acroparesthesia, all from the same family. No affected or hemizygous males were detected and all affected females were heterozygous. Urine Gb3 levels from 2 individuals were elevated to 25 and 27 µGb3 /mg creatinine (Reference value: up to 15 µg Gb3 /mg creatinine). Discrepant enzyme activity was found in one carrier, who presented normal levels in leukocytes (48 nmol/h/mg protein, RV: 43-63) but low levels in plasma (7.6 nmol/h/mL, RV: 12-22). Evaluation of c.195T>C impact by RESCUE-ESE and PESX indicated loss of an enhancer motif. MutationTaster2 classified it as disease causing due to splice site changes. Mutpred Splice general score was 0.82, which represents a high confident call for a Splice Affecting Variant (SAV). HSF displayed conflicting results with overall interpretation of no significant splicing motif alteration detected (raw data in table S2). In order to evaluate potential splicing errors, mRNA was extracted from blood and fibroblasts of a female carrier and cDNA sequencing was performed. No aberrant splice forms were detected, as no frameshift was seen in Sanger sequence, and mutation was present in heterozygosity (Figure S1).

c.967C>A (p.Pro323Thr) was also deposited at ClinVar by a single submitter and displays uncertain clinical significance. This variant was found in a 40-year-old female with history of stroke and normal GLA activity in DBS (6.2 nmol/h/mL, RV: 2.9-14). *In silico* pathogenicity analysis with different tools is described on table 3. From the seven

prediction tools used, five classified this variant as possibly/probably pathogenic. Additionally, MutationTaster2 classified this variant as disease causing due to changes in amino acid sequence and loss of protein features caused by splice site changes (gain of donor site). HSF 3.0 also indicated potential alteration of splicing and alteration of an exonic ESE site. Fabry_CEP resulted in 44% chance of responsiveness to therapy with pharmacological chaperones.

Table 3: *In silico* analysis of p.Pro323Thr.

Prediction tool	**Score (pathogenicity threshold)**	**Prediction**
SIFT	0.01 (≤0.05)*	Damaging
PROVEAN	-5.249 (<-2.5)	Deleterious
M-CAP	0.875 (> 0.025)	Possibly pathogenic
Panther	1237 (>450)	Probably damaging
SNPs&GO	0.885 (>0.5)**	Disease
MutPred2	0.270 (>0.5)	Not pathogenic
Polyphen2	0.091; sensitivity: 0.91; specificity: 0.68 (>0.15)***	Benign

* Median Information Content: 2.59, # Sequences:190; **Reliability Index:8;
***HumVar model

4. Discussion

In this study, 408 individuals (56 males and 352 females) with either clinical suspicion or family history of FD were analyzed. Only males with previous biochemical diagnosis of FD were included, which explains the significant difference in gender frequency. In females with FD, GLA enzyme activity overlaps with the normal range. Therefore, although combined measurement of enzymatic activity from different sources and gender-specific reference values has proven useful in female screening [13], biochemical analysis may only serve as a first tier screening in women, prior to genetic analysis.

Outside the coding region of *GLA*, we identified 4 variants that might modulate phenotype, but individually do not have strong experimental evidence of pathogenicity.

These variants were often found in individuals from both genders with or without known pathogenic variants. Moreover, their frequency in the Brazilian population is higher than expected for pathogenic alleles in rare monogenic diseases. Variant c.1000-22C>T, particularly, has a frequency of 0.3 in the Brazilian population and of 0.25 globally (gnomAD), which is inconsistent with estimated disease frequency. Therefore, we chose to express their frequencies by the number of alleles analyzed, instead of the number of affected families. However, family-based frequencies are described in supplementary table S3. As expected, the most frequent variation detected in this study was c.1000-22C>T, found in 36% of alleles analyzed, which represents a significant increase from the Brazilian population frequency. This variation is within a haplotype also including c.-10C>T and c.640-16A>G, and was reported as pathogenic in a 40-year-old female individual with lysosomal inclusions in renal biopsy, multisystem involvement and early onset of symptoms [49]. Authors did not performed MLPA analysis or cDNA sequencing for detection of whole exons deletions or duplications. Therefore, the possibility of a causative mutation including deletion/duplication of exons 2, 4 or 6 in this patient cannot be excluded. However, c.-10C>T was shown to be the variant in this haplotype responsible for reduced expression of *GLA* mRNA [50]. Also, it was the only variant found in patients with neurological manifestations and abnormal lyso-Gb3 and/or enzyme activity levels (including females) in some studies [50,51]. In this study, c.-10C>T was only detected in females and no correlation was seen between c.-10C>T allele or haplotype presence and GLA activity in either plasma or leukocytes (data not shown). Altogether, our data does not support a causative link between any of the variants found outside the coding region and disease manifestation.

FD is a disorder genetically characterized by private mutations, which translates to near 1,000 variants described at HGMD in a fast increasing rate [52]. Within coding region, 26 variants were identified in 56 males and 79 females, members of 31 families. The vast majority of variants detected were missense mutations (80.8%), which is in accordance with HGMD, Fabry Database, and our previous screening of females [13,53]. Moreover, higher frequency of missense variants detected in exon 6 is also observed in HGMD (figure 1 and table S1).

The most frequent coding alteration was p.Arg118Cys, found in six females from three families. This variant has conflicting interpretations of pathogenicity at ClinVar, ranging from “likely pathogenic” to “uncertain significance”. In the individuals analyzed in this study, it resulted in slightly elevated urinary Gb3 in two of six carriers. A recent report has reviewed the role of p.Arg118Cys in FD and suggested it is either a non-pathogenic or a low-pathogenicity variant that might modulate multifactorial risk of cerebrovascular disease [54]. p.Asp313Tyr was another recurrent variant in females, found in two unrelated individuals with no biochemical analysis. It is reported as a pseudodeficiency or low-pathological allele, and it is detected in multiple patients with high residual enzymatic activity, low levels of lyso-Gb3, and no severe organ manifestation [55–57]. However, it is still classified as pathogenic, without co-occurrence of other factors, by some authors [58,59].

Variants p.Gly271Ala and p.Ala368Thr were identified in a single female each. The individual with p.Gly271Ala had low enzymatic activity and this variant has strong evidence supporting its pathogenicity. It was described in a large family with individuals from both genders presenting low enzyme activity, elevated lyso-Gb3 levels, and classic FD symptoms including patients with renal transplantation and pacemaker implantation [42]. The individual with p.Ala368Thr had normal enzyme activity in both plasma and leukocytes. In previous reports, lyso-Gb3 levels were normal in three female carriers and *in vitro* mutagenesis resulted in enzyme activity comparable to normal levels [47]. This variant was identified in a screening of hemodialysis patients in a 73-year-old male with enzyme activity lower than normal (1.7 µmol/l/h, RV <2.2 µmol/l/h) in DBS [60]. This level is not as reduced as seen in classic affected men and, in our experience, DBS measurements are not as reliable as data from leukocytes and plasma, and therefore require confirmation in other sources [13]. As p.Met290Ile, it shown high frequency in a large screening of dialysis patients, but no specific clinical or biochemical data from carriers were described [58]. Therefore, our data is in agreement with previous publications and does not support an association between p.Ala368Thr and classical FD.

Two variants not previously described in the literature in individuals with FD were detected: c.195T>C (p.Ser65Ser) and c.967C>A (p.Pro323Thr). c.195T>C is located in an

intron-exon junction, affecting the first nucleotide of exon 2. Although synonymous variants are usually automatically regarded as neutral, results from RESCUE-ESE and PESX indicated it abolished an ESE motif, while Mutpred Splice classified it as a splice affecting variant with a high confidence score. ESEs are small sequences within exons that affect splicing efficiency in constitutive and alternative splice regions. They are recognized by members of the family of serine/arginine (SR)-rich proteins which recruit members of the splicing machinery to nearby splice sites and thus, positively regulate exon inclusion. Consequently, mutations in these sequences may lead to splicing defects as exon skipping [61,62]. Exonic variants that alter splicing were already described in lysosomal diseases, including FD. Two missense mutations were experimentally proven to be pathogenic by causing aberrant splicing. c.194G>C (p.Ser65Thr), located in the same codon as c.195T>C but previous exon, activates a cryptic splice site with consequent inclusion of part of intron 1 and creation of a premature stop codon [63]. Likewise, c.639G>T (p.Lys213Asn) induces skipping of exon 4 [64]. Therefore to evaluate possible effect of c.195T>C in splicing, cDNA from a female carrier was analyzed. Sequencing showed the variant in heterozygosity, with no splicing change. Additionally, enzyme activity in leucocytes was within normal range and the melt profile of a fragment including exons 1 to 3 and the overall *GLA* mRNA expression had no significant difference between the index case and healthy female controls (data not shown). Therefore, our data strongly supports that this variant can be considered a non-pathogenic polymorphism.

Variant p.Pro323Thr was found in a female with history of stoke and with no other variants detected in *GLA*, including non-coding regions. It is a non-conservative mutation from a nonpolar, aliphatic to an uncharged polar amino acid. *In silico* analysis of c.967C>A pathogenicity resulted in conflicting predictions. Most predictors, including M-CAP (a predicting tool designed specifically for analysis of rare missense variants in humans) confidently classified this variant as possibly pathogenic. However, Mutpred2 and Polyphen2 classification was benign. A common feature included in prediction tools is sequence conservation, and might be the cause for the results variation. Grade of conservation from PhastCons (phastCons100way track on UCSC Genome browser), which considers flanking positions, was maximum. However, PhyloP score (phyloP100way track

on UCSC Genome browser), which ignores neighborhood effects, was 4.14206 (range: fast-evolving -20 to 7.532 conserved). This might explain the inconsistency observed, since Polyphen2 and Mutpred2 are more focused on protein function than the other programs. Another variant in the same residue (p.Pro323Arg) was described in the literature and classified as having uncertain clinical significance in ClinVar. It reached 60% wild type enzyme activity in transfected HEK-293H cells and lyso-Gb3 levels were normal in a male and only slightly elevated in two female individuals [65]. Interestingly, MutationTaster2 and HSF indicated possible splicing alteration resulting in an in-frame deletion from residues 322 to 333. Unfortunately, no RNA sample from this patient was available to test this hypothesis. Therefore, c.967C>A might be a mild variant that, as p.Arg118Cys, might modulate cerebrovascular phenotype in conjunction with other factors. However, further biochemical and clinical studies, as Gb3 analysis in a tissue biopsy and/or *in vitro* expression [3], are needed to test this hypothesis.

Another study of the Brazilian population reported similar results to ours regarding frequency of missense mutations and distribution of variants across families with two remarkable exceptions: p.Arg342Gln and p.Arg356Trp [36]. Both are pathogenic variants related to the classical form of the disease and were detected in five and six families, respectively, out of a group of 51. Although Arg342 and Arg356 residues are mutational hot spots for including CpG dinucleotides, it cannot be determined if these are independently arising variants or if these patients were distantly related. Founder effect connecting four seemingly unrelated Brazilian families with FD sharing the same c.32delG mutation has been previously reported [66]. In the present study this mutation has been found in twenty-two individuals, all of them related to the original families. Another variant, p.Val269Met, is also recurrent in Brazilian families with FD. In the present study it was found in 32 individuals from a large family composed of two separate groups living in different regions of the country. In this case, a presumably unrelated female carrier from a third region was also detected.

In summary, we detected 30 *GLA* variants in 408 Brazilian individuals with clinical suspicion or family history of FD. In 130 individuals, 26 variants in coding regions were identified. Most were causative of FD and found in families with affected males. However,

variants without consensus in the literature for causative relation to FD, as p.Arg118Cys, and p.Ala368Thr, frequently found in screenings of high-risk populations, were also present. In addition, our data strongly indicates the role of c.195T>C as a non-pathogenic variant, but was insufficient to ascertain pathogenicity of p.Pro323Thr. With the increasing amount of high-risk population screenings and popularization of massive parallel sequencing of isolated individuals with non-specific symptoms of FD, the number of detected mutations in *GLA*, including variants of uncertain significance (VUS), will rapidly increase. Therefore, comprehensive analysis of variants, including non-pathogenic mutations, like the one performed in this study should aid in diagnostic decisions and genetic counseling of families. Particularly in the case of VUS and mild variants, extended clinical and biochemical evaluation, including analysis of environmental and genetic risk factors will be critical for the appropriate management and correct genetic counseling.

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Supporting Information

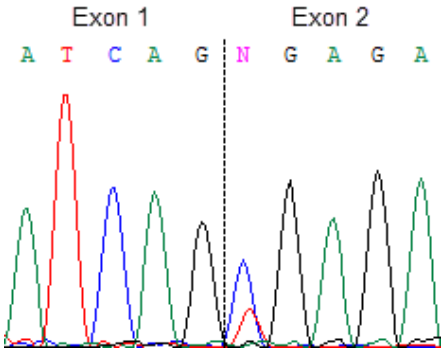

Figure S1: cDNA sequencing of a female individual heterozygous for the c.195T>C mutation.

Table S1: Distribution of variants across coding region of *GLA*

Variant type			Exons							Coding region	P value*
			1	2	3	4	5	6	7		
Missense/Nonsense		n	97	79	87	35	94	136	108	636	<0.001 (0.000-0.000)
		Expected n	96	86	88	45	80	98	143		
		Freq (%)	15%	12%	14%	6%	15%	21%	17%	100%	
		Ratio to exon size	0.5	0.45	0.49	0.38	0.58	0.69	0.37	0.46	
	Missense	n	88	67	77	31	81	124	88	556	<0.001 (0.000-0.000)
		Expected n	84	75	77	40	70	85	125		
		Freq (%)	16%	12%	14%	6%	15%	22%	16%	100%	
		Ratio to exon size	0.45	0.38	0.43	0.34	0.5	0.63	0.3	0.4	
	Nonsense	n	9	12	10	4	13	12	20	80	0.852 (0.843-0.861)
		Expected n	12	11	11	6	10	12	18		
		Freq (%)	11%	15%	13%	5%	16%	15%	25%	100%	
		Ratio to size	0.05	0.07	0.06	0.04	0.08	0.06	0.07	0.06	
Small rearrangements		n	19	18	14	13	29	29	54	164	0.020 (0.016-0.023)**
		Expected n	26	24	24	13	22	27	40		
		Freq (%)	11%	10%	9%	7%	16%	16%	30%	100%	
		Ratio to exon size	0.09	0.1	0.08	0.12	0.17	0.14	0.17	0.13	
	Small deletions	n	14	11	9	10	19	20	41	124	0.031 (0.026-0.035)**
		Expected n	19	17	17	9	16	19	28		
		Freq (%)	11%	9%	7%	8%	15%	16%	33%	100%	
		Ratio to size	0.07	0.06	0.05	0.11	0.12	0.1	0.14	0.09	
	Small insertions	n	3	3	4	2	8	9	10	39	0.402 (0.390-0.415)
		Expected n	6	5	5	3	5	6	9		
		Freq (%)	8%	8%	10%	5%	21%	23%	26%	100%	

	Ratio to exon size	0.02	0.02	0.02	0.02	0.05	0.05	0.03	0.03	
Small indels	n	2	4	1	1	2	0	3	13	0.851 (0.842-0.860)
	Expected n	2	2	2	1	2	2	3		
	Freq (%)	15%	31%	8%	8%	15%	0%	23%	100%	
	Ratio to exon size	0.01	0.02	0.01	0.01	0.01	0	0.01	0.01	
Missense/Nonsense & Small rearrangements	n	116	97	101	48	123	165	162	812	<0.001 (0.000-0.000)
	Expected n	122	110	112	58	102	125	183		
	Freq (%)	14%	12%	12%	6%	15%	20%	20%	100%	
	Ratio to exon size	0.6	0.55	0.57	0.52	0.76	0.83	0.56	0.58	

*Monte Carlo P value (95% IC); **Not significant after Bonferroni correction. Reference: Human Genome Mutation Database Professional, version 2017.2

Table S2: Human Splice Finder 3.0 - Raw data tables for c.195T>C

Elements	Tools	Sequence Position*	cDNA Position*	Linked SR protein	Reference motif	Linked SR protein	Mutant motif	Variation
Enhancer motifs	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins	95	-6			SRp40	tttcagC (86.77)	New site
		98	-3			SF2/ASF (IgM-BRCA1)	cagCGAG (73.08)	New site
		101	1	SRp40	TGAGAAG (81.38)	SRp40	CGAGAAG (79.22)	-2.65%
	RESCUE ESE hexamers	101	1	-	TGAGAA	-		Site broken
	Predicted PESE Octamers from Zhang & Chasin	98	-3	-	cagTGAGA	-		Site broken
		101	1	-	TGAGAAGC	-		Site broken
	EIEs from Zhang et al.	97	-4	-	tcagTG	-		Site broken
		98	-3	-	cagTGA	-	cagCGA	
		99	-2	-		-	agCGAG	New Site
		100	-1	-		-	gCGAGA	New Site
		101	1	-	TGAGAA	-	CGAGAA	

* Variant located at sequence position 101 and cDNA position 1

Table S3: Family-based frequency of variants identified in non-coding regions of *GLA*

Location	cDNA[1]	rsID	N Families analyzed	N families with carriers	Freq	Hemizygotes	Hetero/Homozygotes
5' UTR	c.-12G>A	rs3027585	191	40	0.21	1	39
5' UTR	c.-10C>T	rs2071225	191	38	0.20	0	42
Intron 4	c.640-16A>G	rs2071397	175	37	0.21	3	41
Intron 6	c.1000-22C>T	rs2071228	163	98	0.60	10	98

[1]GenBank: NM_000169.2

3.4. Artigo IV

Pasqualim G, Vairo F, Giugliani R and Matte U, **The cardiac phenotype in Fabry Disease: a search for modifier genes**.

Artigo a ser submetido ao periódico *American Journal of Human Genetics*.

The cardiac phenotype in Fabry Disease: a search for modifier genes

Gabriela Pasqualim, [1,2] Filippo Vairo, [3] Roberto Giugliani, [1,2,3,4,5] Ursula Matte [1,2,4,*]

[1]: Post-Graduation Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[2]: Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[3]: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[4]: Department of Genetics, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[5]: INAGEMP, Porto Alegre, RS, 90035-903, Brazil

*Correspondence: umatte@hcpa.edu.br

Abstract

The manifestation of symptoms in individuals with Fabry Disease (FD) is highly heterogeneous, even in patients with same genetic variant. Therefore, in this study we investigated new modifier genes that could be associated with the variability of cardiac signs and symptoms in patients with FD. Whole exome sequencing was performed in 6 members of a family with a classical mutation of FD. Variants in 5 genes were selected for replication: rs1803250 (*CTSB*), rs2291569 (*FLNC*), rs3858340 (*BAG3*), rs2302190 (*MTMR4*) and rs3188055 (*INPP5F*). Validation cohort included 45 individuals with FD with (n=30) or without (n=15) cardiac disease. No statistically significant association was found between the analyzed SNPs and the development of cardiac manifestations in FD, probably due to small sample size. However, when also compared to the Brazilian population, rs3188055 allele and genotype frequencies were significantly different [*P* = 0.026 (0.022-0.029) and *P* = 0.014 (0.012-0.017), respectively]. The group with cardiac manifestations presented an increase of 50% in the presence of the mutated allele and of 79% in the frequency of heterozygotes. Additionally, while the presence of reference allele (in homo or heterozygosity) was equal between groups, the presence of the mutated allele also was significantly increased in the FD group with cardiac symptoms [*P* = 0.004 (0.003-0.005)]. Therefore our data suggest that *INPP5F*, a negative regulator of cardiac hypertrophy, might be involved in the modulation of development of cardiac manifestations in FD and thus, in its variability in phenotype.

Introduction

Fabry disease (FD [MIM: 301500]) is an X-linked lysosomal disorder caused by mutations in the alpha-galatosidase (*GLA*) gene, which encodes for the enzyme α-galactosidase A (GLA, EC 3.2.1.22). This hydrolase is involved in the catabolism of glycosphingolipids and its deficiency results in progressive accumulation mainly of globotriaosylceramide (Gb_3) throughout the body [1,2]. Typical symptoms and signs of the disease include acroparesthesia, angiokeratomas, sweating abnormalities, gastrointestinal symptoms and corneal dystrophy [3]. Patients with the classical form have childhood onset of symptoms that progresses to renal, cardiac, and/or cerebrovascular complications leading to premature death [4]. Additionally, non-classical phenotypes as renal and cardiac variants affect mostly one organ and are associated with higher residual enzyme activity and later onset of symptoms [5]. Despite being an X-linked disease, these phenotypes are also seen in females, who present a clinical spectrum ranging from asymptomatic to as severe as in men.

Cardiac disease is an important aspect of FD, reported as the most frequent cause of mortality for both male and female patients [6,7]. Frequently detected manifestations include left ventricular hypertrophy, valvar dysfunction, conduction abnormalities, arrhythmia, angina and dyspnea [7,8]. Data from Fabry Outcome Survey (FOS, [7]) indicates that, at baseline examination, frequency of any cardiac symptom ranged from 39% in untreated women to 66.5% in treated men. Moreover, amongst affected patients, including those with the same genetic variant, there is heterogeneity in the degree and specific type of affection. This phenotypic continuum may be related to environmental factors and/or the existence of modifier genes.

Polymorphisms in IL-6, endothelial nitric oxide synthase (eNOS), factor V Leiden and protein Z genes were associated with cerebrovascular manifestations in individuals with FD [9]. Likewise, alterations in eNOS and paraoxonase were also associated with cardiovascular symptoms [10,11]. Therefore, the aim of this study was to investigate new modifier genes that could be associated with the variability of cardiac signs and symptoms in patients with FD.

Material and Methods

Patients

The discovery cohort consisted of six Fabry patients from a family with the c.32delG variant. All patients had genetic analysis of the *GLA* gene and cardiac evaluation by echocardiogram and/or electrocardiogram. The validation cohort consisted of 45 patients with FD from 12 families, with a pathogenic variant confirmed by Sanger sequencing and cardiac evaluation by echocardiogram and/or electrocardiogram. All patients were research consented and the study was approved by the Ethics Research Committee of our institution (# 15-0196). Comparisons with the general population were made with data from ABraOM, a repository with genomic variants exclusively formed by 609 elderly Brazilian individuals [12].

DNA extraction

Whole blood samples from all participants were collected in EDTA tubes. Genomic DNA was extracted with EasyDNA Kit (Thermo Fisher Scientific, USA), according to manufacturer's instructions, and quantified by spectrophotometry with Nanodrop (Thermo Fisher Scientific, USA).

Whole exome sequencing (WES) and variant filtering

Whole-exome sequencing was performed with Nextera Exome Capture System in an Illumina HiSeq 2500 platform at Mendelics Genomic Analysis S.A. (Brazil). Vcf files were analyzed by an *in-house* protocol and gene annotation and variant filtering was made with Enlis Genome Research version 1.10 Software (Figure 1). The following filters were applied (initial filtering): presence in genome set "patients with cardiac symptoms"; absence in genome set "patients without cardiac symptoms"; quality score ≥ 30; read depth ≥ 10; non-synonymous impact on protein. After initial filtering, variants were filtered in parallel by

three general parameters: tissue expression, cellular process and cardiac disease. For tissue expression, preset gene categories related to medium and high expression in left ventricle and atrial appendage as well as a custom gene list including main genes expressed in heart reported at Genotype-Tissue Expression (GTEx) Project [13] were used. For cellular process, preset gene categories including heart development and heart contraction and a custom list of genes associated with autophagy-lysosomal pathway [14] were applied. Finally, for cardiac disease, sets of genes related to "abnormality of the cardiovascular system", cardiomyopathy and hypertrophic cardiomyopathy were obtained from Human Phenotype Ontology [15]. After filtering, selection of genes for validation was made based on literature search of correlation with cardiac disease.

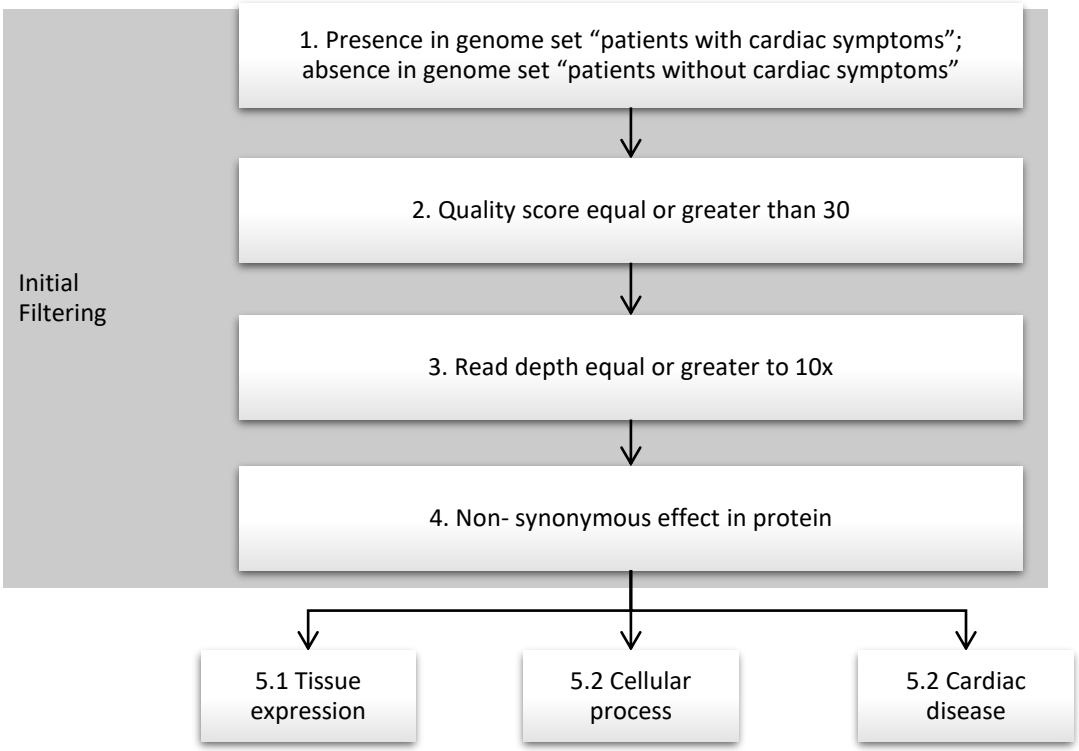

Figure 1: Filtering process of variants from whole-exome sequencing of patients with Fabry disease.

Genotyping

Mutations selected from exome sequencing analysis were evaluated by Sanger sequencing. Interest regions containing SNPs were amplified separately in 25 µl reactions with 50 ng of DNA, 1× PCR buffer, 0.2 mM of dNTPs, 1.5mM $MgCl_2$, 0.2 µM of primers and 0.5 U of Taq DNA polymerase (Life Technologies, USA). Primers sequences and annealing temperatures are described in table S1. Cycling conditions were: 95°C for 5 min, followed by 35 cycles at 95°C for 30s, Tm for 30s and 72°C for 30s and 1 cycle at 72°C for 7 min. After purification with EXO-SAP iT (GE Healthcare Life Sciences, USA) and quantification with Low DNA Mass Ladder (Life Technologies, USA), samples were sequenced in an ABI3500 genetic analyzer using BigDye Terminator v3.1 (Life Technologies, USA). Results were compared to the reference sequences from dbSNP [16] and UCSC Genome Browser [17].

Diagrams and Statistical Analysis

Venn diagrams were designed with jvenn [18]. Statistical analysis was performed in IBM SPSS Statistics version 22.0 software (SPSS Inc., USA). Agreement of genotype frequencies with Hardy–Weinberg (HW) equilibrium was assessed by $\chi 2$ test. In Fabry patients, differences in allele frequencies were assessed with Fischer's Exact Test. Association between genetic alterations and disease outcome were calculated with binary logistic regression. Potential confounders were defined by statistical definition: association with the outcome at $P \leqslant 0.12$. Differences in allele and genotype frequencies between Fabry disease groups and Brazilian population were calculated with Monte Carlo method followed by z-test and Bonferroni correction.

Results

Sample characterization

The pedigree of the family analyzed by WES is shown in figure 2. Patient II.1 is a 79 year-old female with cornea verticillata, angiokeratomas and conduction abnormalities that required

pacemaker implantation. Patient II.2 is a 62 year-old female who has only mild albuminuria (39.5mg/24h; reference value: 0-30mg/24h). Patient II.3 is a 71 year-old female severely affected and treated with enzyme replacement therapy (ERT). Her symptoms include cornea verticilata, stroke, proteinuria, left ventricular hypertrophy, tricuspide insufficiency and secondary changes in ventricular repolarization. Patient III.2 is a 31 years old male, on ERT, who has angiokeratomas, proteinuria, albuminuria and left ventricular overload without hypertrophy. Patient III.2 is a 44 years old female who has proteinuria, albuminuria and left ventricular hypertrophy. Patient IV.1 is a 25 year-old untreated male who has angiokeratomas and mild albuminuria (25mg/L; reference value: <14mg/L) without proteinuria.

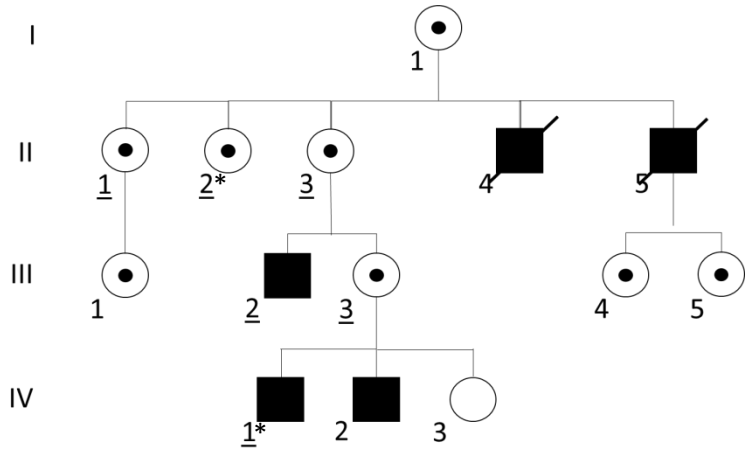

Figure 2: Pedigree of the family with FD included in which WES was performed. Underline numbers indicate selected patients. Asterisks indicate selected individuals without cardiac disease.

WES and variant filtering

Each patient had in average 66,654 genetic variants. Initial filtering resulted in 110 mutations (figure 3, table S2).

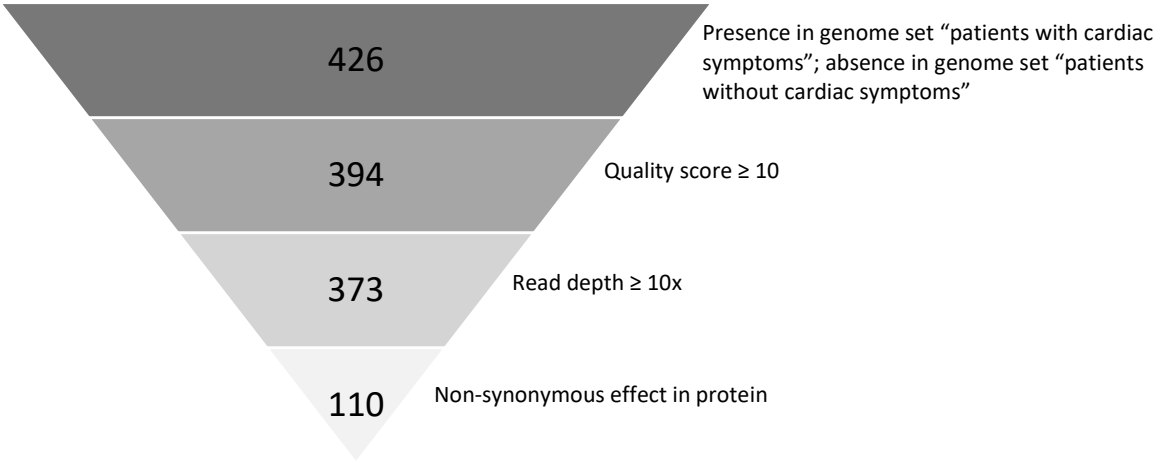

Figure 3: Initial filtering of variants from whole-exome sequencing of patients with Fabry disease

The subsequent filtering by tissue expression yielded 18 different variants in 17 different genes, whereas filtering for both cellular process and cardiac disease resulted in 8 variants in 6 genes (Figure 4).

Selected variants, based on subsequent literature search of experimentally proven gene expression in the cardiovascular system and possible correlation with cardiac disease, are listed on table 1. These variants were then analyzed in the validation cohort.

Table 1: SNPs selected from initial WES analysis for validation

Symbol	Gene name	rsID	cDNA effect	Protein effect	MAF*
CTSB	Cathepsin B	rs1803250	NM_001908.4: c.157A>G	NP_001899.1: p.Ser53Gly	0.11
FLNC	Filamin C	rs2291569	NM_001458.4: c.4700G>A	NP_001449.3: p.Arg1567Gln	0.08
BAG3	BCL2 Associated Athanogene 3	rs3858340	NM_004281.3: c.1220C>T	NP_004272.2: p.Pro407Leu	0.11
MTMR4	Myotubularin Related Protein 4	rs2302190	NM_004687.4: c.838A>G	NP_004678.3: p.Ser280Gly	0.26
INPP5F	Inositol Polyphosphate-5-Phosphatase F	rs3188055	NM_014937.3: c.2989A>G	NP_055752.1: p.Asn997Asp	0.29

*Data from AbraOM, a database for Brazilian genomic variants [12].

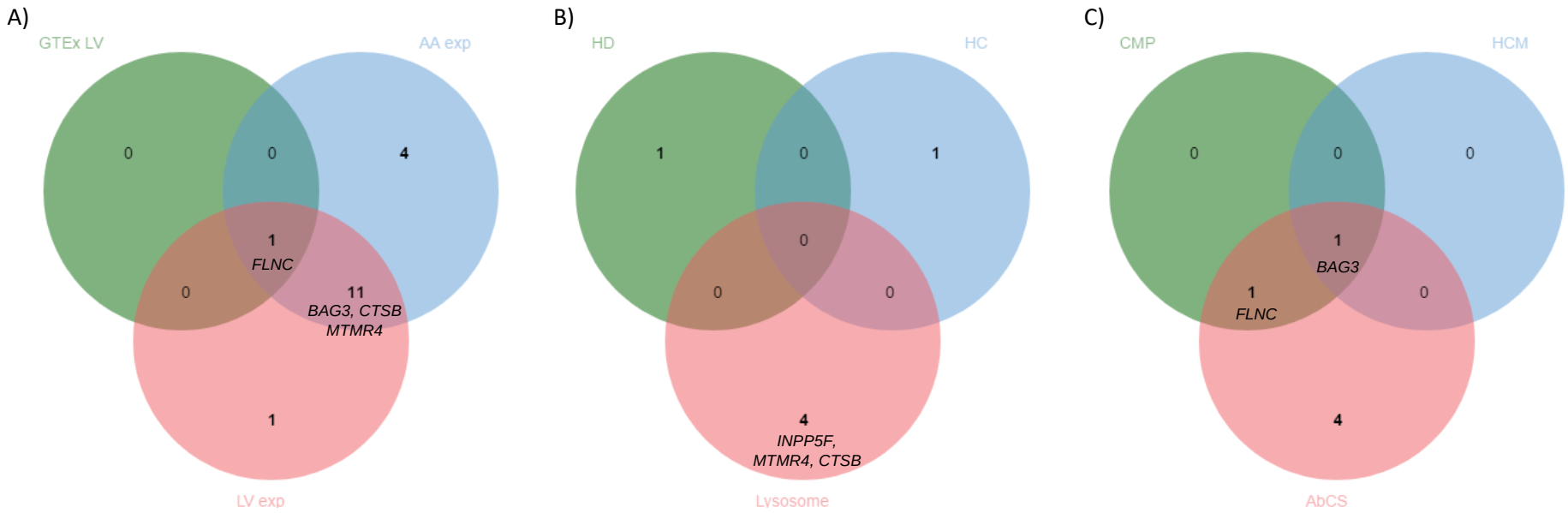

Figure 4: After initial filtering (presence in individuals with FD with cardiac manifestations and absence in individuals without cardiac disease; quality score ≥30; read depth ≥10; and non-synonymous protein impact), variants were filtered in parallel by three parameters: tissue expression (A), cellular process (B) and cardiac disease (C). The following gene sets were used: GTEx LV - top 100 genes overexpressed in the left ventricle; LV exp: genes with medium and high expression in left ventricle. AA exp: genes with medium and high expression in the atrial appendage; HD (heart development); HC (heart contraction); Lysosome (autophagy-lysosomal pathway); CMP (cardiomyopathy), HCM (hypertrophic cardiomyopathy) and AbCS (abnormality of the cardiovascular system). Variants in the listed genes were selected for validation after literature analysis of correlation with heart disease.

Validation cohort

Clinical characteristics of the validation cohort are described in table 2. The group of individuals with FD and cardiac symptoms (n=30) had similar proportion of males and females (57% and 43%, respectively). The mean age was 47 years (54 years in females and 42 years in males). Most of patients were being treated with ERT. In echocardiographic analysis, the most common manifestation was LV hypertrophy, present in approximately 70% of individuals. Electrocardiogram abnormalities as LV dysfunction and arrhythmias were detected in 77% of individuals. The group of individuals with FD without cardiac manifestations (n=15) had a larger proportion of females (67%) and average age of 32 years (36 years in females and 25 years in males).

Table 2: Clinical characterization of the validation cohort of individuals with Fabry disease

	FD with cardiac manifestations	FD without cardiac manifestations
Total	30	15
Gender		
Female	13 (43.3%)	10 (66.7%)
Male	17 (56.7%)	5 (33.3%)
Age (years)	47 ± 14	32 ± 12
ERT	22	6
Cardiac manifestations		
Echocardiographic abnormalities (N / total analysis)	22 (29)	0 (12)
LV hypertrophy	20 (69%)	-
Valvar involvement	12 (41.4%)	-
Electrocardiogram abnormalities (N / total analysis)	23 (30)	0 (13)
LV dysfunction	17 (56.7%)	-
Electrophysiological abnormalities and arrhythmias	16 (53.3%)	-

FD: Fabry disease; LV: left ventricle; ERT: enzyme replacement therapy.

In the analysis of possible confounders, a borderline association (Chi-square with exact *P* = 0.123) was identified between sex and disease outcome. A significant association was found between age (independent T-test; *P* = 0.001) and treatment with ERT (Chi-square with exact *P* value = 0.033) with the development of cardiac manifestations in individuals with FD.

Therefore, these three factors were considered potential confounders and included in the regression analysis. As expected, the type of mutation had no statically significant effect on disease outcome (Chi-square with exact *P* = 0.626).

Results from genotyping are listed in table 3. Genotype distributions of *FLNC* (MIM: 102565)*, INPP5F* (MIM: 609389), *MTMR4* (MIM: 603559)*,* and *CT*SB (MIM: 116810) did not deviate from HW Equilibrium. However, *BAG3* (MIM: 603883) frequencies were statistically different, with fixation of the reference allele (C) in the FD without cardiac symptoms group. No statistically significant association was found between any of the analyzed SNPs and the development of cardiac manifestations in FD. However, a tendency of association was found with the presence of a mutated allele of rs3188055 in *INPP5F* (*P* = 0.070, OR: 4.423, 95% IC (0.886 - 20.316)). The frequency of mutated allele "G" was higher in the group with cardiac manifestations than in the control group (0.45 to 0.30), though it was not statistically significant.

Sample size, particularly significant in the group without cardiac symptoms, was small and thus, most likely hindered identification of associations. Therefore, to further explore the possible association between *INPP5F* and cardiac disease in FD, groups were compared to the general population. Since Brazilians are a highly admixed population, underrepresented in population genomic databases, data from ABraOM, a repository containing variants from exome sequencing of elderly (from 60 to over 90 years old) Brazilians, was used. A significant increase in rs3188055 heterozygosity was identified in the FD group with cardiac manifestations when compared to both control group and the Brazilian population (Table 4, *P* = 0.014, 95% IC 0.012 - 0.017). Similarly, frequency of the mutated allele was also significantly higher in the cardiac group when compared to both control groups (*P* = 0026, 95% IC 0.022 - 0.029)

Table 3: Comparison of genotype and allele frequencies in individuals with Fabry disease with or without cardiac manifestations

Gene	Protein effect	rsID	Genotype / Allele	FD with cardiac manif.		FD without cardiac manif.		P^a	P^b	OR^b (95% IC)
				n	Freq	n	Freq			
CTSB	NP_001899.1:p.Ser53Gly	rs1803250	AA	24	0.80	12	0.80		0.915	0.901 (0.132 - 6.147)
			AG	6	0.20	3	0.20			
			GG	0	0.00	0	0.00			
			A	54	0.90	27	0.90	1.000		
			G	6	0.10	3	0.10			
FLNC	NP_001449.3:p.Arg1567Gln	rs2291569	GG	25	0.83	13	0.87		0.735	1.478 (0.155 - 1.126)
			GA	5	0.17	2	0.13			
			AA	0	0.00	0	0.00			
			G	55	0.92	28	0.93	1.000		
			A	5	0.08	2	0.07			
BAG3	NP_004272.2:p.Pro407Leu	rs3858340	CC	26	0.87	15	1.00		0.999	-
			CT	4	0.13	0	0.00			
			TT	0	0.00	0	0.00			
			C	56	0.93	30	1.00	-[c]		
			T	4	0.07	0	0.00			
MTMR4	NP_004678.3:p.Ser280Gly	rs2302190	TT	15	0.50	10	0.67		0.382	1.676 (0.527 - 5.332)
			CT	11	0.37	3	0.20			
			CC	4	0.13	2	0.13			
			T	41	0.68	23	0.77	0.429		
			C	19	0.32	7	0.23			
INPP5F	NP_055752.1:p.Asn997Asp	rs3188055	AA	6	0.20	7	0.47		0.070	4.243 (0.886 - 20.316)
			AG	21	0.70	7	0.47			
			GG	3	0.10	1	0.06			
			A	33	0.55	21	0.70	0.254		
			G	27	0.45	9	0.30			

FD: Fabry disease; OR: odds ratio; a: Fischer Exact Test; b: Binary logistic regression analysis of association with development of cardiac manifestations corrected by age, sex and ERT. C: Populations not in Hardy-Weinberg equilibrium. In all genes, the upper genotype/allele is the reference. Manif: manifestations.

Table 4: Comparison of allele and genotype frequencies of rs3188055 in individuals with Fabry disease and in the Brazilian population

Gene	rsID	Genotype / Allele	FD with cardiac manif			FD without cardiac manif			AbraOM			*P**
			n	Freq		n	Freq		n	Freq		
INPP5F	rs3188055	AA	6	0.20	[a]	7	0.47	[a,b]	314	0.52	[b]	0.014 (0.012-0.017)
		AG	21	0.70	[a]	7	0.47	[a,b]	238	0.39	[b]	
		GG	3	0.10	[a]	1	0.07	[a,b]	57	0.09	[a]	
		A	33	0.55	[a]	21	0.70	[a,b]	866	0.71	[b]	0.026 (0.022-0.029)
		G	27	0.45	[a]	9	0.30	[a,b]	352	0.29	[b]	

FD: Fabry disease; [a] and [b]: column comparisons with z-test and Bonferroni correction; *: Monte Carlo method: p value (95% IC). A: reference allele. G: mutated allele. AbraOM: Online Archive of Brazilian Mutations. Manif: manifestations

Moreover, significant differences were identified in the presence and absence of rs3188055 alleles between groups (table 5). The presence of the mutated allele either in homo or heterozygosity (GG+AG) was similar among the Fabry group with no cardiac manifestations and the Brazilian population with frequencies of 0.53 and 0.48, respectively. However, in the FD group with cardiac symptoms, it was significantly increased to 0.80 (*P* = 0.004, 95% IC 0.003-0.005).

Table 5: Comparison of the presence and absence of the rs3188055 allele

Combined genotypes		FD with cardiac manif		FD without cardiac manif		AbraOM		*P**
		n	Freq	n	Freq	n	Freq	
Presence of reference allele	AA + AG	27	0.90	14	0.93	552	0.91	1.000 (1.000-1.000)
	GG	3	0.10	1	0.07	57	0.09	
Presence of mutated allele	GG + AG	24	0.80 [a]	8	0.53 [a,b]	295	0.48 [b]	0.004 (0.003-0.005)
	AA	6	0.20 [a]	7	0.47 [a,b]	314	0.52 [b]	

FD: Fabry disease; [a] and [b]: column comparisons with z-test and Bonferroni correction; *: Monte Carlo method: p value (95% IC). A: reference allele. G: mutated allele. AbraOM: Online Archive of Brazilian Mutations. Manif: manifestations

Discussion

LV hypertrophy is one of the most frequent cardiac manifestations of FD, associated to cardiac symptoms, arrhythmias, and valvar disease [7,19]. However, despite being a monogenic disease, the primary enzyme defect and consequent accumulation of glycosphingolipids accounts for less than 5% of the heart mass in the hypertrophy seen in affected individuals [20]. Hence, other genetic and environmental factors must modulate cardiac disease in FD.

In this study, we performed an explorative analysis to search for possible modifiers associated with the cardiac phenotype in FD. Initially, exome sequencing analysis in members of a family with the classical form of the disease with diverse clinical presentations was performed. Patients II.1-3 are 3 sisters in their 6-7th decade of life that well exemplify the large phenotype variation of cardiac manifestations in FD. Patient II.3 has the classic FD phenotype with severe symptoms in cardiac, renal and cerebrovascular systems and is the only sibling under ERT. Patient II.1 presents the cardiac variant with minor involvement in

other organs aside from the heart. Patient II.2 is mostly asymptomatic, presenting just minor albuminuria. This variation might be more pronounced in females, but is also seen in males, since not all male FD patients, as patient IV.1, develop cardiac symptoms. Moreover, among those who do, there is also a variation in the severity of the disease and in the specific manifestations, which are very variable [19]. Patient IV.1 is younger than the average age of cardiac symptoms onset reported in untreated males enrolled in the Fabry Outcome Survey [7]. However, this patient is very mildly affect overall, presenting exclusively angiokeratomas and minor albuminuria. Also, variant filtering stages were also performed excluding this patient and resulted in very similar results, with the same SNPs being selected (Figures S1-S3).

There are several reports in the literature of associations between common variants and cardiovascular diseases, including reports in FD. Variants in *NOS3* were associated with increased left posterior wall thickness in individuals with FD. For instance, the p.Glu298Asp variant has a MAF of 0.25 and was also reported as a risk factor for LV hypertrophy in individuals with hypertension. Other example is the relationship between SNPs in *RYR1 (*ryanodine receptor 1, MIM: 180901) with LV hypertrophy in the Korean population [21]. Therefore, we did not filter out variants based on stringent frequency when selecting variants for validation. Our approach was based on gene function, tissue expression and likelihood of strong relation to heart disease, rather than based primarily on frequency. Also, due to the high frequency of LV hypertrophy in FD, emphasis was given to this manifestation in the variant filtering stages with the use of specific gene lists related to hypertrophic cardiomyopathy. After extensive literature revision, variants in five different genes were selected for replication: *CTSB*, *FLNC*, *BAG3*, *MTMR4* and *INPP5F*.

Cathepsin B (CTSB) is a cysteine protease mostly located inside lysosomes where it participates in nonselective protein degradation. It is involved in several processes such as regulation of apoptosis, immune response and extracellular matrix remodeling [22]. Mouse model and *in vitro* studies shown that CTSB modulates cardiomyocyte hypertrophy and cardiac remodeling in response to stress by regulation of TNF-α/ASK1/JNK signaling pathway [22]. Pharmacological inhibition of CSTB decreases cardiac dysfunction and reduces cardiomyocyte size and fibrosis in the rat model of myocardial infarction [23]. Individuals with end-stage dilated cardiomyopathy (DCM) have high myocardial apoptotic index, expression

and protein levels that negatively correlated to the ejection fraction [24]. DCM patients also have elevation in CTSB activity in blood cells, indicating that overexpression of this protease is not restricted to myocardium [25]. Increases in CSTB activity were also associated with cardiac disease in other lysosomal diseases [26]. The SNP analyzed in this study, rs1803250 (p.Ser53Gly), is located in the propeptide region of the protein and mutations in this area were already related to significant increase in enzyme activity [27]. However, we did not find association between this variant and cardiac disease in FD.

Filamin C (FLNC), or filamin gamma, is a Z-disc protein involved in maintenance of the structural integrity of myocytes. It is expressed mainly in skeletal and heart muscle, where it forms dimers and cross-links actin filaments, binds Z-disc associated proteins and serves as scaffold to signaling proteins. Initially, FLNC mutations were associated with skeletal myofibrillar myopathies, characterized by myofibrils disarray and formation of protein aggregates [28,29]. However, FLNC variants have been increasingly reported as causing cardiac diseases without skeletal muscle involvement, and, for some mutations, without cytotoxic protein aggregation. Examples include familial cases of hypertrophic cardiomyopathy (HCM) [30], restrictive cardiomyopathy (RCM) [31], DCM [28] and arrhythmogenic cardiomyopathy [32]. Inconsistently with these findings, rs2291569 (p.Arg1567Gln) was recently associated with a reduced risk of sporadic DCM [33]. We found a slight increase in the frequency of heterozygotes for rs2291569 in the FD group with cardiac symptoms, but there was no statistically significant difference between groups.

FLNC interacts with BCL2 Associated Athanogene 3 (BAG3) which acts as an anti-apoptotic molecule by interaction with Bcl2, being up-regulated in cancer cells. Normally, it is expressed mainly in cardiac and skeletal muscle, and at lower rate, in the brain and peripheral nervous system. It maintains Z-disc stability by binding to actin capping protein beta 1 (CapZβ1, MIM: 601580) and stimulates filamin transcripton [34]. In addition, it is a co-chaperone to heat-shock proteins in the chaperone assisted selective autophagy (CASA) pathway, removing degraded or misfolded Z-disc proteins. The protein aggregates formed as a consequence of some *FLNC* mutations recruit BAG3. However, CASA pathway's degradation capacity is either exceed or block by them [29,35]. The rs121918312 (p.Pro209Leu) variant in *BAG3* is associated with myofibrillar myopathy with HCM or RCM [36,37]. Other rare variants are usually associated with a milder disease, with adult-onset DCM even without myofibrillar

myopathy [38,39]. In a GWAS study with over 2,000 controls and patients with DCM, an association between sporadic DCM and the common variant rs2234962 (p.Cys151Arg) was found. The study also analyzed the variant rs3858340 (p.Pro407Leu) and reported complete linkage disequilibrium between them. The haplotype formed by the mutated allele in rs2234962 and reference allele from rs3858340 was also described as a protection factor. Familial cases of DCM were also evaluated and rare mutations were identified, suggesting that common mutations may be linked to sporadic DMC, while rare mutations to familial DMC [40]. In a larger DCM study from the same group recently published, rs2234962 was also associated with a reduced risk of disease [OR = 0.62 (0.57–0.68)] [33]. In the present study, the only variant identified in *BAG3* was rs3858340. Moreover, it was not in Hardy-Weinberg equilibrium, with the reference allele fixed in the group without cardiac symptoms. Although the heterozygous allele were more frequent in the group with cardiac symptoms, there was no significant association with development of cardiac symptoms.

Myotubularin Related Protein 4 (MTMR4) is a protein involved in the metabolism of phosphoinositides (PIs), which are active compounds that regulate lipid homeostasis, cell signaling, cytoskeleton dynamics, traffic and interactions of the plasma membrane and organelles membranes, among other processes. It belongs to a family of enzymes that hydrolyse phosphatidylinositol 3-phosphate [PtdIns(3)P] to phosphatidylinositol (PtdIns) [41], and is localized to early and recycling endosomes, aiding in the regulation of endosomal trafficking. An important aspect of this gene for lysosomal diseases, is that MTMR4 may control trafficking of mannose 6-phosphate receptor in the endosomal pathway [42]. In addition, *in vitro* studies showed that inhibition of MTMR4 alters the location of endosomes, which become distributed around the cell periphery, further from the pericentriolar recycling compartment [41]. In this study, we found increased frequencies of both the mutated allele and the heterozygous genotype were found in individuals with FD with cardiac manifestations. However, these results were not statically significant, which may be due to the reduced number of individuals analyzed.

Finally, Inositol Polyphosphate-5-Phosphatase F (*INPP5F*) is also a gene involved in PIs metabolism. Moreover, it is located in the same 9.5 MB region in chromosome 10q25-26 which includes *BAG3* and was described [43] as a locus associated with cardiomyopathy, diffuse myocardial fibrosis, and sudden death. *INPP5F* is a negative regulator of cardiac myocyte size

and of the cardiac stress response, and thus, of cardiac hypertrophy [44]. Data from animal studies shown that overexpression of *INPP5F* reduces hypertrophic responsiveness, while its repression results in increased hypertrophy in response to stress [44]. As *MTMR4*, *INPP5F* acts on PIs metabolizing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), abundant in the plasma membrane, to phosphatidylinositol (PI(4)P). This reaction occurs during the trafficking of clathrin coated vesicles to early endosomes in the endocytic pathway, being critical for endosomal maturation and recycling [45,46]. The connection between *INPP5F* and hypertrophy lies on the negative regulation of the PI 3-kinase–Akt signaling pathway through modulation of endocytic trafficking of signaling receptors and signal magnitude by PIs [45]. In addition, some authors reported that *INPP5F* may also act directly on the AKT pathway, by reducing PI(3,4,5)P3 (PIP3) levels, with consequent down-regulation of Akt (MIM: 164730) and activation of Gsk3β (MIM: 605004) [44,47]. Since FD is a lysosomal disease, additional imbalance in the endosomal-lysosomal system by reduced activity of *INPP5F* may be more harmful in individuals with FD than in the general population. There may be a synergic deleterious effect between GB3 accumulation and INPP5F reduced activity. An increased frequency of the *INPP5F* variant analyzed (rs3188055, p.Asn997Asp) was identified in the group with cardiac manifestations. It was accompanied by increased frequencies of both heterozygous and mutated homozygous genotypes. However, probably due to sample size, this difference did not achieve significant levels (P = 0.07). Therefore, to further investigate the possible association between this gene and the development of cardiac phenotype, FD groups were compared to the Brazilian population. Allele frequencies varied by 1% in the general population and FD group without cardiac manifestations. The affected group, however, presented a significant increase of 50% in the presence of the mutated allele (P = 0.026, 95% IC 0.022-0.029). Genotypes were also significantly different; the frequency of heterozygotes was 79% higher in the cardiac group than in the normal population. Additionally, while the presence of reference allele (in homo or heterozygosity) was equal between groups, the presence of the mutated allele also was significantly increased in the FD group with cardiac symptoms (P = 0.004, 95% IC 0.003-0.005). Taken together, these data indicate that sample sizes may have hindered initial comparison and there might be an association between *INPP5F* and the development of cardiac manifestations in FD.

In summary, we performed an exploratory study of possible modifier genes of the cardiac phenotype in FD and identified a significantly higher frequency of rs3188055 allele

and heterozygous genotype in *INPP5F* in individuals with FD who have cardiac symptoms. *INPP5F* plays a role in the endosomal-lysosomal system and is implicated in the negative regulation of cardiac hypertrophy. Therefore our data suggest that *INPP5F* might be involved in the modulation of development of cardiac manifestations in FD and thus, in its variability in phenotype.

Supplemental Data description

Document S1: Figures S1-S3. Tables S1-S2

Figure S1: Venn diagram of tissue expression based filtering.

Figure S2: Venn diagram of cellular process based filtering.

Figure S3: Venn diagram of cardiac manifestations based filtering.

Table S1: Primers and conditions used for genotyping.

Table S2: Variants identified by exome analysis in Fabry disease patients filtered by presence in patients with cardiac manifestations; quality score ≥30, read depth ≥10 and non-synonymous protein impact.

Conflicts of Interest

Authors declare no conflict of interest.

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Web Resources

ABraOM: Brazilian genomic variants, http://abraom.ib.usp.br/

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

GTEx Portal, https://www.gtexportal.org/home/

GenBank, http://www.ncbi.nlm.nih.gov/genbank/

jvenn, http://jvenn.toulouse.inra.fr/app/index.html

OMIM, http://www.omim.org/

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Supplemental data

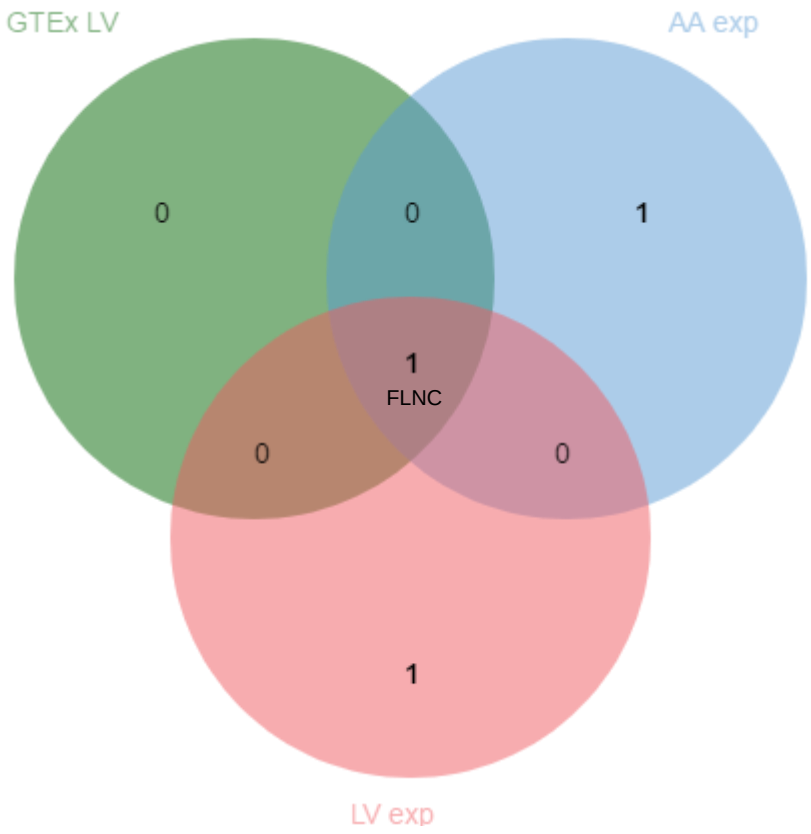

Figure S1: Venn diagram of tissue expression based filtering. Variants filtered by presence in FD patients with cardiac manifestations; quality score ≥30, read depth ≥10, non-synonymous protein impact and presence in in the following gene sets: GTEx LV - top 100 genes overexpressed in the left ventricle; LV exp: genes with high expression in left ventricle. AA exp: genes with high expression in the atrial appendage. Listed genes were selected for validation after literature analysis of correlation with heart disease.

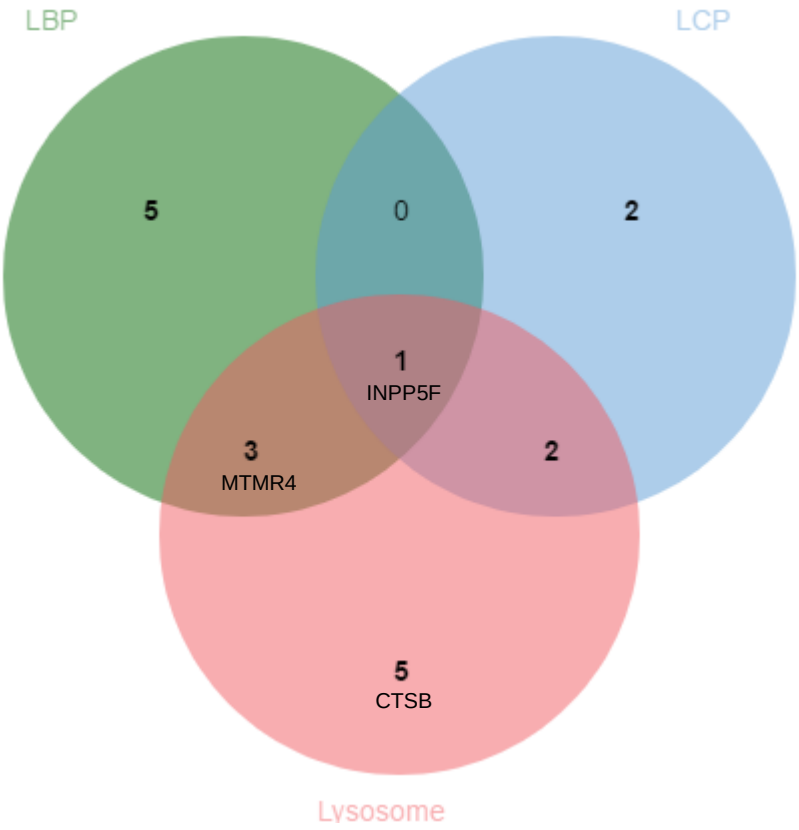

Figure S2: Venn diagram of cellular process based filtering. Variants filtered by presence in FD patients with cardiac manifestations; quality score ≥30, read depth ≥10, non-synonymous protein impact and presence in the following gene sets: LBP (Lipid biosynthetic process); LCP (Lipid catabolic process) and Lysosome (autophagy-lysosomal pathway [14]). Listed genes were selected for validation after literature analysis of correlation with heart disease.

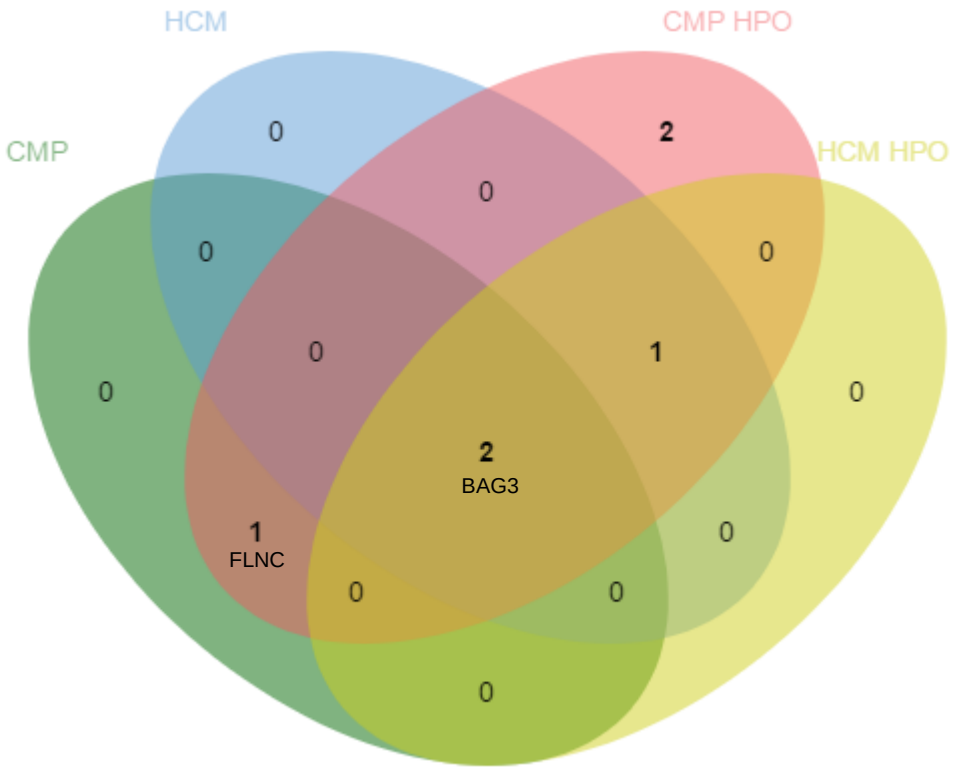

Figure S3: Venn diagram of cardiac manifestations based filtering. Variants filtered by presence in FD patients with cardiac manifestations; quality score ≥30, read depth ≥10, non-synonymous protein impact and presence in the following gene sets: CMP (Cardiomyopathy, Enlis software preset list), HCM (Cardiac muscle hypertrophy, Enlis software preset list), CMP (cardiomyopathy, HPO based list) and HCM HPO (Hypertrophic cardiomyopathy, HPO based list). Listed genes were selected for validation after literature analysis of correlation with heart disease.

Table S1: Primers and conditions used for genotyping.

Gene	rsID	Primer Sequence (5'>3')	Tm
CTSB	rs1803250	F GACCCTCGGACTGAGAACC R ACTGTACCTCAAGCGGTGTC	62°C
FLNC	rs2291569	F GACTCTGGCTCAAGATGAGATCAC R ACCTGTGACGAGGCACTTG	62°C
BAG3	rs3858340	F CAGAACTCCCTCCTGGACAC R GTCTGAACCTTCCTGACACCG	62°C
INPP5F	rs3188055	F GGTCCCAGTCTCTTAGCAGC R AGGAGTTACATGAAGCCCTGTCT	60°C
MTMR4	rs2302190	F CAGAGCACGCAGTCAGAGAA R GCCAAGGGCATTACGGTTTC	58°C

Table S2: Variants identified by exome analysis in Fabry disease patients filtered by presence in patients with cardiac manifestations; quality score ≥30, read depth ≥10 and non-synonymous protein impact.

chr	Chr Start	Chr End	Var Type	Ref Seq	Var Seq	Rsid List	Genes List	Gene Component List	Protein Impact List	Aa Change	Global Allele Freq	Mamm Conserv
17	7951819	7951819	SNP	A	G	rs4792147	ALOX15B	CDS	MISSENSE	Q-656-R; Q-644-R; Q-627-R; Q-582-R	55.9	no
17	66267650	66267650	SNP	T	G	rs3744307	ARSG; SLC16A6	INTRON;CDS	MISSENSE	E-217-D	16.87	no
17	66270082	66270082	SNP	A	G	rs35397826	ARSG; SLC16A6	INTRON;CDS	MISSENSE	I-121-T	12.42	Yes (5.8)
10	121436286	121436286	SNP	C	T	rs3858340	BAG3	CDS	MISSENSE	P-407-L	11.41	Yes (5.7)
15	80263217	80263217	SNP	C	T	rs3826007	BCL2A1	CDS	MISSENSE	G-82-D	23.44	Yes (5.6)
15	80263345	80263345	SNP	A	C	rs1138358	BCL2A1	CDS	MISSENSE	N-39-K	33.65	no
15	80263406	80263406	SNP	C	T	rs1138357	BCL2A1	CDS	MISSENSE	C-19-Y	30.33	no
11	4593411	4593411	SNP	G	A	rs34097396	C11ORF40	CDS	MISSENSE	T-141-M	6.83	no
11	4594545	4594545	SNP	A	G	rs12795289	C11ORF40	CDS	MISSENSE	F-100-S	4.93	no
11	4594608	4594608	SNP	G	A	rs750491811	C11ORF40	CDS	MISSENSE	P-79-L	0.01	no
17	71231868	71231868	SNP	G	A	rs61732265	C17ORF80	CDS;INTRON	MISSENSE	V-83-M	0.85	no
3	14724413	14724413	SNP	G	A	rs9821143	C3ORF20	CDS;INTRON	MISSENSE	D-65-N	33.84	no
17	64876683	64876683	SNP	G	A	rs138881080	CACNG5	CDS	MISSENSE	R-98-H	0.02	no
17	64876761	64876761	SNP	G	A	rs145214481	CACNG5	CDS	MISSENSE	R-124-H	0.01	Yes (3.6)
6	4716037	4716037	SNP	T	C	rs3812178	CDYL	CDS	MISSENSE	S-9-P	21.34	Yes (3.3)
11	89935586	89935586	SNP	G	T	rs1045861	CHORDC1	CDS	MISSENSE	A-329-D; A-141-D; A-310-D	68.84	Yes (4.0)
17	71199781	71199781	SNP	A	G	rs7208207	COG1	CDS	MISSENSE	Y-744-C	0.13	Yes (5.0)
17	61987570	61987570	SNP	G	T	rs2727307	CSHL1	CDS;UTR3	MISSENSE	D-58-E; D-79-E; D-47-E; D-141-E	35.95	Yes (3.0)
17	61988014	61988014	SNP	G	A	rs2006208	CSHL1	INTRON;CDS	MISSENSE	A-94-V	37.76	no
8	11710174	11710174	SNP	T	C	rs1803250	CTSB	CDS	MISSENSE	S-53-G	9.38	Yes (5.8)

11	107299631	107299631	SNP	G	A	rs659040	CWF19L2	CDS	MISSENSE	H-443-Y	16.43	no
17	61515412	61515412	SNP	G	T	rs4968774	CYB561	INTRON;CDS	MISSENSE	Q-60-K	4.84	no
11	6648424	6648424	SNP	G	A	rs4758443	DCHS1	CDS	MISSENSE	T-1949-M	35.4	no
17	56277106	56277106	SNP	T	A	rs33955150	EPX	CDS	MISSENSE	H-496-Q	0.41	no
8	11296049	11296049	SNP	T	A	rs3021518	FAM167A-AS1; FAM167A	CDS;INTRON	NONSTOP	*-105-K	12.31	no
17	66596650	66596650	SNP	A	T	rs79958465	FAM20A; RP11-118B18.1	CDS;INTRON	MISSENSE	L-53-Q	0.41	no
8	12287957	12287957	SNP	C	T	rs148629503	FAM86B2	INTRON;CDS	MISSENSE	E-82-K	16.57	no
7	128488734	128488734	SNP	G	A	rs2291569	FLNC	CDS	MISSENSE	R-1567-Q	7.62	Yes (5.0)
1	171154959	171154959	SNP	A	G	rs2020870	FMO2	CDS	MISSENSE	D-36-G	8.15	Yes (5.6)
11	5372856	5372856	SNP	A	G	rs4910756	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	N-40-S	16.66	Yes (4.0)
11	5373006	5373006	SNP	T	C	rs7483122	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	I-90-T	23.94	Yes (5.0)
11	5373104	5373104	SNP	A	G	rs5006889	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	T-123-A	23.76	Yes (5.0)
11	5373111	5373111	SNP	G	A	rs7479477	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	R-125-H	23.86	no
11	5373129	5373129	SNP	C	T	rs5006887	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	T-131-I	23.86	Yes (4.0)
11	5373170	5373170	SNP	C	G	rs5006886	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	R-145-G	23.9	Yes (5.0)
11	5373242	5373242	SNP	T	G	rs5006885	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	S-169-A	24.03	no
11	5373251	5373251	SNP	C	T	rs5006884	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	L-172-F	23.86	Yes (3.2)
11	5373311	5373311	SNP	T	C	rs5006883	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	F-192-L	23.93	Yes (5.1)
11	5373562	5373562	SNP	C	A	rs5024042	HBG2; HBE1; AC104389.28; OR51B6	INTRON;CDS	MISSENSE	S-275-R	23.8	no
11	5462255	5462255	SNP	C	G	rs11037445	HBG2; HBE1; AC104389.28; OR51I1	INTRON;CDS	MISSENSE	V-164-L	21.07	Yes (3.6)
11	5424050	5424050	SNP	G	A	rs1909262	HBG2; HBE1; AC104389.28; OR51J1	INTRON;CDS	MISSENSE	R-75-H	35.53	Yes (4.3)
11	5424430	5424430	SNP	A	G	rs7929412	HBG2; HBE1; AC104389.28; OR51J1	INTRON;CDS	MISSENSE	I-202-V	11.79	Yes (4.2)

11	5411398	5411398	SNP	T	G	rs2736531	HBG2; HBE1; AC104389.28; OR51M1	INTRON;CDS	MISSENSE	L-257-R	36.83	Yes (5.2)
11	5443867	5443867	SNP	C	T	rs10838092	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	MISSENSE	T-146-I	42.52	Yes (5.0)
11	5443887	5443887	SNP	T	C	rs10838093	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	MISSENSE	C-153-R	42.51	Yes (3.0)
11	5443893	5443893	SNP	G	A	rs10838094	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	MISSENSE	V-155-I	42.51	no
11	5443963	5443963	SNP	G	A	rs10838095	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	MISSENSE	R-178-H	42.5	Yes (5.0)
11	5444061	5444061	SNP	G	A	rs2736586	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	MISSENSE	V-211-M	42.53	no
11	5444136	5444136	SNP	C	T	rs2647574	HBG2; HBE1; AC104389.28; OR51Q1	INTRON;CDS	NONSENSE	R-236-*	42.53	no
11	5510541	5510541	INS		GGCT	rs576495879	HBG2; HBE1; AC104389.28; OR52D1	INTRON;CDS	FRAMESHIFT	G-202-GLA	13.46	no
11	5322737	5322737	SNP	A	G	rs10837771	HBG2; HBE1; OR51B4	INTRON;CDS	MISSENSE	M-147-T	45.6	no
11	5529152	5529152	SNP	A	G	rs2234455	HBG2; UBQLN3	INTRON;CDS	MISSENSE	M-546-T	17.52	no
17	58121453	58121453	SNP	G	A	rs16943991	HEATR6	CDS	MISSENSE	S-1006-L; S-894-L	6.87	Yes (4.1)
10	121586882	121586882	SNP	A	G	rs3188055	INPP5F	CDS	MISSENSE	N-997-D; N-387-D	29.26	no
3	19490425	19490425	SNP	C	T	rs17005976	KCNH8	INTRON;CDS	NONSENSE	Q-102-*	2.14	no
12	32134815	32134815	SNP	G	A	rs16919122	KIAA1551	CDS	MISSENSE	R-309-Q	12.23	no
12	9750669	9750669	SNP	A	G	rs1135816	KLRB1	CDS	MISSENSE	I-168-T	42.89	no
18	47093864	47093864	SNP	C	T	rs2000813	LIPG	CDS	MISSENSE	T-147-I; T-111-I	26.66	no
17	56324988	56324988	SNP	C	T	rs8178318	LPO	INTRON;CDS	MISSENSE	T-105-I; T-46-I	0.82	no
17	62856621	62856621	SNP	T	C	rs9893710	LRRC37A3	CDS	MISSENSE	K-333-E; K-253-E; K-192-E; K-1215-E	4.36	no
3	46501213	46501213	SNP	T	C	rs1126478	LTF	CDS	MISSENSE	K-47-R; K-3-R	43.59	no
5	56177443	56177443	SNP	G	A	rs702689	MAP3K1	CDS	MISSENSE	D-806-N	59.79	Yes (5.5)

5	56177743	56177743	SNP	G	A	rs832582	MAP3K1	CDS	MISSENSE	V-906-I	76.42	Yes (3.5)
14	23312594	23312594	SNP	G	A	rs1042704	MMP14	CDS	MISSENSE	D-273-N	16.35	Yes (5.6)
11	4928841	4928841	SNP	T	C	rs7108225	MMP26; OR51A7	INTRON;CDS	MISSENSE	M-81-T	8.61	Yes (3.8)
11	4936608	4936608	SNP	C	G	rs12419598	MMP26; OR51G2	INTRON;CDS	MISSENSE	E-96-Q	8.5	Yes (5.4)
11	4880992	4880992	SNP	C	G	rs7947547	MMP26; OR51H1	INTRON;CDS	MISSENSE	W-268-S	20.36	Yes (4.4)
11	4870269	4870269	SNP	A	T	rs12417164	MMP26; OR51S1	INTRON;CDS	MISSENSE	I-57-N	17.94	Yes (4.8)
11	10655623	10655623	SNP	C	T	rs11042902	MRVI1	INTRON;ACCEPTOR	DISRUPT		23.96	no
17	56584508	56584508	SNP	T	C	rs2302190	MTMR4	CDS	MISSENSE	S-280-G	24.59	Yes (4.0)
8	18258103	18258103	SNP	G	A	rs1799930	NAT2	CDS	MISSENSE	R-197-Q; R-67-Q	27.68	no
17	65722704	65722704	SNP	C	G	rs76234567	NOL11	CDS	MISSENSE	L-265-V; L-83-V	0.84	Yes (3.1)
3	13361287	13361287	SNP	C	T	rs354478	NUP210	CDS	MISSENSE	V-1787-M	55.33	no
14	19377881	19377881	SNP	G	A	rs138502172	OR11H12	CDS	MISSENSE	M-96-I	2.74	no
17	56232675	56232675	SNP	G	A	rs12602205	OR4D1	CDS	MISSENSE	R-54-Q	8.3	no
14	20296278	20296278	SNP	G	A	rs117025898	OR4N2	UTR3;CDS	MISSENSE	R-224-H	3.01	no
11	4389405	4389405	DEL	G		rs11310407	OR52B4	CDS	FRAMESHIFT	L-41-F	35.42	no
11	5068431	5068431	SNP	G	A	rs17350764	OR52J3	CDS	MISSENSE	V-226-I	21.12	Yes (3.2)
11	6816875	6816875	SNP	G	A	rs7122644	OR6A2	CDS	MISSENSE	A-22-V	29.9	no
11	7727886	7727886	SNP	C	T	rs7927138	OVCH2; RP11-35J10.5; RP11-35J10.4	CDS;INTRON	MISSENSE	R-19-Q	38.99	no
9	71628207	71628207	SNP	G	C	rs3730386	PRKACG	CDS	MISSENSE	H-268-D	27.51	no
1	171486912	171486912	SNP	G	A	rs10913157	PRRC2C	CDS	MISSENSE	A-235-T; A-237-T	13.64	no
9	33796799	33796799	SNP	A	T	rs200709040	PRSS3; RP11-133O22.6	CDS;INTRON	MISSENSE	T-124-S; T-81-S; T-60-S; T-67-S	7.04	no
8	10383138	10383138	SNP	G	A	rs35102108	PRSS51; PRSS55	INTRON;CDS	MISSENSE	G-15-R	20.46	no
14	39784005	39784006	DEL	TA		rs75318507	RP11-407N17.3; CTAGE5	DONOR	DISRUPT		31.25	Yes (5.5)
8	26944733	26944733	SNP	G	A	rs17056759	RP11-521M14.2	CDS	CODING(INCOMPLETE)		25.95	no
9	45733681	45733681	SNP	T	A	rs10909739	RP11-7G23.8; FAM27E2	INTRON;CDS	MISSENSE	L-32-M	29.86	no
9	45733796	45733796	SNP	T	G	rs10796744	RP11-7G23.8; FAM27E2	INTRON;CDS	MISSENSE	M-70-R	50.4	no
8	10465063	10465063	SNP	G	T	rs183570817	RP1L1	CDS	MISSENSE	A-2182-D	0.73	no
8	10465942	10465942	SNP	T	A	rs28446662	RP1L1	CDS	MISSENSE	D-1889-V	24.98	no
8	10480305	10480305	SNP	C	T	rs189960401	RP1L1	CDS	MISSENSE	R-136-H	0.41	no

17	5326145	5326145	SNP	C	G	rs12761	RPAIN	CDS	MISSENSE	N-103-K	36.54	no
17	71380062	71380062	SNP	G	A	rs35467001	SDK2	CDS	MISSENSE	T-1553-M	5.49	Yes (5.4)
5	56207123	56207123	SNP	T	A	rs2257505	SETD9	CDS	MISSENSE	S-76-T	63.14	no
17	2266799	2266799	SNP	G	A	rs745400	SGSM2	CDS	MISSENSE	R-238-K	48.97	Yes (3.7)
17	2268311	2268311	SNP	G	A	rs2248821	SGSM2	CDS	MISSENSE	R-374-Q	49.77	no
18	44773382	44773382	SNP	A	T	rs9956387	SKOR2	CDS;INTRON	MISSENSE	C-725-S	37.36	Yes (3.4)
5	150667016	150667016	SNP	T	C	rs978012	SLC36A3	CDS	MISSENSE	K-167-E; K-208-E	45.9	no
8	10623138	10623138	SNP	T	A	rs1078543	SOX7; CTD-2135J3.3; PINX1	INTRON;CDS;UTR3	MISSENSE	S-254-C	13.35	Yes (4.1)
14	88852166	88852166	SNP	G	A	rs4904448	SPATA7	CDS	MISSENSE	D-2-N	31.7	no
17	4356375	4356375	SNP	G	T	rs11655342	SPNS3	CDS	MISSENSE	A-330-S; A-203-S	35.08	no
14	20876282	20876282	SNP	G	A	rs41310936	TEP1	CDS	MISSENSE	S-106-F	1.33	Yes (5.0)
21	10942756	10942756	SNP	G	A	rs1810540	TPTE	CDS	NONSENSE	R-211-*; R-191-*; R-229-*	36.23	no
21	10942925	10942927	DEL	CTT		rs113444703	TPTE	CDS	DELETE+	RR-202-R; RR-182-R; RR-220-R	32.48	no
11	4621639	4621639	SNP	C	T	rs2231975	TRIM68	CDS	MISSENSE	C-442-Y	3.71	no
14	64988830	64988830	SNP	C	A	rs45512391	ZBTB1; RP11-973N13.4	CDS;INTRON	MISSENSE	T-203-N	9.2	Yes (4.2)
19	57646570	57646570	SNP	T	C	rs4801433	ZIM3	CDS	MISSENSE	I-379-V	48.78	no
19	57648277	57648277	SNP	A	T	rs4801200	ZIM3	CDS	MISSENSE	L-69-M	48.64	no
3	44607013	44607013	SNP	T	C	rs13081859	ZKSCAN7; RP11-944L7.4	CDS;INTRON	MISSENSE	F-153-S; F-3-S	24.55	no
3	44611896	44611896	SNP	A	G	rs9835485	ZKSCAN7; RP11-944L7.4; RP11-944L7.5	CDS;INTRON	MISSENSE	T-432-A; T-281-A	26.11	Yes (3.0)

Mamm Conserv: Mammalian Conservation

3.5. Artigo V

Pasqualim G, Mendoza M, Vairo F, Giugliani R and Matte U – **Gene expression signatures for specific disease manifestations revealed by transcriptome analysis of women with Fabry Disease**.

Artigo a ser submetido ao periódico *Human Genetics*.

Gene expression signatures for specific disease manifestations revealed by transcriptome analysis of Fabry Disease women

Gabriela Pasqualim[1,2], Mariana Mendoza[3], Filippo Vairo[4], Roberto Giugliani [1,2,4,5], Ursula Matte[1,2]

[1]: Post-Graduation Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, 91501-970, Brazil

[2]: Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[3]: Department of Applied Informatics, Informatics Institute, UFRGS, Porto Alegre, RS, 91509-900, Brazil

[4]: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, 90035-903, Brazil

[5]: INAGEMP, Porto Alegre, RS, 90035-903, Brazil

Abstract

Patients with Fabry disease (FD) present wide phenotypic variability. However, biological mechanisms underlying this variability in disease manifestations are still largely unknown. Therefore, in this study we aimed to identify possible differences in gene expression in women with different FD manifestations (cardiac symptoms, renal symptoms or both) by transcriptome analysis. Blood samples were collected from 13 female patients with FD classified according to their manifestations in three groups: renal-only (R), cardiac-only (C) or renal and cardiac (RC). Differential gene expression analysis was performed with GeneChip Human Transcriptome array (HTA) 2.0. Following characterization of expression profiles of each group, functional enrichment analyses of GO terms and validation of selected genes with qRT-PCR were performed. A total of 3107 differentially expressed (DE) transcripts were identified among groups, and resulted in molecular signatures with 6, 8 and 82 distinctive transcripts. GO terms mostly related to immune response, particularly viral response and interferon I signaling, were significantly enriched in the renal and cardiac symptoms group. Quantitative RT-PCR confirmed altered down-regulated expression of *DEFA1, A1B, A3* and *CEACAM8* in group R and *PRKXP1* in group C. Patients with renal and cardiac symptoms shown up-regulation of several interferon type I induced genes (*IFI44, IFI44L, IFIT1, HERC5, EIF2AK2 and RSAD2*). In conclusion, we identified three characteristic expression profiles and validated DE genes that are able to discriminate adult female patients with FD with different disease manifestations. These DE genes indicate possible disease specific pathways involved in phenotypic variability seen in FD, which might aid in patient management.

Keywords: Fabry disease, lisossomal storage disorder, transcriptome, HTA 2.0 microarray, molecular signature

1. Introduction

Fabry disease (FD; OMIM #301500) is an X-linked lysosomal storage disease caused by deficiency of alpha-galactosidase A (GLA, EC 3.2.1.22), encoded by the alpha-galactosidase gene (*GLA*). This lysosomal hydrolase is involved in the catabolism of glycosphingolipids and its deficiency leads to progressive storage of these compounds, mostly globotriaosylceramide (Gb3) and lyso-globotriaosylceramide (lyso-Gb3), throughout the body. Disease manifestations include acroparesthesia, angiokeratomas, sweating abnormalities, gastrointestinal symptoms, *cornea verticillata*, cardiac, cerebrovascular and renal complications (Desnick et al. 2001).

However, there is a wide phenotypic variability. Patients with the classical form of the disease are more severely affected, with lower enzyme activity and multi-organ involvement. Patients with the non-classical form have higher residual activity and show later-onset of symptoms that may be limited to a single organ. Heterozygous females may present a clinical spectrum ranging from asymptomatic to as severely affected as men, although the latter is not as frequent (Schiffmann and Ries 2016).

Biological mechanisms underlying this variability in disease manifestations are still unknown. There are currently no available biomarkers to predict in which different systems a patient will develop symptoms, and within these systems, which specific manifestations. Gb3 and lyso-Gb3 levels in plasma and urine are frequently used as biomarmarkers in FD. Elevated levels of Gb3 are normally seen in classically affected hemizygous, but males with non-classical disease and heterozygous females may have normal levels of this molecule (Smid et al. 2015). Likewise, there are reports of heterozygous females and non-classical males with normal plasmatic values of lyso-Gb3, a more sensitive marker (Smid et al. 2015; Kubo et al. 2017; Talbot et al. 2017). A recent study in a large cohort of patients found association between lyso-Gb3 plasma levels and overall disease severity in nonclassical patients, but this association was absent in patients with the classical form (Arends et al. 2016). On the other hand, others have reported its association with disease severity in classical males (Nowak et al. 2017; Talbot et al. 2017). Nonetheless, lyso-Gb3 is not predictive of specific disease manifestations.

Proteomics approaches have been applied for the identification of other biomarkers that could be used for better understanding the pathophysiology of the disease and the impact of enzyme replacement therapy (ERT) or diagnosis, particularly in females. Proteomic analysis of urine of adult female patients with FD resulted in a diagnostic biomarker profile that also could be applied to monitoring treatment (Kistler et al. 2011). Recently, another proteomics study reported plasma biomarker signatures in adult patients with FD, in comparison to healthy controls, with gender-specific alterations (Hollander et al. 2015). Moreover, a blood transcriptome study on ERT effects also reported gender-specific responsiveness (Ko et al. 2016). These results highlight the need for the gender-based analysis of pathways involved in overall disease burden and treatment monitoring. However, to the best of our knowledge, there are no studies focused on gene expression and altered pathways in FD patients with different phenotypes, as classical, renal and cardiac variants. Therefore, in this study we aimed to identify possible differences in gene expression in women with different FD manifestations (cardiac symptoms, renal symptoms or both) by transcriptome analysis.

2. Material and methods

2.1. Patients and clinical data

All patients included in this study had genetic analysis of the *GLA* gene with a pathogenic variant confirmed by Sanger sequencing. Clinical data were collected from patient's charts and if necessary, supplementary exams were performed. Absence of either cardiac or renal function assessment were exclusion criteria. Cardiac evaluation included echocardiogram and/or electrocardiogram. Renal evaluation included 24-hour urine total protein and microalbuminuria, blood and urine creatinine and Estimated Glomerular Filtration Rate (eGFR). Following clinical revision with stringent criteria, patients were classified accordingly to their manifestations in to three groups: cardiac-only (C), renal-only (R) or renal and cardiac (RC). This study was approved by the Ethics Research Committee of our institution (#15-0196) and the patients gave written informed consent.

2.2. RNA extraction and Array hybridization

Whole blood samples were collected and extracted with PAXgene Blood RNA System (PreanalytiX, Switzerland). Total RNA samples were quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). In addition, agarose gel electrophoresis was performed to verify RNA integrity. Only samples with 260/230 ratio between 1.8-2.1, and 28S and 18S rRNA bands resolved into two discrete bands without significant smearing were included in the analysis.

GeneChip Human Transcriptome array 2.0 (HTA 2.0, Thermo Fisher Scientific, USA) was used to profile mRNA expression. Briefly, 80 ng of each sample were processed with GeneChip WT PLUS Reagent Kit (Thermo Fisher Scientific, USA). Following hybridization, GeneChip Hybridization, Wash and Stain Kit (Thermo Fisher Scientific, USA) was used to process samples in the GeneChip Fluidics Station 450. All procedures were performed according to manufacturer's instructions. HTA 2.0 arrays were scanned using the Affymetrix 3000 7G scanner and the signal intensity of probe hybridization was processed using the Affymetrix GeneChip Command Console (AGCC) software version 4.0. (Thermo Fisher Scientific, USA) to compute the probe cell intensity data for the array and create the CEL files.

2.3. Differential Expression Analysis

The CEL files containing raw expression data for HTA 2.0 arrays were pre-processed and analyzed with R/Bioconductor packages to obtain normalized expression profiles and the list of differentially expressed mRNAs. The RMA algorithm, as implemented in the oligo package (Carvalho and Irizarry 2010), was applied to perform background correction, quantile normalization, and probeset summarization. Probe annotation CSV files were downloaded from Affymetrix support material website, using Transcript Cluster Annotations Release 36 for HTA 2.0 array. To address the fold change compression property of transcriptome arrays and comply with default analysis settings suggested by Affymetrix, HTA 2.0 CEL files intensities were adjusted with the Signal Space Transformation (SST) algorithm provided by the Affymetrix Power Tools through the apt-cel-transformer utility program prior to normalization using RMA.

Normalized expression data, expressed on log-scale, were analyzed with limma R package (Smyth 2005) to investigate differentially expressed (DE) mRNAs among groups of interest. All mRNAs with a defined gene symbol that satisfied a minimum level of statistical significance (p-value < 0.05) and showed a fold change (FC) of at least two were considered as DE. To account for multiple testing, p-values were corrected using the Benjamini and Hochberg False Discovery Rate (FDR) method. Differentially expressed transcripts with |FC|>2 and P< 0.05 present in both comparisons of each group were used to create expression profiles (or molecular signatures) of groups and were evaluated in the following analysis. Hierarchical clustering, venn diagram and expression profile images were created with gplots R Package.

2.4. Functional Enrichment Analyses

Functional enrichment of DE genes in each group expression profile were accessed by analysis of Gene Ontology (GO) terms related to biological processes. The web tool Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al. 2008, 2009) was used and terms with adjusted P Value < 0.05 (Benjamini-Hochberg method) were considered potentially enriched.

2.5 Validation of microarray data

Selected DE genes were validated by qRT-PCR. Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Reactions were performed in duplicates with SYBR GreenER qPCR SuperMix Universal (Thermo Fisher Scientific, USA) in a QuantStudio™ 3 System (Thermo Fisher Scientific, USA). Cycling conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 30s and 60°C for 1 min, and then a melting curve analysis. Primers pairs used are listed on Supplementary Table 1. Expression levels were normalized by expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the $2^{-\Delta\Delta CT}$ method was used. Results were compared using ANOVA and Tukey or Kruskall-Wallis and Dunn-Bonferroni, as indicated, with the IBM SPSS

Statistics version 22 software (SPSS Inc., USA). P values lower than 0.05 were considered statistically significant. GraphPad Prism 5 software was used to graphic design.

3. Results

3.1. Sample characterization

In total, samples from 13 female individuals with FD from 5 families were included. Detailed clinical characteristics are shown in Table 1. No statistical significance was found on either age distribution or ERT treatment between/amongst groups (ANOVA P value= 0.21 and Fischer Exact Test P Value=0.79, respectively). Similarly, no significant differences were identified between groups for *GLA* variant (Fischer Exact Test P value 0.874), α-GAL activity in plasma or leukocytes (ANOVA P value: 0.25 and 0.41, respectively) or urinary Gb3 (Kruskal-Wallis P value: 0.465).

Table 1: Clinical and biochemical characterization of female individuals with Fabry disease included in this study.

Patient#	Group	Age (years)	*GLA* variant	Family	ERT	α-GAL activity in plasma (nmol/h/mL)[1]	α-GAL activity in leukocytes (nmol/h/mg protein)[2]	Urinary Gb3 (µg/mg creatinine)[3]	Cardiac manifestations	Renal manifestations
1	Cardiac	28	c.44C>A (p.Ala15Glu)	1	No	2.7	21	282.69	Arrhythmia	None
2	Cardiac	57	c.32delG (p.Gly11Alafs)	2.1*	Yes	NA	29	17.78	Cardiac hypertrophy, Left anterior fascicular block	None
3	Cardiac	60	c.32delG (p.Gly11Alafs)	2.1*	Yes	3.8	27	14.95	Left ventricular hypertrophy	None
4	Renal	19	c.1033_1034delTC (p.Ser345Argfs)	3	No	NA	33	58.36	None	Albuminuria, reduced glomerular filtration rate
5	Renal	20	c.334C>T (p.Arg112Cys)	4	Yes	4.3	15	84.04	None	Proteinuria, Albuminuria
6	Renal	37	c.334C>T (p.Arg112Cys)	4	No	3.5	20	146.18	None	Proteinuria, Albuminuria
7	Renal	61	c.32delG (p.Gly11Alafs)	2.2*	No	2.8	29	28.41	None	Albuminuria
8	Renal	37	c.32delG (p.Gly11Alafs)	2.2*	Yes	NA	NA	26.79	None	Proteinuria, Albuminuria
9	Renal	38	c.32delG (p.Gly11Alafs)	2.1*	No	14	25	53.73	None	Proteinuria
10	Cardiac and renal	62	c.456C>A (p.Tyr152Term)	5	No	6.4	45	33.58	Cardiac hypertrophy, left ventricular hypertrophy, short PR interval	Reduced glomerular filtration rate
11	Cardiac and renal	70	c.32delG (p.Gly11Alafs)	2.2*	Yes	7	42	10.6	Cardiac hypertrophy, left ventricular hypertrophy	Proteinuria, Albuminuria
12	Cardiac and renal	40	c.334C>T (p.Arg112Cys)	4	Yes	8.1	28	12.3	Left ventricular hypertrophy	Proteinuria, Albuminuria
13	Cardiac and renal	43	c.32delG (p.Gly11Alafs)	2.2*	No	5.1	16	110.36	Cardiac hypertrophy	Proteinuria, Albuminuria

1: Reference value (RV) 12 – 22 nmol/h/mL; 2: RV 43 - 63 nmol/h/mg protein; 3: RV <15 µg/mg creatinine. NA: Not available. *: Seemingly unrelated families connected by founder effect.

3.2. Differential expression analysis

In the initial screen, 3107 DE transcripts were identified amongst the 3 groups. Hierarchical clustering is shown in Figure 1A. Pairwise comparison between R and RC resulted in 98 DE transcripts, 9 up-regulated and 89 down-regulated (Figure 1B). Comparisons between C x RC shown 137 DE mRNAs, 104 down and 33 up-regulated (*Figure 1*C). Finally, when compared to the renal symptoms group, the cardiac symptoms group presented 29 DE transcripts, 27 up-regulated and 2 down-regulated, with clear separation of groups (Figure 1D).

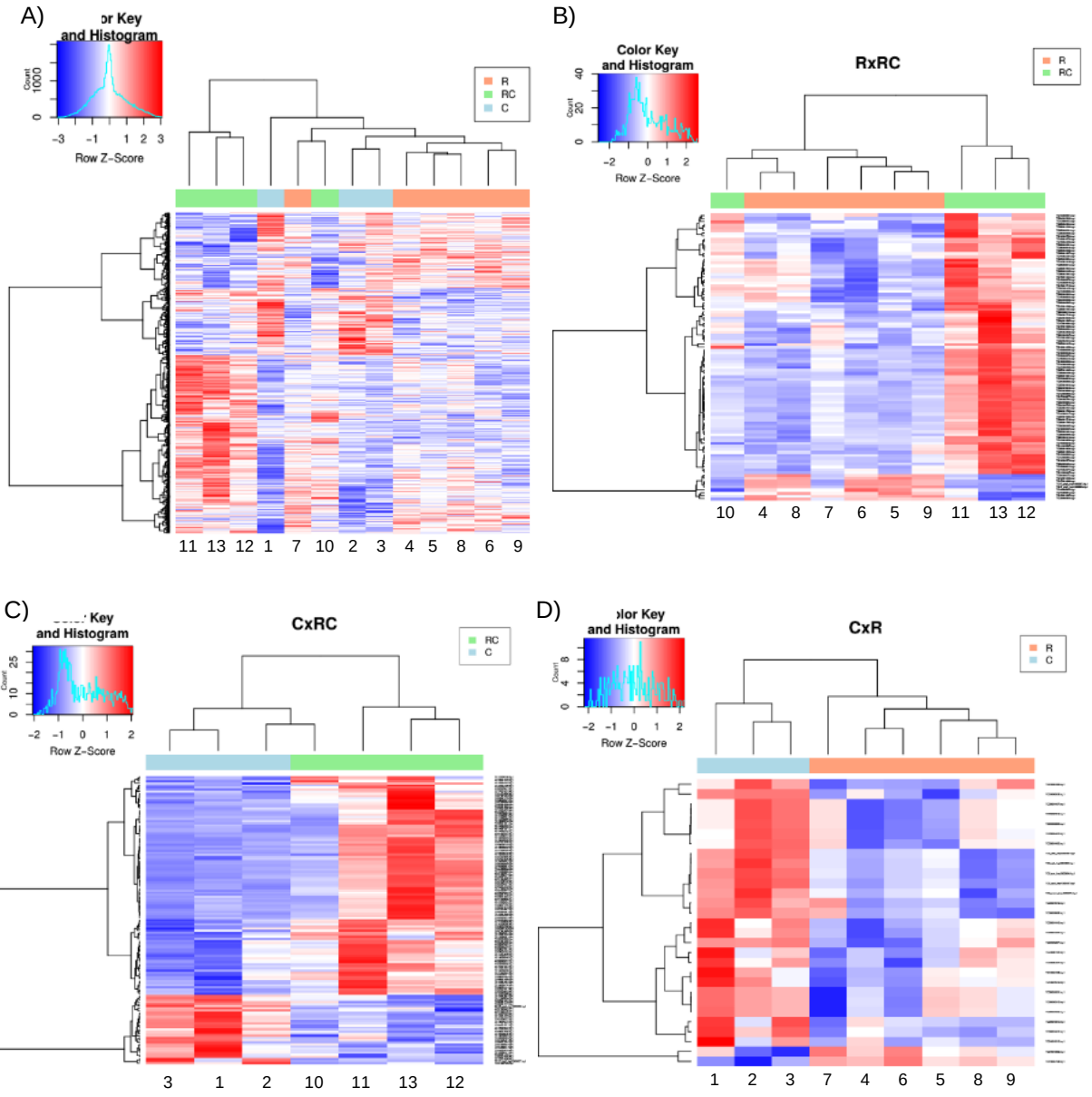

Figure 1: Hierarchical clustering. Clustering analysis of the DE mRNAs between female individuals with FD and different disease symptoms. Patients were classified according to development of renal (R), cardiac (C) or renal and cardiac (RC) manifestations. General (A) and pairwise comparisons (B-D). Red represents upregulation of and blue represents downregulation.

In order to select representative genes from each group to be validated, pairwise results were ploted and subsets of genes with DE in both analysis from each group were made (Figure 2 and Supplementary Table 2). Differences in expression of those subsets are further characterized in *Figure 3*, where a molecular signature of each group can be seen. Interestingly, the 6 transcripts in the R group correspond to different mRNA variants of 3 genes. In the C group the 8 transcripts correspond to 7 genes, while the 82 DE mRNAS in RC group represent 79 genes.

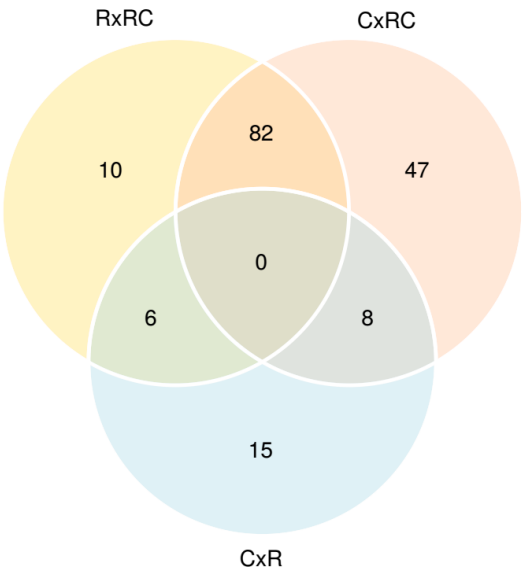

Figure 2: Venn diagram of differentially expressed mRNAs in females with FD (|FC|≥2, P value< 0.05). R: Renal-only symptoms group. C: cardiac-only symptoms group. RC: Renal and cardiac symptoms group.

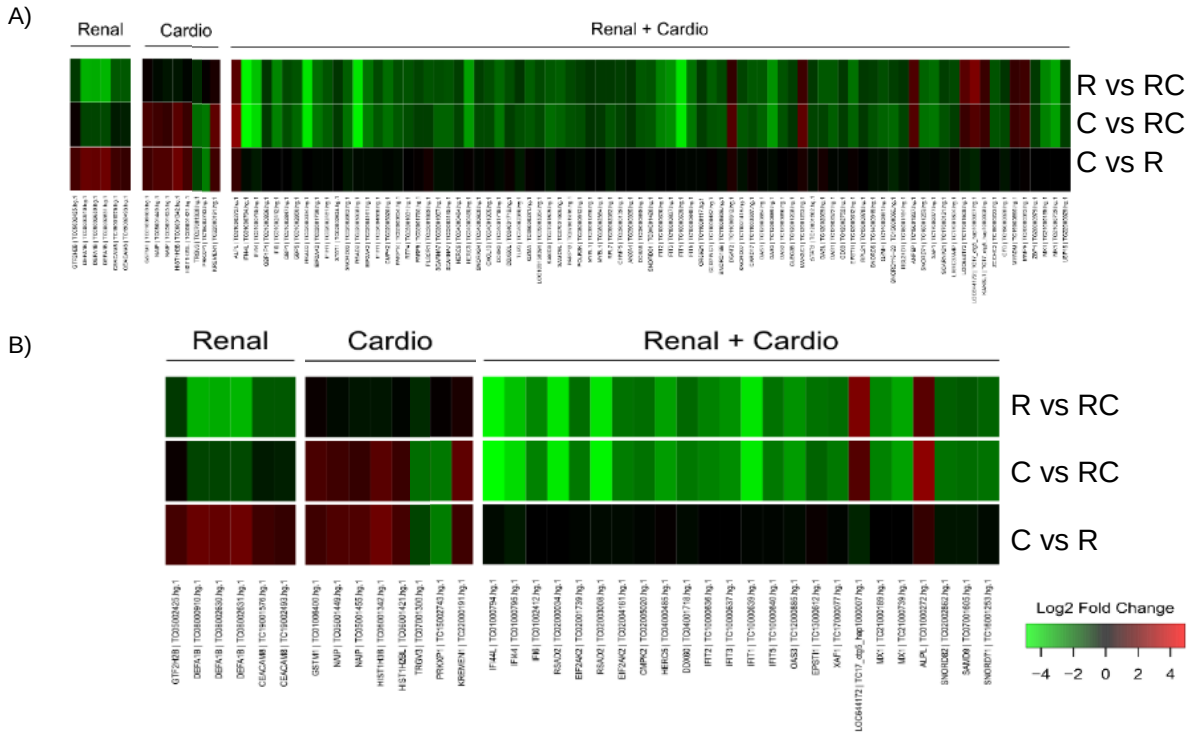

Figure 3: Expression profile of FD female individuals with different disease manifestations. Differentially expressed transcripts with |logFC|>1 (FC >2) for all groups (A) and |logFC|>2 (FC>4) for RC (B). R: Renal symptoms group. C: cardiac symptoms group. RC: Renal and cardio symptoms group. FC: fold change.

3.3. Functional Enrichment Analyses

GO analysis of genes from the expression profiles of either renal-only or cardiac-only symptoms groups did not result in significantly enriched terms after Benjamini-Hochberg correction (Table 2) most likely due to small number of DE genes. On the other hand, 74 terms mostly related immune response, particularly viral response and interferon I signaling, were significantly enriched in the renal and cardiac symptoms group (Table 2).

Table 2: GO analysis of enriched biological process terms for DE genes.

Group	GO term ID	GO term description	Count	%	Fold Enr.	P Value	Adj. P Value*
R	GO:0040011	Locomotion	2	66.7	11.0	9.11 E-02	9.99 E-01
R	GO:0006955	Immune response	2	66.7	10.8	9.24 E-02	9.72 E-01
C	GO:0006334	nucleosome assembly	2	33.3	44.5	3.55 E-02	1.00 E+00
C	GO:0031497	chromatin assembly	2	33.3	40.2	3.92 E-02	9.89 E-01
C	GO:0034728	nucleosome organization	2	33.3	37.7	4.17 E-02	9.60 E-01
C	GO:0006333	chromatin assembly or disassembly	2	33.3	35.2	4.47 E-02	9.25 E-01
C	GO:0006323	DNA packaging	2	33.3	32.8	4.79 E-02	8.92 E-01
C	GO:0065004	protein-DNA complex assembly	2	33.3	28.6	5.48 E-02	8.80 E-01
C	GO:0071824	protein-DNA complex subunit organization	2	33.3	25.6	6.10 E-02	8.69 E-01
C	GO:0071103	DNA conformation change	2	33.3	23.7	6.60 E-02	8.55 E-01
C	GO:0006334	nucleosome assembly	2	33.3	56.4	2.80 E-02	5.98 E-01
RC	GO:0051607	defense response to virus	22	29.7	29.3	9.15 E-26	1.72 E-22
RC	GO:0009615	response to virus	23	31.1	22.6	1.64 E-24	1.54 E-21
RC	GO:0045087	innate immune response	29	39.2	10.6	1.11 E-22	6.94 E-20
RC	GO:0016032	viral process	28	37.8	8.8	9.54 E-20	4.47 E-17
RC	GO:0044764	multi-organism cellular process	28	37.8	8.8	1.15 E-19	4.31 E-17
RC	GO:0044419	interspecies interaction between organisms	28	37.8	8.6	2.20 E-19	6.88 E-17
RC	GO:0044403	symbiosis, encompassing mutualism through parasitism	28	37.8	8.6	2.20 E-19	6.88 E-17
RC	GO:0006955	immune response	32	43.2	6.3	7.89 E-19	2.11 E-16
RC	GO:0002252	immune effector process	25	33.8	10.3	8.08 E-19	1.89 E-16
RC	GO:0098542	defense response to other organism	22	29.7	13.6	9.23 E-19	1.92 E-16
RC	GO:0006952	defense response	31	41.9	6.2	5.60 E-18	1.05 E-15
RC	GO:0060337	type I interferon signaling pathway	13	17.6	53.6	6.16 E-18	1.05 E-15
RC	GO:0071357	cellular response to type I interferon	13	17.6	53.6	6.16 E-18	1.05 E-15
RC	GO:0051707	response to other organism	25	33.8	9.2	1.02 E-17	1.60 E-15
RC	GO:0043207	response to external biotic stimulus	25	33.8	9.2	1.02 E-17	1.60 E-15
RC	GO:0034340	response to type I interferon	13	17.6	50.9	1.21 E-17	1.74 E-15
RC	GO:0009607	response to biotic stimulus	25	33.8	8.7	3.31 E-17	4.43 E-15
RC	GO:0002376	immune system process	36	48.6	4.5	5.19 E-17	6.49 E-15
RC	GO:0034097	response to cytokine	22	29.7	8.4	1.65 E-14	1.93 E-12
RC	GO:0051704	multi-organism process	31	41.9	4.0	8.23 E-13	9.08 E-11
RC	GO:0009605	response to external stimulus	28	37.8	4.1	1.70 E-11	1.77 E-09
RC	GO:0050792	regulation of viral process	13	17.6	15.1	3.10 E-11	3.06 E-09
RC	GO:0071345	cellular response to cytokine stimulus	18	24.3	7.8	3.64 E-11	3.41 E-09
RC	GO:0043903	regulation of symbiosis, encompassing mutualism through parasitism	13	17.6	13.9	8.24 E-11	7.36 E-09
RC	GO:0019221	cytokine-mediated signaling pathway	16	21.6	8.8	1.31 E-10	1.11 E-08
RC	GO:0045071	negative regulation of viral genome replication	8	10.8	47.9	2.47 E-10	2.01 E-08
RC	GO:0048525	negative regulation of viral process	9	12.2	25.4	1.83 E-09	1.43 E-07
RC	GO:0043900	regulation of multi-organism process	13	17.6	10.5	2.02 E-09	1.52 E-07
RC	GO:0031347	regulation of defense response	16	21.6	7.2	2.03 E-09	1.47 E-07
RC	GO:0045069	regulation of viral genome replication	8	10.8	30.2	6.88 E-09	4.78 E-07
RC	GO:1903901	negative regulation of viral life cycle	8	10.8	27.4	1.34 E-08	8.96 E-07
RC	GO:0006950	response to stress	32	43.2	2.7	1.54 E-08	9.97 E-07
RC	GO:0045088	regulation of innate immune response	12	16.2	10.2	1.58 E-08	9.90 E-07
RC	GO:0019058	viral life cycle	13	17.6	8.7	1.66 E-08	1.00 E-06
RC	GO:0002682	regulation of immune system process	20	27.0	4.4	2.34 E-08	1.37 E-06

RC	GO:0043901	negative regulation of multi-organism process	9	12.2	17.5	3.53 E-08	2.00 E-06
RC	GO:0019079	viral genome replication	8	10.8	23.0	4.54 E-08	2.50 E-06
RC	GO:0071310	cellular response to organic substance	24	32.4	3.4	7.18 E-08	3.85 E-06
RC	GO:1903900	regulation of viral life cycle	9	12.2	15.5	8.95 E-08	4.66 E-06
RC	GO:0010033	response to organic substance	27	36.5	2.9	9.38 E-08	4.76 E-06
RC	GO:0050776	regulation of immune response	16	21.6	5.4	1.11 E-07	5.47 E-06
RC	GO:0071346	cellular response to interferon-gamma	8	10.8	18.1	2.40 E-07	1.15 E-05
RC	GO:0070887	cellular response to chemical stimulus	25	33.8	2.9	5.55 E-07	2.60 E-05
RC	GO:0034341	response to interferon-gamma	8	10.8	15.2	7.93 E-07	3.63 E-05
RC	GO:0001817	regulation of cytokine production	12	16.2	6.3	1.79 E-06	8.00 E-05
RC	GO:0001819	positive regulation of cytokine production	10	13.5	7.9	3.48 E-06	1.52 E-04
RC	GO:0042221	response to chemical	30	40.5	2.2	3.83 E-06	1.63 E-04
RC	GO:0001816	cytokine production	12	16.2	5.8	4.25 E-06	1.77 E-04
RC	GO:0050896	response to stimulus	44	59.5	1.6	5.23 E-06	2.13 E-04
RC	GO:0060333	interferon-gamma-mediated signaling pathway	6	8.1	22.9	5.70 E-06	2.27 E-04
RC	GO:0080134	regulation of response to stress	16	21.6	3.8	8.34 E-06	3.26 E-04
RC	GO:0002697	regulation of immune effector process	9	12.2	8.5	8.66 E-06	3.31 E-04
RC	GO:0032606	type I interferon production	6	8.1	17.0	2.47 E-05	9.25 E-04
RC	GO:0035455	response to interferon-alpha	4	5.4	64.3	2.94 E-05	1.08 E-03
RC	GO:0032479	regulation of type I interferon production	6	8.1	16.4	2.94 E-05	1.06 E-03
RC	GO:0034344	regulation of type III interferon production	3	4.1	305.3	3.04 E-05	1.07 E-03
RC	GO:0050778	positive regulation of immune response	11	14.9	4.9	5.55 E-05	1.93 E-03
RC	GO:0035456	response to interferon-beta	4	5.4	50.9	6.07 E-05	2.07 E-03
RC	GO:0032481	positive regulation of type I interferon production	5	6.8	21.5	7.91 E-05	2.65 E-03
RC	GO:0009597	detection of virus	3	4.1	183.2	1.01 E-04	3.31 E-03
RC	GO:0050688	regulation of defense response to virus	5	6.8	17.5	1.74 E-04	5.62 E-03
RC	GO:0002684	positive regulation of immune system process	12	16.2	3.8	2.00 E-04	6.32 E-03
RC	GO:0045089	positive regulation of innate immune response	7	9.5	7.3	3.36 E-04	1.05 E-02
RC	GO:0035457	cellular response to interferon-alpha	3	4.1	101.8	3.60 E-04	1.10 E-02
RC	GO:0007166	cell surface receptor signaling pathway	20	27.0	2.3	4.11 E-04	1.24 E-02
RC	GO:0032101	regulation of response to external stimulus	10	13.5	4.2	4.43 E-04	1.31 E-02
RC	GO:0002831	regulation of response to biotic stimulus	5	6.8	11.2	9.51 E-04	2.75 E-02
RC	GO:0048583	regulation of response to stimulus	23	31.1	1.9	1.19 E-03	3.37 E-02
RC	GO:0032727	positive regulation of interferon-alpha production	3	4.1	53.9	1.34 E-03	3.73 E-02
RC	GO:0002221	pattern recognition receptor signaling pathway	5	6.8	9.6	1.69 E-03	4.63 E-02
RC	GO:0051346	negative regulation of hydrolase activity	7	9.5	5.3	1.75 E-03	4.72 E-02
RC	GO:0031349	positive regulation of defense response	7	9.5	5.3	1.77 E-03	4.71 E-02
RC	GO:0002253	activation of immune response	8	10.8	4.4	1.82 E-03	4.75 E-02
RC	GO:0032647	regulation of interferon-alpha production	3	4.1	45.8	1.86 E-03	4.79 E-02

R: Renal only symptoms group. C: cardiac only symptoms group. RC: Renal and cardiac symptoms group.

*Benjamini-Hochberg method. Fold Enr: Fold enrichment.

3.4. qRT-PCR validation

Genes with the largest differences in fold change from expression profiles subsets of each group (*Figure 3*) were selected for validation by qRT-PCR. Ten genes were analyzed: 2 each from the "renal-only" and "cardiac-only" groups, and 6 from the renal and cardiac symptoms group. Carcinoembryonic antigen related cell adhesion molecule 8 (*CEACAM8*) and defensin alpha 1B (*DEFA1B*) were selected from the renal-only group. There are minimal differences between *DEFA1* and *DEFA1B* sequences, which encode the same protein. Moreover, *DEFA3* differs from *DEFA/A1B* by only one amino acid. Therefore, due to these minimal sequence differences, the three transcripts were quantified simultaneously. All genes shown significantly lower expression from 2 to 6-fold when compared to other groups (Figure 4).

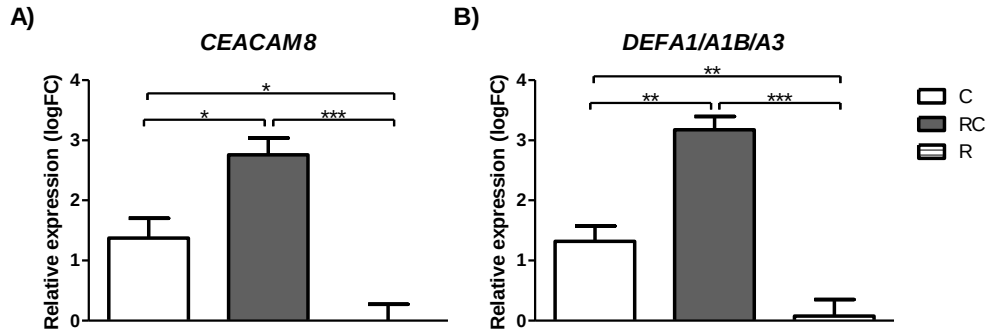

Figure 4: Quantitative RT-PCR validation of DE genes in the renal symptoms group. *CEACAM8* and alpha-defensins (*DEFA*) *1, 1B* and *3* and mRNA expression normalized by *GAPDH*. Data were calculated by the 2-ddCT method and are expressed as mean ± SEM. ANOVA with Tukey post-hoc, *P<0.05, ** P < 0.01, *** P < 0.001. R: Renal symptoms group. C: cardiac symptoms group. RC: Renal and cardiac symptoms group.

Female individuals with cardiac symptoms only presented a significant (P < 0.01) 4-fold reduction in the expression of Protein Kinase, X-Linked, Pseudogene 1 (*PRKXP1*). In addition, expression of Histone Cluster 1 H3 Family Member B (*HIST1H3B*) was increased, with statically significant difference from the individuals with renal symptoms only (P < 0.001, *Figure 5*).

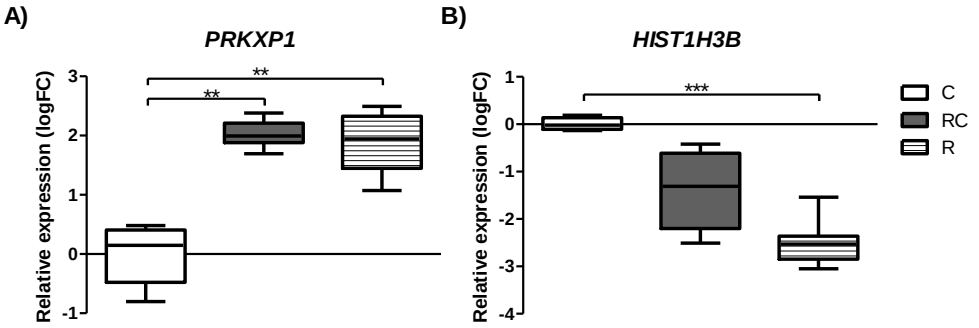

Figure 5: Quantitative RT-PCR validation of DE genes in the cardiac symptoms group. *PRKXP1* and *HIST1H3B* mRNA expression normalized by *GAPDH*. Data were calculated by the 2-ddCT method and are expressed as mean ± SEM. Kruskal-Wallis with Dunn-Bonferroni post-hoc, ** P< 0.01, *** P< 0.001. R: Renal symptoms group. C: cardiac symptoms group. RC: Renal and cardiac symptoms group.

The following genes selected from the RC molecular signature subset were analyzed: Interferon Induced Protein 44 (*IFI44*), Interferon Induced Protein 44 like (*IFI44L*), Interferon Induced Protein With Tetratricopeptide Repeats 1 (*IFIT1*), HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 5 (*HERC5*), Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2 (*EIF2AK2*) and Radical S-Adenosyl Methionine Domain Containing 2 (*RSAD2*). All showed significantly up-regulation in this group, ranging from 4 to more than 32-fold increased expression (*Figure 6*).

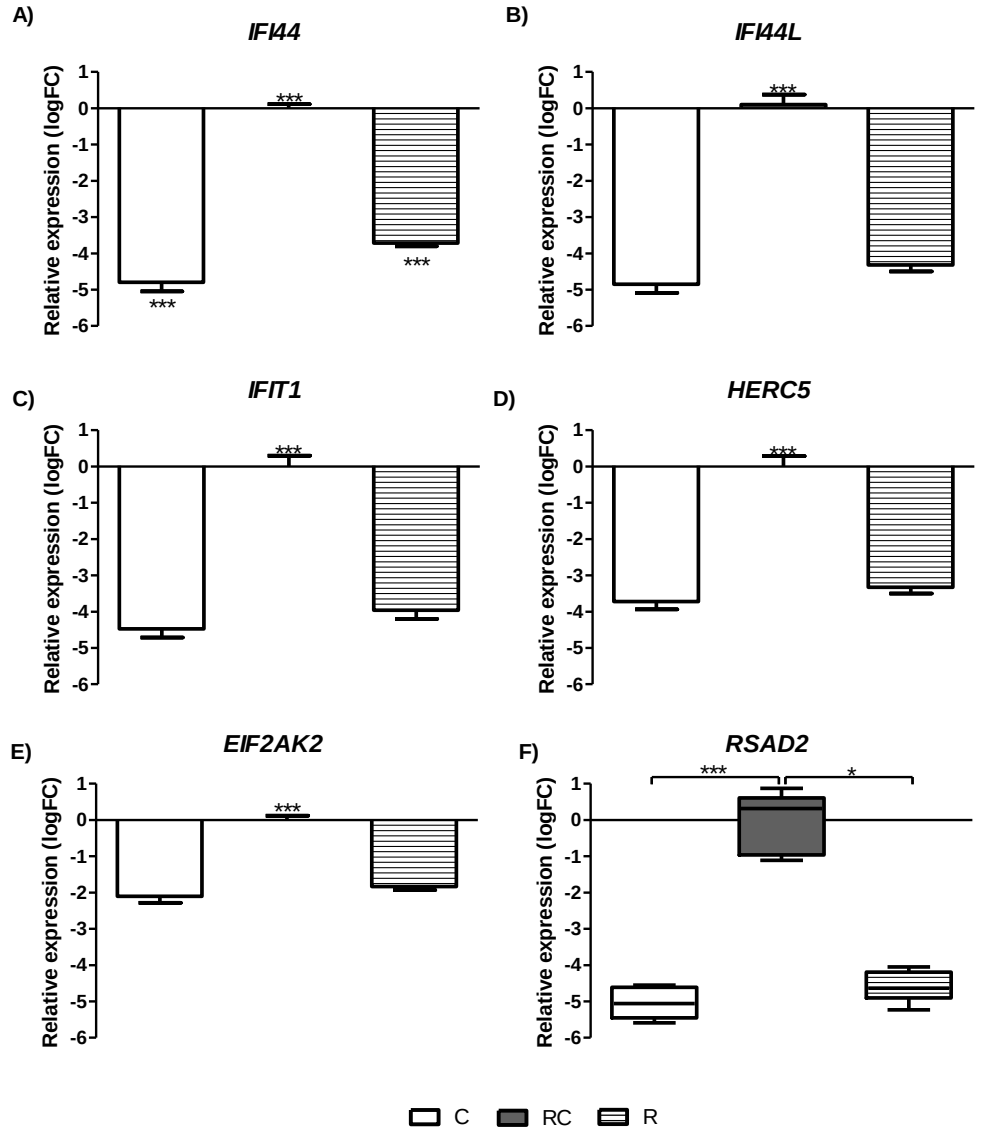

Figure 6: Quantitative RT-PCR validation of DE genes in the Renal and cardiac symptoms group. *IFI44*, *IFI44L, IFIT1, HERC5, EIF2AK2* and *RSAD2* mRNA expression normalized by *GAPDH*. ANOVA with Tukey post-hoc (A-E) and Kruskal-Wallis with Dunn-Bonferroni, *P<0.05, ** P < 0.01, *** P < 0.001. R: Renal symptoms group. C: cardiac symptoms group. RC: Renal and cardiac symptoms group.

4. Discussion

Fabry disease is an X-linked lisossomal disorder with high allelic and phenotypic variability. Recently, transcriptome analysis of peripheral blood mononuclear cells (PBMC) from 6 FD patients, including 4 females, comparing expression immediately before and after

ERT treatment was reported. A significant difference in the number of DE genes was found between males and females (167 and 37, respectively) (Ko et al. 2016). This data corroborates previous findings that there is a sex-biased gene expression across all chromosomes (Jansen et al. 2014). Moreover, plasma biomarker signatures in FD are also distinct between genders (Hollander et al. 2015). However, biological mechanisms involved in distinct symptoms development even in family members with the same gender are unclear. Therefore, this study aimed to identify possible differences in gene expression in women with different FD manifestations (cardiac symptoms, renal symptoms or both) by transcriptome analysis. In order to detect possible transcripts involved in disease development, we used very strict criteria for classifying patients into groups. Any manifestation in either renal or cardiac systems, as 24h proteinuria without significantly reduced eGFR, was considered significant for classification.

Distinct expression profiles, or molecular signatures, were identified for each group. The molecular signature of patients with only renal symptoms included down-regulation of *CEACAM8* and alpha-defensins in comparison to both groups involving cardiac symptoms. CEACAM8 (CD66b) is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein located in lipid rafts and expressed in neutrophils and, at higher rates, at eosinophils. Despite this difference in basal levels, in both cells types its expression is up-regulated in inflammatory processes. Its activation in eosinophils induces cellular adhesion, superoxide production and degranulation (Yoon et al. 2007). This molecule is up regulated in inflammatory disorders as severe asthma (Tsitsiou et al. 2012) and eosinophilic eosophagitis (Nguyen et al. 2011), and in cancers as acute lymphoblastic leukemias (Lasa et al. 2008). Moreover, it is up-regulated in breast cancer patients with decline in left ventricle ejection fraction (LVEF) after chemotherapy (Todorova et al. 2016). The connection between this marker and FD might involve both an activation by the overall inflammatory state of FD patients and/or alterations in lipid rafts caused by Gb3 storage (Brogden et al. 2017). However, the exact mechanism for the overexpression in patients with cardiac symptoms is unclear.

Alpha-defensins (DEFA), also known as human neutrophil peptides (HNPs), are small peptides (~30 aa) secreted mainly by neutrophils and involved in the innate immune response to bacteria, fungi and enveloped viruses (Yang et al. 2007; Jarczak et al. 2013). They are also involved in adaptive immunity, where they mobilize, activate and enhance antigen uptake by

dendritic cells and mobilize T cells (Yang et al. 2007). Furthermore, overexpression of DEFA1-3 play a role in the pathogenesis of multiple auto-immune disorders and types of cancer, for which they can be predictive biomarkers of chemotherapy response (Vordenbäumen and Schneider 2011; Khong et al. 2015; Kohli et al. 2015; Cui et al. 2016). Their mechanisms of action include: direct cytotoxic effect, increased plasma membrane permeability, angiogenesis inhibition, and endothelial cell proliferation inhibition (Li et al. 2014). They can also impair mitochondrial transmembrane potential, reducing energy synthesis (Li et al. 2014). The consequent energy imbalance has increased relevance regarding the cardiac phenotype in FD, since energy depletion was described as a cause for hypertrophic cardiomyopathy (HCM) and the cardiac variant of FD can be considered a mimic of HCM (Ashrafian et al. 2003; Havndrup et al. 2010; Alamo et al. 2017). Recently, alpha-defensins were proposed as potential biomarkers for coronary heart disease in male hyperlipidemia patients, with increased *DEFA1/DEFA3* expression and plasma levels (Maneerat et al. 2016). Also, as *CEACAM8*, alpha-defensins are up-regulated in chemotherapy treated cancer patients with LVEF decline (Todorova et al. 2016).

In FD patients with only cardiac symptoms, a molecular signature including down-regulation of *PRKXP1* and up-regulation of *HIST1H3* were identified. However, significant differences for *HIST1H3* expression were validated qRT-PCR only between cardiac only and renal only groups, indicating that this was not a distinctive gene among the 3 groups. *PRKXP1* is one of the two pseudogenes reported for *PRKX* (Protein Kinase, X-Linked) (Schiebel et al. 1997). It is a processed pseudogene whose function has not yet been determined. However, it was reported as part of an expression signature for Alzheimer's disease (Squillario and Barla 2011). Moreover, altered expression was described in clear cell renal cell carcinoma (Hirota et al. 2006) and specific types of breast tumors (Honeth et al. 2008), with increased expression being correlated with metastatic behavior (Seitz et al. 2006).

Pseudogenes can affect parental gene expression in different ways (as reviewed by Emadi-Baygi et al. 2017). When transcribed in sense orientation, as *PRKXP1*, they can compete with parental genes for the binding of microRNAs, RNA-binding proteins or the translational machinery (Poliseno et al. 2015). Thus, the increased level of *PRKXP1* seen in both groups with renal symptoms may affect *PRKX* expression. *PRKX* is a gene with high protein levels in human fetal kidney tissue that maintains expression at lower dose in adult tissue. On the other hand,

it is not detected in adult heart tissue (Li et al. 2005). Aside from its role in granulocyte/macrophage lineage differentiation, *PRKX* plays a crucial role in kidney development and function (Huang et al. 2016). This gene activates renal epithelial cell migration and morphogenesis (Li et al. 2002), stimulates endothelial cell proliferation, migration and vascular-like structure formation (Li et al. 2011), and restores normal function in polycystic kidney disease-1 gene (*PKD1*) deficiency (Li et al. 2008). Therefore, *PRKXP1* up-regulation might decrease PRKX protein levels in FD patients with renal disease and further affect renal function.

Due to low number of genes in molecular signatures of cardiac-only and renal-only groups, no GO terms were significantly enriched after FDR correction. However, in the group of patients with both cardiac and renal symptoms, several terms related to immune response were enriched. This altered profile related to the immune system is in accordance with gene expression changes seen in men in response to ERT (Ko et al. 2016) and urinary proteomics alterations detected between FD patients and controls (Matafora et al. 2015). In this study, up-regulation of several type I interferon induced genes was identified and the increased expression of all analyzed genes (*IFI44, IFI44L, IFIT1, HERC5, EIF2AK2 AND RSAD2*) was validated.

As other lysosomal diseases, FD has an important inflammatory component to disease progression due to overall lysosomal function impairment, and specific and secondary metabolic changes (Simonaro 2016). Moreover, since the immune stimulus cannot be eliminated, the inflammatory response becomes a chronic process (Rozenfeld and Feriozzi 2017). Glycosphingolipids are recognized by invariant natural killer T cells (iNKTs) as antigens when bound to CD1d of antigen-presenting cells (APCs). After activation, iNKT proliferate and produce large amounts of IFN-γ (interferon-gamma) and IL-4 (Spada et al. 1998). In the mouse model of FD, there is a decrease in the number of T and iNKT cells (Balreira et al. 2008). This reduction was not observed in FD patients, but conflicting results were reported regarding differences in iNKT cell sub-populations (Rozenfeld et al. 2009; Pereira et al. 2013). Moreover, low levels of CD1d and high levels of MHC class II were reported in FD patients (Balreira et al. 2008; Rozenfeld et al. 2009).

Proinflammatory cytokines secretion by PBMCs was described as a result of the IFN-γ release by iNKT cells in response to activation of Toll-like receptor 4 (TLR4) in APCs by

circulating Gb3 (Mauhin et al. 2015). However, the production of IFN-γ by either PBMC or iNKT cells in Fabry disease patients is not significantly altered in comparison to control samples (Pereira et al. 2013; De Francesco et al. 2013). Moreover, it was recently demonstrated that Gb3 inhibits iNKT activation through direct competition for CD1d binding (Pereira et al. 2016). In FD patients, increased plasma levels of proinflammatory cytokines TNF-α and IL-6 were described, regardless of ERT treatment (Biancini et al. 2012). Recently, no significant differences in TNF-α serum levels were identified in FD patients with late onset mutation IVS4+919G>A, but IL-1β, IL-6,IL-2, MCP-1, sVCAM and IL-18 were all increased (Chen et al. 2016; Chien et al. 2016). Accordingly, after 24h of culture, PBMC of FD patients secreted high levels of IL-1β and IL-6, while DC and monocytes had increased TNF-α, IL-1β, and/or IL-6. In unstimulated freshly isolated PBMC of FD patients, only high levels of IL-1β were detected (De Francesco et al. 2013). This increase in IL-1β and TNF-α by Gb3 was replicated in monocyte-derived macrophages, and was reverted by inhibition of TLR4. Therefore, high plasmatic levels of proinflammatory cytokines in FD could be directly caused by TLR4 activation of APC and other cell types, not necessarily including iNKTs. Moreover, activation of TLR4 can also leads to induction of TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway, which activates IRF3 and phase NF-κB and results in type I interferon induction (Kawai and Akira 2010).

Aside from TLR4 mediated signaling, sphingolipids can affect other immune signaling pathways. Previous studies on type I IFN signaling have shown that Gb3 and Gb2, both α-GAL substrates, are able to interact directly with IFNAR, a common receptor for all type I interferons. The membrane content of both sphingolipids alters IFNAR binding capacity to IFN-α without affecting its expression. Reduction of Gb2 or Gb3 alters signal transduction mediated by *ISGF3* (IFN-stimulated gene factor 3) activation and decreases expression of interferon induced genes (Ghislain et al. 1994; Khine and Lingwood 2000). Therefore, the increased expression of several IFN-stimulated genes (*IFI44, IFI44L, IFIT1, HERC5, EIF2AK2* and *RSAD2*) seen on the renal and cardiac group could be a response to Gb3 or Gb2 levels.

It is important to notice that our data is based on gene expression in whole blood and not in the specific organs affected by cardiac or renal disease. It would be interesting to validate the mechanisms hypothesized above in kidney and, especially, heart biopsy tissue. However, the methodology applied in this study allowed the identification of distinctive

expression profiles of different manifestations of the disease in a much less invasive way. Therefore, these findings have greater potential application in clinical practice, where they could help in the clinical management of patients, especially those with variants of uncertain significance.

In conclusion, we identified three characteristic expression profiles and validated DE genes that are able to discriminate adult female patients with FD with different disease manifestations: renal-only, cardiac-only and renal and cardiac. These DE genes (*DEFA1, A1B, A3*; *CEACAM8; PRKXP1; IFI44; IFI44L; IFIT1; HERC5; EIF2AK2* and *RSAD2*) indicate possible disease specific pathways involved in phenotypic variability seen in FD, which might aid in patient management. However, these data must be interpreted with caution due to small sample size, and must be replicated in larger cohorts.

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Supplementary material

Supplementary Table 1: Primer pairs used in qRT-PCR

Gene	Forward (5'>3')	Reverse (5'>3')	Frag (bp)	Tm (°C)
GAPDH	CCCATCACCATCTTCCAGG	GAG ATG ATG ACC CTT TTG GC	150	60
DEFA1/1B/3	CAAAGCATCCAGGCTCAAGGA	AGCGACGTTCTCCTGCAATG	75	60
CEACAM8	TGGCACATTCCAGCAATACACA	ATCATGATGCTGACAGTGGCTCTA	198	60
HIST1H3B	AGACAGCTCGGAAATCCACC	AACGGTGAGGCTTTTTCACG	108	60
PRKXP1	AGTATGTTCCGGGCTGTGAG	AGGATGTTCTCCGGCTTCAA	130	60
IFI44	GAGTTGGTAAACGCTGGTGT	CTCACAGGCTCACATCTCTCTA	110	60
IFI44L	ATTCTCATCCTCTCTGCACTG	AATTGCACCAGTTTCCTCAAGA	87	60
EIF2AK2	CGTGTGAGTCCCAAAGCAAC	CTGAGACCATTCATAAGCAACGA	145	60
RSAD2	CAAGACCGGGGAGAATACCTG	GCGAGAATGTCCAAATACTCACC	143	60
IFIT1	TCAGGTCAAGGATAGTCTGGAG	AGGTTGTGTATTCCCACACTGTA	147	60
HERC5	CACAGGGTAAACCAGGTGAAA	CATTTTCTGAAGCGTCCACA	136	60

Supplementary Table 2: Differentially expressed mRNAs in females with FD (|FC|≥2, P value< 0.05).

Group	PROBEID	ID	SYMBOL	GENENAME	locus.type	RxRC (logFC)	CxRC (logFC)	CxR (logFC)	Ave Expr	F	P.Value	adj.P.Val
R	TC08002630.hg.1	NM_001042500	DEFA1B	defensin, alpha 1B	Coding	-3.28	-1.33	1.95	11.34	12.25	1.36E-03	3.54E-01
R	TC08000910.hg.1	NM_001042500	DEFA1B	defensin, alpha 1B	Coding	-3.35	-1.37	1.97	11.37	12.09	1.43E-03	3.54E-01
R	TC19002493.hg.1	D90064	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	NonCoding	-1.73	-0.67	1.07	8.11	11.81	1.57E-03	3.54E-01
R	TC08002631.hg.1	NM_001042500	DEFA1B	defensin, alpha 1B	Coding	-3.50	-1.42	2.08	11.44	11.60	1.68E-03	3.54E-01
R	TC19001576.hg.1	NM_001816	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	Coding	-1.74	-0.49	1.25	8.46	10.63	2.35E-03	3.66E-01
R	TC05002425.hg.1	NR_033417	GTF2H2B	general transcription factor IIH subunit 2B (pseudogene)	NonCoding	-1.08	0.23	1.31	12.35	6.25	1.43E-02	4.69E-01
C	TC15002743.hg.1	Y10483	PRKXP1	protein kinase, X-linked, pseudogene 1	NonCoding	0.12	-2.31	-2.43	12.22	28.68	3.13E-05	2.09E-01
C	TC06001342.hg.1	NM_003537	HIST1H3B	histone cluster 1, H3b	Coding	-0.40	1.73	2.12	6.34	6.23	1.44E-02	4.71E-01
C	TC01006400.hg.1	NM_000561	GSTM1	glutathione S-transferase mu 1	Coding	0.20	1.43	1.23	7.54	4.42	3.72E-02	5.13E-01
C	TC05001449.hg.1	NM_022892	NAIP	NLR family, apoptosis inhibitory protein	Coding	-0.29	1.28	1.57	14.26	4.57	3.43E-02	5.11E-01
C	TC05001455.hg.1	NM_004536	NAIP	NLR family, apoptosis inhibitory protein	Coding	-0.42	1.05	1.48	13.29	5.76	1.82E-02	4.86E-01
C	TC06001421.hg.1	NM_003519	HIST1H2BL	histone cluster 1, H2bl	Coding	-0.19	1.13	1.33	6.10	13.81	8.39E-04	3.54E-01
C	TC07001300.hg.1	OTTHUMT00000338416	TRGV3	T cell receptor gamma variable 3	Coding	-0.84	-2.12	-1.28	9.15	5.99	1.62E-02	4.79E-01
C	TC22000191.hg.1	NM_001039570	KREMEN1	kringle containing transmembrane protein 1	Coding	0.55	1.81	1.26	8.60	4.43	3.70E-02	5.13E-01
RC	TC02001739.hg.1	NM_001135651	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	Coding	-2.15	-2.28	-0.13	12.91	14.88	6.14E-04	3.54E-01
RC	TC02004161.hg.1	AF086566	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	NonCoding	-2.18	-2.40	-0.22	13.73	13.54	9.10E-04	3.54E-01
RC	TC04000485.hg.1	NM_016323	HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	Coding	-3.13	-3.01	0.12	9.30	11.70	1.63E-03	3.54E-01
RC	TC01000795.hg.1	NM_006417	IFI44	interferon-induced protein 44	Coding	-3.72	-4.21	-0.49	10.27	14.52	6.82E-04	3.54E-01
RC	TC01000794.hg.1	NM_006820	IFI44L	interferon-induced protein 44-like	Coding	-4.66	-4.91	-0.25	10.03	13.03	1.06E-03	3.54E-01
RC	TC10000639.hg.1	NM_001548	IFIT1	interferon-induced protein with tetratricopeptide repeats 1	Coding	-4.34	-4.49	-0.15	9.28	14.59	6.68E-04	3.54E-01
RC	TC02000034.hg.1	NM_080657	RSAD2	radical S-adenosyl methionine domain containing 2	Coding	-4.40	-4.47	-0.07	9.08	12.08	1.44E-03	3.54E-01
RC	TC02003008.hg.1	AF026942	RSAD2	radical S-adenosyl methionine domain containing 2	NonCoding	-4.39	-4.66	-0.27	8.98	14.07	7.76E-04	3.54E-01
RC	TC04002368.hg.1	DQ470079	TLR3	toll-like receptor 3	NonCoding	-1.02	-1.54	-0.52	6.81	22.42	1.01E-04	3.28E-01
RC	TC01000272.hg.1	NM_000478	ALPL	alkaline phosphatase, liver/bone/kidney	Coding	1.72	2.74	1.02	10.37	15.09	5.81E-04	3.54E-01
RC	TC02005020.hg.1	NM_207315	CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	Coding	-2.13	-2.26	-0.13	7.68	12.15	1.40E-03	3.54E-01
RC	TC07001605.hg.1	NM_001193307	SAMD9	sterile alpha motif domain containing 9	Coding	-1.69	-2.11	-0.42	12.02	11.71	1.62E-03	3.54E-01
RC	TC07001606.hg.1	NM_152703	SAMD9L	sterile alpha motif domain containing 9-like	Coding	-1.80	-1.89	-0.08	14.77	13.26	9.90E-04	3.54E-01
RC	TC09000139.hg.1	NM_001195536	CHMP5	charged multivesicular body protein 5	Coding	-1.26	-1.30	-0.03	9.37	15.87	4.69E-04	3.54E-01
RC	TC10000636.hg.1	NM_001547	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	Coding	-2.46	-2.48	-0.02	11.35	12.34	1.32E-03	3.54E-01

RC	TC10000640.hg.1	NM_012420	IFIT5	interferon-induced protein with tetratricopeptide repeats 5	Coding	-2.16	-2.28	-0.12	9.52	17.08	3.41E-04	3.54E-01
RC	TC12001253.hg.1	NM_018050	MANSC1	MANSC domain containing 1	Coding	1.23	1.77	0.54	9.87	13.50	9.20E-04	3.54E-01
RC	TC12002752.hg.1	NR_026671	CD69	CD69 molecule	NonCoding	-1.09	-1.28	-0.19	8.30	12.46	1.27E-03	3.54E-01
RC	TC15001837.hg.1	NM_001150	ANPEP	alanyl (membrane) aminopeptidase	Coding	1.54	1.63	0.09	12.81	13.33	9.69E-04	3.54E-01
RC	TC17_ctg5_hap 1000008.hg.1	NM_015443	KANSL1	KAT8 regulatory NSL complex subunit 1	Coding	1.10	1.13	0.03	11.47	17.09	3.41E-04	3.54E-01
RC	TC20000979.hg.1	NM_001160417	ZBP1	Z-DNA binding protein 1	Coding	-1.18	-1.19	-0.02	8.47	11.64	1.66E-03	3.54E-01
RC	TC21000189.hg.1	NM_001144925	MX1	MX dynamin-like GTPase 1	Coding	-2.56	-2.51	0.04	9.80	11.90	1.52E-03	3.54E-01
RC	TC21000739.hg.1	AY186254	MX1	MX dynamin-like GTPase 1	NonCoding	-3.18	-3.10	0.08	12.31	12.51	1.25E-03	3.54E-01
RC	TC12000885.hg.1	NM_006187	OAS3	2-5-oligoadenylate synthetase 3	Coding	-2.81	-3.04	-0.24	8.27	11.27	1.88E-03	3.66E-01
RC	TC04001718.hg.1	NM_017631	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	Coding	-2.02	-2.29	-0.27	10.12	10.79	2.22E-03	3.66E-01
RC	TC10000637.hg.1	NM_001031683	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	Coding	-2.77	-2.67	0.09	11.61	11.04	2.03E-03	3.66E-01
RC	TC17000077.hg.1	NM_017523	XAF1	XIAP associated factor 1	Coding	-2.15	-2.29	-0.14	11.85	10.85	2.17E-03	3.66E-01
RC	TC16001253.hg.1	NR_003059	SNORD71	small nucleolar RNA, C/D box 71	Coding	-1.91	-2.16	-0.25	15.12	10.53	2.43E-03	3.70E-01
RC	TC03001705.hg.1	NM_001146102	PARP9	poly(ADP-ribose) polymerase family member 9	Coding	-1.31	-1.21	0.10	10.99	10.00	2.95E-03	3.75E-01
RC	TC09000999.hg.1	NM_014314	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Coding	-1.69	-1.67	0.02	11.96	10.09	2.85E-03	3.75E-01
RC	TC11000117.hg.1	NM_001005180	OR56B1	olfactory receptor, family 56, subfamily B, member 1	Coding	-1.15	-1.25	-0.10	12.20	10.14	2.81E-03	3.75E-01
RC	TC11000808.hg.1	NR_000025	SNORD15B	small nucleolar RNA, C/D box 15B	Coding	-1.39	-1.68	-0.29	12.37	10.16	2.78E-03	3.75E-01
RC	TC18000223.hg.1	NM_017742	ZCCHC2	zinc finger, CCHC domain containing 2	Coding	-1.62	-1.66	-0.04	10.73	10.10	2.84E-03	3.75E-01
RC	TC11000812.hg.1	NM_001253891	DGAT2	diacylglycerol O-acyltransferase 2	Coding	1.04	1.64	0.61	10.72	9.69	3.31E-03	3.75E-01
RC	TC12002059.hg.1	NM_003733	OASL	2-5-oligoadenylate synthetase-like	Coding	-1.71	-1.31	0.40	9.20	9.85	3.12E-03	3.75E-01
RC	TC07001916.hg.1	NM_022750	PARP12	poly(ADP-ribose) polymerase family member 12	Coding	-1.41	-1.59	-0.18	10.22	9.65	3.36E-03	3.76E-01
RC	TC01002412.hg.1	NM_002038	IFI6	interferon, alpha-inducible protein 6	Coding	-2.60	-2.51	0.09	14.46	9.49	3.57E-03	3.77E-01
RC	TC05000231.hg.1	NM_006144	GZMA	granzyme A	Coding	-1.84	-1.67	0.17	11.64	9.58	3.45E-03	3.77E-01
RC	TC04001719.hg.1	NM_001012967	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	Coding	-1.51	-1.23	0.28	12.42	9.06	4.22E-03	3.83E-01
RC	TC22000029.hg.1	NM_017414	USP18	ubiquitin specific peptidase 18	Coding	-1.17	-1.19	-0.02	6.76	8.88	4.53E-03	3.94E-01
RC	TC01002850.hg.1	NM_001134486	GBP5	guanylate binding protein 5	Coding	-1.62	-1.98	-0.36	13.79	8.36	5.59E-03	4.11E-01
RC	TC17_ctg5_hap 1000007.hg.1	NR_026901	LOC644172	mitogen-activated protein kinase 8 interacting protein 1 pseudogene	Coding	2.40	1.66	-0.74	8.15	8.31	5.69E-03	4.13E-01
RC	TC19000865.hg.1	NM_001020818	MYADM	myeloid-associated differentiation marker	Coding	1.01	1.09	0.08	12.93	8.08	6.28E-03	4.14E-01
RC	TC12000884.hg.1	NM_001032409	OAS1	2-5-oligoadenylate synthetase 1	Coding	-1.75	-1.90	-0.14	9.06	7.92	6.70E-03	4.19E-01
RC	TC02002481.hg.1	NM_022168	IFIH1	interferon induced, with helicase C domain 1	Coding	-1.65	-1.83	-0.18	10.05	7.71	7.35E-03	4.26E-01
RC	TC04000484.hg.1	NM_001165136	HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	Coding	-1.19	-1.37	-0.17	9.24	7.69	7.41E-03	4.26E-01
RC	TC11003507.hg.1	NM_001007232	CARD17	caspase recruitment domain family, member 17	Coding	-1.74	-1.52	0.22	10.03	7.71	7.35E-03	4.26E-01
RC	TC17001608.hg.1	NR_026901	LOC644172	mitogen-activated protein kinase 8 interacting protein 1 pseudogene	Coding	1.68	1.18	-0.51	6.27	7.52	7.97E-03	4.26E-01
RC	TC08001286.hg.1	NM_001080416	MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1	Coding	-1.06	-1.05	0.00	11.23	7.43	8.30E-03	4.27E-01

RC	TC03001869.hg.1	NM_021105	PLSCR1	phospholipid scramblase 1	Coding	-1.65	-1.19	0.46	10.95	7.34	8.62E-03	4.28E-01
RC	TC12001208.hg.1	NM_005127	CLEC2B	C-type lectin domain family 2, member B	Coding	-1.21	-1.07	0.14	12.05	7.29	8.83E-03	4.28E-01
RC	TC12001602.hg.1	NM_005419	STAT2	signal transducer and activator of transcription 2	Coding	-1.15	-1.37	-0.23	10.99	7.27	8.89E-03	4.28E-01
RC	TC02002862.hg.1	NR_004398	SNORD82	small nucleolar RNA, C/D box 82	Coding	-1.80	-2.21	-0.41	11.83	6.95	1.03E-02	4.39E-01
RC	TC08002364.hg.1	X13294	MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1	NonCoding	-1.11	-1.14	-0.03	11.73	6.90	1.05E-02	4.39E-01
RC	TC12002572.hg.1	DQ914956	OAS1	2-5-oligoadenylate synthetase 1	NonCoding	-1.71	-1.82	-0.10	8.48	6.83	1.09E-02	4.42E-01
RC	TC03001031.hg.1	NM_022147	RTP4	receptor (chemosensory) transporter protein 4	Coding	-1.59	-1.55	0.04	7.18	6.67	1.17E-02	4.50E-01
RC	TC15001464.hg.1	NM_016304	RSL24D1	ribosomal L24 domain containing 1	Coding	-1.02	-1.36	-0.34	9.09	6.46	1.29E-02	4.59E-01
RC	TC14000919.hg.1	NR_002916	SNORD8	small nucleolar RNA, C/D box 8	Coding	-1.27	-1.59	-0.32	12.57	6.22	1.45E-02	4.71E-01
RC	TC09000335.hg.1	NM_000700	ANXA1	annexin A1	Coding	-1.05	-1.08	-0.03	12.74	6.14	1.51E-02	4.78E-01
RC	TC01000836.hg.1	NR_003133	GBP1P1	guanylate binding protein 1, interferon-inducible pseudogene 1	Coding	-1.01	-1.10	-0.09	7.19	6.05	1.57E-02	4.79E-01
RC	TC03000634.hg.1	NM_017554	PARP14	poly(ADP-ribose) polymerase family member 14	Coding	-1.54	-1.76	-0.22	13.26	6.00	1.62E-02	4.79E-01
RC	TC11001418.hg.1	NR_004403	SNORD97	small nucleolar RNA, C/D box 97	Coding	-1.32	-1.68	-0.36	13.74	5.71	1.87E-02	4.86E-01
RC	TC01002847.hg.1	NM_002053	GBP1	guanylate binding protein 1, interferon-inducible	Coding	-1.43	-1.51	-0.08	12.16	5.48	2.10E-02	4.89E-01
RC	TC15000066.hg.1	NR_003337	SNORD116-23	small nucleolar RNA, C/D box 116-23	Coding	-1.25	-2.00	-0.74	9.85	5.54	2.04E-02	4.89E-01
RC	TC20000363.hg.1	NM_004994	MMP9	matrix metallopeptidase 9	Coding	1.22	1.38	0.16	9.64	5.48	2.10E-02	4.89E-01
RC	TC02004668.hg.1	AL080107	IFIH1	interferon induced, with helicase C domain 1	NonCoding	-1.39	-1.68	-0.29	9.29	5.20	2.43E-02	5.00E-01
RC	TC02002624.hg.1	NM_007315	STAT1	signal transducer and activator of transcription 1	Coding	-1.07	-1.15	-0.08	13.76	5.12	2.53E-02	5.01E-01
RC	TC03003185.hg.1	AY077740	SCARNA7	small Cajal body-specific RNA 7	NonCoding	-1.58	-1.96	-0.37	13.28	5.08	2.59E-02	5.02E-01
RC	TC12000886.hg.1	NM_001032731	OAS2	2-5-oligoadenylate synthetase 2	Coding	-1.63	-1.61	0.02	11.27	5.05	2.63E-02	5.02E-01
RC	TC0X001420.hg.1	NR_002735	SNORD61	small nucleolar RNA, C/D box 61	Coding	-1.60	-1.76	-0.16	16.46	5.01	2.69E-02	5.03E-01
RC	TC17001604.hg.1	uc002ije.3	LRRC37A4P	leucine rich repeat containing 37, member A4, pseudogene	Coding	-1.38	-1.19	0.19	10.05	5.00	2.70E-02	5.03E-01
RC	TC04001305.hg.1	NM_001565	CXCL10	chemokine (C-X-C motif) ligand 10	Coding	-1.50	-1.40	0.10	6.25	4.89	2.87E-02	5.06E-01
RC	TC13000612.hg.1	NM_001002264	EPSTI1	epithelial stromal interaction 1 (breast)	Coding	-2.19	-1.94	0.25	9.70	4.87	2.90E-02	5.06E-01
RC	TC19000020.hg.1	NM_001928	CFD	complement factor D (adipsin)	Coding	-1.04	-1.45	-0.41	8.74	4.85	2.94E-02	5.06E-01
RC	TC03001957.hg.1	NR_003001	SCARNA7	small Cajal body-specific RNA 7	Coding	-1.54	-1.90	-0.35	13.31	4.78	3.05E-02	5.07E-01
RC	TC04000600.hg.1	NR_002963	SNORA24	small nucleolar RNA, H/ACA box 24	Coding	-1.27	-1.39	-0.12	13.01	4.71	3.16E-02	5.08E-01
RC	TC11000467.hg.1	NM_000062	SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Coding	-1.79	-1.61	0.17	8.11	4.54	3.47E-02	5.11E-01
RC	TC14000981.hg.1	NM_033423	GZMH	granzyme H	Coding	-1.50	-1.89	-0.39	10.14	4.56	3.45E-02	5.11E-01
RC	TC05003354.hg.1	AF275799	LOC100130394	translation machinery associated 7 homolog (S. cerevisiae) pseudogene	NonCoding	-1.47	-1.44	0.03	12.75	4.42	3.71E-02	5.13E-01
RC	TC08000612.hg.1	NM_005034	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	Coding	-1.21	-1.48	-0.28	8.91	4.23	4.15E-02	5.24E-01
RC	TC13000978.hg.1	AY320407	RPL21	ribosomal protein L21	NonCoding	-1.11	-1.11	0.00	12.79	4.13	4.40E-02	5.28E-01
RC	TC08002382.hg.1	BC070215	RPL7	ribosomal protein L7	NonCoding	-1.14	-1.59	-0.45	9.90	3.93	4.96E-02	5.38E-01

R: Renal symptoms group. C: cardiac symptoms group. RC: Renal and cardiac symptoms group.

4. Discussão

A Doença de Fabry é uma das doenças lisossômicas mais frequentes, com estimativas de incidência baseadas em triagem neonatal tão altas quanto 1:1.250 homens em Taiwan (Hwu et al., 2009). Além disso, os pacientes podem apresentar sintomas não específicos, sendo, por exemplo, considerada um mímico da cardiomiopatia hipertrófica (Havndrup et al. 2010). Por esse motivo, muitos pacientes são diagnosticados tardiamente a despeito da presença de sintomas inespecíficos precoces (Mehta et al. 2004).

O Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, um centro de referência para o diagnóstico de doenças raras, recebe um grande número de amostras para análise tanto bioquímica quanto genética de homens e mulheres com suspeita clínica, histórico familiar e de populações de risco para DF. Por ter herança ligada ao X, os níveis de atividade enzimática em mulheres são muito variáveis e se sobrepõem aos níveis normais. Assim, apesar de a determinação bioquímica isolada não ser uma metodologia confiável para o diagnóstico em mulheres, ela é solicitada de forma corriqueira por médicos atendentes. Além disso, é uma técnica mais amplamente disponível quando comparada ao sequenciamento completo do gene *GLA*. Dessa forma, o primeiro artigo apresentado nesta tese buscou responder de forma sistemática perguntas de cunho prático na rotina de diagnósticos: é adequada a determinação da atividade enzimática de α-GAL em mulheres antes da análise molecular? Qual o tipo de amostra mais apropriado?

O principal método de coleta utilizado para envio de amostras de sangue entre centros é a impregnação em papel filtro. Esta técnica é rápida, prática e não necessita de refrigeração. Porém, nossos dados indicam que, para mulheres, este é o tipo de amostra menos confiável dentre os testados, com sensibilidade inferior a 45%. Isto resulta em um alto número de falsos positivos, o que é contraditório ao propósito de *screening*. Apesar de ser amplamente utilizado, outros estudos também descreveram as deficiências desse material na análise de heterozigotas (Linthorst et al. 2005; Massaccesi et al. 2011). Recentemente, um dos principais grupos internacionais de diagnóstico molecular estendeu essa conclusão à análise de pacientes do sexo masculino. Os autores indicam diferenças na contagem de leucócitos entre indivíduos diferentes ou do mesmo individuo em coletas

distintas; manipulação da amostra e os níveis de hematócrito como principais razões para alta variabilidade intra e inter-amostral (Boettcher et al. 2017).

A metodologia proposta no capítulo 3.1, de uso combinado de atividade em leucócitos e plasma com valores de referência gênero-específicos, pode reduzir o número de amostras a serem analisadas por sequenciamento de Sanger em 35%. Tomando-se por exemplo o HCPA e considerando-se apenas os custos das reações de PCR e sequenciamento, isso representa uma economia de cerca de R$7.500,00 a cada 100 amostras. Além disso, a quantidade de centros com capacidade de realizar atividade enzimática é muito maior. Fora do âmbito acadêmico, o acesso ao diagnóstico de DF por sequenciamento é muito reduzido, restrito a laboratórios especializados. Grandes redes nacionais de diagnóstico laboratorial, por exemplo, oferecem apenas dosagem da atividade enzimática da α-GAL. Para outras DLs como MPS I e MPS II, análise da variante familiar ou do gene completo por sequenciamento são oferecidos com preços em média 5 e 18x vezes, respectivamente, superiores à dosagem enzimática.

A metodologia para sequenciamento do gene *GLA* desenvolvida durante esta tese permite a amplificação de todos os éxons e regiões flanqueadoras do gene com o mesmo *mix* de reação, em tubos individuais por éxons, e com a mesmo programa no termociclador. Além disso, quando amostras coletadas em tubos com EDTA são utilizadas, dispensa a verificação do PCR antes da purificação, que pode ser feita tanto por método enzimático (EXO-SAP) quanto por precipitação com polietilenoglicol (PEG). Dessa forma, o tempo necessário para análise é otimizado. Porém, os custos associados ao sequenciamento de Sanger dos 7 éxons podem representar uma despesa elevada, principalmente para análise de um grande número de pacientes. Por isso, uns dos objetivos subsequentes desta tese foi o estabelecimento de uma metodologia de *screening* molecular, descrita no capítulo 3.2.

Dentre as técnicas de *screening* já usadas em DF pode-se citar o polimorfismo conformacional de fita simples (SSCP) (Madsen et al. 1996; Blaydon et al. 2001; Auray-Blais et al. 2008) e a cromatografia líquida desnaturante de alta performance (dHPLC) (Shabbeer et al. 2005) . Recentemente, o uso de um novo ensaio com espectrometria de massas foi

reportado em Taiwan (Lee et al. 2014). Nesta técnica, 29 variantes encontradas em *screeening* de neonatos taiwaneses foram avaliadas. A metodologia obteve sensibilidade máxima. Porém, requer 3 etapas de adição de reagentes e incubações antes de análise propriamente dita e é restrita a análise de mutações pré-determinadas. De forma similar, as outras metodologias citadas também são trabalhosas e requerem manipulação pós-PCR. Assim, buscando-se uma metodologia rápida, não direcionada a mutações específicas e com pouca manipulação, foi desenvolvido um protocolo com a técnica de HRM (*high resolution melting*). Esta metodologia é indicada para amostras de pacientes de ambos sexos provenientes de qualquer tipo de projeto: de *screening* de populações de risco a análise de casos índices. O objetivo principal é a identificação de diferenças nas curvas de dissociação em comparação com controles sequenciados previamente. Estas diferenças representam genótipos distintos, que são confirmados posteriormente por sequenciamento convencional. Tendo em vista que apenas os éxons com perfis diferentes dos controles são sequenciados, há uma consequente redução significativa de custos envolvidos. Além disso, esta técnica permite o envio direto de amostras de interesse para sequenciamento por simples diluição, o que também elimina custos de purificação.

Existem doze estudos na literatura que utilizam HRM para DF (Tabela 5), com 3 protocolos distintos (Bono et al. 2011; Tai et al. 2012; Ezgu et al. 2014). A maioria aplica esta técnica para *screening* molecular, mesmo propósito visado na presente tese, e um estudo o utiliza como uma confirmação alternativa para sequenciamento de Sanger (Tuttolomondo et al. 2012). Todos os estudos publicados utilizam reagentes comerciais (*master mixes*) e mais de 80% utilizam equipamento específico para HRM (*Light Cycler* 480, LC480, Roche), com períodos curtos para aquisição dos dados de dissociação. Essas duas características podem diminuir a variação entre experimentos e, principalmente, aumentar o número de amostras processadas simultaneamente. A velocidade da rampa de temperatura e do tempo de aquisição; e, consequentemente, do tempo necessário a coleta de dados na curva de dissociação, é extremamente variável entre equipamentos. Por exemplo, o LC480 tem uma taxa da rampa de 0,04°C/s, com 25 aquisições por °C, e leva entre 10 a 15 minutos para analisar 96 amostras (Tai et al. 2012; Li et al. 2014). O *StepOnePlus* (*Thermo Fisher Scientific*), equipamento similar ao utilizado nesta tese, tem

uma taxa de rampa de +0,3%, aproximadamente 0,005°C/s, e leva cerca de 1 hora e 40 minutos para analisar 96 amostras (Li et al. 2014). Levando-se em conta também o tempo da ciclagem do PCR, há uma limitação de 3 a 4 corridas diárias. Este aumento no tempo de dissociação em alguns equipamentos é um recurso utilizado por algumas companhias, como a *Thermo Scientific*, para aumentar a quantidade de coletas por grau Celsius, e com isso, a acurácia do equipamento (Farrar and Wittwer 2017). Dessa forma, apesar do *throughput* reduzido, não há necessariamente uma perda na sensibilidade do experimento.

Tabela 5: Estudos publicados com *high resolution melting* em Doença de Fabry.

Local	Objetivo do estudo	Propósito	Equipamento	*Master mix*	N	Regiões analisadas[1]	Fragmentos analisados[1]	Variantes		Referência
								Total	Média/éxon	
Itália	Estabelecimento método	*Screening* pré-seq.	*Light Cycler* 480	Sim	740	7	7	10	1,4	(Bono et al. 2011)
Itália	Diagnóstico diferencial com Febre mediterrânea familial	*Screening* pré-seq.	*Light Cycler* 480	Sim	42 (11*)	7 [1]	7[1]	2	0,3	(Zizzo et al. 2013)
Itália	Diagnóstico de paciente com suspeita clínica	Confirmação de sequenciamento	*Light Cycler* 480	Sim	1	1	1	1	0,1	(Tuttolomondo et al. 2012)
Itália	Diagnóstico de paciente com suspeita clínica	*Screening* pré-seq.	*Light Cycler* 480	Sim	1	7	7	4	0,6	(Pisani et al. 2012)
Itália	Diagnóstico de pacientes com suspeita clínica	*Screening* pré-seq.	*Light Cycler* 480	Sim	6	7	7	1	0,1	(Colomba et al. 2012)
Itália	Diagnóstico de paciente com suspeita clínica	*Screening* pré-seq.	*Light Cycler* 480	Sim	1 (4*)	7[1]	7[1]	1	0,1	(Iemolo et al. 2014)
Itália	*Screening* familiar	*Screening* pré-seq.	*Light Cycler* 480	Sim	15	7	7	1	0,1	(Cammarata et al. 2015)
Itália	Diagnóstico de paciente com suspeita clínica / *Screening* familiar	*Screening* pré-seq.	*Light Cycler* 480	Sim	4	7	7	4	0,6	(Tuttolomondo et al. 2015)
Itália	Diagnóstico de pacientes com suspeita clínica	*Screening* pré-seq.	*Light Cycler* 480	Sim	5 (13*)	7[1]	7	5	0,7	(Zizzo et al. 2016)
Taiwan	*Screening* neonatal	*Screening* pré-seq.	*Light Cycler* 480	Sim	299007 (218*)	8[1]	9[1]	20	2,5	(Tai et al. 2012)
Reino Unido	Prevalência de DF em pacientes jovens com AVC	*Screening* pré-seq.	ABI7500 *Fast*	Sim	1247	8[1]	9 [1]	1	0,1	(Kilarski et al. 2015)
Turquia	Estabelecimento método	*Screening* pré-seq.	ABI7500 *Fast*	Sim	15	7	8	5	0,7	(Ezgu et al. 2014)
Brasil	Estabelecimento método	*Screening* pré-seq.	*StepOne*	Não	103	7	8	27	3,9	Esta tese

AVC: acidente vascular cerebral; Pré-seq: pré-sequenciamento; *Familiares analisados; [1]: Apenas a região/fragmento da variante familiar foi analisada em familiares.

Um ponto divergente nos estudos publicados é a formação de "heterozigotos artificiais", que consistem da mistura de amostras de hemizigotos com controles sequenciados e sem variantes do sexo masculino. Homozigotos/hemizigotos mantém o mesmo formato da curva de dissociação com alteração da temperatura de dissociação (*melt)*, que pode ser muito pequena (inferior a 0,25°C) ou até mesmo não detectável em deleções, inserções ou SNPs de classe 3 (C>G) ou 4 (A>T) (Von Ahsen et al. 2000; Liew et al. 2004; Erali and Wittwer 2010). Além disso, estudo de Li e colaboradores (2014) demonstrou que equipamentos que utilizam blocos *peltier* ao invés de capilares de vidro tem acurácia reduzida na genotipagem de homozigotos em fragmentos maiores de 250 pb. Por outro lado, heterozigotos apresentam mudanças de formato por serem o resultado da dissociação de 4 duplexes distintos (Taylor 2009); e, portanto são mais facilmente identificados. Dessa forma, "heterozigotos artificiais" podem facilitar e aumentar as taxas de detecção de hemizigotos. Enquanto estudos do grupo italiano utilizam esta abordagem, a mesma é evitada pelo grupo Taiwanês principalmente em função do tempo adicional requerido, já que parte das amostras de DNA não era quantificada, mas usada em reações padronizadas por volume de amostra (Bono et al. 2011; Tai et al. 2012). No artigo apresentado nesta tese, o protocolo utilizado incluía a quantificação e diluição das amostras. Além disso, o número de amostras era significativamente reduzido em relação às 299.007 do estudo taiwanês, de forma que o tempo necessário ao preparo dos "heterozigotos" não era impeditivo. Portanto, esta metodologia foi utilizada para aumentar a detecção dos hemizigotos e também evitar possíveis deficiências em acurácia inerentes do equipamento e/ou relacionadas aos tamanhos dos fragmentos analisados. Além disso, também pode minimizar a perda de sensibilidade do ensaio devido a variações inter-amostrais.

O tamanho dos amplicons é um fator importante para sensibilidade da análise, de forma que amplicons menores tem diferenças maiores de temperatura de *melt*. O gene *GLA* tem apenas 7 éxons, que são de tamanho reduzido. Dessa forma, incialmente testaram-se amplicons únicos para todos os éxons, conforme metodologia utilizada por outros grupos. Porém, a replicação dos resultados descritos na literatura não foi possível para os éxons 2, 6 e 7. A análise com o software *uMelt* (Dwight et al. 2011), um preditor

de curvas de dissociação de alta resolução, indicou a presença de múltiplos domínios de *melt* nestes amplicons. Apesar do éxon 7 apresentar tamanho similar aos demais fragmentos, três domínios de *melt* foram identificados nesta região. Isso se deve a distribuição desigual do conteúdo GC, com excesso na metade inicial do éxon (54%) em relação ao final (40%). Essa característica reduziu a sensibilidade e impediu a detecção de todos os heterozigotos neste éxon, criando a necessidade da separação em dois fragmentos. Este achado está de acordo com o relato de Tai e colaboradores (2012), único outro estudo a relatar mais de duas variantes neste éxon. Isso corrobora que não se trata de uma deficiência do protocolo, mas uma característica inerente da sequência. De forma similar, após redesenho dos amplicons dos éxons 2 e 6 levando-se em consideração os domínios de *melt*, todos os heterozigotos foram identificados com clareza.

Todas as variantes analisadas no estudo apresentado nesta tese resultaram em perfis distintos dos controles normais. Com exceção das variantes localizadas no éxon 7, também foi possível a identificação de todos os genótipos diferentes analisados. Isso demonstra que a metodologia desenvolvida está adequada para o perfil de mutações visto em DF, podendo ser aplicada em pacientes de ambos os sexos. Do mesmo modo, possibilita o uso de amostras extraídas com diferentes metodologias, o que pode ser importante em colaborações de centros diferentes ou no uso de materiais diferentes adquiridos por licitações em laboratórios públicos. Apesar do uso do HRM para genotipagens ser controverso, também indica a possibilidade de uso para análises familiares, após identificação da varainte específica no caso índice, de forma menos dispendiosa que Sanger. Outro ponto importante é a redução no tempo para obtenção do diagnóstico. Embora o desenho e padronização da técnica requeiram maior planejamento e cuidado em comparação ao sequenciamento convencional, uma vez padronizado o ensaio, os resultados podem ser liberados em 48h.

Assim como outras técnicas baseadas em PCR, dentre as limitações da análise por HRM pode-se citar a detecção de grandes deleções ou inserções (Wittwer 2009), como éxons inteiros ou regiões localizadas além dos locais de anelamento dos primers. Nesses casos, há necessidade de aplicação de técnicas complementares como MLPA ou qRT-PCR.

Felizmente, esse tipo de mutação corresponde a apenas 5,5% do total de mutações já descritas no gene *GLA* e é identeificado em frequencias muito baixa em pacientes.

No terceiro artigo incluído nesta tese, capítulo 3.3, é apresentada a análise molecular de 408 pacientes brasileiros, provenientes de 213 famílias, com histórico familiar ou suspeita clínica de DF. Conforme o esperado para uma doença com mutações privadas, a maioria das variantes foi encontrada em famílias únicas. Das 26 variantes identificadas em regiões codificantes, cerca de 80% eram do tipo *missense,* o que está de acordo com a literatura e HGMD. Por ser um centro de referência, o HCPA recebe amostras de diversas partes do mundo. Dessa forma, pacientes de outros países da América Latina como Peru, Bolívia e México também foram analisados, mas não incluídos neste artigo. O artigo contido no anexo VI (Uribe et al. 2015) apresenta o perfil mutacional de 9 famílias colombianas estudadas. Apesar do tamanho amostral reduzido, também pode ser observada uma maior proporção de mutações *missense* (38%) em relação aos demais tipos. Além disso, pequenos rearranjos estão restritos ao éxon 7. Outros estudos também relatam uma alta proporção desse tipo de mutação nesta região (Turaça et al. 2012; Pan et al. 2016). Conforme pode ser observado em dados compilados a partir do HGMD (tabela S1 do capítulo 3.3), este éxon parece ser mais suscetível a pequenos rearranjos, apresentando a maior diferença entre as frequências observada e esperada (30% vs. 23%, respectivamente). Também há grande diferença na proporção de pequenos rearranjos observados em relação às mutações *missense/nonsense* observadas; enquanto essa razão é 0,5 no éxon 7, ela varia entre 0,2 e 0,3 no resto do gene. A razão deste aumento ainda é incerta. Existem muitas pequenas repetições que poderiam estar envolvidas em erros de pareamento, como já descrito para algumas mutações de *GLA* (Kornreich et al. 1990; Eng et al. 1993). É interessante ressaltar que os pequenos rearranjos descritos (pequenas inserções, deleções, indels) no éxon 7 estão concentrados na primeira metade do éxon (Figura 12), que apresenta maior conteúdo GC (53% x 39%) e os dinucleotídeos CpG, associados a mutações frequentes.

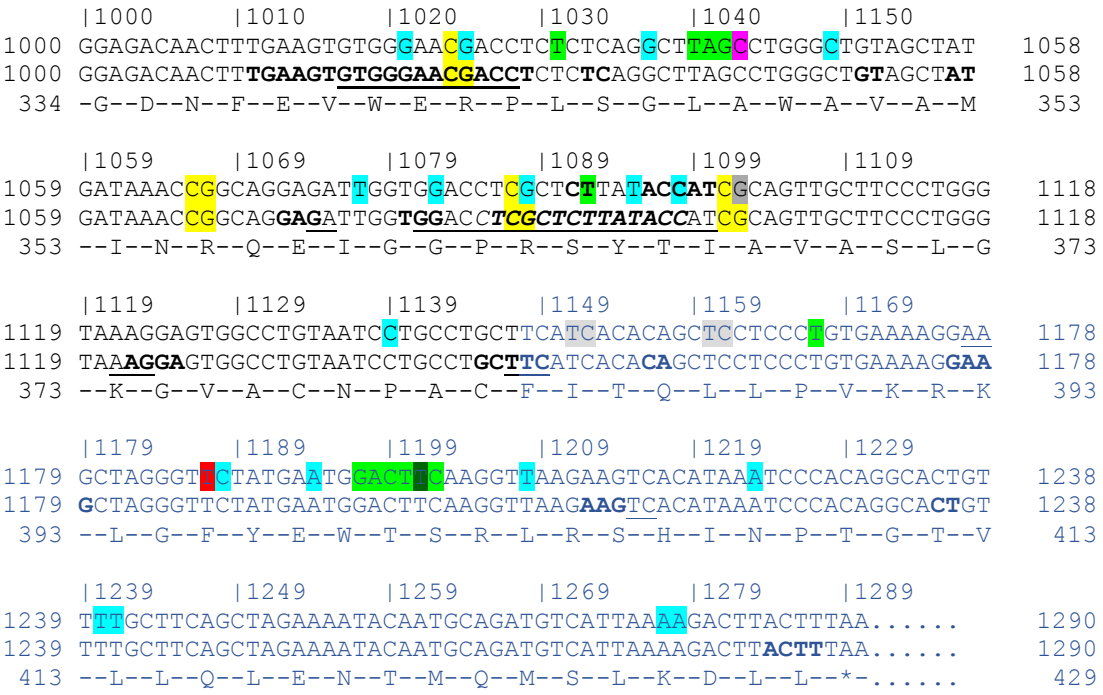

Figura 12: Distribuição de pequenos rearranjos no éxon 7 do gene *GLA* descritos no HGMD v2017.2. As cores indicam os tipos de alteração: **N**/ **NN** / NN / *NN* : deleção; N/N: duplicação; N: insersão; N : duplicação e deleção independentes na mesma base; N: indel; NNNN: %GC = 53%, NNNN: %GC = 39%.

Neste artigo, a presença de variações em regiões não codificantes é ressaltada. De forma geral, em estudos que detectam outras variantes conhecidamente patogênicas, não há menção das variações em regiões não codificantes. Isso não corresponde ao que se observa no cotidiano do diagnóstico molecular para DF, onde elas são identificadas em conjunto com variantes patogênicas conhecidas. A frequência destas variantes varia entre 6 a 30% a população saudável, o que não é condizente com variantes causais em doenças raras. Porém, Schelleckes e colaboradores (2014) sugeriram uma relação causal entre um haplótipo formado por quatro variantes intrônicas e manifestações neurológicas em DF. Da mesma forma, Pisani e colaboradores (2012) atribui o desenvolvimento da forma clássica de DF em uma heterozigota a um haplótipo intrônico com a variante c.-10C>T. Outros estudos também relataram a associação desta mutação ou haplótipo ao desenvolvimento de sintomas cerebrovasculares (Oliveira et al. 2008b; Tanislav et al. 2011; Zeevi et al. 2014;

Song et al. 2016) ou de manifestações variadas de DF (Gervas-Arruga et al. 2015; Tuttolomondo et al. 2015; Tuttolomondo et al. 2017). Inclusive, há relato de uma heterozigota do haplótipo c.-10C>T/c.640-16A>G/c.1000-22 C>T com declínio da função renal em tratamento por TRE (Üçeyler et al. 2011). Em muitos destes estudos, não há necessariamente uma avaliação através de técnicas complementares ao sequenciamento de DNA, como MLPA, PCR quantitativo em tempo real ou sequenciamento de cDNA, que possam detectar outras possíveis variantes causais, como grandes deleções. Porém, há demonstração experimental do efeito da variante c.-10C>T na redução da expressão gênica e da atividade enzimática de *GLA* possivelmente pela alteração do sítio de ligação com a família de fatores de transcrição "fator regulador X" (RFX) (Oliveira et al. 2008a; Schelleckes et al. 2014; Ferreira et al. 2015b). Outra variante da região 5' UTR, c.-30G>A, também é relacionada com alteração na expressão genica por alteração da ligação de fatores de transcrição (Fitzmaurice et al. 1997). Dessa forma, estas e outras variações poderiam influenciar a amplitude fenotípica vista em DF. Portanto, descrições de todas as variantes identificadas nos pacientes são importantes pois podem ajudar a explicar a variabilidade de fenótipo que ainda não é completamente compreendida.

A metodologia utilizada para genotipagem nesta tese foi o sequenciamento de Sanger. Tendo em vista que não há mutações patogênicas recorrentes e que 95% das alterações em *GLA* são mutações de ponto ou que afetam poucas bases, Sanger é considerado padrão ouro para diagnóstico molecular de DF. Porém, este cenário tem sido alterado nos últimos anos. A tecnologia de sequenciamento paralelo maciço, também conhecido como sequenciamento de nova geração (NGS), vem se tornando mais acessível mundialmente. Dessa forma, sequenciamentos de painéis de genes alvo, exomas e genomas vem se tornando mais comuns, com crescente aplicação na prática clínica em países desenvolvidos. Porém, em países com menos recursos, é necessário manter métodos com maior custo-efetividade.

Nestas técnicas, especialmente no sequenciamento de exomas e genomas, milhares de variantes são identificadas em cada paciente. Consequentemente, o número de variantes de significado clínico incerto, ou VUS (*variant of uncertain significance*), também cresce de forma exponencial. Neste aspecto, um fato interessante é a recomendação da

ACMG (*American College of Medical Genetics and Genomics*) de incluir *GLA* da lista de 59 genes cujos achados incidentais devem ser reportados em todos os sequenciamentos clínicos feitos por laboratórios, independente da solicitação do médico atendente ou da idade do paciente (Green et al. 2013; Kalia et al. 2017). Dessa forma, o número de VUS em *GLA* identificados tanto em pacientes com suspeita de DF ou com outras doenças relacionadas ou não deve aumentar de forma significativa, o que pode influenciar o diagnóstico de um grande número de indivíduos.

Para diagnóstico, é fundamental que o efeito dos VUS identificados seja definido. Para isso, Lukas e colaboradores (2013) propuseram a comparação da avalição *in silico* do efeito com o preditor *Polyphen2* com efeitos *in vitro* da superexpressão da enzima e dos níveis de liso-Gb3 para definição do diagnóstico. Recentemente, Schiffmann e colaboradores (2016) propuseram um algoritmo distinto para homens e mulheres com VUS em *GLA* que também envolve o uso de biomarcadores. Em homens, atividade enzimática inferior a 25% do normal seria confirmatório de DF. Acima deste limiar, sugeriram a determinação dos níveis de Gb3 por espectrometria de massas ou imunohistoquimíca em biópsias de órgãos mais afetados, como rim e coração. Os autores comentam que não existe um limiar universal para atividade enzimática, e que a mesma pode variar entre diferentes tecidos. Dessa forma, a utilização do limiar de 25% é arbitrário e não necessariamente reflete corretamente os processos biológicos envolvidos. Em mulheres sem parentes do sexo masculino afetados, é sugerido estudo de expressão *in vitro,* com o mesmo limiar de atividade em homens, ou biópsias para determinação dos níveis de Gb3.

Apesar de serem amplamente utilizadas para avaliação do efeito de mutações *missense* na proteína madura, as técnicas de análise *in silico* e *in vitro* apresentam algumas limitações para o uso indiscriminado em todos os tipos de variantes. Programas de predição de patogenicidade geralmente utilizam informação sobre estrutura da proteína e/ou conservação evolutiva. Porém, existe uma gama muito grande de preditores com algoritmos diferentes que muitas vezes apresentam resultados diversos para uma mesma variante. No exemplo avaliado nesta tese, variante p.Pro323Thr, se obteve 70% concordância entre 7 programas utilizados. Além disso, foram obtidos resultados com alto nível de confiabilidade predita tanto para a classificação patogênica (M-CAP; 0,875 com

limiar de 0,025), quanto para não patogênica (*Polyphen2*: 0,091, limiar >0,15). Essas inconsistências demostram que o uso isolado de uma ferramenta pode levar a conclusões equivocadas e que a avaliação de acurácia entre softwares diferentes pode ser necessária para estabelecimento do protocolo mais adequado a cada gene. Trabalhos em andamento do nosso grupo indicam que, por exemplo, para o gene envolvido na Mucopolissacaridose do tipo I (*IDUA*) o preditor *MutPred* apresenta maior sensibilidade e especificidade dentre 8 preditores avaliados (Pasqualim et al. 2017). Outra limitação é a avaliação de mutações de ponto no peptídeo sinal, região que dirige a proteína para a membrana do retículo endoplasmático, onde é clivado por enzimas específicas e, portanto, não está presente na forma madura da enzima. Para avaliação do efeito de mutações *missense* no peptídeo sinal, programas específicos como *Phobius* (Käll et al. 2004) e *SignalP* (Petersen et al. 2011) devem ser utilizados. Um exemplo prático dessa situação está descrito no anexo VII desta tese (Pasqualim et al. 2015b). Foram identificados 4 pacientes de 3 famílias sul-americanas com diagnóstico bioquímico de Mucopolissacaridose do tipo I mas com fenótipo atípico. Apesar dos níveis aumentados de excreção de glicosaminoglicanos e muito reduzidos de atividade enzimática, nenhum dos pacientes apresentava visceromegalia, uma das manifestações mais clássica da doença. A análise molecular revelou a mutação p.Leu18Pro em homozigose em 3 pacientes e heterozigose em um. Os programas de predição mais frequentemente usados como *Polyphen2*, *MutPred*, *Provean*, *SNP&GO*, SIFT, *Panther*, *PhD-SNP* e *Pmut* classificaram a variante com não patogênica ou patogênica com baixo índice de confiança. Por outro lado, *Phobius* indicou mudança no tamanho do peptídeo e alteração na região hidrofóbica, envolvida no reconhecimento pela partícula de reconhecimento de sinal e eficiência do transporte (Jarjanazi et al. 2008).

Estudos de superexpressão *in vitro* não são indicados para avaliação de mutações envolvendo alterações de *splicing*. Além de mutações intrônicas, também estão incluídas nesta categoria algumas mutações exônicas com exemplos descritos em DF, como p.Ser65Thr e p.Lys213Asn (Okumiya et al. 1995; Lai et al. 2003). Apesar de serem frequentemente denominadas como silenciosas ou neutras, mutações sinônimas também podem ter este efeito patológico. Há um crescente número de estudos que as descrevem como patogênicas por alteração de *splicing* em doenças humanas como osteoporose

autossômica recessiva (Palagano et al. 2017), Doença de von Willebrand (Yadegari et al. 2016), distrofia muscular (Joshi et al. 2014) e diversos tipos de câncer (Soussi et al. 2017; Yamaguchi et al. 2017).

Outros efeitos das mutações sinônimas devem-se ao uso de códons não preferenciais. Embora o código genético seja degenerado, com até 6 códons diferentes para um único aminoácido, os códons não são igualmente distribuídos pelo genoma e existe um viés de uso. A presença de mutações sinônimas altera a distribuição desses códons. Assim, por alteração na disponibilidade de tRNA, pareamento oscilante e interações ente códons adjacentes; altera a eficiência de tradução e consequente alteração na estabilidade do RNAm, dobramento e estrutura tridimensional (Buhr et al. 2016). Além disso, outras propriedades também são alteradas, como: atividade enzimática, interação com drogas e inibidores, perfis de fosforilação, propensão a agregação, interação com a partícula de reconhecimento de sinal (Brule and Grayhack 2017; Livingstone et al. 2017).

A análise de liso-Gb3 e Gb3 é outro critério sugerido para definição do diagnóstico em VUS. Tendo em vista que a magnitude da diferença dos níveis de liso-Gb3 entre pacientes e controles é maior do que de Gb3, este tem sido o principal biomarcador utilizado nos últimos anos. De forma geral, ele apresenta boa correlação com a gravidade das manifestações em hemizigotos com a forma clássica de DF. Ainda que os limites inferiores de variação deste grupo possam se sobrepor a hemizigotos com a forma não-clássica, são invariavelmente superiores aos controles normais (Lukas et al. 2013; Smid et al. 2015; Talbot et al. 2017; Nowak et al. 2017b). Porém, em hemizigotos com forma não-clássica e heterozigotas, o aumento nos níveis desse biomarcador é modesto, com sobreposição aos níveis normais (Smid et al. 2015; Nowak et al. 2017a; Nowak et al. 2017b). Consequentemente, liso-Gb3 pode não ser informativo em alguns casos, principalmente em heterozigotas, onde níveis normais não excluem o diagnóstico de DF nem se correlacionam com sintomas (Smid et al. 2015).

Além disso, aumentos nos níveis dessas moléculas não são características exclusivas da DF. Níveis plasmáticos significativamente elevados de liso-Gb3 podem ser identificados em pacientes com Doença de Gaucher (DG) tipo I não tratados, na mesma magnitude de

pacientes com a forma clássica de DF (Ferraz et al. 2016). Os autores sugerem que a alteração na especificidade da enzima ceramidase ácida, que seria responsável pela deacetilação de Gb3 em liso-Gb3, como possível causa. Um estudo recente, não detectou essa elevação em pacientes com DG ou outras doenças lisossômicas (Talbot et al. 2017), porém não há informação relativa ao tratamento. De forma similar, níveis urinários de Gb3 em pacientes sem mutações em *GLA* e com diferentes doenças cardíacas comuns em DF (arritmias, doença arterial coronariana, etc) também pode ser tão elevados quanto os de pacientes com a forma clássica (Schiffmann et al. 2014).

Conforme o exposto acima, pode-se concluir que a definição do significado clínico de novas variantes pode ser uma tarefa complexa. Os algoritmos gerais sugeridos pelos autores citados acima são frequentemente seguidos e podem levar a resultados satisfatórios em muitos casos. Porém, a metodologia necessária a ser aplicada em cada caso pode requerer algumas modificações, de forma que deve ser avaliada individualmente. Além dos testes *in vitro, in silico* e comparações com frequências populacionais, a avaliação clínica abrangente do paciente e histórico familiar é fundamental. Isso fica mais evidente em casos de mulheres com suspeita de DF, onde a avaliação de parentes afetados do sexo masculino pode reduzir o tempo de diagnóstico, além de facilitá-lo.

A identificação da mutação específica pode auxiliar no manejo dos pacientes e direcionar o tratamento para o uso de terapia de reposição enzimática ou terapias alterativas, como as chaperonas em pacientes com mutações *missense* específicas. De forma geral, pacientes com variantes *nonsense* ou que levem a troca de fase de leitura tem menor atividade residual da enzima e apresentam fenótipo clássico. Já os pacientes com mutações *missense* que retenham maior atividade residual normalmente apresentam a forma não-clássica, com fenótipo mais brando. Porém, assim como a heterogeneidade alélica, a heterogeneidade fenotípica vista em DF é muito grande. Existe grande variabilidade no tipo específico de sintoma e na intensidade de manifestação.

Esta amplitude fenotípica é mais comumente (e com maior magnitude) observada em mulheres. Por ser uma doença ligada ao X, muitos autores consideram a inativação não-

aleatória do cromossomo X como seu principal motivo. O primeiro relato sobre este tema em DF identificou associação entre altos níveis de inativação preferencial (acima de 95%) e o fenótipo clínico em fibroblastos de gêmeas monozigóticas com fenótipos discordantes (Redonnet-Vernhet et al. 1996). Em seguida, níveis de inativação foram correlacionados com a gravidade do fenótipo em 4 heterozigotas de uma mesma família (Morrone et al. 2003).

Um estudo europeu com 38 heterozigotas de famílias diferentes encontrou inativação não-aleatória do X em 11 pacientes (28%). Destas, 6 com até 30 anos e o alelo normal inativo não apresentavam sintomas graves; 4 tinham entre 40 e 60 anos e sintomas graves e 1 paciente era idosa com sintomas leves. Estes resultados foram similares em análises de diferentes tecidos: leucócitos, células do sedimento urinário e células do epitélio bucal. Os autores relacionaram a inativação com um aumento na taxa de progressão da doença (escore de gravidade / idade) (Dobrovolny et al. 2005).

Um estudo subsequente com 28 heterozigotas não encontrou relação entre o nível de enviesamento com o fenótipo clínico (Maier et al. 2006). A taxa de enviesamento acima de 75% encontrada foi similar ao visto na população normal, cerca de 40% (Busque et al. 2009). Esses autores relacionaram o aparecimento de sintomas e a progressão da doença com um mecanismo de seleção clonal e ineficiência de correção cruzada, definida como a capacidade de uma célula secretar a enzima e a mesma ser captada por uma célula deficiente. Diferenças de taxas de crescimento entre populações de células com diferentes alelos inativados podem levar à desvantagem seletiva. Diferenças muito pronunciadas levariam a perda precoce das células com o alelo mutante. Com diferenças mais sutis, essa perda seria gradual e o aparecimento dos sintomas seria tardio, o que explicaria o fato de algumas mulheres apresentarem sintomas mais intensos com o passar do tempo. De forma similar, um estudo em 77 heterozigotas também relatou 30% de enviesamento e ausência de correlação com fenótipo (Elstein et al. 2012). Juchniewicz e colaboradores (2017) descreveram em um grupo de 11 pacientes sintomáticas, apenas 9% de enviesamento e ausência de correlação com sintomas.

Recentemente um estudo avaliou o enviesamento em 56 heterozigotas (Echevarria et al. 2016). Os resultados em leucócitos, fibroblastos, células do sedimento urinário e células do epitélio bucal foram similares, indicando correlação entre taxas de inativação em diferentes tecidos. A frequência de inativação acima de 75% foi de 28%: 10% com expressão do alelo normal e 18% com expressão do alelo mutado. Os autores propuseram processos patológicos diferentes de acordo com o nível de inativação preferencial. Na maioria das heterozigotas a inativação seria aleatória e a doença se desenvolve progressivamente devido aos efeitos deletérios do acúmulo lisossomal e consequente ativação de cascatas patogênicas, sem efeitos de seleção clonal ou outra forma de pressão seletiva. Porém, na pequena parcela onde há inativação enviesada, a manifestação da doença dependeria da direção do enviesamento. Mulheres com o alelo normal predominantemente expresso teriam poucos ou nenhum sintoma. As que apresentassem expressão predominantemente do alelo mutado, teriam fenótipo grave.

Em uma abordagem diferente, Fuller e colaboradores (2015) propuseram que a ineficiência na correção cruzada é o motivo pelo qual heterozigotas são afetadas, apesar de produzirem enzima funcional em algum nível. Correção cruzada é o mecanismo no qual a deficiência enzimática de uma célula afetada é corrigida pela captação da enzima funcional produzida por outra célula. A ineficiência proposta por Fuller seria resultado da quantidade insuficiente de secreção da enzima funcional ou da incapacidade de absorção adequada pelas células afetadas. Apesar de ser um mecanismo plausível, algumas ressalvas podem ser feitas com relação a este trabalho. Os autores utilizaram fibroblastos, que não estão entre os tipos celulares mais afetados em DF. Além disso, a absorção da enzima se dá por diferentes receptores (M6PR, receptor de manose, megalina, sortilinia), cuja distribuição varia entre os tecidos (Prabakaran et al. 2011; Prabakaran et al. 2012; Marchesan et al. 2012; Meng et al. 2016; Shen et al. 2016). Shen e colaboradores (2016), por exemplo, demonstraram *in vitro* que fibroblastos são mais dependentes da via de M6PR do que células endoteliais. Dessa forma, esses resultados podem não ser replicados em outros tipos celulares. Finalmente, é possível que exista um desbalanço entre a taxa com a qual liso-Gb3 (pequeno e solúvel em água) se difunda e estimule a produção de Gb3

das células vizinhas com a velocidade com a qual níveis normais em 50% das células pode reverter. Nesse caso, a indução-cruzada superaria a correção.

Dessa forma, não há consenso na literatura sobre os motivos que acarretam a variabilidade vista em heterozigotas. Além disso, embora sejam mais evidentes em mulheres, variações fenotípicas entre os tipos específicos e gravidade de sintomas também são descritas em hemizigotos com idades próximas em diversas famílias (MacDermot et al. 2001; Elliott et al. 2006) (MacDermot et al. 2001; Elliott et al. 2006; Gomez et al. 2012; Cammarata et al. 2015; Adalsteinsdottir et al. 2017; Tuttolomondo et al. 2017). Assim, fica evidente que outros fatores além da alteração genética primária em *GLA* e da inativação preferencial do cromossomo X devem alterar o curso da doença.

Além do efeito modulador da expressão dos polimorfismos da 5'UTR descritos acima, a influência de outros genes modificadores já foi descrita em DF e outras DLs. Na Doença de Gaucher, o SNP rs11986414 do gene *CLN8*, com frequência mundial de 0,42 (Lek et al. 2016), foi associado a um efeito protetor contra os danos cognitivos da forma grave da doença em judeus Ashkenazi. Da mesma forma, a superexpressão do gene *CLN8* foi associado com desenvolvimento mais brando dessa esfingolipidose (Zhang et al. 2012). Este gene codifica uma proteína de membrana sem função definida que é possivelmente envolvida no tráfego e monitoramento de esfingolipídios e cuja deficiência causa lipofuscinose ceróide neuronal.

Não foi detectado um efeito significativo dos polimorfismos de 5'UTR na amostra estudada que pudesse contribuir para uma correlação genótipo-fenótipo. Dessa forma, buscou-se uma abordagem distinta para a identificação de possíveis genes modificadores a partir da análise de exoma em uma família com heterogeneidade clínica (capítulo 3.4). Para que o desenho experimental fosse válido era preciso incluir apenas indivíduos com informação clínica detalhada, tanto na família quanto na coorte de validação. Por isso, o número de amostras que puderam ser incluídas nesta coorte foi reduzido. Assim, para evitar uma redução ainda maior no número amostral pela ausência de exames de outros sistemas e maior estratificação com a inclusão de mais grupos, neste estudo se optou por focar em uma avaliação mais completa de um fenótipo mais específico de DF. Tendo em

vista que as manifestações cardíacas são a principal causa de morte em ambos os sexos (Linhart et al. 2007; Mehta et al. 2009; Waldek et al. 2009), o fenótipo cardíaco foi selecionado para estudo.

A família analisada neste estudo exemplifica perfeitamente a variação fenotípica em DF. As 3 irmãs da geração II, que apresentam os extremos fenotípicos, estão acima das idades média para aparecimentos de sintomas cardíacos, cerebrovasculares e renais (Waldek et al. 2009). Além disso, o padrão de inativação do cromossomo X foi avaliado com o ensaio HUMARA e nenhuma apresentou enviesamento. Apesar desta avaliação ter sido realizada em amostras de sangue e não nos tecidos alvo, há correlação dos níveis de enviesamento entre diferentes tecidos (Echevarria et al. 2016).

Na análise de exoma foram identificados 24 potenciais genes modificadores. Destes, 5 foram escolhidos para validação na coorte de pacientes com DF baseados na sua relação com doença ou desenvolvimento cardíaco. Não houve associações estatisticamente significativas entre as variantes identificadas com os grupos na coorte de validação. Porém, quando a comparação foi estendida à população brasileira idosa e saudável, se observou aumento significativo da variante rs3188055 do gene inositol polifosfato 5-fosfatase (*INPP5F)* no grupo de pacientes com sintomas cardíacos. Este gene é envolvido na maturação e reciclagem dos endossomos e regula negativamente a hipertrofia cardíaca, um dos sintomas mais frequentes de DF. Portanto, pode apresentar um efeito sinérgico deletério com os danos causados pelo acúmulo de Gb3/liso-Gb3 visto em DF. Devido ao tamanho amostral limitado, é necessária a replicação deste estudo em coortes maiores para confirmação destes achados. Do mesmo modo, a realização de estudos funcionais pode auxiliar na identificação dos mecanismos de interação entre os genes *INPP5F* e *GLA* e o desenvolvimento de sintomas cardíacos.

Após identificação de um possível gene modificador do fenótipo cardíaco, buscou-se entender melhor os possíveis mecanismos moleculares envolvidos na variabilidade de desenvolvimento de sintomas de DF em mulheres. Existem alguns estudos de proteômica na literatura que buscam elucidar os mecanismos fisiopatológicos da doença. Alguns avaliam diferenças entre pacientes com DF e controles saudáveis com relação a

marcadores no plasma (Hollander et al. 2015), células mononucleraes sanguineas (Cigna et al. 2013) ou urina (Vojtová et al. 2010). Outros também avaliaram o impacto da TRE em amostras de urina (Kistler et al. 2011; Manwaring et al. 2013; Matafora et al. 2015) ou soro (Moore et al. 2007). Porém, nenhum estudo é focado nas diferenças que existem em pacientes com diferentes manifestações de DF, as quais podem auxiliar no entendimento da expressividade variável da doença. Dessa forma, o artigo do capítulo V avaliou, através da análise do transcriptoma com microarranjos, a existência de perfis de expressão específicos para as principais manifestações de DF (cardíacas e renais) em mulheres. A limitação do estudo ao gênero feminino se baseou em estudos prévios que indicam a existência de diferenças significativas de expressão gênica e proteica em DF entre os gêneros quando comparados a controles normais e em resposta a TRE (Hollander et al. 2015; Ko et al. 2016). Essa diferença de expressão também é vista em genes localizados em todos os cromossomos na população normal (Jansen et al. 2014). Além disso, o gênero feminino é o que apresenta biomarcadores menos eficientes atualmente.

Neste estudo foram incluídas 13 heterozigotas, provenientes de famílias com mutações associadas ao fenótipo clássico que apresentavam grande variabilidade fenotípica. Seis pacientes apresentavam manifestações restritas ao fenótipo renal; 3 ao fenótipo cardíaco e 4 apresentavam os dois tipos de manifestações. É importante salientar que qualquer alteração em um dos dois sistemas, inclusive pequenos valores de proteinúria e albuminúria, eram suficientes para inclusão naquele fenótipo, pois não há como excluir a relação causal com DF. Novamente, os critérios de inclusão previam pacientes com análises detalhadas dos dois sistemas, sem dados ausentes, o que restringiu o número de participantes. O número reduzido de pacientes em cada grupo é uma das limitações deste trabalho, que acarretou em valores ajustados de P elevados nas comparações feitas (Pawitan et al. 2005). Porém, a abordagem utilizada onde se selecionaram os transcritos concomitantemente significativos em 2 comparações par a par e que apresentavam as maiores diferenças entre grupos resultou na ausência de validação de apenas 1 dos 10 genes selecionados.

Nas pacientes com fenótipos não clássicos (apenas renal ou apenas cardíaco) foi identificada uma quantidade reduzida de transcritos distintivos dos grupos, quando

comparado às pacientes clássicas. Além disso, a magnitude das diferenças entre as pacientes clássicas e não clássicas foi maior do que entre as não clássicas entre si: variações de até 64x versus 16x, respectivamente.

No grupo dos indivíduos apenas com sintomas renais foi identificada regulação negativa dos genes expressos principalmente em granulócitos: molécula de adesão celular relacionada ao antígeno carcinoembrionônico 8 (*CEACAM8)* e α-defensinas A, A1 e 3 (*DEFA/A1B/A3).* Isso significa que a superexpressão destes genes pode estar relacionada com a patofisiologia dos sintomas cardíacos. Essa hipótese é corroborada pelo fato da suprexpressão de ambos genes já ter sido relacionada com declínio da função cardíaca avaliada pela fração de ejeção do ventrículo esquerdo (Todorova et al. 2016). Além disso, níveis plasmáticos elevados de α-defensinas já foram propostos como biomarcadores de doença coronariana (Maneerat et al. 2016).

Nas pacientes que apresentam exclusivamente doença cardíaca foi identificada significativa repressão da expressão do pseudogene *PRKXP1* (Proteína quinase ligada ao X, Pseudogene 1). Novamente, além de ser uma característica distintiva deste grupo, este dado também pode significar que a superexpressão de *PRKXP1* está relacionada com o desenvolvimento de manifestações renais. A expressão deste pseudogene pode reduzir a expressão de seu gene parental, que é fundamental no desenvolvimento do tecido renal, podendo inclusive restaurar a função renal alterada por mutações no gene da doença renal policística (PKD1) (Li et al. 2002; Li et al. 2008; Li et al. 2011; Huang et al. 2016).

Finalmente, em pacientes com os dois tipos de manifestações há superexpressão de diversos genes induzidos por interferon do tipo I: *IFI44, IFI44L, IFIT1, HERC5, EIF2AK2 e RSAD2*. Apesar da DF, assim como as demais DLs, ter um componente inflamatório importante com inflamação sistêmica crônica (Rozenfeld and Feriozzi 2017), os mecanismos envolvidos nessa resposta imune ainda não foram completamente elucidados. Este aumento poderia ser induzido tanto pela interação de Gb3 com TLR4, que pode induzir a expressão de interferons (IFN) do tipo I, quanto pela interação direta de Gb2 e Gb3 com o receptor comum dos IFN tipo I (Ghislain et al. 1994; Khine and Lingwood 2000; Kawai and Akira 2010).

O tecido utilizado neste estudo não inclui as células alvo afetadas na DF, como podócitos e cardiomiócitos, cuja coleta envolve procedimentos invasivos. Porém, a metodologia aplicada permitiu a identificação de perfis de expressão distintivos de diferentes manifestações da doença de forma muito menos invasiva. Portanto, esses achados tem maior potencial aplicação na prática clínica, como possíveis biomarcadores de prognóstico ou seguimento. Assim, os perfis identificados poderiam auxiliar no manejo clinico de pacientes, principalmente daqueles que apresentam VUS.

Entre as perspectivas de seguimento deste trabalho está avaliação da expressão de pequenos RNAs não codificadores (sncRNAs), como microRNAs (miRNAs). Estas moléculas podem modular a expressão dos transcritos analisados, podendo ser parcialmente responsáveis pelos perfis de expressão identificados. Estes experimentos já estão em andamento com a utilização do microarranjo GeneChip miRNA 4.0 (Thermo Fischer Scientific). Os dados referentes a expressão dos sncRNAs, assim como suas interações com os dados de transcriptomas já apresentados, serão incluídos no artigo do capítulo V desta tese. Além disso, os níveis proteicos dos genes alterados serão avaliados por western blot, ELISA ou Luminex.

Em conjunto, os trabalhos apresentados nesta tese contribuem para uma análise ampla dos diferentes aspectos envolvidos na DF, desde os desafios para o diagnóstico de mulheres, as distintas abordagens para avaliação molecular até a busca por elementos que auxiliem no entendimento da variabilidade fenotípica apresentada pelos pacientes. Espera-se que essas informações possam contribuir para um melhor manejo dos pacientes e para uma avaliação efetiva das alternativas terapêuticas existentes.

5. Conclusões

O método para diagnóstico enzimático proposto utiliza medições combinadas de atividade em leucócitos e plasma, sendo capaz de reduzir em 35% o número de pacientes com suspeita de Doença de Fabry direcionadas ao diagnóstico genético.

Para triagem de mutações em indivíduos com suspeita de Doença de Fabry foi proposto um método que utiliza a análise por HRM do gene *GLA*. Esta metodologia consiste em uma adaptação da reação convencional já utilizada para sequenciamento de Sanger, que não necessita de manipulações adicionais pós-PCR e que foi capaz de identificar todas as variantes da população brasileira coletadas em tubos com EDTA.

Os pacientes brasileiros com Doença de Fabry apresentam principalmente mutações do tipo *missense*, presentes em famílias únicas.

O gene inositol polifosfato 5-fosfatase (*INPP5F*), envolvido na maturação e reciclagem dos endossomos e regulação negativa da hipertrofia cardíaca, foi identificado como um possível gene modificador do fenótipo cardíaco em pacientes brasileiros com Doença de Fabry.

A partir da análise de transcriptoma, foram identificados perfis de expressão específicos de doença cardíaca e renal em heterozigotas com Doença de Fabry. Nas pacientes que apresentam exclusivamente a doença cardíaca, foi identificada significativa repressão da expressão do pseudogene *PRKXP1* (Proteína quinase ligada ao X, Pseudogene 1). Em pacientes que apresentam exclusivamente doença renal, há regulação negativa dos genes molécula de adesão celular relacionada ao antígeno carcinoembrionônico 8 (*CEACAM8)* e α-defensinas A, A1 e 3 (*DEFA/A1B/A3).* Em pacientes com dois tipos de manifestações, há superexpressão de diversos genes induzidos por interferon do tipo I.

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7. Anexos

Nesta seção estão incluídas produções bibliográficas produzidas durante o período do doutorado não diretamente ligadas ao tema principal da tese e/ou de autoria principal de terceiros e a carta de aprovação ética do projeto envolvido.

I. Lysosome: The Story Beyond the Storage.

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Lysosome: The Story Beyond the Storage

Ursula Matte, BSc, PhD[1,2,3] and Gabriela Pasqualim, BSc, MSc[1,2,3]

Abstract

Since Christian de Duve first described the lysosome in the 1950s, it has been generally presented as a membrane-bound compartment containing acid hydrolases that enables the cell to degrade molecules without being digested by autolysis. For those working on the field of lysosomal storage disorders, the lack of one such hydrolase would lead to undegraded or partially degraded substrate storage inside engorged organelles disturbing cellular function by yet poorly explored mechanisms. However, in recent years, a much more complex scenario of lysosomal function has emerged, beyond and above the cellular "digestive" system. Knowledge on how the impairment of this organelle affects cell functioning may shed light on signs and symptoms of lysosomal disorders and open new roads for therapy.

Keywords

lysosomal biology, autophagy, lysosomal disorders, lysosomal cell death

Lysosomal Composition and Biogenesis

Lysosomes are membrane-bound compartments formed by a lipid bilayer that contains a number of characteristic proteins, such as lysosome-associated membrane proteins (LAMPs) 1 and 2, lysosome integral membrane protein (LIMP2), and tetraspanin CD63.[1] Their biogenesis and functions are shared by lysosome-related organelles (LROs) whose composition and denomination vary according to cell type. Such organelles include melanosomes in melanocytes, lytic granules in lymphocytes, and delta granules in platelets, among others. Both lysosomes and LRO participate in many cellular processes such as signal transduction, antigen presentation and immune response, cell death, autophagy, and membrane repair.[2] Lysosome-associated membrane protein cytosolic tails interact with microtubules, thus having an important role in lysosome exocytosis, dynamics, and protein translocation.[1]

Lysosomal biogenesis relies on a complex trafficking mechanism from the trans-Golgi network (TGN) that is responsible for sorting the intraluminal and membrane-bound proteins that form the lysosomes. Acid hydrolases are modified with mannose 6-phosphate (M6P) residues, allowing their recognition by M6P receptors in the TGN and ensuing transport to the endosomal/lysosomal system. On the other hand, other soluble enzymes and nonenzymatic proteins are transported to lysosomes in an M6P-independent manner by LIMP-2 or sortilin. Sorting of cargo receptors and lysosomal transmembrane proteins requires sorting signals present in their cytosolic domains.[3]

Lysosome function is heavily dependent on its fusogenic and acidic properties. The first makes it possible for the organelle to merge not only with the endocytic vesicle but also with the autophagosome and the plasma membrane. The latter is responsible for regulating the optimal pH for substrate degradation, a measure to ensure that the lytic pathway is only activated at the precise moment. Both characteristics are due to the presence of families of lysosome membrane proteins such as proton-pumping ATPases or vesicle-associated membrane proteins (VAMPs) and other soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE). It is important to recognize that the addition of such proteins changes the properties of the compartment itself. For instance, acidity can be altered by the delivery of vesicular type H+ ATPase complex, whereas fusogenicity can be altered by the delivery of VAMP7. Therefore, the multiple delivery pathways must act together to allow the regulated and sequential deposition of lysosomal components.[1] The increased acidic pH is a transition marker

[1] Gene Therapy Center, Hospital de Clínicas de Porto Alegre, Brazil
[2] Post-graduation Program on Genetics and Molecular Biology
[3] Genetics Department, Universidade Federal do Rio Grande do Sul, Brazil

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Corresponding Author:
Ursula Matte, Gene Therapy Center, Hospital de Clinicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul 90040-060, Brazil.
Email: umatte@hcpa.edu.br

from early to late endosomal vesicles (multivesicular bodies) and then to lysosomes.[4]

Gene Expression

A body of work led by Andrea Ballabio group identified transcription factor EB (TFEB) as a master regulator of genes related to lysosome biogenesis, acidification, and function.[5] Moreover, they characterized a network of coregulated genes involved in many cellular processes directly or indirectly related to lysosomes (including autophagy, vesicle-mediated transport, and cell response to stress). This Coordinated Lysosomal Expression and Regulation (CLEAR) network includes over 470 genes with a consensus TFEB-binding site.[6] TFEB is a basic helix–loop–helix (bHLH) leucine zipper transcription factor that also regulates autophagy-related genes. Phosphorylated TFEB is retained at the cytoplasm, whereas nuclear translocation is regulated by sensing mechanisms via mammalian (or mechanistic) target of rapamycin complex 1 (mTORC1, see below).[7,8] Another transcription factor from the same family as TFEB, TFE3, was also shown to regulate the expression of genes associated with autophagy and lysosomal biogenesis, even though not binding to CLEAR motifs, but still regulated by mTORC.[9] Evidence suggests that increasing TFEB expression and nuclear translocation may result in reduced storage in lysosomal diseases and increased autophagic activity that is beneficial for a variety of neurodegenerative diseases.[10] Similar preliminary results were also shown for TFE3.[9]

The TFEB and the TFE3 are part of a family of 4 closely related bHLH leucine zipper transcription factors also composed by Microphtalmia-associated transcription factor (MITF) and TFEC, which can bind target DNA both as homodimers or heterodimers.[11] Transcription factor E3 or TFEB proteins also regulate several molecular pathways in carcinogenesis, other than metabolism regulation, including the activation of transforming growth factor β, E-cadherin expression, and retinoblastoma-dependent cell cycle arrest.[12] The MITF was recently reported as able to increase expression of late endosomal proteins, such as Rab7, LAMP1, and CD63 in a cell model of melanoma. These late endosomes, however, were not functional lysosomes as they were less active in proteolysis. However, the accumulation of multiple vesicular bodies led to enhanced Wnt signaling generating a positive-feedback loop that contributes to the proliferation of melanoma.[13]

Brignull et al[14] using expression correlation analyses found that signal transducer and activator of transcription 6 (Stat6)/interleukin (IL)-4 are novel regulators of lysosome-specific genes. The Stat6 is itself regulated by IL-4, and these results were further confirmed in primary mouse macrophages derived from Stat6 deficient or wild-type animals. The authors suggest that in cells cultured with IL-4, the expression of 103 lysosomal genes was dependent on Stat6, reflecting 40% of the known lysosomal proteome and 54% of lysosomal genes expressed in this cell type. Furthermore, Stat6, under IL-4 induction, seems to control 14 of the 15 different subunits of vacuolar H^+ ATPase thus proving fundamental for lysosomal pH maintenance and therefore lysosomal function.

Recently, a database of human lysosomal genes (http://lysosome.unipg.it) and its regulation have been established to collect information on the several genes related to lysosomal biogenesis and function. Genes are included based on the presence of TFEB-binding sites and the presence of the nucleotide motif (GTCACGTGAC) characteristic of genes belonging to the CLEAR network. The database focuses not only on lysosomal genes but also on putative microRNAs that can act as their posttranscriptional regulators.[15]

Lysosomal Function in Substrate Degradation

Lysosomal Storage Disorders

In the field of medical genetics, lysosomes are primarily associated to lysosomal storage disorders (LSDs), a group of more than 50 inborn errors of metabolism that has an incidence of about 1:7000.[16] Although most LSDs are due to deficiencies in lysosomal hydrolases, defects in membrane-associated proteins and nonenzymatic lysosomal proteins have also been described. Soluble lysosomal enzymes, such as glycosidases, sulfatases, phosphatases, and lipases, are involved in substrate degradation and are crucial to the turnover of cellular components. Other causes of LSD include errors in enzyme targeting (such as in mucolipidosis II and III), errors in posttranslational processing (multiple sulfatase deficiency), and activator deficiencies (as saposin deficiencies). Also, defects in transport proteins are related to LSDs (such as Niemann-Pick disease type C1 [NPC1], ceroid-lipofuscinosis neuronal 3 [CLN3], and cystinosis).[1,17,18]

For many years, a simplistic mechanism of substrate accumulation leading to disturbance of cell homeostasis was applied to LSD. Over the years, however, and especially from the 1990s with the development of animal models, a more complex understanding of these diseases has emerged. Disturbances in lysosomal degradation of macromolecules were viewed not isolated but in conjunction with other lysosomal functions in the cell—and in some instances, the very study of LSD contributed to the knowledge of lysosome biology as well.[19] More than grouping LSD by the type of stored substrate, primary or secondary storage, and enzyme or nonenzyme defects, researchers are recognizing the many common pathogenic cascades elicited by the dysfunction in one or many cellular processes involving the lysosomal compartment.[17,20] Moreover, phenocopies of lysosomal diseases can be made by silencing RagA/B in mice, showing that RagA/B GTPases are key regulators of lysosomal function and cardiac protection.[21]

Lysosomal Functions Other Than Substrate Degradation

Signal Transduction

Endocytosis of ligand-bound transmembrane receptors is a general mechanism by which cells control signal transduction.

Tyrosine kinase receptors, for instance, are internalized following ligand-induced activation. The specific internalization pathway may vary between clathrin-dependent and clathrin-independent routes, depending on the specific type of receptor.[22] For Epidermal Growth Factor Receptor (EGFR), the canonical process elicited by EGF binding initiates with the receptor migrating to a clathrin-coated pit, where it is internalized by endocytosis in a clathrin-coated vesicle. Afterward, the pathway includes migration through early and late endosomes, with ligand release. Part of EGFR enters the recycling pathway, being shuttled back to the plasma membrane. The remaining receptor molecules and ligands are digested in the lysosomes. Interestingly, decorin, a small leucine-rich proteoglycan found in the extracellular matrix, changes the endocytic pathway upon EGFR ligation. Internalization occurs via caveolae-mediated endocytosis, and EGFR is transferred directly to late endosomes for final degradation. This leads to a decreased number of EGF-binding sites on cell surface, thus promoting growth suppression and anti-oncogenic activities.[23]

Antigen Presentation and Immune Response

Antigen presentation and immune response show lysosome involvement. Antigen presentation involves processing foreign peptides for major histocompatibility complex (MHC) class II presentation, and the role of lysosomes spans from epitope liberation to the processing of MHC class II molecules. Antigens that enter the cell via endocytosis move through the endo-lysosomal pathway by a series of pH changes and vesicle aggregation. As the antigen is processed in the early endosome, MHC class II molecules are transferred from the TGN into the nascent late endosome/early lysosome vesicle. There, proteases perform a stepwise maturation of the invariant chain of MHC class II that prepares the molecule for receiving the foreign peptide. Finally, epitopes are mounted on the MHC molecules for antigen presentation. The whole vesicle then moves along microtubules toward the cell membrane where MHC class II molecules bound to epitopes are exposed for $CD4^+$ T cells in the cell surface. A very rigid mechanism of control based on low levels of protease expression and slow acidification kinetics has a profound impact on antigen presentation.[24]

In addition, cells of the immune system, such as T cells and Natural Killer (NK) cells, possess secretory lysosomes or LROs, which are also called lytic granules. These contain not only MHC class II molecules but also Fas ligand, granzyme A, and perforin. Upon activation, secretory lysosomes move along microtubules toward the plasma membrane in direction to the target cell. The lytic granules are then released to the immunological synapse that is formed between the lymphocyte and the target cell.[25]

Autophagy

Lysosomes are also involved in autophagy. Autophagy is a vacuolar self-digesting mechanism responsible for the removal of long-lived proteins and damaged organelles. It has a role in quality control of cellular components, degrading aberrant protein aggregates, and defective organelles, as depolarized mitochondria.[26] It is also essential in metabolic stress situations, as nutrient deprivation, providing energy by recycling components for protein synthesis. In mammalian cells, autophagy can be classified in 3 types of events—microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Chaperone-mediated autophagy involves direct engulfment of proteins with KFERQ motifs contents by the lysosomal membrane or interaction with specific proteins, such as heat shock protein (hsp)70 and LAMP2A.[27] Macroautophagy, commonly referred to simply as autophagy, is the most well-studied type and requires the formation of an autophagosome and its subsequent fusion to endosomes–lysosomes.[28,29]

The autophagy mechanism itself may be directly involved in the pathogenesis of lysosomal disorders. In cystinosis, caused by defects in cystine transporter cystinosin (CTNS), there is a decrease and mislocalization of LAMP2A and consequent impairment of CMA. Correction of CMA is only achieved by the expression of *CTNS* and not by the decrease in lysosomal storage of cysteine, indicating the independence of these processes.[18]

One of the major suppressors of autophagy is the mTORC1 signaling pathway. In normal cellular conditions, in the presence of sufficient nutrients, mTORC1 is recruited to the lysosomal membrane and activated by another complex formed by v-ATPase, Ragulator, and Rag GTPases. It then phosphorylates TFEB at Ser142 and Ser211 and TFE3 at Ser321, which induces ligation with 14-3-3 proteins and retention at the cytoplasm.[8,9,30] In situations of starvation and lysosomal stress, mTORC1 is released from lysosomes. The unphosphorylated TFEB and TFE3 are able to translocate to the nucleus and directly induce transcription of the CLEAR network genes, including several genes related to LSDs, as discussed above.

Other kinases also interfere with TFEB signaling, and extracellular signal-regulated kinase 2 (ERK2) has a similar effect to mTORC1 on TFEB. However, in osteoclasts, ERK2 phosphorylation of TFE3 induces its promoter activity on target genes.[9,29]

The binding of Rag GTPases to the lysosomal vacuolar-type H^+-ATPase is thought to be a way for the Rags to sense the amino acid content inside lysosomes and, by extension, the nutritional state of the cell.[31] It is also known that TFEB and MITF interact directly with Rags, and that the binding is dependent on the nucleotide binding state of Rags.[32] This mechanism is important in many disease-related situations, such as cancer. Pancreatic ductal adenocarcinoma, for instance, heavily depends on MITF/TFE family of transcription factors (MITF, TFE3, and TFEB) that induce high levels of lysosomal catabolic function essential for tumor growth.[33]

Cell Death

Besides participating in the autophagic cell death, lysosomes are associated with the 2 other major types of cell death—apoptosis and necrosis.[34] It has been shown that in response

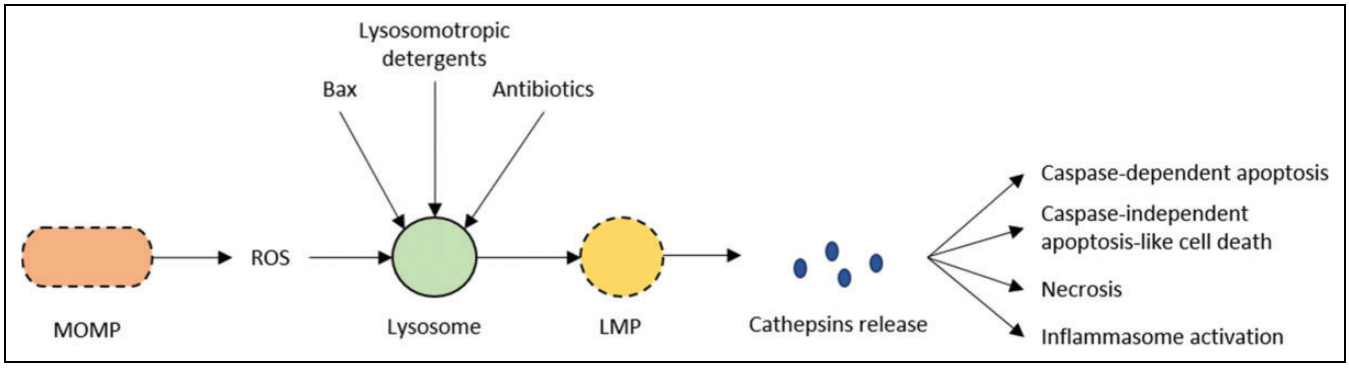

Figure 1. Lysosomes and cell death. Proapoptotic molecules (as Bax, lysosomotropic detergents, and some antibiotics) directly target the lysosomal membrane, causing lysosomal membrane permeabilization (LMP). The mitochondrial outer membrane permeabilization (MOMP) and consequent reactive oxygen species (ROS) generation also affect the lysosomal membrane, being the major physiologically relevant route to late-onset LMP. It leads to the release of cathepsins, which mediate caspase-dependent apoptosis, caspase-independent apoptosis-like cell death, and necrosis. It can also activate the inflammasome, resulting in caspase 1-dependent pyroptosis.

to endogenous or exogenous stress, the lysosomal membrane can leak, leading to the release of catabolic hydrolases (cathepsins) that mediate caspase-dependent apoptosis, caspase-independent apoptosis-like cell death, or even necrosis[35] (Figure 1). Apparently, the extent of lysosomal membrane permeabilization (LMP) is related to the ensuing type of cell death, with a limited release of lysosomal contents triggering apoptosis or apoptosis-like cell death, whereas generalized lysosomal rupture results in rapid cellular necrosis.[36]

Different agents are reported to induce LMP. Proapoptotic molecules, such as Bax, directly target the lysosomal membrane as well as lysosomotropic detergents and some antibiotics.[37] Lysosomal membranes are particularly sensitive to reactive oxygen species (ROS)-mediated damage, which can be generated by destabilized mitochondria leading to peroxidation of membrane lipids. The mitochondrial outer membrane permeabilization (MOMP), which is accompanied by ROS generation, seems to be the major physiologically relevant route to late-onset LMP.[35] In addition, osmotic lysis or direct destabilization by surfactant activity is also known to cause LMP. Cathepsin release after LMP results in both caspase-dependent and caspase-independent pathways of apoptosis.[34] It can also activate the inflammasome, resulting in caspase 1-dependent pyroptosis.[37] Moreover, LMP also induces the formation of protein aggregates in classic late-infantile CLN2.[38]

Lysosomal cell death occurs in tissue remodeling, immune response to intracellular pathogens, aging, and neurodegenerative diseases. The limitation of neutrophil population during infection and inflammation is mainly achieved by LMP.[37] Aging alters the physical and chemical properties of lysosomes, rendering them more sensitive to stress, and thus to LMP[39] and neurodegenerative conditions such as Parkinson, Alzheimer, and Huntington diseases.[37] It also mediates death of cancer cells, and a target for cancer therapy uses lysosomotropic drugs that enhance LMP. Dielschneider et al[40] showed that patients with chronic lymphocytic leukemia (CLL) are more susceptible to LMP due to increased levels of sphingosine and the overexpression of sphingosine-1-phosphate phosphatase 1 compared to normal B cells. The CLL cells were found to be sensitive to a lysosomotropic agent, despite other prognostic factors. Similarly, in NPC, the storage of sphingosine leads to LMP and cell death, particularly in the cerebellum, with consequent neurodegeneration.[41] Cell death by LMP has also been reported in mucopolysaccharidosis type I.[42] Moreover, since this mechanism can be induced by immunological reactions and ROS-mediated damage, common characteristics of LSDs, it may be more frequent than reported and an important factor in several diseases.

Even though lysosomal cell death is being increasingly studied and viewed as a potential alternative for anticancer therapies, many doubts remain about the underlying mechanisms. For instance, it is not known whether LMP results in the selective release of certain cathepsins and whether a subpopulation of lysosomes is especially susceptible to LMP.[36] Moreover, it remains to be elucidated whether LMP is a primary event in the cascade of cell death, upstream of MOMP, and other signals or whether it is an intermediate mechanism. Finally, even though Bid is considered the main physiological substrate of lysosomal cathepsins, there is evidence of cathepsin-independent signaling in lysosomal-associated cell death.[35]

Other Functions

Lysosomes are also involved in cell membrane repair. The mechanism of wound repair involves Ca^{2+} influx that triggers lysosomal exocytosis. In some circumstances, this leads to a patch repair of the wound with lysosomal markers such as LAMP-1 detected at the outer leaflet of the plasma membrane. More recent evidence, though, suggests that lysosomal patching and endocytic events may act simultaneously during membrane repair.[43] In this model, lysosome exocytosis in fact promotes remodeling of the plasma membrane by releasing acid sphingomyelinase (ASM). Acid sphingomyelinase cleaves the phosphocholine head group of sphingomyelin, generating ceramide which, in turn, drives membrane invagination and endocytosis. The simultaneous translocation of lysosomal V1

H^+-ATPase to the plasma membrane may help to achieve the low pH necessary for ASM activity. This suggests that, at least for some types of lesions, plasma membrane wound repair involves not only patching but also lesion removal.[44,45] Either way, lysosomes are pivotal players for maintaining cell integrity.

Lysosomes may play a role in tumor invasion and metastasis, as cancer cells show altered lysosomal trafficking and increased expression of cathepsins. In cells located to the edge of the tumor, lysosome localization shifts from a perinuclear to a peripheral pattern, indicating that lysosomal contents are secreted into the extracellular space, where they help degrading the extracellular matrix.[36]

This location of lysosomes may be related to the modulation of components of the adhesion machinery by lysosomal proteases, although it remains to be determined if the subcellular localization alters the lysosomal membrane stability and hydrolytic capacity.[46] Recent work from Johnson et al[47] described reduced acidity and proteolytic activity in peripheral lysosomes when compared to juxtanuclear ones in HeLa cells. This findings, however, must be confirmed in other cellular types.

In addition, it has been shown that lysosome exocytosis may represent an important mechanism of drug resistance in tumor cells by which lysosomotropic drugs are extruded to the extracellular compartment. Furthermore, different mechanisms of lysosomal sequestration of chemoterapics have been shown, either passive or mediated by ATP-binding cassette (ABC) transporters. In addition, TFEB-mediated lysosomal biogenesis is triggered by drug sequestration, resulting in enhanced capacity of chemoresistance.[48] The inhibition of lysosomal activity could emerge as a new therapeutic strategy to overcome drug resistance in cancer.[49] Machado et al[50] showed that the inhibition of lysosomal exocytosis reversed invasiveness and chemoresistance in aggressive sarcoma cells.

Finally, lysosomes are involved in double-stranded DNA degradation mediated by Dnase2a, a lysosomal endonuclease. In Dnase2A-deficient cells, damaged DNA originated in the nucleus accumulates in small speckles that colocalize with the autophagy machinery. However, the lysosome-autophagosome fusion fails to happen in the absence of the enzyme, and the DNA fragments accumulated in the cytosol trigger inflammation via the Sting cytosolic DNA-sensing pathway.[51]

Conclusion and Future Prospects

This interaction between lysosomal biology and LSD pathophysiology has shown to be mutually beneficial, both for the basic science of cell biology as to the applied field of medical genetics. For example, the role of lysosomes in membrane repair led the observation that cells from patients with Niemann-Pick disease type A (NPA), lacking ASM activity, show signs of reduced injury-induced endocytosis and deficient membrane repair that may account for some aspects of the pathology.[52] The broader understanding of cellular mechanisms involving the lysosomes may lead to secondary therapeutic targets of LSD other than the enzyme replacement/substrate reduction binomium. On the other hand, the implication of lysosomes in pathogenic mechanisms of common diseases, such as Alzheimer and cancer, opened new roads for research in the field of lysosomal biology that eventually will also benefit the LSD.

Declaration of Conflicting Interests

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Roberto Giugliani, Ana-Carolina Brusius-Facchin, Gabriela Pasqualim, Sandra Leistner-Segal, Mariluce Riegel & Ursula Matte

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Current molecular genetics strategies for the diagnosis of lysosomal storage disorders

Roberto Giugliani[1,2]

Ana-Carolina Brusius-Facchin[1,3]

Gabriela Pasqualim[3,4]

Sandra Leistner-Segal[1,5]

Mariluce Riegel[1,3]

Ursula Matte[2,3,4]

[1] Medical Genetics Service, HCPA, Porto Alegre, Brazil

[2] Department of Genetics, UFRGS, Brazil

[3] Postgraduate Program in Genetics and Molecular Biology, UFRGS, Porto Alegre, Brazil

[4] Gene Therapy Center, Experimental Research Center, HCPA, Porto Alegre, Brazil

[5] Postgraduate Program in Medical Sciences, UFRGS, Porto Alegre, Brazil

Corresponding Author:
Roberto Giugliani
Medical Genetics Service
Hospital de Clínicas de Porto Alegre
Rua Ramiro Barcelos 2350
90035-903 – Porto Alegre – RS
Brazil
Tel + 55 51 3359 8011
Email rgiugliani@hcpa.edu.br

Summary

Lysosomal storage disorders (LSDs) are a group of almost 50 monogenic diseases characterized by mutations causing deficiency of lysosomal enzymes or non-enzyme proteins involved in transport across the lysosomal membrane, protein maturation or lysosomal biogenesis. Usually, affected patients are normal at birth and have a progressive and severe disease with high morbidity and reduced life expectancy. The overall incidence of LSDs is usually estimated as 1:5000, but newborn screening studies are indicating that it could be much higher. Specific therapies were already developed for selected LSDs, making the timely and correct diagnosis very important for successful treatment and also for genetic counseling. In most LSD cases the biochemical techniques provide a reliable diagnosis. However, the identification of pathogenic mutations by genetic analysis is being increasingly recommended to provide additional information. In this paper we discuss the conventional methods for genetic analysis used in the LSDs (restriction fragment length polymorphism [RFLP], amplification-refractory mutation system [ARMS], single strand conformation polymorphism [SSCP], denaturing high performance liquid chromatography [dHPLC], real-time polymerase chain reaction [RT-PCR], high resolution melting [HRM], multiplex ligation-dependent probe amplification [MLPA], Sanger sequencing) and also the newer approaches (massive parallel sequencing, array comparative genomic hybridization [CGH]).

Keywords: Lysosomal Storage Diseases, Enzyme Deficiency, Massive Parallel Sequencing, Molecular Diagnosis, Mutation identification

Introduction

Lysosomal storage disorders (LSDs) are a group of almost 50 genetic diseases (table 1) characterized by mutations and loss of activity of lysosomal enzymes or, less frequently, non-lysosomal proteins that are involved in transport across the lysosomal membrane, protein maturation or lysosomal biogenesis.

Most LSDs have an autosomal recessive inheritance, with some the exceptions of Hunter syndrome, Danon disease and Fabry disease that are X-linked [1].

LSDs are usually characterized by their progressive course with high morbidity and reduced life expectancy, although there are significant variations between different diseases, and even among patients with the same disease [2].

Generally these diseases are multisystemic, and clinical features may include organomegaly, coarse facies, central nervous system dysfunction, skeletal dysplasia, abnormalities in eyes and ears, problems in vital organs like heart, liver, kidney, and lungs, among other alterations.

The current view is that the primary gene defect and substrate storage are triggers of a complex cascade of events that lead to many of the disease manifestations [3]. In this context, secondary substrate storage, perturbations of calcium homeostasis and lipid trafficking would contribute to disease pathogenesis. Other manifestations, related to the lysosome's role in vesicle trafficking, including antigen presentation, innate immunity, and signal transduction would cause inflammatory and auto-immune disturbances observed in the LSD [4]. In addition, general mechanisms such as unfolded protein response, reticulum stress, oxidative stress and autophagy blockade would also play a role in the pathogenesis [5].

Large studies suggest that the overall incidence of the LSDs vary from 1:5,000-1:7,700 [6]. However, data emerging from neonatal screening programs indicate that this incidence could be considerably higher [7]. In addition, the incidence and prevalence of these diseases varies from different countries and regions. For example, the overall incidence of GM1 Gangliosidosis is considered to be 1:100,000-1:200,000, however in some countries as Malta (1:3,700) and the South of Brazil (1:13,317) it is considerably higher [8].

Specific therapies were already developed for selected LSDs, including hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (ERT)

and substrate inhibition therapy (SRT), available for Gaucher, Fabry, Pompe, acid lipase deficiency, and some mucopolysaccharidoses. These approaches and the development of new ones for further LSDs (as chaperones, gene therapy, read through, exon skipping) make the timely and correct diagnosis very important to introduce treatment early enough to prevent irreversible organ damage. Also, diagnosis is important for genetic counseling, detection of carriers and prenatal/preimplantation diagnosis.

In the case of LSDs, specific enzyme assays usually identify a severe deficiency in the affected patients, and this is, in most cases, considered sufficient for the diagnosis. However, a confirmation of pathogenic mutations by genetic analysis is being increased recommended to provide additional information. The following sections will detail the methods currently used for these analyses.

Conventional Molecular Genetics Approaches

Traditional mutation analysis techniques focus mainly on the identification of only one specific mutation at a time.

Restriction fragment length polymorphism (RFLP) for example, is based on differences on amplicon fragment lengths caused by mutations that either eliminate or create new recognition sites of a specific restriction enzyme. Amplification-refractory mutation system (ARMS) uses sets of allele-specific primers that inhibit polymerase extension in the absence of perfect complementarity [9]. They allow low- and medium-throughput genotyping at a low cost with minimal equipment requirements and are still presently applied [10–12]. Similarly, classic screening analysis such as single strand conformation polymorphism (SSCP) and denaturing high performance liquid chromatography (dHPLC), can be the methods of choice in several laboratories depending on personnel expertise and equipment available [8,13–15].

More modern approaches are able to increase the number of variations analyzed at the same time, enhance throughput and reduce length of diagnosis or screening. Thus, Sanger sequencing, multiplex ligation-dependent probe amplification, Real-time PCR, and High Resolution melting will be addressed in details as follows.

Sanger Sequencing

Sanger Sequencing is also known as dideoxy sequencing or chain termination. It is based on the addition of dideoxynucleotides (ddNTPs), which prevents extension by DNA polymerase, to an amplification reaction [16]. The addition of different fluorophores to each ddNTP allowed the development of automated DNA sequencing [17]. In this technique, after cycles of amplification, fragments are purified to remove unincorporated components, separated by capillary electrophoresis, excited by a laser and the different fluorescence signals are translated into an electropherogram. Finally, results are compared to reference sequences present in databases such as NCBI (National Center for Biotechnology Information).

Automated sequencing of both strands is widely used as a gold standard for molecular diagnosis and for validation of next-generation sequencing data. It can detect point mutation and small insertions and deletions. However, caution has to be given when interpreting sequencing results. In recessive disorders is important to determine if both pathogenic mutations found are in different alleles (trans), since it can affect diagnostic conclusions and especially genetic counseling and prenatal diagnosis. If both mutations are present in the same allele (cis), the patient might be an unaffected carrier due to expression of the normal allele or he might be affected by another unidentified mutation. This analysis can be easily made by sequencing parental DNA [18]. Also, diagnostic reports of variants of unknown significance (especially when previously unreported) should preferably be accompanied of *in silico* analysis of possible effects on splicing or protein activity or localization, including signal peptide alterations. Alternative splicing can be confirmed by cDNA sequencing.

Amongst the limitations of this method is the identification of large deletions/duplications or rearrangements. Unless breakpoints are located inside the region delimited by the primers used for amplification, these types of alterations will not be noticed. In patients with confirmed biochemical diagnosis, complementary approaches such as MLPA, cDNA sequencing or real-time PCR should always be performed to detect causal variations missed by DNA sequencing. In addition, genetic variants located at the primers annealing region can lead to allelic dropout, where one strand of DNA fails to amplify, and result in an inaccurate finding of homozygosity. Therefore, parental testing [19], cDNA sequencing [20] or the use different sets of primers should always be performed to confirm apparent homozygosity.

Real-time PCR

Real-time PCR is based on simultaneous PCR amplification and detection or quantification of a fluorescently labeled target sequence. SNP genotyping, analysis of Copy Number Variation (CNV) and gene expression are amongst its major applications.

There are two main strategies for fluorescent labeling: double-stranded DNA (ds-DNA)-binding dyes and fluorescent labeled primers or probes (Figure 1). The most used dsDNA dye is Sybr® Green I. It binds nonspecifically to dsDNA and emits a fluorescent signal that is considerably stronger than when the dye is free in solution. It allows for easy assay design and is very cost-effective for low throughput applications. Also, it can be used for analysis of melt curves, plots of fluorescence intensity versus temperature, and to check for amplicon specificity. However, its use is limited to singleplex reactions, since fluorescence emitted from different amplicons is undistinguishable. Likewise, the amplification of non-intended targets and primer dimers will also affect quantification [21]. Regarding labeled probes, Taqman® probes are the most widely used. They consist of target-specific oligonucleotides with a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. In intact probes, fluorescence from reporter is quenched by its proximity with the quencher. During amplification, probes hybridize with DNA and are cleaved by the 5'>3' exonuclease activity of the polymerase. This releases the reporter and fluorescence levels increases proportionally to amplicon amounts [22]. Although initially more expensive than dsDNA dyes, probes can increase throughput and reduce assay time with multiplex reactions. They also have greater sensitivity to low number of copies of target sequences.

Real-time PCR can be either a qualitative or quantitative (qPCR) technique, evaluating the presence/absence of a target sequence (SNP genotyping) or its number of copies (CNVs [23–25] and gene expression analysis [26,27]). Molecular diagnosis by qPCR usually employs relative quantification. In this approach, the cycle in which fluoresce exceeds background threshold (C_t) of the target gene and the reference gene are compared in controls samples and patients. Higher initial amounts of target sequences, as seen in duplications, will result in lower C_ts, since the number of cycles needed to reach threshold will be lower. In contrast, heterozygous deletions will result in higher C_ts.

High Resolution melting (HRM)

HRM is a PCR-based technique in which the analysis of the melting (dissociation) profile of a target sequence allows genotyping and screening [28]. It might be seen as an improvement of regular melt curve analysis from real-time PCR reactions. After amplification, fragments are denatured and cooled to facilitate heteroduplex formation (double strands with different alleles) in heterozygotes. Then melt analysis is performed by slow denaturation (0.1 to 0.3ºC/seg) and the use of specific saturating dyes. These dyes have high fluorescence only when bound to double stranded DNA (dsDNA). Hence, an increase in temperature increases denaturation of DNA strands and decreases fluorescence. HRM dyes are improved to avoid redistribution during melting stage and consequent increase in successive peak heights [29].

Melting curve profiles are plots of fluorescence intensity versus temperature. They are mainly affected by GC percentage, sequence length and composition. Therefore, it can be altered by sequence variations. Small variants, such as point mutations, are identified easier in smaller amplicons, since they have a more significant impact on melt temperature. However, larger amplicons might contain distinct melting domains affected independently by DNA alterations. Thus, HRM may also be effective for larger target sequences [29].

Similar to sequencing and other PCR-based methods, HRM has limited power to detect large deletions/duplications or rearrangements. It requires the use of complementary techniques, such as MLPA, when such variations are suspected [30]. Moreover, reactions should be standardized for each amplicon. Since they can inhibit amplification, dye selection and concentration can greatly affect results [31]. Also, DNA and salt concentration can also exert considerable influence by reducing melt temperature and reducing heteroduplex formation, respectively [29].

To date, HRM has been successfully applied for screening of Fabry and Niemann-Pick C patients [32,33].

Multiplex ligation-dependent probe amplification (MLPA)

MLPA® (MRC Holland, Amsterdam, The Netherlands) is a high-throughput multiplex PCR technique that is able to detect abnormal copy numbers, such as deletions and duplications, in genomic sequences [34]. Reverse Transcriptase MLPA (RT-MLPA) and Methylation-Specific MLPA (MS-MLPA) are variations of this technique, intended for mRNA and methylation profiling, respectively [35,36]. These methods are based

on the hybridization of several probes with different lengths to the target sequence, which are then ligated and amplified by a single primer pair (Figure 1C). Subsequently, amplicons are separated by capillary electrophoresis and analyzed by comparison of peek height patterns between unknown and reference samples.

One important limitation of MLPA is the effect of genetic variants on the hybridization of probes. Mismatches with the target sequences can lead to reduced or absent probe signal, falsely indicating a deletion. Therefore, it is necessary to confirm results either using another method or a different set of probes, particularly when the possible deletion involves only one probe. Also, it is recommended to analyze probe sequences, available at the manufacturer's website, in search for known genetic variants of the population to be studied.

MLPA has been applied for patients with cryptic sanger sequencing mutations or to verify coexisting gross deletions and/or duplications in Fabry [37–39], MPS II [40] and Niemann-Pick type C [41,42] patients. Currently, there are also MLPA probemixes available for Krabbe (#P446), Tay-Sachs (#P199) and Pompe Disease (#P453).

Massive Parallel Sequencing

Over the past 30 years, Sanger sequencing, either with gel or capillary electrophoresis, has been regarded as the gold standard for identifying sequence alterations in target regions, being an accurate approach for molecular diagnosis. However, this traditional analysis of individual genes and exon-by-exon sequencing has been surpassed by more cost- and time- effective alternatives. The ability to simultaneously sequence several genes or regions from different patients revolutionized the ways molecular diagnosis is performed.

This Massive Parallel Sequencing (MPS) technology, also known as Next Generation Sequencing (NGS), allowed the sequencing of large genomic regions in short time and at relatively low cost. Different platforms that use different chemistry for sequencing millions of small fragments of DNA in parallel are commercially available. Among the most widely used are: the pyrosequencing method by 454 (later acquired by Roche); the sequencing by synthesis method by Solexa (now the base of all Illumina NGS platforms); and pH-based semiconductor technology by Ion Torrent (Thermo Scientific). All these methods, despite their intrinsic differences, rely on the production of a DNA library consisting in small DNA fragments, which then will be

sequenced and assembled against a reference sequence [43]. In addition, bioinformatics becomes a very important, if not crucial, part of data analysis [44].

MPS applications include the sequencing of PCR-amplified set of genomic regions, whole exome sequencing (WES) and whole genome sequencing (WGS). WGS is still not part of routine clinical diagnosis and is often used in research context or to answer very specific questions in the diagnosis of complex alterations. WES, on the other hand, is standard diagnostic procedure in many places and, as prices keep decreasing, may become the primary choice for molecular diagnosis. Ethical aspects related to disclosure of incidental findings (that is the discovery of pathogenic mutations in genes not related to the investigated phenotype), however, are one of the main challenges of WES [45].

Targeted sequencing of gene panels, on the other hand, poses less ethical problems as the only genes investigated are those known to be related to the patient's phenotype. It also requires less effort in terms of bioinformatics and computational power, since significantly lesser amount of data is analyzed [46]. This approach, however, is heavily dependent on a diagnostic hypothesis that determines which set of genes will be analyzed for each patient, acknowledging that if the diagnosis changes new analysis must be performed. Also, the laboratory must determine its panels and establish the optimal conditions for each panel, whereas for WES the same procedure may be applied to a wide variety of situations.

For LSDs both targeted sequencing and WES have been successfully applied. In many cases, targeted panels can be designed for phenotypically similar disorders, such as the MPS III spectrum that involves mutations in four different genes (table 1). Recently, a targeted panel for 57 lysosomal genes that was able to detect even gross macrodeletions that were missed by Sanger sequencing and Lysoplex, a panel with impressive 891 genes involved in lysosomal, endocytic, and autophagic pathways, were reported [47,48]. The Lysoplex study, in particular, provided a comprehensive catalogue of sequence variants in autophagy-lysosomal pathway genes allowing the assessment of their relevance in cell biology. This approach, however, is not able to provide a diagnosis for those patients with overlapping symptoms caused by non-lysosomal storage disorders. WES, on the other hand, has led to the diagnosis of atypical forms of MPS III [49] and galactosialidosis [50]. Interestingly, in both cases, traditional biochemistry tests (GAG dosage and enzyme assay, respectively) were performed post-WES to confirm the molecular diagnosis, in an inversion of the traditional diagnostic algorithms.

NGS has a broad utility into clinical practice for molecular diagnosis of many human genetic disorders, which include single gene disorder, multigene disorders and diseases caused by a group of related genes. In the case of targeted sequencing, the main advantage is the simultaneous analysis of genes that lead to a common phenotype. For WES it is the possibility to define a diagnosis without a clear hypothesis, or at least one that encompasses many different genes. Together with WGS, these technologies are increasing our knowledge of genetic disorders by helping to define causative mutations for previously unrecognized Mendelian diseases and expanding the phenotypic spectrum of rare syndromes [50,51].

Oligonucleotide array-based comparative genomic hybridization

As mentioned above, DNA sequencing is the primary clinical technique for identifying mutations in human disease, but sequencing often does not detect intragenic or whole-gene deletions/duplications.

Comparative genomic hybridization (CGH) using oligonucleotide arrays has been implemented in cytogenetic and molecular diagnostic laboratories as a robust, rapid and sensitive assay for detecting targeted gene deletions [52].

Oligonucleotide array-based comparative genomic hybridization (aCGH) targeted to coding exons of genes of interest has been proven to be a valuable diagnostic tool to complement with sequencing for the detection of large deletions/duplications. Targeted oligonucleotides arrays can be designed to detect both whole-gene deletions and small intragenic deletions in genes related to metabolic disorders, including LSDs [53–55].

This technology may be particularly useful as an additional diagnostic test to the gene sequencing analyses in situations where intragenic deletions, duplications and rearrangements are causative. It could also be beneficial in the context of a recessive disease when only one mutant allele was found by conventional methods. And also in an X-linked disease, when the supposed carrier seems to not present the mutation by sequencing analysis, targeted aCGH should be considered for the detection of large heterozygous deletions.

Furthermore, aCGH can be helpful when traditional methods for genetic analysis used for lysosomal diseases cannot determine genomic positions of the breakpoints and the size of the deletion/duplication within the target gene.

The targeted CGH array is rapid, highly sensitive and accurate method that can be useful for detecting single- and multi- exon deletions and duplications in a large set of LSD genes simultaneously [54]. As gene CGH array is a recent technology proposed to be used as a ancillary diagnostic tool in LSDs and not yet a standard test, the intragenic deletions/duplications within the target genes detected by the array should be confirmed by PCR amplification followed by sequencing to determine the exact deletion/duplication breakpoints and the sequence characteristics of the junction fragment. Methods such as fluorescent in situ hydridization (FISH), MLPA or real time qPCR could be used in combination, especially when one of the breakpoints falls in the non-targeted gene region.

Oligonucleotide array-based comparative genomic hybridization targeted to coding exons of genes of interest has been proven to be a valuable diagnostic tool to complement with Sanger sequencing for the detection of large deletions/duplications [55]. This method is best applied to cases that are suggestive of a possible large deletion (figure 2). As well as in X-linked recessively inherited LSDs, such as MPS II, when single or multiple-exons failed to amplify in affected male patients or female carriers, or when direct DNA sequencing fails to identify a mutation in females who are either affected or have a family history. For female carriers of X-linked disorders, if sequencing analysis does not detect point mutations, targeted aCGH should be considered for the detection of large heterozygous deletions.

Expert Commentary

LSDs are monogenic conditions related to the deficiency of a specific lysosomal enzyme, in most cases, and the diagnosis is largely based on the identification of the deficient enzyme activity, usually in leucocytes or fibroblasts. For a few LSDs where the deficiency is not enzymatic or where the enzyme is not easily measurable, other tests have been important in the diagnostic investigation. A couple of examples are the electron microscopy of skin biopsies (in the investigation of some types of neuronal ceroid lipofuscinosis) and the Filipin test in fibroblasts (performed in the investigation of Niemann-Pick disease type C). The developments occurred in the last decades, especially in the field of molecular biology lead to an intense use of these techniques to determine the causative genetic defect in LSDs.

The information about the genotype is important for several reasons, such as:

Confirmation of the biochemical diagnosis, as in some cases the specific enzyme may show high residual activity, especially if an artificial substrate was used in the

enzyme assay; also there are some LSDs which show multiple enzyme deficiencies (multiple sulphatase deficiency, mucopolidoses II and II, for example), making the biochemical diagnosis difficult;

Confirmation of the diagnosis in cases where the specific enzyme deficiency cannot be easily verified in clinical laboratories; as in the mucolipidoses II and III, whose specific enzyme assay involves radioactive substrates, for example;

Confirmation of the diagnosis in cases where the specific enzyme deficiency is not informative, as in the cases of females affected by Fabry disease, an X-linked condition, whose enzyme activities can overlap the control range;

Confirmation of the diagnosis in cases where the functional defect does not involve an enzyme deficiency, as in most ceroid lipofuscinosis, in Niemann-Pick C disease and in activator deficiencies, for example;

Prediction of the phenotype in cases where the enzyme deficiency and the clinical information do not allow for a clear distinction between severe and more attenuated forms, especially when this distinction is important for a treatment decision. A well known example is Mucopolysaccharidosis I, where patients with the severe form would be indicated for HSCT, while for patients with the attenuated forms ERT is usually indicated. Gene deletions/rearrangements, stop-codon and frameshift mutations are often associated to the severe form, while most missense mutations can be associated with a clinical spectrum which varies from severe to attenuated forms.

Detection of carriers, as in most cases carriers present some degree of enzyme deficiency but usually activities overlap with the low range showed by normal controls. This is especially important in X-linked conditions, as Mucopolysaccharidosis II and Fabry disease, when female carriers could transmit the disease to their children independent of the male partners genotypes;

Genetic counseling, as the detection of carriers allows for a more precise information regarding genetic risks for family members;

Prenatal diagnosis, as measurement of enzyme deficiency in chorionic villi or amniotic fluid cells may be tricky, and genetic data provides a more precise information about the status of the fetus;

Pre-implantation diagnosis, a tool being increasingly used by at-risk couples Table 2 summarizes the most important points related to the use of molecular diagnosis in the LSDs, and Figure 3 provides a tentative flow-chart for the use of the different molecular genetics tools to achieve a precise molecular diagnosis in the LSDs.

Five-year view

In the opinion of the authors, dramatic changes will happen in the next five years regarding the laboratory diagnosis of LSDs. Technologies of genomic analysis becoming cheaper, more precise, and easily accessible. Therefore, there is a growing possibility that they would become the first approach to the diagnosis, moving upside down the present paradigm of doing first biochemical investigation and later the molecular analyses. In cases where clinical and/or biochemical hypothesis is limited to a few diseases, for example, targeted panels will become more appealing due to lower sequencing costs, shorter sequencing time, simpler data analysis, and greater sensitivity per gene due to the greater coverage achieved when compared to WES. On the other hand, laboratories with high throughput may use WES or WGS as a primary method, since they do not required multiple standardizations and allows multiple types of patients to be analyzed together. Depending on equipment available and personnel expertise, particularly in cases with family history and/or strong clinical and biochemical evidence, techniques such as Sanger sequencing, MLPA and real-time PCR will continue to be applied.

Nonetheless, biochemical techniques will not be less important. They will be essential, for example, to confirm the functional consequences of the molecular pathology found by the genomic investigation, especially when genetic variants with unknown significance (GVUS) are identified. So, the laboratories involved with the diagnosis of LSDs, will need to continue to deal with biochemical and molecular techniques to provide to the affected families the best information available.

Key issues

- Availability of specific therapies in selected LSDs makes the timely and correct diagnosis very important
- Introduction of treatment early enough could prevent irreversible organ damage

- Confirmation of pathogenic mutations by genetic analysis is being increased recommended to provide additional information related to genetic counseling, treatment options and prognosis
- Traditional mutation analysis is precise but limited as usually explores one copy of one ene sequence at a time.
- Ability to simultaneously sequence several genes or regions from different patients revolutionized the way molecular diagnosis is performed.
- Targeted sequencing and Whole Exome Sequencing have been successfully applied for LSDs diagnosis.
- aCGH can be a helpful and important addition to traditional methods for genetic analysis.
- Clinical and biochemical information continue to be essential to understand the full picture and to confirm the functional consequences found in the molecular genetics investigation.

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*** Of considerable interest*

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* **The develpment of an Oligonucleotide-array platform provided tiled coverage**

of the entire mitochondrial genome and high-density coverage of a set of nuclear genes involving mitochondrial and metabolic disorders and was used to evaluate large deletions in targeted genes.

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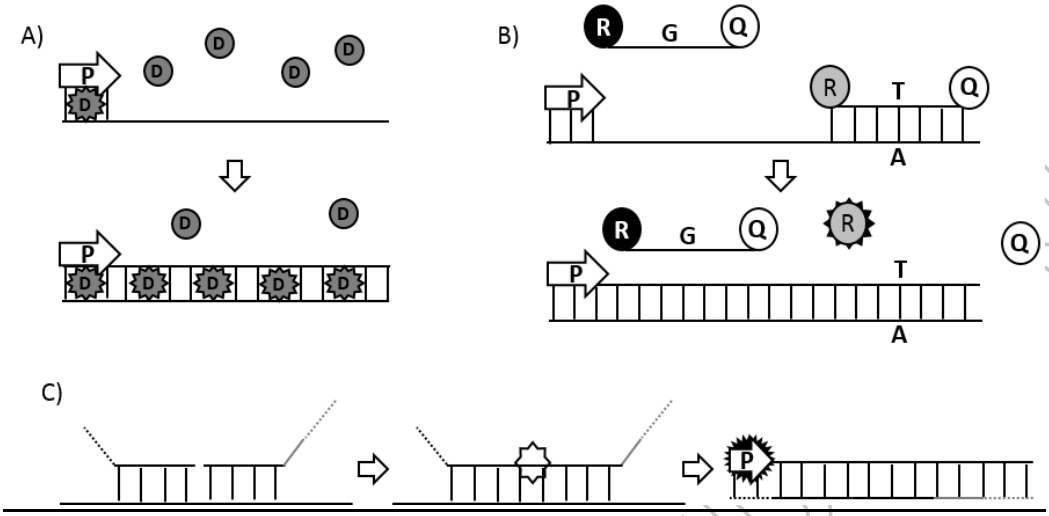
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

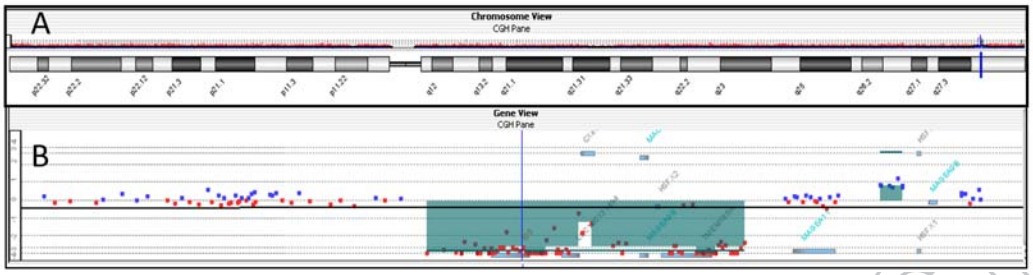
Figure Legends

Figure 1: Real-time PCR and Multiplex ligation-dependent probe amplification (MLPA) basis



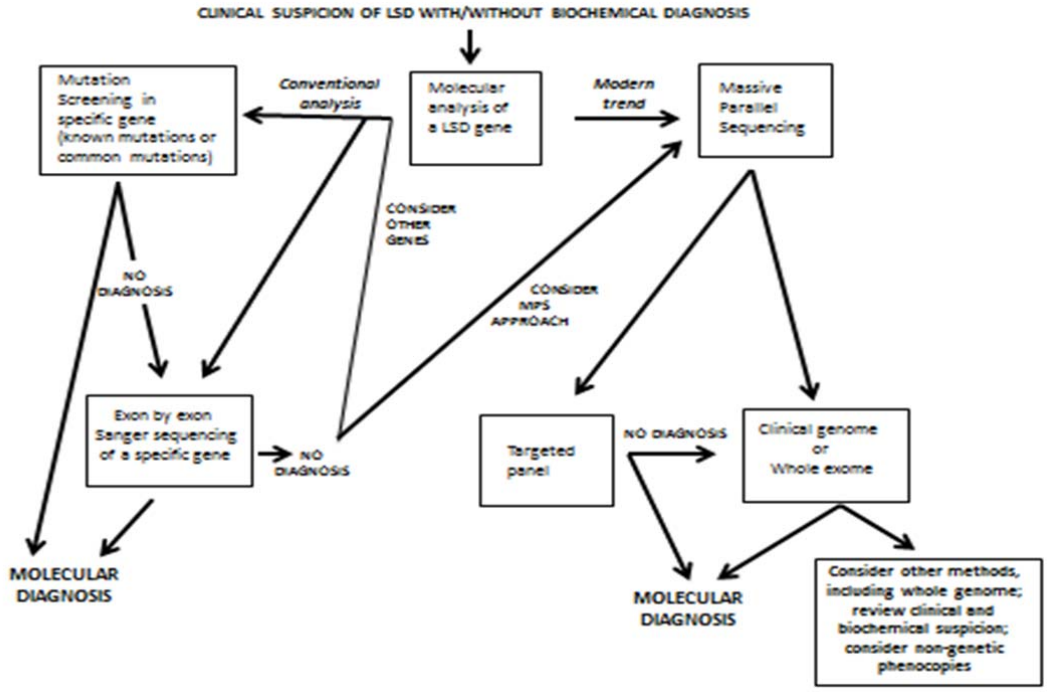
Real-time PCR is based on simultaneous PCR amplification and detection or quantification of a fluorescently labeled target sequence. It has two main strategies for fluorescent labeling: double-stranded DNA (dsDNA)-binding dyes, as Sybr Green, and fluorescent labeled primers or probes, as Taqman. (A) Sybr Green binds nonspecifically to dsDNA and emits a fluorescent signal that is considerably stronger than when the dye is free in solution. (B) Taqman probes consist of target-specific oligonucleotides with a reporter dye and a quencher, that quenches fluorescence from the reporter. During amplification, probes hybridize with DNA and are cleaved by the polymerase, and reporter fluorescence is no longer quenched. C) MLPA is based on the hybridization of several probes with different lengths to the target sequence, which are then ligated and amplified by a single fluorescent primer pair. P: Primer, D: dye, R: reporter, Q: quencher.

Figure 2. Example of an oligonucleotide array-based comparative genomic hybridization analysis using genomic DNA from a MPS II patient and a normal male as a test (red dots) and a reference (blue dots):



the 1 M Array (Agilent Technologies, Santa Clara, CA) test/reference ratio data for the X chromosome are shown. (A): The ideogram of X chromosome shows the location of probes for the IDS gene region at Xq28 (vertical blue bar).(B) A high resolution view of the Xq28 deletion region in the patient. An approximately 19,767 bp deletion (chrX:148,564,332-148,584,098) is shown (green bar).

Figure 3 - Molecular analysis of LSDs today.



Patients with suspected LSD or with clinical/biochemical diagnosis could be investigated by conventional analysis or by massive parallel sequencing. Conventional molecular analysis could start with the search for specific (the most common or the familial ones) disease-causing mutations, followed by Sanger sequencing of the suspected gene(s) when the mutation is not identified. When results are negative, conventional analysis of other genes could be considered, or the analysis could continue with massive parallel sequencing. Alternatively, a more modern trend is to start the molecular analysis with massive parallel sequencing, usually studying a panel of genes selected according to the phenotype/previous results, followed by whole exome or clinical genome (4800+ known disease-related genes) analysis if mutations are not identified. If the analysis is unsuccessful after this last step, the case should be reviewed in the clinical, biochemical aspects, and further molecular analysis could be considered, as whole genome analysis, CGH-array, MLPA, RNA studies and others. The possibility of non-genetic phenocopies should also be considered.

Table 1. List of main lysosomal storage diseases with their respective Online Mendelian Inheritance in Man (OMIM) accession number, gene and enzyme deficiency.

Disease	OMIM	Gene	Enzyme deficient
Aspartylglucosaminuria	208400	*AGA*	N-aspartyl-beta-glucosaminidase
Canavan disease	271900	*ASPA*	Aspartocylase
Cystinosis	219800	*CTNS*	Cystinosin
Danon disease	300257	*LAMP2*	Lysosomal-associated membrane protein 2
Fabry disease	301500	*GLA*	A-galactosidase A
Farber disease	228000	*ASAH1*	Ceramidase
Fucosidosis	230000	*FUCA1*	α-L-fucosidase
Galactosialidosis	256540	*CTSA*	Cathepsin A
Gaucher disease	230800	*GBA*	acid β-glucosidase
GM1 gangliosidosis	230600	*GLB1*	β-Galactosidase
Krabbe disease	245200	*GALC*	galactocerebrosidase
Lysosomal Acid Lipase Deficiency	278000	*LIPA*	Lysosomal acid lipase
α- mannosidosis	248500	*MAN2B1*	α -D -mannosidase
β- mannosidosis	248510	*MANBA*	β -D-mannosidase
Metachromatic leucodystrophy	250100	*ARSA*	Arylsulphatase-A
Metachromatic leucodystrophy	249900	*ARSA*	Saposin-B
Mucolipidosis type I	256550	*NEU1*	Sialidase
Mucolipidosis types II/III	252500	*GNPTAB*	N-acetylglucosamine-1-phosphotransferase
Mucolipidosis type IIIC	252605	*GNPTG*	N-acetylglucosamine-1-phosphotransferase γ-subunit
Mucolipidosis type IV	252650	*MCOLN1*	Mucolipin 1
Mucopolysaccharidosis type I	607014	*IDUA*	α-L-iduronidase
Mucopolysaccharidosis type II	309900	*IDS*	Iduronate sulfatase
Mucopolysaccharidosis type IIIA	252900	*SGSH*	Heparan-N-sulfatase
Mucopolysaccharidosis type IIIB	252920	*NAGLU*	α- N-acetylglucosaminidase
Mucopolysaccharidosis type IIIC	252930	*HGSNAT*	AcetylCoa-glucosamine-N-acetyltransferase
Mucopolysaccharidosis type IIID	252940	*GNS*	N-acetylglucosamine-6-sulfatase
Mucopolysaccharidosis type IVA	253000	*GALNS*	N-acetylgalactosamine-6-sulphatase
Mucopolysaccharidosis type IVB	253010	*GLB1*	β-Galactosidase
Mucopolysaccharidosis type VI	253200	*ARSB*	N-acetylgalactosamine-4-sulphatase
Mucopolysaccharidosis type VII	253220	*GUSB*	β-Glucuronidase
Mucopolysaccharidosis type IX	601492	*HYAL1*	Hyaluronidase
Multiple sulphatase deficiency	272200	*SUMF1*	Formylglicine-generating-enzyme
Neuronal ceroid lipofuscinosis 1	256730	*PPT1*	Palmitoyl protein thioesterase-1
Neuronal ceroid lipofuscinosis 2	204500	*TPP1*	Tripeptydil-peptidase I
Neuronal ceroid lipofuscinosis 3	204200	*CLN3*	CLN3 protein
Neuronal ceroid lipofuscinosis 5	256731	*CLN5*	CLN5 protein
Neuronal ceroid lipofuscinosis 6	601780	*CLN6*	CLN6 protein
Neuronal ceroid lipofuscinosis 8	600143	*CLN8*	CLN8 protein
Niemann-Pick disease A/B	257200	*SMPD1*	Acid sphingomyelinidase
Niemann-Pick disease C1	257220	*NPC1*	NPC1 protein
Niemann-Pick disease C2	607625	*NPC2*	NPC2 protein
Pompe disease	232300	*GAA*	Alpha-glucosidase
Prosaposin deficiency	176801	*PSAP*	Prosaposin
Pycnodysostosis	265800	*CTSK*	Cathepsin K
Sandhoff disease	268800	*HEXB*	Hexosaminidase B
Schindler disease	609241	*NAGA*	Alpha-N-acetylgalactosaminidase
Sialic acid storage disease	269920	*SLC17A5*	Sialin
Sialuria	269921	*GNE*	UDP-N-acetylglucosamine-2-epimerase
Tay-Sachs disease	272800	*HEXA*	Hexosaminidase A

Table 2. Summary of the potential uses of genetic tests in the LSDs

POTENTIAL USE	EXAMPLES
Confirmation of the biochemical diagnosis, especially if a borderline result was found with an artificial substrate, or when multiple enzyme deficiencies are found	Niemann-Pick A/B, Multiple Sulphatase Deficiency, Mucopolidoses II and II
Confirmation of the biochemical diagnosis in cases where the specific enzyme deficiency cannot be easily verified in clinical laboratories	Mucolipidoses II and III, activator deficiencies
Confirmation of the diagnosis in cases where the specific enzyme deficiency is not informative	Females at risk for Fabry disease, where the enzyme activities can overlap the control range
Confirmation of the diagnosis in cases where the functional defect does not involve an enzyme deficiency	Most ceroid lipofuscinosis, Niemann-Pick C disease
Prediction of the phenotype in cases where the enzyme deficiency and the clinical information do not allow for a clear distinction between severe and more attenuated forms.	Mucopolysaccharidosis I: gene deletions/rearrangements, stop-codon and frameshift mutations are often associated to the severe form, while most missense mutations associated to attenuated forms.
Detection of carriers, especially important in X-linked conditions when female carriers could transmit the disease to their children independent of the male partners genotypes	Mucopolysaccharidosis II, Fabry disease
Exclusion of pseudodeficiency	Fabry disease, Pompe disease and Metachromatic Leukodystrophy
Genetic counseling for family members	Detection of carriers by genetic tests brings a more precise information regarding genetic risks
Prenatal diagnosis for couples at risk	Detection of fetal status by genetic tests provides a faster and more precise diagnosis, especially when the mutation to be searched is previously known
Pre-implantation diagnosis	Selection of unaffected embryos for implantation with genetic tests

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

Bravo H, Neto EC, Schulte J, Pereira J, Filho CS, Bittencourt F, Sebastião F, Bender F, de Magalhães APS, Guidobono R, Trapp FB, Michelin-Tirelli K, Souza CFM, Rojas Málaga D, Pasqualim G, Brusius-Facchin AC, Giugliani R. Mol Genet Metab Rep. 2017 Jul 4;12:92-97. doi: 10.1016/j.ymgmr.2017.06.006. eCollection 2017 Sep.

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

Heydy Bravo[a], Eurico Camargo Neto[b], Jaqueline Schulte[b], Jamile Pereira[b], Claudio Sampaio Filho[c], Fernanda Bittencourt[d], Fernanda Sebastião[d], Fernanda Bender[d], Ana Paula Scholz de Magalhães[d], Régis Guidobono[d], Franciele Barbosa Trapp[d], Kristiane Michelin-Tirelli[d], Carolina F.M. Souza[d], Diana Rojas Málaga[a], Gabriela Pasqualim[a,f], Ana Carolina Brusius-Facchin[d], Roberto Giugliani[a,d,e,f,*]

[a] Post-Graduate Program of Genetics and Molecular Biology, UFRGS, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, RS, Brazil
[b] CTN Diagnósticos, Av. Ipiranga 5000, Porto Alegre, RS 90610-000, Brazil
[c] Intercientifica, Av. Ouro Fino, São José dos Campos, SP 12233-401, Brazil
[d] Medical Genetics Service, HCPA, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903, Brazil
[e] Post-Graduate Program in Medical Sciences, UFRGS, Rua Ramiro Barcelos 2400, 90035-003 Porto Alegre, RS, Brazil
[f] Gene Therapy Center, Experimental Research Center, HCPA, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903, Brazil

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ABSTRACT

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

1. Introduction

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

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

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

* Corresponding author at: Medical Genetics Service, HCPA, Rua Ramiro Barcelos 2350, Porto Alegre, RS, Brazil.
E-mail address: rgiugliani@hcpa.edu.br (R. Giugliani).

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

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

2. Materials and methods

2.1. Samples

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

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

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

2.2. Enzyme activity analyses

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

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

2.3. Urinary glycosaminoglycans (GAGs) analysis

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

2.4. Gene analysis

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

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

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

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

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

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

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

3. Results

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

3.1. Case 1: suspicion of MPS I

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

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

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

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

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

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

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

3.2. Case 2: suspicion of Pompe disease

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

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

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

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

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

3.3. Case 3: suspicion of Gaucher disease

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

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

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

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

3.4. Case 4: suspicion of MPS I

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

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

biochemical results were considered inconclusive.

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

Author contributions

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

Conflicts of interest

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

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*IV. Emerging drugs for the treatment of mucopolysaccharidoses***.**

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REVIEW

Emerging drugs for the treatment of mucopolysaccharidoses

Roberto Giugliani[a,b,c,d], Andressa Federhen[a,c], Filippo Vairo[a], Cláudia Vanzella[a,e], Gabriela Pasqualim[a,b], Letícia Machado Rosa da Silva[a], Luciana Giugliani[a], Ana Paula Kurz de Boer[a], Carolina Fishinger Moura de Souza[a], Ursula Matte[b,d,f] and Guilherme Baldo[d,f,g]

[a]Medical Genetics Service and Clinical Research Group in Medical Genetics, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil; [b]Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [c]Post-Graduate Program in Child and Adolescent Health, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [d]Post-Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [e]Post-Graduate Program in Biological Sciences: Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [f]Gene Therapy Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil; [g]Department of Physiology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

ABSTRACT

Introduction: Despite being reported for the first time almost one century ago, only in the last few decades effective have treatments become available for the mucopolysaccharidoses (MPSs), a group of 11 inherited metabolic diseases that affect lysosomal function. These diseases are progressive, usually severe, and, in a significant number of cases, involve cognitive impairment.

Areas covered: This review will not cover established treatments such as bone marrow/hematopoietic stem cell transplantation and classic intravenous enzyme replacement therapy (ERT), whose long-term outcomes have already been published (MPS I, MPS II, and MPS VI), but it instead focuses on emerging therapies for MPSs. That includes intravenous ERT for MPS IVA and VII, intrathecal ERT, ERT with fusion proteins, substrate reduction therapy, gene therapy, and other novel approaches.

Expert opinion: The available treatments have resulted in improvements for several disease manifestations, but they still do not represent a cure for these diseases; thus, it is important to develop alternative methods to approach the unmet needs (i.e. bone disease, heart valve disease, corneal opacity, and central nervous system (CNS) involvement). The work in progress with novel approaches makes us confident that in 2017, when MPS will commemorate 100 years of its first report, we will be much closer to an effective cure for these challenging conditions.

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1. Background

Mucopolysaccharidoses (MPSs) are a group of inborn errors of metabolism (IEM) caused by deficiency of specific lysosomal enzymes that affect the degradation of mucopolysaccharides or glycosaminoglycans (GAGs). The accumulation of GAGs in various organs and tissues of patients affected by MPS results in a series of signs and symptoms that lead to a multisystemic clinical picture.[1]

MPSs were first described in the last century when Charles Hunter reported in 1917 that two young male siblings presented similar characteristics, including coarse facies, a large abdomen, and bone dysplasia. Shortly thereafter, in 1919, Dr. Gertrud Hurler, a German pediatrician, described the visceromegaly and bone abnormalities present in two unrelated boys with similar features, which later would be classified as 'Hurler syndrome'.[1] In 1929, Luis Morquio described a form of 'familial skeletal dystrophy' affecting four out of five children born to consanguineous parents of Swedish descent. This report,[2] along with another publication on similar cases published in the same year by the radiologist Brailsford [3] led to the recognition of a new form of skeletal dysplasia. The cases described by Hunter, Hurler, and Morquio-Brailsford would be later identified as MPS diseases when the storage of GAG in tissues was identified as a common abnormality. In the early 1960s, an attenuated form of Hurler syndrome was described by Scheie *et al.* [4], and another form of mental retardation associated with mucopolysaccharidoses, but with less pronounced visceral and skeletal manifestations, was identified by Sanfilippo *et al.* [5]. In the same year, Maroteaux *et al.* [6] described a new form of dysostosis with mucopolysaccharidoses, but without cognitive impairment in France, that became known as Maroteaux–Lamy syndrome. Ten years later, Sly and colleagues [7] reported another MPS type called Sly syndrome, and at the end

CONTACT Roberto Giugliani rgiugliani@hcpa.ufrgs.br Medical Genetics Service, Hospital de Clinicas de Porto Alegre, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903, Brazil

Table 1. Characteristics of MPSs – adapted from Giugliani [9] and Neufeld and Muenzer.[1]

MPS	Name	Increased GAGs	Inheritance	Enzyme deficiency	Gene location	Main organs affected
I	Hurler, Hurler–Scheie or Scheie	HS + DS	AR	α-iduronidase	4p16.3	Heart, liver, lungs, CNS (severe form), skin, blood vessels, extracellular matrix
II	Hunter	HS + DS	X-linked	Iduronate sulfatase	Xq28	Heart, liver, lungs, CNS (severe form), skin, extracellular matrix
III A	Sanfilippo A	HS	AR	SGSH	17q25.3	Liver, lungs, CNS
III B	Sanfilippo B	HS	AR	NAGLU	17q21.1	
III C	Sanfilippo C	HS	AR	AcetylCoA α- glucosamine acetyltransferase	14p21	
III D	Sanfilippo D	HS	AR	*N*-acetylglucosamine-6-sulfatase	12q14	
IV A	Morquio A	KS	AR	Galactosamine-6-sulfate sulfatase	16q24.3	Bones, cartilage, cornea
IV B	Morquio B	KS	AR	α-galactosidase	3p21.3	
(V)	Scheie syndrome, initially proposed as type V, was recognized to be the attenuated end of the MPS I spectrum					
VI	Maroteaux–Lamy	DS	AR	*N*-acetylgalactosamine-4-sulfatase	5q11–q13	Skin, blood vessels, heart, tendons, extracellular matrix
VII	Sly	HS + DS	AR	β-glucuronidase	7q21.11	Skin, blood vessels, heart, tendons, liver, lungs, CNS
(VIII)	An enzyme defect was found and proposed as MPS VIII, but shortly thereafter recognized as a laboratory pitfall; the proposal was withdrawn					
IX	Natowicz	Hyaluronan	AR	Hyaluronidase 1	3p21.3	

MPS: mucopolysaccharidosis; GAGs: glycosaminoglycans; HS: heparan sulfate; DS: dermatan sulfate; KS: keratan sulfate; AR: autosomal recessive; NAGLU: α-N-acetylglucosaminidase; CNS: central nervous system; SGSH: N-sulfoglucosamine sulfohydrolase.

of the century, the most recent MPS type was described by Natowicz *et al*. [8]. MPS diseases and their characteristics are summarized in Table 1.

The identification of specific enzyme deficiencies allowed a more definitive classification of MPS, with some types being fused (Hurler and Sheie syndromes, identified as different forms of MPS I) and others split apart (the four types of Sanfilippo disease resulting from four different enzyme defects leading to the same phenotype – A, B, C, and D, and the two types of Morquio disease resulting from two different enzyme defects causing the same phenotype – A and B). The identification of the genes that encode each of the 11 enzymes involved in the MPS diseases allowed the production of recombinant enzymes, generation of animal models, and even gene therapy projects aiming for the specific treatment of these diseases. Thus, though the biochemical and molecular aspects of MPS began to be understood in the second half of the twentieth century, the beginning of the twenty-first century was when many options to treat these conditions emerged.

The first effective therapy for these severe and progressive diseases was reported in 1980, when bone marrow transplantation (BMT) was performed on an MPS I patient. [10] Hematopoietic stem cell transplantation (HSCT) became the standard treatment for MPS I, particularly for the neuronopathic form. This treatment was expanded to other MPS types with variable efficacy and with a considerably high risk of mortality and morbidity. The first specific therapy approved for MPS was intravenous enzyme replacement therapy (ERT) for MPS I, followed by MPS VI, MPS II, and, recently, MPS IVA. Despite bringing a significant, positive change to the natural history of these conditions with a corresponding improvement and/or stabilization of several disease manifestations, intravenous ERT does not represent a cure for these severe conditions. Although there is no cure currently available for any MPS type, a range of different therapeutic strategies is being developed, including ERT with alternative drugs or alternative infusion routes, substrate inhibition therapy, gene therapy, and other approaches.

2. Medical need

Despite the acceptable safety profile and significant improvements related to intravenous ERT that lead to the positive long-term outcomes reported for MPS I, [11] MPS II, [12] and MPS VI, [13] this treatment approach does not constitute a cure for the disease and is not available for all MPS. Even for the MPS for which intravenous ERT is available, the fact that the recombinant enzyme does not cross the blood–brain barrier (BBB) precludes its access to the central nervous system (CNS); thus, it does not prevent or treat the cognitive decline present in several MPS types. BMT and HSCT are also not curative; additionally, these approaches have a high mortality and morbidity risk associated with the procedure, and their efficacy seems to be restricted to some MPS types. This procedure would be able to provide cells to the CNS that could produce the enzyme, but there is evidence that treatment should be performed very early in life to prevent cognitive decline. The reason for the limited efficacy in some types of MPS (such as MPS III) is still unknown, but one hypothesis is that the transplant does not provide enough enzyme for an alleviation of neural

cell dysfunction. In other cases, such as MPS II, results are still conflicting.[14,15]

In addition to the CNS, there are other 'hard-to-treat' organs such as bone, heart valves, and cornea, which show a small response to ERT or BMT/HSCT, due to other biological barriers (such as the blood–ocular barrier) or due to poor vasculature of some organs (such as the bones and heart valves). Therefore, despite the major advances in the treatment of MPS in the last decades, there is a need for innovative therapies to ultimately reach the goal of the cure of these diseases.

3. Existing treatments

For many years, only supportive treatments were available for the different types of MPS. In the early 1980s, BMT was introduced as the first successful treatment for MPS, and it is still used as the standard therapy for the severe form of MPS I (Hurler) if it is performed before the age of 2.5 years and/or the patient presents an intelligence quotient greater than 70. BMT can prevent cognitive decline, improve upper airway disease, reduce hepatosplenomegaly, and improve mobility; however, the cornea is not cleared, cardiac valve disease persists and skeletal changes progress. BMT performed later in life will not prevent cognitive decline; at this stage, other forms of treatment, such as intravenous ERT, are preferred due to the risks associated with the procedure. In a recent review, the authors showed that the results are even better if BMT is performed before 9 months of age in patients with Hurler disease.[16] BMT does not seem to be an option for patients with MPS III and MPS IV, and its efficacy is controversial for patients with MPS type II. There are some reports of the success of BMT for the attenuated forms of MPS I and for MPS VI, but since the 2000s, ERT has arisen as an effective approach to alleviate some of the symptoms of MPS I,[17] MPS II, [18] and MPS VI,[19] and more recently, for MPS IVA.[20] Since 2012, another recombinant enzyme for MPS II, idursulfase beta (Hunterase, Green Cross, Seoul, South Korea), has been used in Korea, with reports of an adequate safety profile and improvements in endurance.[21,22] For MPS VII, intravenous ERT was successfully attempted for the first time in a 12-year-old boy [23] but has not yet been licensed.

4. Market review

MPSs present an attractive opportunity for clinical development because of factors such as orphan drug exclusivity and the increased knowledge on the mechanisms of the disease. Furthermore, premium pricing (may reach US$ 300,000.00/patient, or even more) compensates the small market size.[24]

Although underdiagnosed, epidemiological studies available show that MPS (all types) has an estimated frequency varying from 1/21.000 to 1/57.000 newborns, [25,26] which represents a market varying from 105,000 to 285,000 patients, who can potentially benefit from treatment. We should note that, in developed countries, where laboratory techniques are easily available to diagnose patients, disease estimation is more accurate when compared with developing countries.

5. Current research goals

The main current research goal is the development of potential alternatives that could correct the disease symptoms that presently approved treatments are unable to appropriately address, such as the neurological and skeletal manifestations. Most alternatives are being tested in a preclinical set, but some potential treatments are already being studied in humans and are described in more detail below. The main characteristics of the therapies that reached clinical stages are summarized in Table 2.

6. Scientific rationale

6.1. Intravenous ERT

This section will not cover ERT for MPS I, MPS II, and MPS VI, for which long-term outcome reports are already available, and will concentrate on MPS IVA (recently approved) and MPS VII (still in clinical development).

6.1.1. For MPS IVA

MPS IVA (MPS IVA; Morquio A syndrome) (OMIM 253000) is a lysosomal storage disorder caused by a deficiency in *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) activity (EC 3.1.6.4), leading to the cellular storage of undegraded keratan sulfate.[27] Morquio patients can often be distinguished from other MPS types by the preservation of intelligence and characteristic skeletal changes, such as knock-knee, kyphosis, protrusion of chest, prominent forehead, spondyloepiphyseal dysplasia, unique laxity of joints, and cervical instability. Other complications include airway and pulmonary compromise, muscle weakness, valvular heart disease, hearing loss, and fine corneal clouding.[28] In the beginning of 2014, a recombined GALNS enzyme (elosulfase alfa) was approved in USA and in Europe to treat this disease. Based on the outcomes from the Phase I/II clinical trials, a multicenter, double-blinded, placebo-controlled phase 3 study was conducted to

Table 2. Approved and emerging treatments for MPS.

MPS type	Approved treatments/regimen	Emerging treatments/Status
I	• HSCT/for Hurler patients aged up to 2.5 years • Intravenous ERT with laronidase/ 0.58 mg/kg weekly*	• Intrathecal ERT with laronidase/Preclinical studies, and case reports • Intrathecal ERT with laronidase before and after HSCT/Phase I clinical trial ongoing • Intravenous ERT with recombinant IDUA fused to a monoclonal antibody/ Phase I/II clinical trial • Gene therapy with intracerebral AAV2/5/Preclinical studies • Gene therapy with RV/Preclinical studies • Gene therapy with transposons/Preclinical studies • Gene therapy with cells in alginate beads/Preclinical studies • Gene therapy using BHK cells in microcapsules/Preclinical studies • Nanoparticles of laronidase/Preclinical studies
II	• Intravenous ERT with idursulfase alpha/0.5 mg/kg weekly	• Intravenous ERT with idursulfase beta/Phase III clinical trial completed • Intravenous plus intrathecal ERT with idursulfase alfa/Phase II/III clinical trial • Intravenous ERT with recombinant IDS fused to a monoclonal antibody/ Phase I/II clinical trial • Gene therapy with intravenous AAV2/8/Preclinical studies • Gene therapy using C2C12 myoblasts cells in microcapsules/Preclinical studies
III (all subtypes)	None	• Oral genistein/Clinical trial recruiting
III-A	None	• Intrathecal ERT with recombinant SGSH/Phase I/II clinical trial ongoing • Gene therapy with intracerebral AAV2/5/Preclinical studies • Gene therapy with intracerebral or intravenous AAV2/9/Preclinical studies • Gene therapy with LV/Preclinical studies • SRT with rhodamine/Preclinical studies
III-B	None	• Intracerebroventricular ERT with NAGLU fused in insulin-like growth factor II receptor/Preclinical studies completed • Intravenous ERT with recombinant NAGLU/Preclinical studies completed • Gene therapy with intracerebral AAV2/5/Preclinical studies • Gene therapy with intracerebral or intravenous AAV2/9/Preclinical studies • Gene therapy with LV/Preclinical studies
IV-A	• Intravenous ERT with elosulfase alfa/ 2.0 mg/kg weekly	
VI	• Intravenous ERT with galsulfase/ 1.0 mg/kg weekly	• Gene therapy with intravenous AAV2/8/Preclinical studies • Gene therapy with RV/Preclinical studies • SRT with rhodamine/Preclinical studies • Oral PPS/Preclinical studies • Nanoparticles of ASB/Preclinical studies
VII	None	• Intravenous ERT with recombinant GUS/Phase III clinical trial ongoing • Gene therapy with intracerebral or intravenous AAV2/9/Preclinical studies • Gene therapy with RV/Preclinical studies • Gene therapy using 2A50 fibroblast in microcapsules/Preclinical studies

* The double dose every 2 weeks is also safe and effective.

AAV: adeno-associated virus; ASB: arylsulfatase B; ERT: enzyme replacement therapy; GUS: β-glucuronidase; HSCT: hematopoietic stem cell transplantation; IDS: iduronate sulphatase; IDUA: α-iduronidase; LV: lentivirus; NAGLU: α-*N*-acetylglucosaminidase; PPS: pentosan polysulfate; RV: retrovirus; SGSH: N-sulfoglucosamine sulfohydrolase; SRT: substrate reduction therapy.

evaluate 176 patients older than 5 years. The clinical trial design included three different treatment regimens. In the first group, 58 patients were treated weekly with 2.0 mg/kg of recombinant hematopoietic GALNS; in the second group, 59 patients received the same dose every other week; and in the third group, 59 patients were infused with placebo.[20] After 24 weeks of the study, patients who were receiving weekly elosulfase alfa showed a significant improvement in endurance of 22.5 meters in 6 minute walk test (6MWT) distance compared with the placebo group (p = 0.017). No impact was observed with the every other week dosing regimen, and no significant change was observed in the 3 minute stair climb test (3MSCT) when the groups were compared. Both treatment groups showed a reduction in urinary GAG levels, and the exploratory endpoints, such as maximum voluntary ventilation (MVV) and forced vital capacity, also improved. Elosulfase alfa had a favorable safety profile at both doses, with major adverse events classified as mild or moderate.[20] Analyses of prespecified composite endpoints (combining changes from baseline in equally weighted 6MWT, 3MSCT and MVV Z-scores) showed a modest positive impact of elosulfase alfa weekly treatment versus the placebo group (p = 0.053). As a pre-specified supportive analysis, the O'Brien Rank Sum composite endpoint (changes from baseline in 6MWT, 3MSC, and MVV) analysis also showed that the weekly group performed better than the placebo group (p = 0.011), indicating that treatment with weekly elosulfase alfa led to improvements across most efficacy measures and resulted in clinically meaningful benefits in a heterogeneous study population.[30] A phase 2 open-label clinical trial is being

conducted in patients under 5 years of age in centers in the USA, Europe, and Asia to evaluate the safety and efficacy of weekly 2.0 mg/kg infusions of BMN 110 in pediatric patients up to 208 weeks of age (NTC01515956).[31] This trial will most likely be completed in the coming months. As already observed in previous ERT studies for MPS I, MPS II, and MPS VI, ERT provides limited impact on bones and joints, primarily because its efficacy depends on its biodistribution, and cartilage and ligaments are not vascularized.[32] The correction of skeletal features is a challenge that current treatments have not yet solved.

6.1.2. For MPS VII

Mucopolysaccharidosis type VII (OMIM 353220) is caused by deficiency of the enzyme β-glucuronidase (GUS) (EC 3.2.1.31), which is required for the degradation of dermatan, heparan, and chondroitin sulfate. The accumulation of these GAGs in lysosomes leads to a dysfunction in several tissues and organs.[7] Currently, there is no treatment approved for MPS VII. However, many studies with animal models of MPS VII evaluating the effect of ERT were conducted over the last few years.[33,34] The first was performed using newborn MPS VII mice in which the recombinant enzyme was administered by intravenous injection. The results demonstrated that the enzyme was distributed to many tissues, including the brain, heart, and bone.[35] However, except during the newborn period, the improvement of ERT in brain was limited by the BBB. Considering this observation, subsequent studies showed that treatment with high doses of the recombinant enzyme in adult MPS VII mice resulted in the delivery of the enzyme across the BBB.[36] More recently, studies using a form of chemically modified GUS demonstrated that this enzyme was able to cross the BBB and clear neuronal storage in a murine model of MPS VII.[37] Additionally, the enzyme was delivered more effectively to the heart, kidneys, and muscle.[34] A recent publication [23] described the experience of the first human treatment using ERT with investigational GUS in an advanced-stage MPS VII patient. This patient was a 12-year-old boy who presented many health complications related to the underlying disease, such as significant pulmonary restriction and obstruction, loss of the ability to walk, progressive heart valve disease, and an enlarged liver and spleen. The patient was treated by an intravenous infusion of 2 mg/kg of the enzyme provided by Ultragenyx Pharmaceutical (Novato, CA, USA) every 2 weeks for 24 weeks. During the treatment period, no serious adverse events, hospitalizations, or hypersensitivity reactions were observed. Furthermore, the urinary GAG levels decreased rapidly upon treatment, with the maximum reduction occurring in the sixth week of treatment. Pulmonary function improved, because the patient's liver and spleen size decreased significantly after a single infusion. Based on the experience of the study cited above and information available at the clinical trials site, in 2013, an open-label phase 1/2 study began in the United Kingdom to assess the safety, efficacy, and optimal dose of recombinant GUS (named UX003) in patients with MPS VII; this trial is currently recruiting participants (NCT01856218).[31] In 2014, several states in the United States of America initiated patient recruitment for a phase 3 blinded and randomized study designed to evaluate the safety and efficacy of UX003 (NCT02230566).[31]

6.2. Intrathecal ERT

This section will cover the developments in intrathecal (IT) ERT that are occurring for four different MPS types with CNS involvement (MPS I – Hurler, MPS II – severe, MPS IIIA and MPS IIIB). One of the products under development for MPS IIIB may be used intravenously, depending on the results of the ongoing preclinical studies.

6.2.1. For MPS I

MPS type I (MPS I) is caused by a deficiency of the enzyme α-L-iduronidase (IDUA EC 3.2.1.76). It is commonly classified into three clinical phenotypes, i.e. Hurler (OMIM 607014), Hurler–Scheie (OMIM 607015), and Scheie (OMIM 607016), but because of the high variability of the disease and the overlapping of symptoms, it is more appropriate to classify patients as having the attenuated form or the severe form.[38] Patients with Hurler syndrome, the most severe form of MPS I, can develop CNS manifestations, including cerebral abnormalities, spinal cord compression, and obstruction of cerebrospinal fluid (CSF) reabsorption, in addition to abnormalities such as affected bones, joints, heart, airways, and eyes. Because the BBB prevents the enzyme from reaching the brain in significant quantities, intravenous ERT does not address the neurological complications of the disease. IT ERT with recombinant human α-L-iduronidase (rhIDUA) has been investigated as a treatment for GAG storage in the CNS in MPS I dogs [39] and MPS I patients.[40] Preclinical studies have demonstrated that high concentrations of rhIDUA injected via IT achieved normal IDUA enzyme levels in the brain, spinal cord, and spinal meninges, which persisted for 3 months after the final IT dose in canine models with MPS I.[39] Furthermore, another study that tested whether rhIDUA could reach and treat the MPS I-affected dog's CNS concluded that the treatment

reduced GAG levels in the brain and meninges.[41] Some of the dogs developed a CNS inflammatory response to IT rhIDU with mild to moderate aseptic meningitis.[39,41] CSF anti-rhIDU IgG titers in MPS I dogs correlated with diminished enzyme penetration into the brain and diminished efficacy in reducing GAG storage.[41,42] In humans, the IT ERT with laronidase was first tested in an adult MPS I patient with signs and symptoms of spinal cord compression. After treatment, the patient showed a slight reduction in CSF GAG values and an improvement in 12 minute walk test (12MWT), with better performance on stability and gait control and better results in many parameters of the pulmonary function test. No major adverse events were observed, and no clinically significant changes in serum chemistries were reported.[40] A phase I clinical trial was conducted by Dickson and colleagues (NCT00215527) [31] in four MPS I patients 8 years of age and older, aiming to test the delivery of ERT to the spinal fluid via IT injection. In this pilot study, rhIDUA was administered intrathecally once per month for 4 months in Hurler–Scheie and Scheie patients with spinal cord compression. Another important clinical trial (NCT00852358) [31] is a 24-month open-label, prospective, randomized trial in 16 MPS I patients aged 6 years or older who have documented evidence of cognitive decline. This study will test the safety and efficacy of IT rhIDUA to reduce or stabilize cognitive decline and is still recruiting patients.

6.2.2. For MPS II

MPS type II (MPS II), also known as Hunter syndrome (OMIM 309900), is caused by a deficiency of iduronate-2-sulfatase (EC 3.1.6.13), which results in the accumulation of GAGs. The clinical spectrum of MPS II is divided into the severe or attenuated form according to the involvement of the CNS. Approximately 75% of patients have the severe phenotype, [43,44] leading to neurologic manifestations such as hydrocephalus, spinal cord compression, seizures, and cognitive impairment, which progress to neurological deterioration. The onset of the disease usually occurs between the second and fourth years of age. Cognitive and neurological impairments are devastating problems for patients with the severe type of MPS II. Death generally occurs between the ages of 10 and 14 years. Although intravenous ERT is an effective treatment, the amelioration of CNS with ERT is limited by the BBB.[36] To date, ERT seems to be an ineffective method for the treatment of the severe spectrum of patients with MPS II. Various methods of effective treatment for CNS have been studied in an effort to circumvent the BBB, including direct IT injection into the CSF. The first study involving the chronic IT administration of ERT in nonhuman primates was conducted by Shire (Lexington, MA, USA) [45] and published in 2011. To support the chronic use of IT-idursulfase in humans, the study examined the safety of the combination of repeat-dose lumbar IT injections of idursulfase with bolus of intravenous idursulfase in 48 male cynomolgus monkeys. The study provided confidence for pursuing IT therapy as a treatment strategy for neurological manifestations. Calias *et al*. [46] showed evidence of the effectiveness of intermittent IT ERT in the MPS II mouse model, resulting in widespread enzyme distribution in the brain parenchyma. One year later, a study was conducted [46] treating MPS II mice with continuous IT infusion rather than with intermittent bolus infusion of the recombinant enzyme. This study showed decreased brain GAG accumulation and vacuolization, and the incidence of complications (i.e. pain at the pump site, skin erosion, wound dehiscence, wound infection, CSF leakage, and pump migration) was less than 0.1% per patient-month, which was consistent with the results of previous studies.[47] Currently, the HGT-HIT-094 study (NCT02055118) [31] sponsored by Shire is recruiting patients to determine the effect of monthly IT administration of 10 mg of idursulfase for 12 months on clinical parameters of neurodevelopmental status in pediatric patients with Hunter syndrome and cognitive impairment, who have previously received and tolerated a minimum of 4 months of therapy with Idursulfase.

6.2.3. For MPS IIIA

MPS IIIA (Sanfilippo Syndrome A or MPS IIIA) (OMIM 252900) is an autosomal recessive neurodegenerative lysosomal disorder caused by deficiency of the N-sulfoglucosamine sulfohydrolase (SGSH) enzyme (EC 3.10.1.1), which leads to the accumulation of GAG heparan sulfate. MPS III is characterized by progressive mental deterioration and behavioral problems with only mild visceral disease.[1] Clinical symptoms are primarily related to the CNS and include speech delay, sleep disturbance, hyperactivity, aggression and anxiety, with progressive neurological and behavioral deterioration, and early death.[48,49] Treatment of patients with MPS III is supportive because to date, there is no effective disease-modifying treatment.[50] The use of risperidone has been shown to be effective for behavioral problems.[51] Additionally, sleep disturbances are common, severe, and often difficult to manage,[52] but melatonin in high doses has been demonstrated to be an efficacious treatment.[53,54] Treatment of the CNS is challenging, and several potential therapeutic approaches have been suggested.[55–57] The strategy of increasing brain sulphamidase activity via injection of a recombinant human enzyme (rhSGSH) directly into

the CSF was first explored using MPS IIIA mice.[58–60] Subsequent evaluation in an MPS IIIA Huntaway dog model indicated that the replacement with the recombinant enzyme mediated a reduction of primary and secondary substrate accumulation and other lesions (e.g. neuroinflammation).[58,61–63] Phase I/II clinical trials examining this approach are currently underway in children with MPS IIIA (NCT01155778, NCT01299727).[31] In these trials, one of three doses of rhSGSH (10, 45, 90 mg) is injected into the lumbar CSF via an indwelling delivery device on a monthly basis. The trials and data collection are ongoing, and no peer-reviewed publications have been published to date. To explore the effect of dose/regimen, King *et al*. [64] developed a study with MPS IIIA Huntaway dogs that provided recombinant human sulphamidase (rhSGSH) to young presymptomatic animals from an age at which the brain exhibited significant primary (HS) and secondary (glycolipid) substrate accumulation.[61] The enzyme was infused into the CSF via the cisterna magna (3 mg or 15 mg/infusion), with the higher dose supplied every 2 or 4 weeks. Dose- and regimen-dependent reductions in HS were observed in the CSF and deeper layers of the cerebral cortex. Examination of the amount of LIMP (lysosomal integral membrane protein)-2, a general endo/lysosomal marker, or quantification of activated microglia by immunostaining demonstrated that the higher fortnightly dose resulted in superior outcomes in affected dogs. Secondary accumulation of GM3 ganglioside had a similar response to both rhSGSH regimens. The study findings indicate that the lower fortnightly dose is suboptimal for ameliorating existing deposition and preventing further development of the disease-related pathology in the brains of young MPS IIIA dogs. However, increasing the dose fivefold but halving the frequency of administration enabled the near-normalization of disease-related biomarkers. Thus, the authors suggested that an initial high concentration and frequent enzyme infusions could be followed by reduced enzyme dose/frequency treatments once the initial disease burden is ameliorated. However, these strategies require further preclinical testing.

6.2.4. For MPS IIIB

MPS type IIIB (Sanfilippo B syndrome) (OMIM 252920) is a lysosomal disorder caused by α-*N*-acetylglucosaminidase (EC 3.2.1.50) deficiency, which leads to the buildup of abnormal amounts of HSs in the brain and other organs. The accumulation of abnormal HS, particularly in the CNS, leads to severe cognitive decline, behavioral problems, loss of speech, increasing loss of mobility, and a lifespan of approximately two decades.[65] MPS IIIB is currently untreatable, partly due to the difficulty of developing an enzyme able to cross the BBB.[65] Given the above information, two laboratories (Alexion Pharmaceuticals, Cheshire, CT, USA and BioMarin Pharmaceutical, Novato, CA, USA) are developing recombinant forms of human NAGLU. The enzyme developed by BioMarin Pharmaceutical is produced in Chinese hamster ovary (CHO) cells; however it presents a limitation since it contains little or no mannose-6-phosphate (Man6-P), which is the signal for receptor-mediated endocytosis and targeting to lysosomes. This lack of Man6-P could be overcome by binding to the insulin-like growth factor II (IGFII) receptor at a site distinct from the Man6 binding site (NAGLU–IGFII).[66] In this context, preclinical assays using a mouse model of MPS IIIB with the fusion protein administered by intracerebroventricular injection were performed. The results demonstrated that the NAGLU activity was 30 times higher on the first day postinjection compared with the brains of the heterozygous mice controls, and the HS content in the tissues remained significantly reduced for at least 28 days postinjection. Furthermore, the enzyme was distributed into many areas of the brain and was found in substantial amounts in the liver, where the stored HS was almost completely abolished. More specific experiments showed that the enzyme was localized in the cytoplasm, as expected based on its lysosomal location; the half-life of the enzyme was approximately 10 days in the brain.[67] Alexion Pharmaceuticals also developed a recombinant enzyme named SBC-103 that encodes the same amino acid sequence as the native human enzyme (NCT02324049) [31] and is produced using an egg-based platform. In *in vitro* studies, SBC-103 demonstrated a Man6-P-dependent cellular uptake and was able to cross the BBB.[68] Subsequent assays using a mouse model of MPS IIIB with groups of animals receiving IT or intravenous administration were performed. Both administrations decreased HS in the brain.[69] Additionally, the intravenous administration of SBC-103 reduced HS storage in other tissues such as the liver and kidney in the MPS IIIB animal model. This *in vivo* study confirmed the findings from the *in vitro* studies and demonstrated that SBC-103 was able to cross the BBB (NCT02324049).[31] Both laboratories are beginning natural history studies followed by treatment studies in humans.

6.3. Intravenous ERT for MPS I and MPS II with fusion proteins

Because MPS I and MPS II affect both peripheral tissues and the CNS and because the ERT that is currently available is not able to treat CNS manifestations due to the BBB, a new strategy of treatment was developed to penetrate the brain. The recombinant enzyme IDUA,

used to treat MPS I patients, was reengineered based on its fusion to a genetically engineered monoclonal antibody (MAb) against the human insulin receptor (HIR). Because the insulin receptor is expressed at the BBB, the fused IDUA enzyme (HIRMAb–IDUA) binds the insulin receptor and, acting as a 'Trojan horse', crosses the BBB.[70] Preclinical studies using both male and female juvenile rhesus monkeys were performed to test four different doses that were administered intravenously. The monkeys received weekly infusions in 50 ml of normal saline or in 50 ml of 10% dextrose in normal saline over 30 minutes during a 26-week period. The higher dose (30 mg/Kg) caused hypoglycemia in the monkeys that received the infusion in normal saline; this effect was eliminated when glucose was added to the infusion solution. Pharmacokinetic analyses did not show a change in the rate of clearance of HIRMAb–IDUA from the blood at week 1 and week 26. The results allowed for the estimation of a dose for humans of approximately 0.6 mg/kg, which is predicted to replace >50% of the IDUA enzyme activity in the brain.[71] The same strategy was tested for MPS II in preclinical studies.[72] In the same way, the iduronate sulfatase (IDS) recombinant enzyme was reengineered as an IgG–IDS fusion protein using a genetically engineered MAb against the HIR. This molecule was produced in the CHO cell line. Thirty juvenile male rhesus monkeys were divided into four groups (three dosing groups and one control group) and received weekly infusions of HIRMAb–IDS in three different doses in 50 ml of normal saline with 5% dextrose solution over a 26-week period. No differences were detected in any of the parameters evaluated in the study between the baseline and week 26. Furthermore, no adverse events were observed with the three different doses. Therefore, the safety profiles of BBB-penetrating IgG fusion proteins in mice and monkeys support future clinical trials of these agents for the treatment of CNS disease.

6.4. Substrate reduction therapy

Substrate reduction therapy (SRT) aims to inhibit GAG synthesis using small molecules, thereby compensating for the impaired enzyme activity. Unlike ERT, whose efficacy is restricted mostly to the peripheral symptoms, the small molecules used in SRT are expected to cross the BBB upon oral administration and thereby alleviate neuronopathy, making this strategy particularly interesting for MPS III. Genistein (4,5,7-trihydroexyisoflavone), a naturally occurring plant isoflavone, blocks the epidermal growth factor (EGF)-mediated signal transduction that is responsible for the expression of the genes encoding the GAG synthesizing enzymes. Known as 'gene expression-targeted isoflavone therapy' (GET-IT), genistein reduced GAG levels in various organs, including the brain, of MPS IIIB mice and corrected neuroinflammation and the behavior of these animals.[73] Piotroswska *et al.* [74] enrolled eight pediatric patients with Sanfilippo disease to evaluate cognitive functions using 5 mg of genistein/kg of body weight administered twice daily. During the first year of the treatment, an improvement in cognitive functions in seven patients and stabilization in one patient was assessed, whereas after the third year (2-year follow-up), further improvement was observed in two patients, stabilization in three patients and some deterioration in three patients.[74] In this case report, genistein was effective in either inhibition (in some patients) or slowing down (in other patients) of behavioral and cognitive issues over a longer period. In an open-label study, 19 children with MPS III (age range 2.8–19 years) were supplemented with genistein (5 mg/kg/day) for 1 year. An improvement in the disability scale was not observed, but the authors reported a relative decrease in the recurrence of infections and gastrointestinal symptoms, as well as improvement in skin texture and hair morphology.[75] More recently, a randomized, crossover, placebo-controlled intervention with 10 mg/kg/day of genistein followed by an open-label extension study was conducted to evaluate the effects of genistein in 30 MPS III patients. A significant decrease was observed in the urinary excretion of total GAGs ($p = 0.02$, slope −0.68 mg GAGs/mmol creatinine/mo) and in plasma concentrations of HS ($p = 0.01$, slope −15.85 ng HS/ml/mo), but no effects on total behavior scores or on hair morphology were observed after 12 months of treatment.[76] In the studies conducted to date, no serious adverse events related to genistein therapy have been reported, despite the use of high doses. Based on the results, high-dose oral genistein therapy appears to be safe in MPS patients, but additional testing in a larger, randomized placebo-controlled trial is needed to further assess its safety and efficacy.[77]

6.4.1. Rhodamine B

Rhodamine B ([9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride) is among the main SRT molecules used for MPS.[78,79] *In vitro* experiments have shown decreased lysosomal storage of GAG in MPS VI and MPS IIIA skin fibroblasts. In the MPS IIIA mouse model, a dose as low as 1 mg/kg was able to reduce liver size and GAG levels in urine and tissues (liver and brain), leading to improvements in neurological function assessed by the water cross

maze.[80,81] Moreover, the long-term administration of this low dose presented no adverse effects in mice (i.e. reduction in liver size or hepatotoxicity, both of which are observed with high dosages). The safety and efficacy of this low dosage in humans on disease progression are still unclear.[9] Moreover, the mechanism of action of this molecule is unknown.[79] Because it is nonspecific, its direct effects on GAG biosynthesis cannot be distinguished from possible effects on other pathways affecting lysosomal storage.[82]

6.5. Gene therapy

Several possible approaches can be considered for the gene therapy of MPS. The first possibility is the direct injection of a vector that carries the transgene and allows for its expression (*in vivo* gene therapy). Another possibility is to harvest a patient's cells, insert the transgene, select the corrected cells, and inject them back into the patient (*ex vivo* gene therapy). Additionally, different types of vectors can be used; although a few nonviral approaches have been tested, viral vectors allow high levels of enzyme to be produced long-term.[83] These vectors can be delivered through different routes, including intravenous,[84] intramuscular, [85] and intracranial [86] injections. Neonatal treatment seems to be more efficient and reduces the cost of the procedure, but in a clinical scenario, this type of treatment would only be possible in cases with an early diagnosis of MPS based either on a previous MPS case in the family or on neonatal screening results. In all cases, gene therapy for MPS relies on the same properties for treatments as ERT and HSCT, in that the corrected cells can secrete the enzyme and cross-correct deficient cells.[83] Different types of viral vectors have been used both in animal models and to correct patients' cells. Among these vectors, the ones used in the gene therapy protocols for MPS are mostly derived from adeno-associated viruses (AAVs), retroviruses (RVs) or lentiviruses (LVs). AAVs are non-integrative and produce long-term expression of the transgene. Their different serotypes allow for the efficient transduction of a myriad of cell types. In that scenario, an intracerebral injection of AAV2/5 has been used with success in animal models of MPS I, MPS IIIA, and MPS IIIB.[87,88] Another AAV pseudotype (AAV2/8) was able to correct disease phenotypes in MPS II mice [89] and MPS VI cats [90] when injected intravenously. AAV9 vectors are another promising alternative, and reports have demonstrated their efficiency in MPS IIIA, MPS IIIB, and MPS VII mice using either intravenous or intracerebrospinal injections. [91–93] RVs and LVs are able to insert their genetic material into the host genome, but RVs can only transduce dividing cells. RVs were able to correct disease phenotypes in MPS I [94] and MPS VII mice,[84] MPS I dogs, [95] and MPS VI cats.[96] Interestingly, these studies have shown that although these vectors are not expressed in the brain, very high enzyme levels are achieved in the serum (up to 100-fold the normal levels), and somehow, a fraction of this enzyme can be found in the brain tissue.[94] However, because the RVs are unable to transduce quiescent cells, only LVs have been used to correct neurons and improve brain disease in MPS IIIA and IIIB animal models.[97,98] Studies involving nonviral approaches are less common, but different techniques have been used. The use of a transposable element to integrate exogenous DNA into the host cell (transposons) showed benefits in MPS I mice.[99] Also, new techniques such as minicircle vectors (a DNA vector designed to be resistant to epigenetic gene silencing) was able to produce sustained high levels of IDUA expression in MPS I mice.[100] Moreover, techniques that protect modified cells with alginate beads have shown partial correction in the same animal model.[101] Finally, the use of genome editing using systems such as the CRISPR-CAS-9, although promising, still has to be tested [102] but certainly transforms nonviral gene therapy for MPS into a promising approach.

One important point to be considered for gene therapy is the development of the immune response by the host against both the protein itself and the vector. Both scenarios have been described in preclinical studies for MPS. A cytotoxic T lymphocyte (CTL) response was detected after gene therapy in adult animals,[103,104] whereas neonatal treatment seemed to reduce the incidence of this adverse effect. Antibody responses were detected after both viral and nonviral gene therapy approaches for MPS.[101,105] Immunosuppressive agents and immunomodulation can be used to reduce or prevent these effects, [104] which suggests that gene therapy may be efficacious in older patients if transient immunosuppression is offered. Although no clinical trials on gene therapy for MPS have been published to date, at least two approaches seem to hold great promise (AAVs and LVs), and multicentric efforts should be made by researchers to bring this technology to a Phase 1 trial.

7. Other novel therapies

Particularities of each MPS, including main sites of pathology and disease progression, must be taken into account as key factors that limit the applicability of the novel therapies discussed so far. For example,

MPS IV manifests mainly as bone disease, to which fusion proteins that reach the CNS will probably not be a potential treatment. On the other hand, all MPS with brain symptoms could, in theory, be treated by this approach. Also, gene therapy and classical intravenous ERT has proven to be ineffective to reach the bone, unless the protein is modified to have affinity for this tissue.[106] Same difficulties are found when treating other hard-to-correct organs, such as the aorta and the heart valves. In these sites, it is likely that small molecules that have the affinity for these tissues or that have a better tissue distribution (compared to large proteins) could be used. In this context, several alternative approaches are being proposed for the treatment of MPS. Some of these techniques are summarized below.

7.1. Pentosan polysulfate (PPS)

Pentosan polysulfate (PPS) is an FDA-approved anti-inflammatory medication that is used for the treatment of interstitial cystitis and osteoarthritis, with extensive long-term human safety data.[107] PPS is able to reduce joint inflammation, promote fibrinolysis, and stimulate hyaluronan synthesis by synovial fibroblasts and proteoglycan synthesis by chondrocytes.[108] Its use for MPS has been suggested based on animal data showing an important inflammatory component in joint and bone disease. This process involves the activation of the toll-like receptor 4 (TLR4) signaling pathway and the release of pro-inflammatory cytokines, leading to tissue damage and apoptosis.[109] The oral administration of PPS in the rat model of MPS VI resulted in a reduction in both systemic and joint inflammation, with a reduction in serum inflammatory markers and advanced glycan end products. It led to marked improvements in motility, grooming behavior, and skull malformations as well as a reduction in tracheal deformities.[108] The weekly subcutaneous administration of PPS not only has the same therapeutic effects as the daily oral regimen but also leads to a reduction in urine and tissue GAG. Moreover, no adverse effects were noted over a 6-month period.[107] The mechanism by which PPS reduces GAG levels is still unknown. It might slow its synthesis or increase its degradation, have a direct effect on lysosomal function, or exhibit a chaperone function on lysosomal enzymes.[107] Nevertheless, data from preclinical studies are encouraging regarding the use of PPS alone or in combination with other therapies, and a clinical trial is planned to begin shortly.

7.2. Stop-codon read through

Stop-codon read through therapy (SCRT) aims to suppress the effect of premature termination-codon mutations, which lead to mRNA degradation by nonsense-mediated mRNA decay (NMD) and generate truncated nonfunctional peptides. Some compounds, such as aminoglycosides, can promote SCRT by adding a random amino acid and lead to the generation of full-length protein.[110] There is also the additional benefit of crossing the BBB [111] and addressing issues that ERT is unable to. Although the mechanism of action is not yet fully understood, it involves the binding to ribosomal RNA and altering its structure to allow the addition of an amino acid. [111,112] Moreover, it can directly stabilize mRNA and avoid NMD.[113] *In vitro* studies have shown increased enzyme activity in MPS I fibroblasts and/or cell linages with common MPS I mutations after treatment with chloramphenicol,[110] gentamicin,[111,114,115] amikacin, lividomycin, and paromomycin.[111] There are toxic side effects to the long-term administration of aminoglycosides, such as hearing loss and nephrotoxicity.[116] Therefore, specifically designed less-toxic compounds like PTC124 (Ataluren), NB30, and NB54 (paromomycin derivatives), have been studied and also showed a positive effect in MPS VI fibroblasts [116] and MPS I cells.[111] Currently, Ataluren (Translarna®, PTC Therapeutics, South Plainfield, NJ, USA) is already licensed in the European Union for the treatment of patients with nonsense mutation Duchenne muscular dystrophy (nmDMD). In addition, there are ongoing Phase 3 clinical trials for patients with nmDMD and Cystic Fibrosis. Phase I studies were also conducted for MPS I. It is important to point out that results obtained with different drugs vary according to each patient's specific mutations. Therefore, for this therapy, the patient's genotype must be always taken into account for achievement of the best therapeutic outcome possible.

7.3. Nanotechnology

Nanoparticles that can cross the BBB can be applied to enzyme delivery to improve the therapeutic efficacy of ERT. The feasibility of producing polymer-based nanoparticles for MPS VI was evaluated *in vitro* with commercially available arylsulfatase B (Naglazyme®; BioMarin Pharmaceutical) adsorbed onto poly (butyl cyanoacrylate) (PBCA) nanoparticles. The compound was shown to be stable for at least 60 minutes in human blood serum, indicating its potential as a promising candidate for ERT of MPS VI.[117] Nanoencapsulation can increase treatment responses in different ways, such as masking drug properties

and reducing dosage, increasing drug absorption, and modifying drug biodistribution and pharmacokinetics. [118] A multiple-wall lipid-core nanocapsule coated with chitosan and Fe^{2+} ions to allow the chemical linkage of laronidase (Aldurazyme®, Genzyme Corporation, Boston, MA, USA) has recently been described.[118] The modified enzyme was not cytotoxic and presented improved catalytic properties, the same half-life, and an improved clearance profile compared with conventional laronidase. Moreover, higher enzyme activity was found in the liver, spleen, and heart of treated MPS I mice 4 h after injection. After 24 h, the nanoencapsulated enzyme maintained higher activity levels in both the liver and kidneys. Although the formulation was unable to reach the CNS with the dose tested, a lower dose than conventional ERT can achieve higher activity and could result in the reduction of immunological responses to the treatment.

7.4. Cell microencapsulation

Cell microencapsulation is an approach in which cells are trapped in a semipermeable membrane, which allows for the exchange of metabolites and nutrients between them and the external environment (Figure 1). The membrane prevents the access of the immune system to the cells without the need for the continued immunosuppression of the host. The use of genetically modified cells that overexpress the deficient enzyme makes this a very promising approach for the treatment of IEM.[119] Three *in vitro* studies were performed in lysosomal storage disorders other than the MPS: one in Fabry disease [120] and two in Metachromatic Leukodystrophy.[121,122]

For MPS types I, II, and VII, both *in vitro* and *in vivo* studies have been performed using different cell types and implantation sites. For instance, Ross *et al.* [123] implanted encapsulated 2A50 fibroblasts directly into the lateral ventricles of the brain of MPS VII mice and showed GUS delivery throughout most of the CNS, thereby reversing the histological pathology. In the MPS II mouse model, C2C12 myoblasts implanted in the peritoneum led to a reduction in urinary and tissue GAG levels.[124] Similar results were obtained with the MPS I mouse model using BHK cells, although in this case, the response was diminished by an inflammatory response against the microcapsules.[101] The combined use of prednisolone reduced the inflammatory infiltrate associated with the capsules and significantly increased the enzyme levels in the serum in a short-term study.[125]

8. Competitive environment

The approved and emerging treatments for each MPS as well as the competitive environment are summarized in Tables 3 and 4.

9. Potential development issues

One of the major problems that the development of new treatments for MPS faces is the different patients'

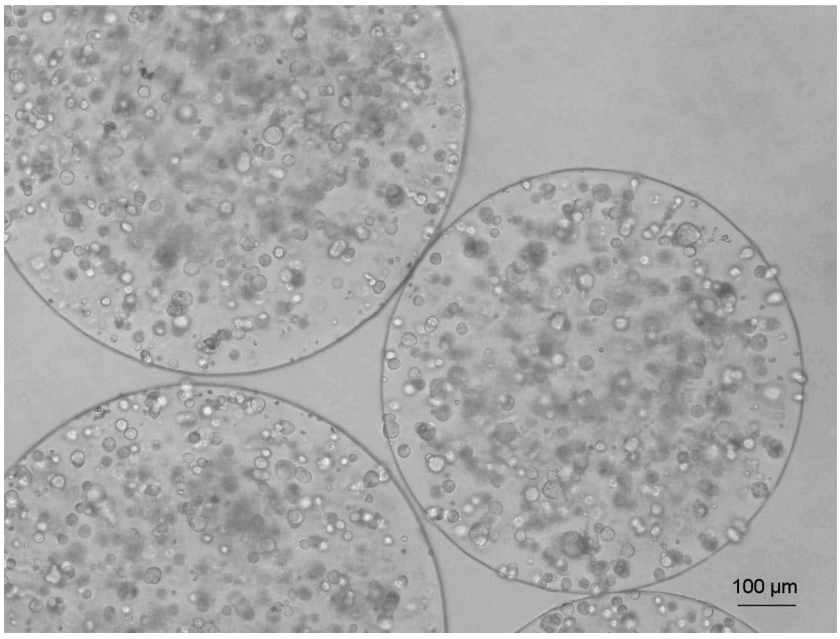

Figure 1. Sodium alginate capsules with genetically modified cells overexpressing IDUA. The capsule is made by a semipermeable membrane that allows the exchange of metabolites and nutrients with the external environment. It prevents the access of the immune system to the cells, without the need for continued immunosuppression of the host.

Table 3. Approved and emerging treatments for MPS.

MPS type	Approved treatments/regimen (Reference)	Emerging treatments/Status (Reference)
I	• HSCT/for Hurler patients aged up to 2.5 years [10] • Intravenous ERT with laronidase/ 0.58 mg/Kg weekly* [17]	• Intrathecal ERT with laronidase/Preclinical studies and case reports [30 (NCT00215527 and NCT00852358), 39] • Intravenous ERT with recombinant IDUA fused to a monoclonal antibody/ Phase I/II clinical trial [70,71] • Gene therapy with intracerebral AAV2/5/Preclinical studies [88] • Gene therapy with RV/Preclinical studies [94] • Gene therapy with transposons/Preclinical studies [99] • Gene therapy with cells in alginate beads/Preclinical studies [101] • Gene therapy using BHK cells in microcapsules/Preclinical studies [101] • Nanoparticles of laronidase/Preclinical studies [118]
II	• Intravenous ERT with idursulfase alfa/ 0.5 mg/Kg weekly [18]	• Intravenous ERT with idursulfase beta/Phase III clinical trial completed [21,22] • Intravenous plus intrathecal ERT with idursulfase alfa/Phase II/III clinical trial [30(NCT02055118)] • Intravenous ERT with recombinant IDS fused to a monoclonal antibody/ Phase I/II clinical trial [72] • Gene therapy with intravenous AAV2/8/Preclinical studies [89] • Gene therapy using C2C12 myoblasts cells in microcapsules/Preclinical studies [124]
III (all subtypes)	None	• Oral genistein/Clinical trial recruiting [74–76]
III-A	None	• Intrathecal ERT with recombinant SGSH/Phase I/II clinical trial ongoing [30 (NCT01155778 and NCT01299727)] • Gene therapy with intracerebral AAV2/5/Preclinical studies [87] • Gene therapy with intracerebral AAV9/Preclinical studies [93] • Gene therapy with LV/Preclinical studies [98] • SRT with Rhodamine/Preclinical studies [78–82]
III-B	None	• Intracerebroventricular ERT with NAGLU fused in insulin-like growth factor II receptor/Preclinical studies completed [66] • Intravenous ERT with recombinant NAGLU/Preclinical studies completed [30 (NCT02324049), 67, 68] • Gene therapy with intracerebral AAV2/5/Preclinical studies [88] • Gene therapy with intravenous AAV9/Preclinical studies [91] • Gene therapy with LV/Preclinical studies [97]
IV-A	• Intravenous ERT with elosulfase alfa/ 2.0 mg/kg weekly [20]	
VI	• Intravenous ERT with galsulfase/ 1.0 mg/kg weekly [19]	• Gene therapy with intravenous AAV2/8/Preclinical studies [90] • Gene therapy with RV/Preclinical studies [96] • SRT with Rhodamine/Preclinical studies [78,79] • Oral PPS/Preclinical studies [107–109] • Nanoparticles of ASB/Preclinical studies [117]
VII	None	• Intravenous ERT with recombinant GUS/Phase III clinical trial ongoing [30 (NCT01856218 and NCT02230566)] • Gene therapy with RV/Preclinical studies [92] • Gene therapy using 2A50 fibroblast in microcapsules/Preclinical studies [123]

* The double dose every 2 weeks is also safe and effective.

AAV: adeno-associated virus; ASB: arylsulfatase B; ERT: enzyme replacement therapy; GUS: β-glucuronidase; HSCT: hematopoietic stem cell transplantation; IDS: iduronate sulphatase; IDUA: α-iduronidase; LV: lentivirus; NAGLU: α-*N*-acetylglucosaminidase; PPS: pentosan polysulfate; RV: retrovirus; SGSH: N-sulfoglucosamine sulfohydrolase; SRT: substrate reduction therapy.

response to the same treatment, which is caused, in part, by the disease heterogeneity. The combination of two different types of treatment – like intravenous ERT and BMT or intravenous and IT ERT – could potentially bring benefits to some patients.

Especially in developing countries, where the challenges posed by more frequent conditions are still a priority, there is a lack of government incentives to the development of basic and clinical research in rare diseases. Additionally, the time to get approval to conduct a research is usually long, precluding the participation of these countries in multinational clinical trials, with usually no special treatment to rare diseases in the regulatory review.

10. Conclusion

The advances in genetic technologies that occurred in the last decades of the twentieth century have enabled the identification of the genes encoding each one of the enzymes involved in the MPS diseases. This allowed the production of recombinant enzymes to replace the missing enzymes in the patients and the development of several genetic strategies to correct the basic defect. It also enabled the generation of animal models to test the new therapeutic interventions, including ones not involving enzymatic or genetic therapy. However, the challenge of effectively approaching the unmet needs of MPS patients still remains.

Table 4. Competitive environment.

Compound	Company	Structure	Indication	Stage of development	Mechanism of action
Laronidase	Genzyme, a Sanofi Company	Recombinant α–L-iduronidase	MPS I	Approved intravenous Phase I intrathecal	Replacement of the missing enzyme
Idursulfase	Shire HGT	Recombinant iduronate-2-sulfatase	MPS II	Approved intravenous Phase I intrathecal	Replacement of the missing enzyme
Elosulfase-α	Biomarin	Recombinant *N*-acetylgalactosamine-6-sulfatase	MPS IVA	Approved	Replacement of the missing enzyme
Galsulfase	Biomarin	Recombinant arylsulfatase B	MPS VI	Approved	Replacement of the missing enzyme
rhGUS	Ultragenix	Recombinant GUS	MPS VII	Phase III	Replacement of the missing enzyme
rhHNS	Shire HGT	Recombinant heparan-N-sulfatase	MPS IIIA	Phase I	Replacement of the missing enzyme
HIRMAb-IDUA	Armagen	Fusion protein	MPS I	Phase I/II	Replacement of the missing enzyme, modified to reach the CNS
HIRMAb-IDS	Armagen/Shire	Fusion protein	MPS II	Phase I/II	Replacement of the missing enzyme, modified to reach the CNS
Genistein-rich soy isoflavone extract	Academic Study	Isoflavone	MPS III	Phase III	GAG synthesis inhibitor
SAF301	Lysogene	Adeno-associated virus containing the heparan-N-sulfatase gene	MPS IIIA	Phase I/II	Insertion of a new copy of the mutated gene
Ataluren	PTC Therapeutics	3-[-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl] benzoic acid	MPS I	Phase I	Nonsense allele inhibitor

MPS: mucopolysaccharidosis; CNS: central nervous system; GAG: glycosaminoglycans.

11. Expert opinion

Just over one decade after the first specific treatment for an MPS was launched (intravenous ERT with laronidase for MPS I), a set of therapeutic tools became available for several MPS types (especially intravenous ERT for MPS VI, MPS II, and, recently, for MPS IVA), and a much larger group of treatment strategies is in development. The long-term outcome reports `of intravenous ERT available for MPS I, MPS II, and MPS VI indicate that this treatment is successful and results in improvements in several disease manifestations (e.g. liver and spleen enlargement, respiratory parameters, joint mobility) but does not significantly impact the bone disease, corneal opacity, and heart valve problems. Moreover, intravenous ERT does not cross the BBB nor change the natural history of the CNS decline that occurs in some MPS types. Thus, in addition to having the intravenous ERT available for a larger number of MPS types, it is important to consider the development of alternative methods to approach the unmet needs regarding MPS therapy. IT ERT is being developed for MPS I, MPS II, MPS IIIA, and MPS IIIB and may impact the natural history of the CNS involvement in these diseases. Trials involving intravenous ERT with enzymes that are able to cross the BBB are in the planning stages for MPS I, MPS II, and MPS IIIB and could provide a different approach to access the CNS. SRT, which has proven to be effective in Gaucher and Niemann–Pick C disease, is being proposed for MPS (particularly MPS III), but still needs to be assessed by a controlled clinical trial to be validated. Gene therapy will most likely represent the ultimate treatment for MPS diseases; this possibility is now closer than ever to clinical practice, but some challenges must still be faced before it becomes a standard treatment. In parallel, some novel approaches (e.g. rhodamine B, PPS, stop-codon read through, nanotechnology, and cell microencapsulation) are being investigated and could provide alternative therapies for specific problems associated with selected MPS types. In conclusion, much work has been done in the past few years, and there is no doubt that, when the MPSs will, in 2017, commemorate 100 years of their first report in the literature, we will be much closer than today to find an effective cure for these challenging conditions.

Declaration of interest

R Giugliani is principal investigator of clinical trials on MPS. He has received investigator fees, speaker honoraria, and travel grants from Amicus Therapeutics, Actelion Pharmaceuticals, BioMarin Pharmaceutical Inc, Shire, Genzyme, and Synogeva. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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V. *Deleterious effects of interruption followed by reintroduction of enzyme replacement therapy on a lysosomal storage disorder.*

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Deleterious effects of interruption followed by reintroduction of enzyme replacement therapy on a lysosomal storage disorder

ANA PAULA SCHNEIDER, URSULA MATTE, GABRIELA PASQUALIM, ANGELA MARIA VICENTE TAVARES, FABIANA QUOOS MAYER, BARBARA MARTINELLI, GRAZIELA RIBAS, CARMEN REGLA VARGAS, ROBERTO GIUGLIANI, and GUILHERME BALDO

PORTO ALEGRE, BRAZIL

Temporary interruption of enzyme replacement therapy (ERT) in patients with different lysosomal storage disorders may happen for different reasons (adverse reactions, issues with reimbursement, logistic difficulties, and so forth), and the impact of the interruption is still uncertain. In the present work, we studied the effects of the interruption of intravenous ERT (Laronidase, Genzyme) followed by its reintroduction in mice with the prototypical lysosomal storage disorder mucopolysaccharidosis type I, comparing to mice receiving continuous treatment, untreated mucopolysaccharidosis type I mice, and normal mice. In the animals which treatment was temporarily interrupted, we observed clear benefits of treatment in several organs (liver, lung, heart, kidney, and testis) after reintroduction, but a worsening in the thickness of the aortic wall was detected. Furthermore, these mice had just partial improvements in behavioral tests, suggesting some deterioration in the brain function. Despite worsening is some disease aspects, urinary glycosaminoglycans levels did not increase during interruption, which indicates that this biomarker commonly used to monitor treatment in patients should not be used alone to assess treatment efficacy. The deterioration observed was not caused by the development of serum antienzyme antibodies. All together our results suggest that temporary ERT interruption leads to deterioration of function in some organs and should be avoided whenever possible. (Translational Research 2016;176:29–37)

Abbreviations: MPS = mucopolysaccharidosis; ERT = enzyme replacement therapy; GAG = glycosaminoglycan

Guilherme Baldo, PhD, *is an assistant professor in the Department of Physiology at the Federal University of Rio Grande do Sul in Brazil. Dr. Baldo is now focusing on developing better treatments for lysosomal storage disorders, including gene therapy and improvements in enzyme replacement therapies. He is working on gene editing studies using the CRISP-Cas9 system to allow autologous stem cell transplantation of genetically modified cells in selected disorders, such as Hurler and Hunter syndromes. He is also studying mechanisms by which these disorders lead to multisystemic features, aiming to find out new disregulated pathways and potential new treatments based on modulation of these alterations.*

From the Centro de Terapia Gênica- HCPA, Porto Alegre, Brazil; Programa de Pós-Graduação em Genética e Biologia Molecular, UFRGS, Porto Alegre, Brazil; Programa de Pós-Graduação em Ciência Biológicas: Fisiologia, UFRGS, Porto Alegre, Brazil; Serviço de Genética Médica-HCPA, Porto Alegre, Brazil.

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Reprint requests: Ursula Matte, Centro de Terapia Gênica-HCPA, Ramiro Barcelos, 2350, Porto Alegre 90035-903; e-mail: umatte@hcpa.edu.br.

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AT A GLANCE COMMENTARY

Schneider AP, et al.

Background

This article deals with the deleterious effects of interrupting enzyme replacement, the main therapy for lysosomal storage disorders, on an animal model of the multisystemic disease mucopolysaccharidosis type 1 (MPS I). This is highly important since we frequently observe treatment interruption in patients due to several reasons, and for years anecdotic cases have tried to show which disease parameters can or cannot be reversed after treatment reintroduction.

Translational Significance

Based on the results, we demonstrate that some organs (such as the brain and the aorta) may be irreversibly impaired due to ERT interruption. We also show that the main biomarker used to follow patients does not reflect disease status after treatment withdrawal. We hope these results will have a high impact on dealing with patients in cases where treatment is discontinued.

INTRODUCTION

The lysosomal storage disorders (LSDs) are a group of genetic diseases, usually caused by the deficient activity a lysosomal enzyme due to mutations in the specific codifying gene, leading to the intracellular storage of undegraded or partially degraded substrates.[1] Many of these disorders can be now treated using different approaches, depending on the characteristics of the disease. Potential approved treatments for selected LSDs include hematopoietic stem cell transplantation, substrate reduction therapy, and enzyme replacement therapy (ERT).

ERT is already available for several LSDs, including Gaucher disease, Fabry disease, Pompe disease, and MPS types I, II, IV, and VI, and Pompe disease. Although effective for several aspects of the MPS (especially organomegaly and respiratory problems), ERT is only partially effective considering the multisystemic features of the diseases.[2] For example, alterations in the heart valves, aorta, bones, and especially the brain are not completely corrected[3–5] probably due to the poor inaccessibility of the enzyme to these tissues when applied intravenously. Together with the treatment limitations, the high cost of these treatments (varying from U$150,000–500,000/year/patient) is an important point to be considered from a public health perspective.[6]

It is not uncommon to find reports in the literature about ERT interruption/withdrawal in the LSDs. Reasons for that include reimbursement issues (difficulties in obtaining the high-cost medication from the health system), medical recommendation (adverse reactions to infusions, pregnancy), and logistic problems (difficulties to travel or to skip school/work to have the infusion, for example). Also, shortage of the recombinant enzyme due to production problems may occur.[7,8] In these cases, some level of deterioration in the patient condition is usually reported. However, due to differences in phenotypes across diseases and even within the same disease, it is hard to obtain accurate data on which organs/systems are more or less affected when treatment is interrupted, and how to monitor any potential deterioration. Therefore, in the present work, we aimed to study the effects of ERT withdrawal followed by reintroduction in an animal model of MPS type I, as a prototypical multisystem lysosomal storage disorder.

MATERIALS AND METHODS

Experimental design. MPS I mice were produced by insertion of a neomycin resistance gene that interrupts the IDUA gene and therefore produces no enzyme, leading to a multisystemic disease that resembles the phenotype of Hurler syndrome in humans and affects all major organs in the body.[9] Furthermore, in a previous study,[3] we have shown that treatment with ERT from birth corrects most of MPS I symptoms, which allows a direct comparison of treated animals with or without interruption in the present study.

The article conforms to the relevant ethical guidelines for human and animal research. All animal studies were approved by our local Ethics Committee and complied with National Guidelines on Animal Care. MPS I mice on a C57BL/6 background (kindly donated by Dr Elizabeth Neufeld, UCLA) were used.

Animals with treatment interruption (ERT-stop group) were submitted to the following protocol: animals were injected intravenously with 1.2 mg/kg of Laronidase (Genzyme) every 2 weeks from birth up to 2 months of age. At 2 months, treatment was interrupted until mice were 4 months old. Finally, treatment was reintroduced and mice were sacrificed at 6 months of age for analyses.

These mice were compared with normal mice (Normal group), untreated MPS I mice (MPS I group), and mice treated from birth without interruption (ERT-neo), as previously described.[3] All animals were sacrificed at 6 months of age, 2 weeks after the last injection, in case of treated animals. At the time of sacrifice, mice were anesthetized with isoflurane, serum was

collected by retro-orbital puncture, and mice were sacrificed by cervical dislocation. Liver, lungs, kidneys, heart, testicles, aorta, and brain cortex were isolated and systematically divided in 2 pieces. One was flash frozen in liquid nitrogen for biochemical analysis and the other portion was fixed in buffered formalin. Thin cross sections were submitted to routine histologic processing, stained with hematoxylin–eosin/alcian blue and analyzed.

Echocardiographic assessment. Six-month-old mice were anesthetized with isofluorane and placed in left lateral decubitus position to obtain cardiac images. An EnVisor HD System, Philips Medical (Andover, Mass, USA), with a 12-4–MHz transducer was used, at 2-cm depth with fundamental and harmonic imaging. Images were captured by a trained operator with experience in echocardiography of small animals.

As measures of left ventricle function, we evaluated left ventricular ejection fraction, LV fraction shortening and fractional area change. As a measure of heart dilatation, we used the average of 3 measures of the heart diameter in systole (SD) and diastole (DD) using M-mode. In the pulmonary valve, the measures of the ejection and acceleration times were obtained using Doppler echocardiography, and their ratio was used as an index of pulmonary vascular resistance. All details of echocardiographic assessment were previously described.[3]

Behavioral tests. ***Open field test.*** Locomotor and exploratory activities were assessed using an open field test. The test consisted of a square arena (52×52 cm^2) with 60-cm high walls. The floor was divided into 16 squares by parallel and intersecting lines, obtaining 4 centered squares and 12 periphery squares. Mice were placed in one of the corners of the open field and (a) ambulation (number of times a mouse crossed with 4 paws one of the lines in the floor), and (b) exploratory behavior (rearings) were observed during 5 minutes for both control and MPS I animals.

Repeated open field. This test is used as a measure of habituation memory.[10] In this test, mice are put in the open field apparatus for 5 minutes and activity (number of crossings and rearings) is measured. The test is repeated 30 and 60 minutes after the first trial to evaluate habituation to the new environment (a reduction in the activity should be observed in mice after each trial), and the results from the third trial are compared with the first one.

Total GAG and dermatan sulfate levels. After euthanasia, tissues were homogenized in phosphate buffer and GAGs content was quantified using the dimethyl blue technique. In this technique, 25 μL of supernatant was mixed with freshly prepared dimethyl blue solution (dimethyl blue 0.3 mol/L with 2-mol/L Tris) and absorbance was read at 530 nm. Results were calculated as μg glycosaminoglycan (GAG)/mg prot and expressed as percentage of normal mice values. Urine samples were centrifuged and 25 μL were used for measuring GAG levels, and results were expressed as μg GAG/mg creatinine. Creatinine was measured using the Picric acid method.[11]

In available samples of liver, lung, and hearts, we measured dermatan sulfate (DS) specific content using MS/MS. Aliquots of 100 μL were evaporated to dryness under nitrogen and the residues were incubated with anhydrous 3M HCl-methanol at 65°C for 75 minutes, and again evaporated to dryness. Each residue was vortex-mixed with 100 μL of internal standard solution and 100 μL of acetonitrile. The solutions were filtered under centrifugation though a 0.2-μm membrane, transferred into an injection vial, dried under nitrogen, and reconstituted in the mobile phase (10 mmol/L ammonium acetate in acetonitrile: H_2O [90:10 vol/vol]). Electrospray ionization-MS/MS was performed using a Waters Quattro Micro API tandem mass spectrometer (Waters, Mass). A volume of 20 μL for each resuspended sample was injected into a Binary HPLC Pump using a Waters 2777C Sample Manager. Chromatographic separation was performed using the Acquity UPLC BEH Amide column (1.7 μm, 2.1 by 50 mm; Waters Corp.) which was heated to 30°C. The capillary voltage was 3.5 kV; cone voltage was 20 V, the source and desolvation temperatures were 120°C and 500°C, respectively. Data were acquired by selected reaction monitoring using the protonated molecular ion transition mass-to-charge ratio (m/z) 426 → 236.[12] Results are shown as nmol DS/mg prot.

Histology. GAG storage was observed by alcian blue staining. Thin sections (6 μm) were obtained from paraffin-embedded tissues and were stained with H-E and alcian blue (1%), to visualize GAGs in blue. At least 2 different slides from each animal were analyzed. Wall thickness of the ascending aorta was measured by obtaining the average of at least 5 wall measurements in different points of the cut, as previously described.[3]

Immunohistochemistry. Immunohistochemistry for glial fibrillary acidic protein (GFAP) was performed in brain cortex using specific antibody (Dako Cytomation, Polyclonal Rabbit anti-GFAP) and a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase. Slides were analyzed by a researcher blinded to the groups, counting positive cells in 5 high-power fields (40 ×). Negative controls were performed in each slide by not incubating the tissue with the primary (anti-GFAP) antibody.[10]

Antibody detection. We evaluated the formation of antibodies against the recombinant enzyme in serum collected at time of sacrifice. For the assay, 96-well ELISA plates were coated with Laronidase (4 μg/mL) in acid phosphate buffer saline overnight and blocked with 3% bovine serum albumin. Diluted serum was added (diluted 1:50) and incubated for 2 hours. A secondary antibody (Goat anti-mouse IgG, Sigma) conjugated to peroxidase was diluted 1:1000, incubated for 3 hours and revealed with 3,3′,5,5′-tetramethylbenzidine (TMB) for 6 minutes. The reaction was stopped with H_2SO_4 1 M and the absorbance was read at 450 nm.[3]

Ethics and statistics. All experiments were approved by our Ethics Committee of our institution (project number 08-658) and all procedures were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, monitored by our veterinarian and designed to minimize animal suffering.

Statistical analysis was performed using ANOVA and Tukey or Student *t* test, as indicated, and shown as average with errors bars indicating one standard deviation. A $P < .05$ was considered as statistically significant.

RESULTS

ERT-neo mice were evaluated in most aspects in a previous paper.[3] In another previous study, we showed that the half-life of the enzyme applied intravenously is 11.5 minutes, and that most of it is taken up by liver cells, although it is also detected in spleen, kidney, lungs, and the heart[13] 24 hours after the infusion. We also showed that a very small fraction can be detected in the brain, in the 1.2 mg/kg dose.[3] The main goal of the present study was to compare the results previously obtained with mice that had their treatment interrupted and reintroduced, and to further expand the analyses previously performed.

Urinary and tissue GAG. First, we evaluated urinary GAG in the mice during treatment interruption with the same method (dimethyl blue) used to monitor effectiveness of ERT therapy in patients. GAG levels were measured every 2 weeks, starting 2 weeks after treatment was interrupted, up to 4 months of age, when treatment was reintroduced. Results were compared to 2-month normal and MPS I untreated mice.

We had previously demonstrated that this dose and regimen was able to normalize GAG levels in mice treated from birth. Therefore, as expected, GAG levels were within normal range soon after treatment interruption. Surprisingly, levels remained low even after 8 weeks of treatment interruption, within normal range, and significantly lower than untreated MPS I mice (Fig 1*A*).

Tissue GAG were extracted and also quantified at 6 months. GAG levels were elevated in the liver (15-fold), whole kidney (18-fold), heart (3-fold), and lungs (5-fold) of untreated MPS I mice, compared with normal. ERT-neo mice showed normal GAG levels in all these tissues. ERT-stop mice showed normal liver and kidney GAG levels, but in heart and lungs the GAG levels were slightly elevated (Fig 1*B*).

Based on these results, we decided to quantify DS in the liver, heart, and lungs available using tandem mass spectrometry, a more precise method of GAG measurement. Results confirmed major increase in DS in all tissues in untreated MPS mice, and a complete normalization in DS levels in treated groups (Supplemental Figure 1), suggesting that both treatment regimens lead to a normalization of GAG levels in visceral tissues.

Histologic analysis of these tissues confirmed those findings. MPS I mice had abundant blue vacuoles in analyzed tissues but no visible vacuoles were observed in the liver, kidney, lung and testis from normal, ERT-neo, or ERT-stop groups. Very mild alterations could be observed in the heart. Interestingly, liver tissue of ERT-stop mice had an aspect similar to hydropic degeneration, possibly resulting from remnants of GAG storage (Fig 1*C*).

It is important to point out that we did not evaluate joint/bone disease in the present work because we have previously shown no effect of ERT on this parameter in the mouse model.[3]

Heart function. Echocardiographic analysis was performed in 6-month-old mice to assess heart function. A total of 6 parameters were analyzed (Table I). Evaluating left ventricular function and contractility, we obtained measures of left ventricular ejection fraction, fractional area change, and shortening fraction. These 3 parameters were reduced in MPS I mice and were restored in both treatment groups.

We also obtained measures of systolic and diastolic diameters, to assess heart dilatation. Both were increased in MPS I mice, and restored back to normal in treated groups.

Finally, untreated mice also had a reduced acceleration/ejection time (AT/ET ratio) in the pulmonary valve, which indicates pulmonary vascular resistance and right ventricle dysfunction, and this parameter was corrected only in ERT-neo mice but not in ERT-stop group, which suggests a deleterious effect in the mice that could not be reverted after treatment reintroduction.

Aorta. Histologic analyses in the aorta revealed that MPS I mice presented increased aortic wall thickness, with numerous white vacuoles in the tissue, which correspond to GAG storage. GAG levels were indeed increased in MPS I mice aortas and were only partially

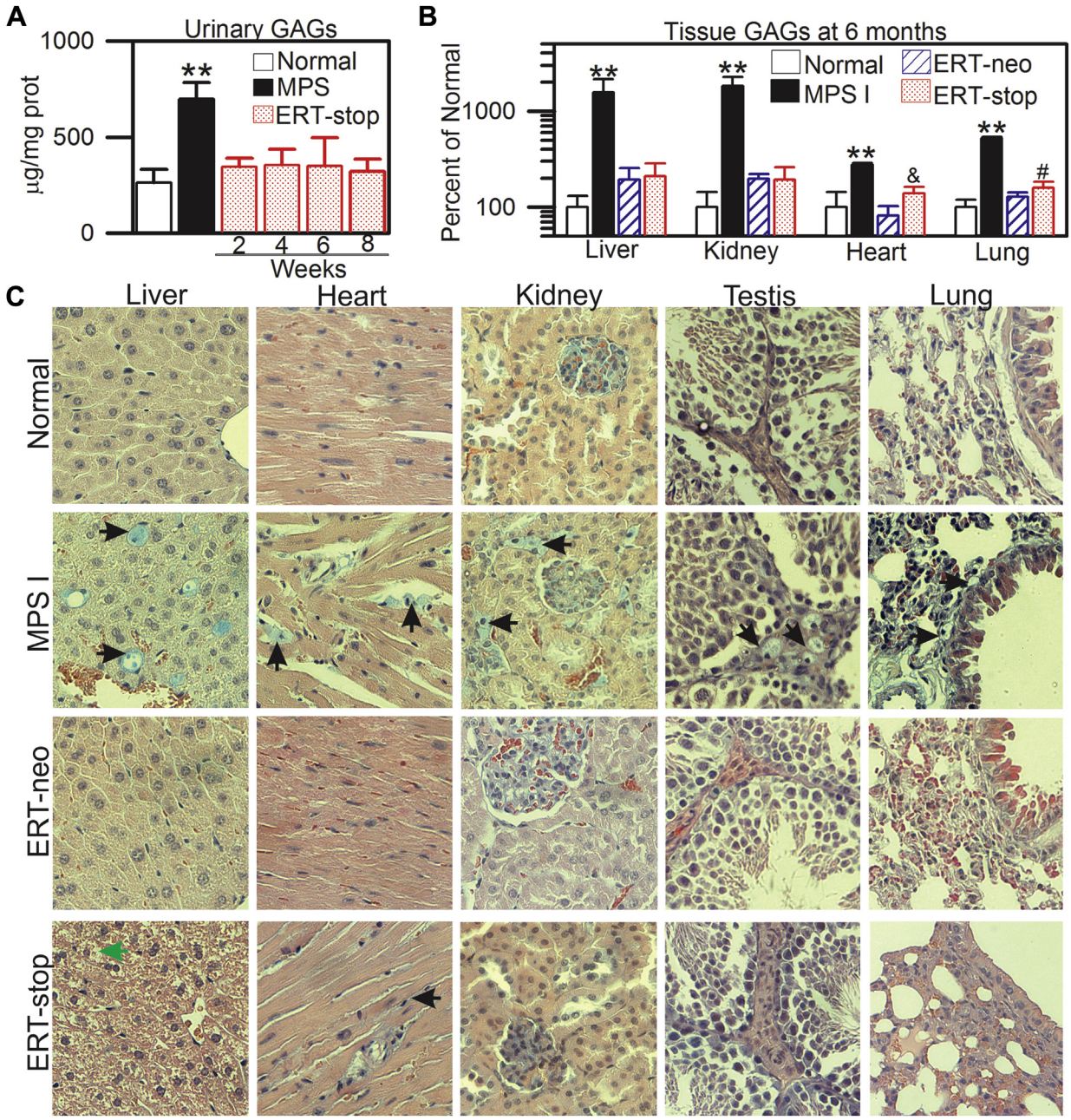

Fig 1. GAG levels. **(A)** Urinary GAG. Total GAG levels were measure in 2-month-old normal and MPS I mice, as well as in animals after treatment interruption every other week. Results are expressed as μg GAG/mg creatinine. $^{**}P < .05$ compared with normal. **(B)** Tissue GAG at 6 months. Results are expressed as percentage of normal levels. $^{**}P < .01$ compared with normal. #- $P < .05$ treatment compared with normal. &- $P < .05$ treatment compared with TRE-neo. **(C)** Histologic analysis of GAG storage. H–E and alcian blue staining in several tissues. Black arrows indicate cells with GAG storage. Green Arrow indicates histologic alterations in liver tissue, similar to an hydropic degeneration. All magnifications are 100X. GAG, glycosaminoglycan; MPS I, mucopolysaccharidosis type I.

restored in both ERT-neo and ERT-stop groups, and that was confirmed by histologic analyses. Also, only ERT-stop mice presented increased aortic wall thickness compared with normal mice, which suggest a deleterious effect of treatment interruption in this organ (Fig 2).

Behavior analysis and brain histology. As a measure of locomotor activity and exploratory behavior, animals

Table I. Heart function parameters assessed by echocardiography

Parameter	Normal	MPS I	ERT-neo	ERT-stop
LVSF (%)	37.52 ± 8.74	24.51 ± 4.78*	41.64 ± 7.71#	35.84 ± 7.32
LVEF (%)	60.23 ± 8.66	49.57 ± 12.19*	58.55 ± 6.97	59.61 ± 15.91
FAC (%)	51.53 ± 10.41	42.52 ± 11.06	53.32 ± 7.10	48.05 ± 6.52
AT/ET ratio	0.26 ± 0.04	0.18 ± 0.04**	0.24 ± 0.04#	0.19 ± 0.03*
SD (cm)	0.25 ± 0.05	0.35 ± 0.08*	0.22 ± 0.04##	0.23 ± 0.05#
DD (cm)	0.39 ± 0.03	0.46 ± 0.08*	0.37 ± 0.03#	0.36 ± 0.04#

Abbreviations: LVEF, left ventricular ejection fraction; *LVSF*, left ventricular shortening fraction; *FAC*, fractional área change; *AT/ET*, acceleration time/ejection time at the pulmonary valve; *SD*, systolic diameter of the left ventricle; *DD*, diastolic diameter of the left ventricle; *ERT*, enzyme replacement therapy; *MPS I*, mucopolysaccharidosis type I.
*$P < .05$ and **$P < .01$, compared with normal; #$P < .05$ and ##$P < .01$, treatments compared with MPS I. n = 5–16 animals/group.

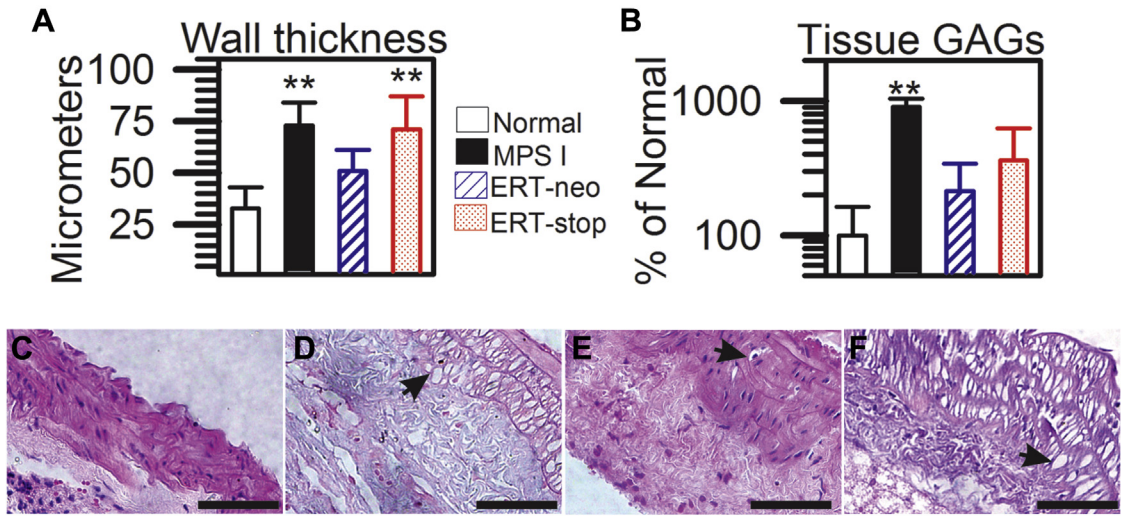

Fig 2. Pathology in the aorta. **(A)** Ascending aorta wall thickness at 6 months. **$P < .01$ compared with normal. **(B)** GAG levels in the aortic tissue at 6 months. **$P < .01$ compared with normal. **(C–F)** Representative sections of an aorta from **(C)** Normal; **(D)** MPS I; **(E)** ERT-neo; and **(F)** ERT-stop. Black arrows indicate GAG storage in the tissue. GAG, glycosaminoglycan; MPS I, mucopolysaccharidosis type I.

were submitted to the open field test. MPS I mice presented reduced activity in both parameters, which were corrected in ERT-neo mice and had an intermediate performance in ERT-stop mice, although not significantly different from untreated mice (Fig 3*A* and *B*).

The repeated open field test is a measure of nonaversive memory, and MPS I mice showed impaired behavior in horizontal activity in this test. Both treated groups had intermediate results, which suggest a limited efficacy on the correction of this parameter (Fig 3*C* and *D*).

GFAP positive cells were increased in MPS I mice, and both treatment groups were partially corrected, not being able to reduce neuroinflammation to normal levels (Fig 4).

Antibodies. Antibodies against the recombinant enzyme were detected by ELISA. As previously reported, animals treated from birth develop an immune tolerance, with only 1 mouse out of 7 developing antibodies at 6 months of age. Similarly, animals treated from birth that had treatment interrupted did not develop antibodies against the enzyme when it was reintroduced (Fig 5). Normal and MPS I-untreated mice, as expected, did not develop anti-IDUA antibodies.

DISCUSSION

Although scenarios of treatment interruption are not uncommon in patients, few studies in the literature address its effects.[14,15] Heterogeneity among patients' phenotypes makes it hard to systematically study which organs/systems can be completely normalized and which disease parameters cannot be improved, once treatment is not provided for a certain amount of time and then reintroduced. Therefore, in this study, we used the animal model of MPS I, a prototype

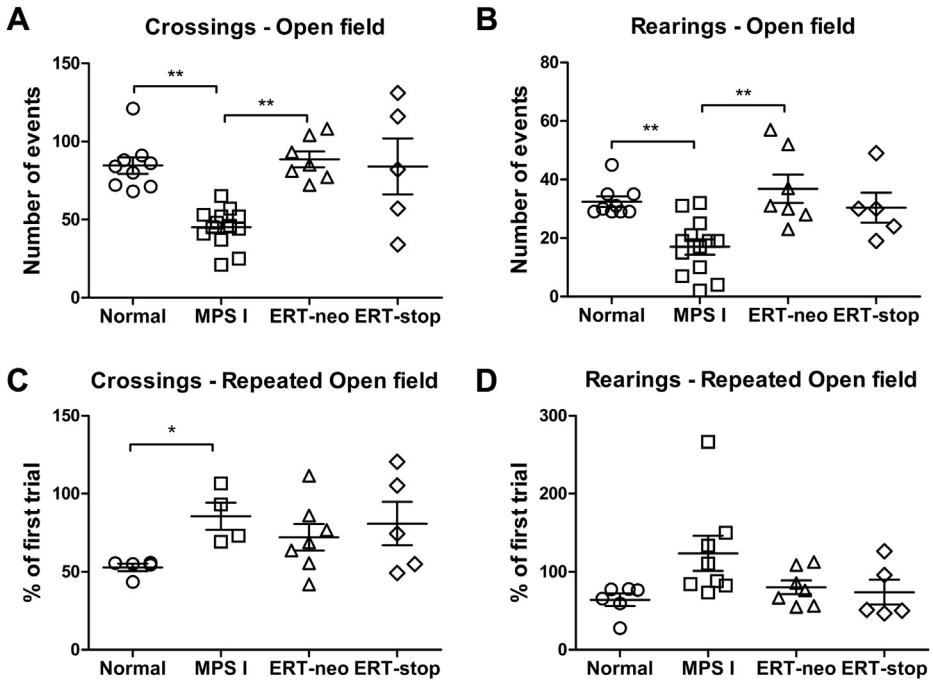

Fig 3. Behavior analysis. (**A** and **B**) open field test. Mice were analyzed at 6 months and **(A)** Locomotor activity and **(B)** Exploratory behavior were compared among groups. (**C–D**) Repeated open field test. Three trials (with interval between trials of 30 minutes) were performed in the open field test and the activity of the third trial was compared with the first one in the **(A)** number of crossings and **(B)** number of rearings. $*P < .05$ and $**P < .01$, compared with normal. Dots represent individual mice and the trace indicated average and standard deviation.

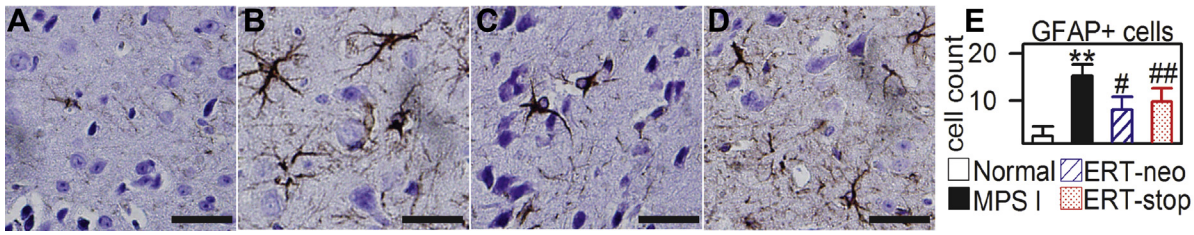

Fig 4. GFAP immunostaining. Glial fibrillary acidic protein (GFAP) was detected in the brain cortex of **(A)** Normal; **(B)** MPS I; **(C)** ERT-neo, and **(D)** ERT-stop mice. **(E)** Quantification of GFAP positive cells in 5 high-power fields. $**P < .01$ compared with normal. $\#P < .05$ and $\#\#P < .01$ compared with both normal and MPS I. ERT, enzyme replacement therapy; MPS I, mucopolysaccharidosis type I.

multisystemic progressive LSD trying to address these questions.

First, we evaluated urinary GAG every 2 weeks after treatment interruption. We have previously shown that this same dose and regimen is able to reduce urinary GAG to normal levels on the long term[3] and that treatment with ERT in older mice normalizes urinary GAG even with only 2 months of treatment.[5] Therefore, we could expect to find normal GAG levels in the first weeks after treatment interruption, as we did. However, surprisingly, urinary GAG did not increase significantly throughout the whole 2 months of interruption. Considering that this exact same test is used to monitor treatment effectiveness in patients, our results raise a concern because although treatment interruption produced significantly alterations in organs, the biomarker usually used to monitor treatment in patients did not reflect that, at least in these first 2 months after interruption. Other biomarkers, such as serum heparin cofactor II-thrombin complex[16] could be potentially used along with urinary GAG as better measures of treatment outcome. Unfortunately, we were unable to perform analysis of DS in urine due to insufficient sample, but future studies will address if any GAG alone is a better marker than a general assay.

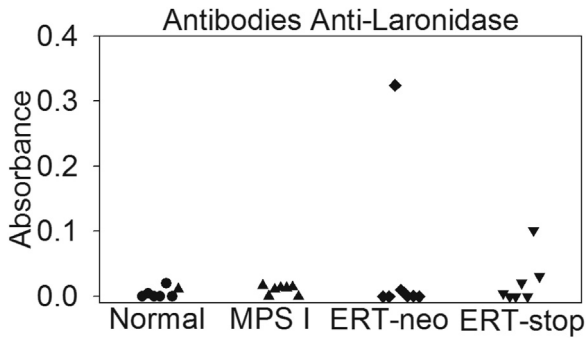

Fig 5. Serum antibodies. Serum antienzyme antibodies were measured in 6-month-old mice. Each dot represents a single animal. Results are shown as intensity of absorbance. No differences were found between groups.

Tissue GAG at 6 months revealed that organs such as the liver and the kidney remain completely corrected after interruption and reintroduction of therapy, whereas results in the lungs and the heart showed a slight elevation, a finding that was not confirmed by the additional specific measurement of DS, the main GAG accumulating in those tissues. So, we conclude GAG levels remain normal in the visceral organs despite the interruption of therapy.

Reduction in liver size is one of the first clinical benefits observed in patients with LSD after ERT.[17] Despite tissue GAG reduction, alterations in liver histology (apparent hydropic degeneration) were still present in ERT-stop mice, revealing that a complete histologic recovery of the organs may require a longer period to be achieved. Small histologic abnormalities were also seen in the heart. It showed remnants of GAG in the ERT-stop tissue by histologic analysis, despite our GAG analysis showing that DS levels were similar to the observed in normal mice. We have previously shown that treatment of 6-month-old mice for 2 months with ERT is able to completely normalize heart GAG levels,[5] which suggest that, for some reason, treatment interruption followed by its subsequent reintroduction for 2 months could possibly be less effective than treatment of a naïve mice for 2 months.

The heart function was assessed by echocardiography. MPS I patients frequently develop both left and right ventricle disease,[18] therefore we aimed to look at both aspects of cardiac function. Three parameters of contractility of the left ventricle as well as heart dimensions in systole and diastole suggest that reintroduction of ERT is able to prevent both left ventricle dysfunction and heart enlargement. Interestingly AT/ET ratio at the pulmonary valve, a commonly used indicator of vascular pulmonary resistance showed deterioration in ERT-stop treated mice, which suggest that in MPS patients, treatment interruption could lead to a right ventricle dysfunction.

The ascending aorta is one of the organs considered as "difficult-to-treat" by current therapies. ERT-neo mice presented reduction in wall thickness (as previously described by our group)[3] and GAG levels (current work). ERT-stop mice presented very little alteration in wall thickness and intermediate results of GAG levels, which suggests that treatment interruption leads to a rapid progression of the disease in this organ and once again highlights the deleterious effect of discontinuous ERT.

It is general consensus that the enzyme administered by intravenous ERT does not cross the blood-brain-barrier in significant amount in human patients, although we[3] and others[19] have shown that, in mice, a fraction of the enzyme is able to reach the brain, through mechanisms still uncertain. Based on that, behavioral tests were performed, and results show an improvement only in ERT-neo mice in the open field test. Our hypothesis is that during the first 2 months, a small fraction of the enzyme reaches the brain in ERT-stop mice, but with treatment interruption for the next 2 months, some deterioration occurs, hence the differences observed in behavioral tests. We also have shown an increase in GFAP cells in MPS I brain.[10] This neuroinflammation indicator was investigated and results from both treatment groups were intermediate between normal and MPS I mice. Altogether these results suggest that ERT is able to reduce inflammatory markers in the central nervous system, although a complete functional correction was achieved only in ERT-neo mice. It is possible that ERT is only delaying the mental deterioration in MPS I mice, and ERT-stop mice had a faster deterioration due to the interruption period.

Finally, one hypothesis why treatment interruption could be less effective after reintroduction was the development of anti-IDUA antibodies. These antibodies could potentially neutralize the enzyme, and therefore cause an immune response in animals once treatment is reestablished, making ERT less available to the tissues, and losing part of its efficacy.[20] As shown in our previous study,[3] starting ERT in the neonatal period leads to an immune tolerance in mice. According to our current results, this tolerance is maintained even if treatment is interrupted, which suggest that the development of antibodies is not a reason why ERT could be less effective when reintroduced.

Taken together, our results suggest deterioration of function in some organs (particularly some aspects of the cardiovascular system and the brain) after ERT withdrawal followed by reintroduction. Other organs, such as the liver and the kidney, seem to be able to recover completely from the temporary absence of the enzyme.

Also, monitoring therapy based only on urinary GAG can be risky based on our results. The development of antibodies is not responsible for the deterioration observed after reintroduction of therapy in some organs, which suggest that the organs susceptible to withdrawal are the ones in which there are either structural irreversible changes in the tissue (such as aorta dilatation) or tissues in which a functional worsening cannot be reverted (such as the brain). These aspects should be taken in consideration when evaluating patients who faced, for any reason, temporary interruption of ERT.

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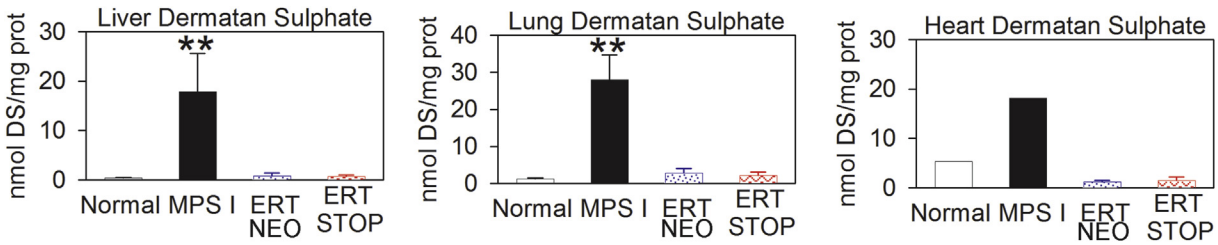
Appendix



Supplemental Figure 1. Dermatan MS/MSsulfate levels measured by MS/MS in liver, lungs, and heart of 6-month mice. $^{**}P < .01$ compared with other groups.

VI. *Identification of mutations in Colombian patients affected with Fabry disease.*

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Research paper

Identification of mutations in Colombian patients affected with Fabry disease

Alfredo Uribe [a,*,1], Heidi Eliana Mateus [b,1], Juan Carlos Prieto [c], Maria Fernanda Palacios [c], Sandra Yaneth Ospina [b], Gabriela Pasqualim [d], Ursula da Silveira Matte [d,e], Roberto Giugliani [d,e]

[a] Centro de Investigaciones en Bioquímica (CIBI), Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 No. 18° - 12, Bogotá, Colombia
[b] Unidad de Genética, Escuela de Medicina y Ciencias de la Salud, Universidad del Rosario, Carrera 24 N° 63C-69, Bogotá, Colombia
[c] Instituto de Genética, Facultad de Medicina, Universidad Javeriana, Carrera 7 No. 40-62 Ed. 32, Bogotá, Colombia
[d] Centro de Terapia Gênica, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, RS 90035-003, Brazil
[e] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, RS 90035-003, Brazil

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ABSTRACT

Fabry Disease (FD) is an X-linked inborn error of glycosphingolipid catabolism, caused by a deficiency of the lisosomal α-galactosidase A (AGAL). The disorder leads to a vascular disease secondary to the involvement of kidney, heart and the central nervous system. The mutation analysis is a valuable tool for diagnosis and genetic counseling. Although more than 600 mutations have been identified, most mutations are private. Our objective was to describe the analysis of nine Colombian patients with Fabry disease by automated sequencing of the seven exons of the GLA gene. Two novel mutations were identified in two patients affected with the classical subtype of FD, in addition to other 6 mutations previously reported. The present study confirms the heterogeneity of mutations in Fabry disease and the importance of molecular analysis for genetic counseling, female heterozygotes detection as well as therapeutic decisions.

1. Introduction

Fabry disease (FD; OMIM: 301500) is an X-linked lysosomal storage disorder caused by the deficiency of the enzyme alpha-galactosidase A (AGAL, EC 3.2.1.22) (Brady, 1967). This enzymatic defect leads to the systemic accumulation of glycosphingolipids (mainly globotriaosylceramide -GL3-) in blood vessels from the skin, kidney, heart and brain (Desnick et al., 2003).

Today two main FD subtypes have been described. The first one, or "classical phenotype", affects males who have a markedly reduced AGAL activity. In these patients the onset of disease occurs in childhood or adolescence and is characterized by acroparesthesias, angiokeratomas, corneal opacities, hypohydrosis and progressive vasculopathy of the kidney, heart, and central nervous system (Desnick and Brady, 2004). The second phenotype, or "milder", has been described in patients with a higher residual AGAL activity and a later onset characterized by cardiac and renal symptoms (Nakao et al., 2003, 1995; Terryn et al., 2013).

Due to the late appearance of symptoms in patients with milder FD, the prevalence of this phenotype seems to be higher in relation to patients with classical FD, whose incidence has been estimated to be between 1:40.000 to 1:117.000 male births, approximately (Lin et al., 2009; Meikle et al., 2006; Spada et al., 2006).

Patients with FD can be diagnosed by evaluating AGAL activity in plasma or white blood cells, however this analysis often fails to distinguish between Fabry heterozygotes with high residual AGAL activity and normal individuals. For this reason, mutation analyses are required to detect female heterozygotes, define genotype/phenotype correlations, and to make an accurate prenatal diagnosis and take therapeutic decisions (Yoshimitsu et al., 2011; Lukas et al., 2013).

The genomic sequence of the *GLA* gene is 12 kb in length and contains 7 exons (OMIM: 300644). To date, more than 600 mutations have been described including missense, nonsense and splice-site mutations, as well as gene rearrangements (Ashley et al., 2001; Schirinzi et al., 2008; Shabbeer et al., 2006). Most of the described mutations are private (with some few exceptions found in several unrelated subjects) and are usually associated with modifications in CpG dinucleotides, known hotspots for the disease (Barker et al., 1984; Cooper and Youssoufian, 1988).

In this study, we describe the first clinical and genetic analysis of nine Colombian FD patients. Direct sequencing of the complete *GLA* open reading frame revealed eight mutations, two new and six previously reported. Patients with the classical phenotype of FD were identified,

Abbreviations: FD, Fabry disease; AGAL, alpha-galactosidase A; *GLA*, alpha-galactosidase A gene; GL3, globotriaosylceramide; DBS, dried blood sample.

* Corresponding author.
E-mail addresses: jeuribe@uniandes.edu.co (A. Uribe), heidi.mateus@urosario.edu.co (H.E. Mateus).

[1] A. Uribe and H. E. Mateus contributed equally to this work.

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thereby providing additional information about the genotype–phenotype correlation.

2. Materials and methods

2.1. Patients

Nine FD Colombian patients were included in this study. All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). All participants completed a consent form for diagnostic testing approved by the Institutional Ethics Committee of each participating institution.

2.2. AGAL activity assays

AGAL enzymatic activity was first determined in dried blood spot (DBS) samples (as previously described by Chamoles et al., 2001; Civallero et al., 2006;Uribe and Giugliani, 2013) and the results were compared with the reference values reported for the Colombian population (range: 2.0–21.8 nmol/ml/h; Uribe and Giugliani, 2013). To confirm the deficiency, AGAL activity was also measured in leukocytes by a fluorometric assay following the procedure described by Shapira et al. (1989).

2.3. GLA mutation analysis

The mutation analyses were performed as described by Pasqualim et al. (2014). Briefly, genomic DNA was extracted from FTA Classic cards (Whatmann, USA) according to the manufacturer's instructions. In all patients the *GLA* open reading frame (exons 1–7 and their flanking regions) was amplified by PCR, as previously described by Shabbeer et al. (2005), with minor modifications. Each amplicon was purified with EXO-SAP (GE Healthcare Lifesciences, USA) and quantified with Low Mass Reader (Invitrogen, USA).

Forward and reverse sequences were analyzed with the same primers used for PCR amplification in an ABI3500 genetic analyzer and BigDye Terminator v3.1 (Applied Biosystems, USA). The obtained sequences were compared to the one reported on GenBank (accession number X14448.1) and sequence variation was confirmed by repeated PCR amplification and sequencing. Sequence variations were described according to the international mutation nomenclature guidelines as set forth by the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

Data from the Fabry Database (http://www.fabry-database.org) and Human Genome Mutation Database (http://www.hgmd.cf.ac.uk) were used to define novel *GLA* sequence variants and their associated phenotype. Each mutation was analyzed to determine the relative conservation of the substituted amino acid by comparison with other vertebrate species. To predict the effect of newly identified mutation we used SIFT, PolyPhen2, MutPred, MutationTaster, NetGene2 and Fabry CEP software.

SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Ng and Henikoff, 2003). In this way, if a score is smaller than 0.05, the corresponding neutral substitutions (NS) are predicted as "damaging", while if it is greater, the NS are predicted as "tolerated". PolyPhen2 is an automatic tool for prediction based on several features, including the sequence, phylogenetic and structural information characterizing the substitution (Adzhubei et al., 2010). The score of PolyPhen2 ranges from 0 to 1, and the corresponding prediction is "probably damaging" (coded as "D" if the score is greater than 0.85); "possibly damaging" (coded as "P" if it is between 0.85 and 0.15) and "benign" (coded as "B" if it is smaller than 0.15).

MutPred is a web application tool developed to classify an amino acid substitution as disease-associated or neutral in human. In this way, it can be used to predict the molecular cause of disease. Another application (MutationTaster) integrates information from different biomedical databases and uses established analysis tools to predict evolutionary conservation sites, splice-site changes, loss of protein features and changes that might affect the amount of mRNA within the cell (Schwarz et al., 2010). We also used the NetGene2 server to predict possible splice sites in human GAL gene (Hebsgaard et al., 1996) and, in order to determine if the mutations found were candidates for the treatment with chaperones, the program Fabry CEP.

3. Results

3.1. AGAL enzymatic activity

The nine Colombian FD patients included in this study had low or undetectable levels of AGAL activity in DBS (0.0 to 0.4 nmol/ml/h) and leukocytes (0.0 to 0.4 nmol/protein mg/h) (Table 1), showing an AGAL residual activity ranging from 0.0 to 0.6%.

3.2. GLA mutations

The sequence analysis of the complete coding region of *GLA* in the nine evaluated patients revealed six previously described mutations: c.1024C > T (p.Arg342*), c.804-2_-3delCA (IVS5-2_-3delCA), c.195-1G > C (IVS1-1G > C), c.1072_1074delGAG (p.Glu358del), c.334C > A (p.Arg112Ser) and c.1072G > A (p.E358K) (Table 1). Patients FD-2 and FD-8 displayed two novel variants: c.1051_1052delGT (p.Val351SerfsX23) and c.806G > T (p.Val269Gly) (Fig. 1A and B). The mutations and enzymatic activities are summarized in Fig. 2.

At the protein level, comparative *in silico* analysis of the novel c.806G > T mutation (p.Val269Gly) revealed a strict conservation of valine at position 269 among vertebrate species. Polyphen2 predicted that this missense mutation is probably damaging with a score of 0.999, and SIFT software showed probabilistic scores compatible with a potential deleterious effect (p.Val269Gly = 0,0). For the c.1051_1052delGT variant the MutationTaster software predicted a pathogenic effect with a changed splice site and a truncated protein, while NetGene2 predicted that an acceptor splice site would be lost with this mutation.

3.3. Clinical correlation

All the patients showed a classical FD phenotype (Table 2). In relation to the patients who showed new variants, patient FD-2 was a 50-year-old male who had experienced intermittent neuropathic pain in the lower extremities since 6 years of age, fever crisis, heat intolerance and hypohidrosis. Later, when he turned 33, he showed an increase in proteinuria that quickly progressed to an end-stage renal disease and kidney transplantation.

Patient FD-8 was a 51-years-old male who presented with angiokeratoma at age 6, and suffered from acroparesthesias, fever crisis, hypohidrosis and heat intolerance from the age of 8.

At the age of 30 he showed increases in proteinuria and developed chronic renal failure.

4. Discussion

This is the first study carried out in Colombia that evaluates the molecular aspects of FD. The mutation analysis of the *GLA* gene in nine FD Colombian patients allowed us to identify eight mutations, six of them previously described and two new variants, illustrating the genetic heterogeneity underlying this disease at the molecular level.

Table 1
Mutations found in nine Colombian patients with Fabry disease. FD: Fabry disease patient. NA: not available.

Proband	Phenotype	GLA activity		Mutation site	Nucleotide change	Amino acid change	Mutation type	Reference
		DBS	Leukocyte					
FD-1	Classical	0.0	0.1	Exon 7	c.1024C>T	p.Arg342*	Missense	(Davies et al., 1993b)
FD-2	Classical	0.1	0.0	Exon 6	c.806G>T	p.Val269Gly	Missense	This study
FD-3	Classical	0.2	0.4	Intron 5	c.804-2_-3delCA (IVS5-2_-3delCA)	—	Aberrant splicing	(Eng et al., 1993)
FD-4	Classical	0.2	0.3	Intron 1	c.195-1G>C (IVS1-1G>C)	—	Aberrant splicing	(Chen et al., 1998)
FD-5	Classical	0.4	NA	Exon 7	c.1072_1074delGAG	p.Glu358del	Deletion	(Blanch et al., 1996)
FD-6	Classical	0.1	0.3	Intron 1	c.195-1G>C (IVS1-1G>C)	—	Aberrant splicing	(Chen et al., 1998)
FD-7	Classical	0.1	0.1	Exon 7	c.1072G>A	p.Glu358Lys	Missense	Miyazaki et al., 1998
FD-8	Classical	0.2	0.4	Exon 7	c.1051_1052delGT	p.Val351SerfsX23	Deletion	This study
FD-9	Classical	0.02	NA	Exon 2	c.334C>A	p.Arg112Ser	Missense	(Ishii et al., 1992)

NA = Not available.

We identified a novel mutation in patient FD-8: c.1051_1052delGT (p.Val351SerfsX23). This deletion causes a frame shift in the open reading frame and creates a premature termination codon at residue 373, probably leading to the degradation of the protein. The c.1051_1052delGT mutation is characterized by an almost complete lack of *GLA* activity in the patient and a classical phenotype of FD.

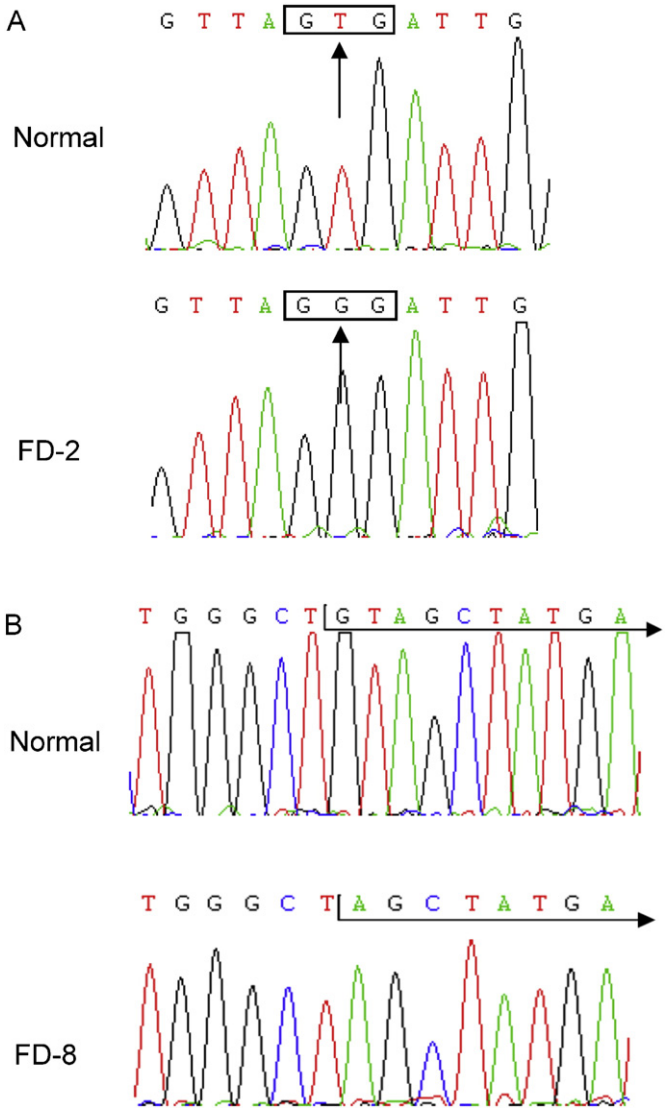

Fig. 1. A. Partial genomic DNA sequence of *GLA* gene, indicating the single-base substitution of G to T (c.806G > T; p.Val269Gly) in the hemizygous patient FD-2. B. Partial genomic DNA sequence of *GLA* gene, indicating the deletion of two nucleotides (c.1051_1052delGT; p.V351SFsX23) in the hemizygous patient FD-8.

Another novel mutation c.806G > T (p.Val269Gly) in exon 6 was identified in patient FD-2. The substitution of valine for glycine results in a change in the small hydrophobic pocket of the protein, probably leading to misfolding and impairment of substrate binding. Davies et al. (1993a) and Shabbeer et al. (2006) have previously reported different missense mutations (p.Val269Ala; p.Val269Met, respectively) at the same amino acid location in patients with Fabry disease. In these reports the patients also showed a classical FD phenotype, but no more clinical information is given (Davies et al., 1993a; Shabbeer et al., 2006). SIFT and Polyphen2 bioinformatic tools predicted a potential deleterious effect of the p.Val269Gly mutation, thus reinforcing the relation genotype–phenotype. However, it was not possible to perform functional studies on the newly identified mutations, therefore we cannot predict the true implications of this variant in the function of the protein.

The genetic analysis of FD-5 revealed a deletion of 3 bp from nucleotides 1072–1074 in exon 7, resulting in the removal of a glutamic acid codon at residue 358. This amino acid has an ion pair linkage to lysine and hydrogen bonds to tryptophan. Patient FD-7 had a similar kind of mutation in the residue 358, where the wildtype glutamine changes to lysine: c.1072G > A (p.E358K). Given that charged residues in ion pairs promote the proper folding of proteins, a mutation of one-half of an ion pair introduces an unpaired charge that is energetically highly unfavorable in the interior of the protein, leading to a destabilization of protein's folded conformation (Blanch et al., 1996; Miyazaki et al., 1998).

Patient FD-3 had a deletion of 2 nucleotides (−2 and −3) in the acceptor splice site of intron 5 of the *GLA* gene that disrupted the 3′ acceptor splice site of intron 5. This mutation gives rise to a rare aberrant splicing variation (simultaneous 3′ destruction and 3′ creation) and then introduced a termination signal and deletion of 162 residues. This mutation (IVS5-2_-3delCA) had already been described in an Irish patient with classic Fabry disease (Eng et al., 1993), but no correlation genotype/phenotype has been described for it.

The c.195-1G > C (IVS1-1G > C) was the most frequent mutation, found in two presumably unrelated families with FD (FD-4 and FD-6). This mutation was previously reported in Japanese classical hemizygotes with FD and provokes aberrant pre-mRNA splicing. This abnormally processed RNA is subjected to accelerated degradation, resulting in a marked decrease in the amount of mRNA (Okumiya et al., 1996).

Most mutations causing FD are private, occurring in a single or few families even though several mutations at CpG dinucleotides (known mutational hotspots for the disease) occur more often in unrelated families. In this study we found p.Arg112Ser, and p.Arg342* mutations, which have been previously reported in unrelated classically affected families (Shabbeer et al., 2006). Arginine-112 is located on the α2-helix of the N-terminal β/α-barrel, suggesting that a substitution to serine is predicted to destabilize the disulfide bond and the pocket that the arginine occupies, leading to protein misfolding (Shabbeer

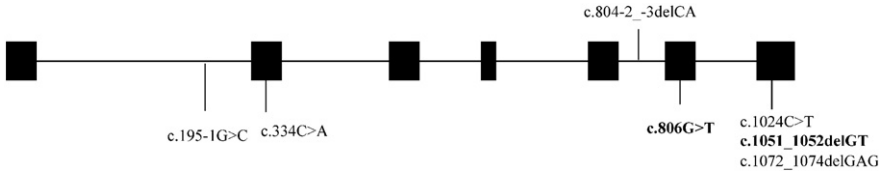

Fig. 2. Map of the *GLA* gene with alterations found in Colombian patients with FD.

et al., 2006). Meanwhile, the p.Arg342* mutation will result in the generation of a truncated protein lacking exon 7, which has been known to be crucial for normal enzymatic function (Ishii et al., 1994, 1995; Miyamura et al., 1996).

The mutation profile here described is consistent with the classical disease presentation and the complete lack of AGAL enzyme activity seen in all the analyzed patients. When comparing the mutations present in the Colombian population with the mutation profile identified in other Latin American countries (Argentina, Mexico and Brazil) (Politei et al., 2013; Ramos-Kuri et al., 2014; Rozenfeld et al., 2006) and Spain (Pereira et al., 2007; Turaça et al., 2012; Rodríguez-Marí et al., 2003) no association is found. This is probably a consequence of the limited sample size analyzed in most of the previous studies that evaluate the mutation profile, such as Argentinean and Mexican studies. Our results are consistent with the correlation between the presence of splicing site mutations and the manifestation of a classical FD phenotype previously described by Saito et al. (2013), since all the patients bearing this type of mutation showed a classical phenotype.

The molecular analysis is an important diagnostic tool to determine the possible benefits of treatment with therapeutic strategies such as pharmacological chaperones. Given that this therapy can only be effective in patients with misfolding mutations, the decision of using it depends on an adequate genotyping based on a case-by-case scenario (Andreotti et al., 2010). In order to see which of these patients would be candidates for this kind of therapy we used the Fabry_CEP program. According to the results obtained, only patients bearing two of the eight mutations found (p.Val269Gly and p.Arg112Ser) had a probability of 59% to respond to this therapy.

In summary, this work intends to make an important contribution for the understanding of the molecular basis of FD in Colombia. Here we showed that there is a high variability in the mutations leading to disease, which highlights the importance of the identification of mutations in the *GLA* gene for the diagnosis, carrier identification, genetic counseling and use of new therapeutic strategies (such as chaperone therapy). Given that most mutations in patients with FD are private, it is necessary to use molecular analyses as a diagnostic tool in order to offer a wider spectrum of therapeutic options for FD patients and families.

5. Conclusions

- Six previously described mutations were found and two new ones are described for the Colombian population.
- There is a high variability of mutations in Colombian patients with Fabry disease.
- A small correlation between the predicted consequence of the mutation in the protein and the classical FD phenotype was found.
- The prevalence of the mutations here described, differs from what has been reported in other Latin American countries and Spain.
- Only two of the eight detected mutations seem to be candidates,

Table 2
Clinical and molecular characteristic of Colombian patients with Fabry Disease.

Patient	FD-1	FD-2	FD-3	FD-4	FD-5	FD-6	FD-7	FD-8	FD-9
Mutation	c.1024C > T	c.806G > T	c.804-2_-3delCA	c.195-1G > C	c.1072_1074delGAG	c.195-1G > C	c.1072G > A	c.1051_1052delGT	c.334C > A
Symptoms									
Neurologic									
Neuropathic Pain	+	−	+	+	−	+	+	+	−
Acroparesthesia	+	+	+	+	−	+	+	+	−
Heat or cold intolerance	+	+	+	+	−	+	+	+	+
Fever Crisis	+	+	+	−	−	−	+	+	−
Strokes	−	−	−	+	−	−	+	−	−
Seizures	−	−	−	−	−	−	−	−	−
Tegumentary									
Angiokeratoma	+	−	+	+	−	+	+	+	+
Hypohydrosis	+	−	+	+	−	+	+	+	+
Kidney									
Renal Failure	−	−	+	+	+	+	+	+	−
Dialysis	−	−	+	−	+	−	−	−	−
Hematuria	+	−	−	+	+	+	+	−	−
Proteinuria	+	+	+	+	+	+	−	+	+
Cardiovascular									
Chest Pain	+	−	−	+	−	−	−	−	−
Dyspnea	+	−	−	+	−	−	−	−	−
Syncope	−	−	−	+	−	−	−	−	−
Hypertension	+	−	−	+	−	−	+	−	−
Left ventricular hypertrophy	+	−	−	+	−	−	−	−	−
Ophthalmologic									
Corneal opacities	+	−	−	+	−	−	−	−	−
Gastrointestinal									
Nauseas	+	−	−	−	−	−	−	−	−
Abdominal Pain	+	+	−	+	−	+	+	−	+
Chronic diarrhea	+	−	−	+	−	−	−	−	−
Metabolic									
Dyslipidemia	−	−	−	+	−	+	−	−	−

with a 59% probability, for new therapeutic treatments such as chaperone therapy.

Compliance with ethical standards

Conflict of interest:

Alfredo Uribe, Heidi Eliana Mateus, Juan Carlos Prieto, Maria Fernanda Palacios, Sandra Yaneth Ospina, Gabriela Pasqualim, Ursula da Silveira Matte and Roberto Giugliani declare that they have no conflict of interest.

Informed consent:

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients included in the study.

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VII. *p.L18P: a novel IDUA mutation that causes a distinct attenuated phenotype in mucopolysaccharidosis type I patients.*

Pasqualim G, Ribeiro MG, da Fonseca GG, Szlago M, Schenone A, Lemes A, Rojas MV, Matte U, Giugliani R. Clin Genet. 2015 Oct;88(4):376-80. doi: 10.1111/cge.12507. Epub 2014 Oct 21. PMID: 25256405

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Short Report

p.L18P: a novel IDUA mutation that causes a distinct attenuated phenotype in mucopolysaccharidosis type I patients

Pasqualim G., Ribeiro M.G., da Fonseca G.G.G., Szlago M., Schenone A., Lemes A, Rojas M.V.M., Matte U., Giugliani R. p.L18P: a novel IDUA mutation that causes a distinct attenuated phenotype in mucopolysaccharidosis type I patients.
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Mucopolysaccharidosis type I is a rare autosomal recessive disorder caused by deficiency of α-L-iduronidase (IDUA) which leads to a wide spectrum of clinical severity. Here, we describe the case of four male patients who present the previously undescribed p.L18P mutation. Patient 1 (p.L18P/p.L18P) presents, despite multiple joint contractures, an attenuated phenotype. Patient 2 (p.L18P/p.W402X) was diagnosed at 4 years of age with bone dysplasia, coarse facies, limited mobility, claw hands and underwent bilateral carpal tunnel surgery at 6 years of age. Patients 3 and 4 (both p.L18P/p.L18P) are brothers. Patient 3 was diagnosed at 4 years of age, when presented claw hands, lower limb and shoulder pain, restricted articular movement and bilateral carpal tunnel syndrome. Patient 4 was diagnosed at 17 months of age when presented lower limb pain at night, respiratory allergy and repeated upper airways infections. Bioinformatics analysis indicates that p.L18P mutation reduces the signal peptide to 25 amino acids and alters its secondary structure. In conclusion, we report a new *IDUA* variant that alters the structure of the signal peptide, which likely impairs transport to lysosomes. Moreover, it leads to a distinct attenuated phenotype with mainly bone and cartilage symptoms, without visceromegalies, heart disease, or cognitive impairment.

Conflict of interest

R. G. has received travel grants, speaker honoraria and investigator fees from Actelion, Amicus, BioMarin, Genzyme, Shire and Synageva. M. V. M. R is an employee of Genzyme, a Sanofi Company. The other authors have no conflicts to disclose.

G. Pasqualim[a,b], M.G. Ribeiro[c], G.G.G. da Fonseca[c], M. Szlago[d], A. Schenone[d], A. Lemes[e], M.V.M. Rojas[f], U. Matte[a,b,g,h] and R. Giugliani[a,b,g,h,i]

[a]Post-Graduate Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, Brazil, [b]Gene Therapy Center, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil, [c]Instituto de Puericultura e Pediatria Martagão Gesteira, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, Brazil, [d]Fundación para el Estudio de las Enfermedades metabólicas (FESEN), Buenos Aires, Argentina, [e]Instituto de Genética Médica, Hospital Italiano, Montevideo, Uruguay, [f]Genzyme, Genzyme Latin America – A Sanofi Company, Rio de Janeiro, Brazil, [g]Department of Genetics, UFRGS, Porto Alegre, Brazil, [h]INAGEMP, Porto Alegre, Brazil, and [i]Medical Genetics Service, Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil

Key words: lysosomal storage disorders – mucopolysaccharidosis type I – Scheie syndrome – signal peptide mutation

Corresponding author: Roberto Giugliani, Medical Genetics Service, Hospital de Clinicas de Porto Alegre, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903 RS, Brazil.
Tel.:+55 51 33598011;
fax: +55 51 33598010;
e-mail: rgiugliani@hcpa.ufrgs.br

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Case report

Mucopolysaccharidosis type I (MPS I) is a rare autosomal recessive disorder caused by deficiency of α-L-iduronidase (IDUA, EC 3.2.1.76). This leads to progressive accumulation of partially degraded heparan sulfate and dermatan sulfate, with subsequent multiorgan dysfunction and damage (1). There is a wide spectrum of symptoms, ranging from a severe Hurler form [OMIM (Online Mendelian Inheritance in Man) #67014] to the more attenuated Hurler–Scheie (OMIM #607015) and Scheie (OMIM #67016) phenotypes. To date, there are more than 200 mutations described in the *IDUA* gene that can cause MPS I (2). Here, we describe four male patients from three different Latin American families who present a new mutation, p.L18P, which seems to lead to an unusual phenotype.

Patient 1 is an Argentinian male born in 1990, homozygous for the p.L18P mutation. There is no record of consanguinity between his parents. Pregnancy and delivery were normal but he was born with torticollis and hydrocele, for which he underwent intervention at 14 months of age. He had normal growth until 4 years of age, except for primary enuresis, and was referred for diagnostic investigation at the age of 4 years and 8 months due to joint stiffness. Radiographs revealed J-shaped sella turcica with normal spine and multiple joint contractures. His weight was in the 50th percentile, height in the 25th percentile and cephalic perimeter in the 50th percentile. He had no visceromegaly, and intellectual performance was appropriate at 11 years of age.

Patient 2 is a male, compound heterozygous for p.L18P and p.W402X. There is no record of consanguinity between his parents. He was born in Uruguay in 1997 after normal pregnancy and delivery without neonatal complications. At 9 months old, a low dorsal kyphosis was identified, and he underwent surgery at 1 year of age for kyphosis. He was referred for diagnosis at 4 years and 4 months for bone dysplasia and hemivertebra. By the time, he presented coarse face, short neck, limited mobility of large and small joints, claw hands, *pectus carinatum* but absence of corneal opacity. IDUA enzyme activity in dried blood spots at 4 years and 6 months was 0.26 nmol/h/L (reference values: 2.20–11.70). He had repeated upper respiratory infections and performed surgery for adenoids and tonsils when he was 5 year old. Carpal tunnel surgery on both hands was performed at 6 years of age. Polysomnography at age of nine was without remarks. At 13 years, he was hospitalized for pneumonia with abscess and had chest drain. Bone radiographs showed clear signs of dysostosis multiplex. Normal echocardiogram and no visceromegalies found in several ultrasounds of abdomen over the years, as well as maintained normal cognitive development. He is currently graduating as a computer technician.

Patients 3 and 4 are Caucasian brothers from Rio de Janeiro, Brazil, sons of consanguineous parents (first degree cousins), they are both homozygous for p.L18P.

Patient 3 was born in 2005, after normal pregnancy and delivery. At 4 years and 4 months, he was referred for diagnosis for having 'crooked fingers' since birth. He was diagnosed with MPS I at 4 years and 8 months, when presented mild phenotype (Fig. 1a,b) with claw hands (Fig. 1f), lower limb and shoulder pain, restricted articular movement in fingers, wrists, elbow flexion and hip rotation. Also, biochemical analysis revealed IDUA activity in leukocytes of 0.73 nmoles/h/mg protein (reference range 32–56) and glycosaminoglycans (GAG) excretion in urine of 188 μg GAGs/g creatinine (age-related reference values: 67–124). Radiographs from this same age revealed apparent increase in bone density in the skull, enlargement of the proximal and middle phalanges of both hands (Fig. 1i), bilateral structural changes of the femoral epiphysis' medial region (Fig. 1l), lumbar scoliosis with left convexity and rectification of physiological lumbar lordosis. By the age of 5 years, he had no ophthalmologic alterations and is still without corneal opacity but requires the use of corrective lens. He started enzyme replacement therapy (ERT) with laronidase at 5 years and 3 months, resulting in improvement of joint movement. This patient presents normal heart function, as shown by electrocardiograms from 5 to 9 years old and normal echocardiogram at 9 years old. He has respiratory allergy and repeated upper airways infections, currently with very mild obstructive lung disease and negative bronchodilator test. Patient has frequent vomiting and difficulty of weight and height gain of possible functional cause. At 6 year old, he had normal endoscopy, moderate esophagitis, gastric mucosa with minimal inflammatory changes and negative test for *Helicobacter pylori*, duodenal mucosa with minimal inflammatory changes. Currently, symptoms decreased and patient does not require medication. At this same age, electromyography results showed lack of sensory nerve action potentials (SNAPs) in right and left median nerve. In addition, patient had normal sensory conduction velocity and amplitude of SNAPs in the radial, ulnar, sural, right and left plantar medial nerves. The compound motor action potentials amplitude was slightly decreased in the right and left median nerves. There was prolonged distal motor latency in the left and right median nerves. Tibial and fibular nerves were normal, and patient had bilateral carpal tunnel syndrome, which was surgically corrected at the age of 8 years. At the age of 7 years, he had normal skull magnetic resonance imaging (MRI) and cervical spine MRI indicated the possibility of storage of GAGs in the synovia/dural membrane and hypertrophy of odontoids ligaments. Patient also presented positive Romberg test at 7 years but otherwise normal cognitive development. He is currently taking classes at a regular school in the appropriate level for his age.

Patient 4 was born in 2008, after normal pregnancy and delivery. Along with his brother, he was referred for diagnosis at 1 year and 2 months of age for having curved bones in hands and feet and sporadic pain in both hands. By 17 months of age, he was diagnosed with MPS I, IDUA activity in leukocytes was 0.61 nmoles/h/mg protein (reference range 32–56) and GAG excretion in urine was 218 μg GAGs/g creatinine (age-related reference values: 79–256). At this same age, he had lower limb pain at night, respiratory allergy and repeated upper

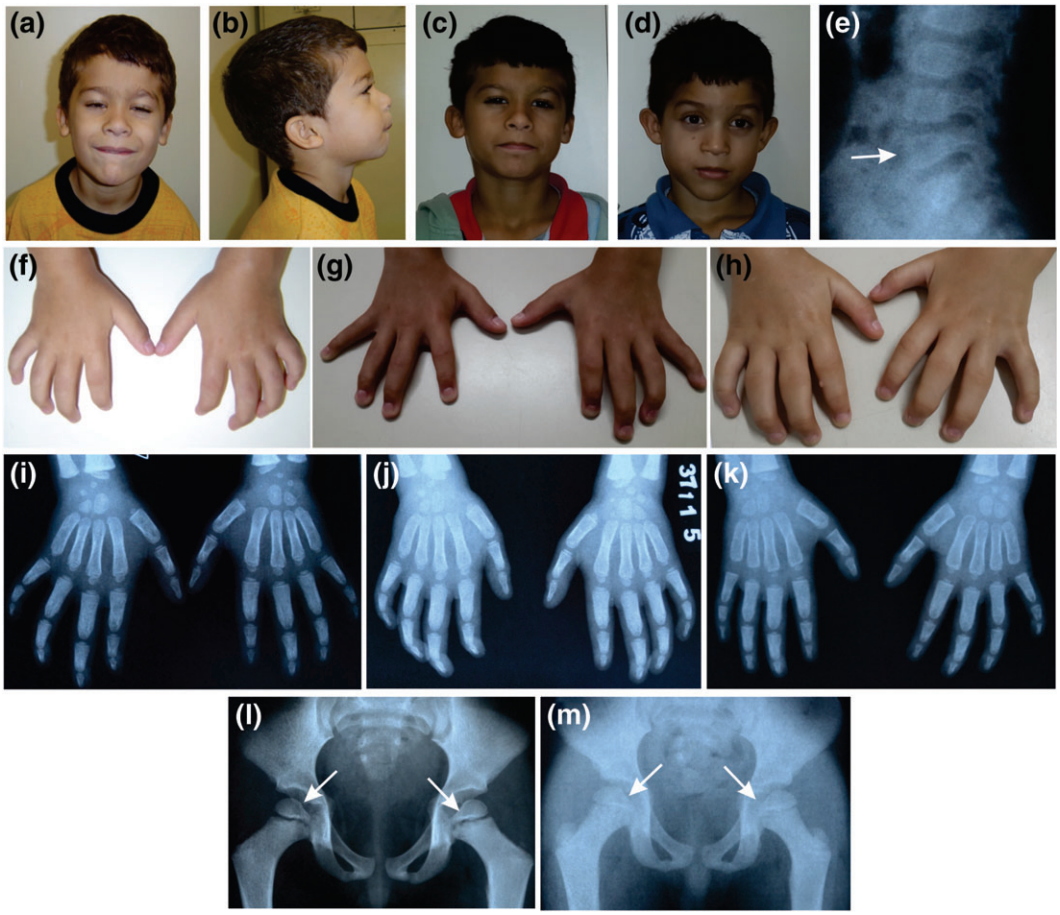

Fig. 1. Clinical and radiological features of patients 3 and 4 – facial features: patient 3 at 5 (**a**, **b**) and at 9 (**c**) years of age. Note the mild phenotype, with absence of coarse facial features. The same phenotype can also be observed in his brother, patient 4, at 6 years of age (**d**); Spine: radiograph from patient 3 (**e**) at 16 months of age showing L2 vertebral body (arrow) with smaller dimensions; Hands photos: both patients present claw hands, patient 3 at 5 (**f**) and 9 (**g**) years of age and patient 4 at 6 years of age (**h**); Hand radiographs: patient 3 at 4 (**i**) and 6 (**j**) years of age and patient 4 at 3 years of age (**k**). Note the enlargement of proximal and middle phalanges and the increase in bone density; Hip radiographs: patient 3 at 4 (**l**) and at 7 years of age (**m**). Note the bilateral morphostructural changes of the femoral epiphysis' medial region (arrows).

airways infections, slight claw hands but no visceromegalies or ophthalmologic symptoms. Radiographs of the spine showed L2 vertebral body with smaller dimensions (Fig. 1e). Neurodevelopment was also normal. ERT was started at 24 months of age and evolved with no adverse effects until the 23rd infusion. From then on, he presented urticarial reaction that got progressively more intense. In 2011, a desensitization protocol without premedication was performed. Patient reacted with cough, edema of the upper lip, hands, feet and upper eyelids and drooling. In 2012, a different protocol, with premedication, was attempted but also did not had the expected results. Therefore, the patient's family decided to interrupt ERT and perform routine assessments. By the age of 3 years old, radiographs showed normal thorax, cervical column without signs of atlantoaxial instability and enlargement and increased bone mineral density of the proximal and middle phalanges of both hands (Fig. 1k). Measurement of serum immunoglobulin was negative. In 2014, a new protocol, with premedication before and during ERT (3, 4), was successfully implemented and patient has already had 11 infusions. Currently, at the age of 6 years old, patient remains with mild phenotype (Fig. 1d). The last echocardiogram showed no cardiologic alterations. Patient has movement restriction in fingers (Fig. 1h) and wrists that got better since the restart of ERT; there are no overt ophthalmologic or neurologic symptoms. As his brother, he is also taking classes at a regular school in the appropriate level for his age.

Materials and methods

Whole blood genomic DNA was extracted from ethylenediaminetetraacetic acid tubes with EasyDNA Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Amplification of exonic regions of the *IDUA* gene was performed as previously described (5) in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). Following purification with EXO-SAP and quantification with Low Mass Reader (Invitrogen, Carlsbad, CA), sequencing was done in 3500 Genetic Analyzer using BigDye Terminator (both from Applied

Biosystems, Foster City, CA). Sequences were analyzed by comparison to genomic reference sequence (GenBank accession number NG_008103.1), and all alterations were confirmed by reverse strand sequencing.

The study was approved by the Ethics Research Committee of our institution, and the patients gave written informed consent.

Results and discussion

The mutation p.L18P (c.53C>T) was identified in all patients. It affects the signal peptide of the enzyme, which potentially ranges from amino acid 1 to 26 of 27. Another mutation was already described in this site, the p.S16_A19del (c.46_57del12, also known as 134del12) that leads to the deletion of amino acids 16–19 (6). This deletion leads to expression of a larger (77 kD) yet enzymatically active IDUA precursor but impairs correct post-translational processing and transport to the lysosome (7). Two compound heterozygous p.S16_A19del/p.W402X patients previously attended by our group at the Medical Genetics Service presented severe Hurler phenotype. Another patient with the genotype p.S16_A19del/p.N350I also presented severe organ involvement and no cognitive impairment. Many cases reported in literature with this mutation were also described as having severe phenotype (5, 8–10). Hence, the critical role of this region in the maintenance of normal levels of activity is highlighted.

Signal peptides in N-terminal region of proteins sequences range in size in average from 16 to 30 amino acids. They are characterized by maintenance of three structural domains. The N hydrophilic terminal (N-region) is composed of 5–8 positively charged residues. The following is the hydrophobic core or 'H-region', composed of 7–15 highly hydrophobic residues and very important for cotranslational processing. Finally, the 'C-region' is composed of around six polar residues and contains the signal peptide cleavage site (11, 12). Bioinformatics analysis in different commonly used single nucleotide polymorphism (SNP) prediction tools (Polyphen2, MutPred, Provean, SNP&GO, SIFT, Panther, PhD-SNP and Pmut) resulted in mixed predictions, with most programs classifying this alteration as either non-pathological/neutral or pathological with low reliability (data not shown). These tools, however, are not specific for predicting mutation effects in signal peptides. Therefore, the alteration was also analyzed with Phobius, a combined transmembrane topology and signal peptide predictor (available at http://phobius.sbc.su.se/) that is currently used by UniProt (Universal Protein Resource) database to predict signal peptides. Phobius results indicate that p.L18P mutation alters the H-region structure, reducing it from 12 to 10 residues. Also, the C-region is enlarged from 7 to 8 residues. Therefore, the signal peptide is compressed from 26 to 25 amino acids and has its hydrophobic core altered. The proline residue introduce by the p.L18P mutation is more rigid than other amino acids due to its cyclic side chain. This characteristic leads to the disruption of α-helices. Moreover, it is an hydrophilic residue, which makes it unsuitable for and rarely found in the hydrophobic cores of signal sequences (12). A study by Zanen et al.13 showed that hydrophobicity of the h-region is very important for its recognition by the signal recognition particle, and changes in this criterion affect the efficiency of protein transportation. Therefore, p.L18P alters not only the size of the signal peptide but also its secondary structure as it affects the protein secretion.

Interestingly, such an important disruption on signal peptide would predictably result in severe phenotype. However, none of the patients in this report presents visceromegaly, or heart or cognitive impairment. Their main presentation is bone, joint and respiratory involvement. This finding, along with other reports about mainly joint involvement in MPS I patients, reinforces the importance of the rheumatologists to be aware of MPS (14–16). The presence of claw hands, even in the absence of other classical signs and symptoms, should lead to the hypothesis of MPS in the differential diagnosis. The reasons why this mutation leads to such a particular phenotype are still unknown. Differential affinity for particular substrates may be a cause although other mechanisms cannot be ruled out.

In conclusion, we report a new *IDUA* variant that alters the structure of the signal peptide, which likely impairs transport to lysosomes. Moreover, the p.L18P mutation leads to a distinct attenuated phenotype.

Acknowledgements

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VIII. Carta de aprovação ética do projeto 15-0196 pela Comissão Científica do HCPA.

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

COMISSÃO CIENTÍFICA

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

Projeto: 150196
Data da Versão do Projeto: 27/04/2015

Pesquisadores:
FILIPPO PINTO VAIRO
MARILUCE RIEGEL
URSULA DA SILVEIRA MATTE
JACQUES AVILA ANGREZANI
CARMEN REGLA VARGAS
ROBERTO GIUGLIANI
MAIRA GRAEFF BURIN
GABRIELA PASQUALIM

Título: Por que as mulheres heterozigotas Apresentam Doença de Fabry ? Um estudo clínico e experimental para abordar esta questão intrigante

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

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

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

Porto Alegre, 12 de agosto de 2015.

Prof. José Roberto Goldim
Coordenador CEP/HCPA