UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Tese de Doutorado

Efeitos de alterações no gene da glicocerebrosidase e em genes modificadores do fenótipo da doença de Gaucher e de patologias associadas à proteína glicocerebrosidase

Marina Siebert

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Doutor em Ciências.

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Orientadora: Profa. Dra. Maria Luiza Saraiva Pereira

Porto Alegre, junho de 2014.

INSTITUIÇÕES E FONTES FINANCIADORAS

Este trabalho foi realizado no Laboratório de Identificação Genética, localizado no Centro de Pesquisa Experimental (CPE) do Hospital de Clínicas de Porto Alegre (HCPA), e no Serviço de Genética Médica do HCPA com apoio financeiro parcial do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), do Instituto Nacional de Genética Médica e Populacional (INAGEMP), do Fundo de Incentivo à Pesquisa e Eventos do HCPA (FIPE-HCPA) e da Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). A aluna recebeu bolsa de estudos concedida pelo CNPq durante a realização dos seus estudos no Brasil.

O período de doutorado-sanduíche foi realizado no *Medical Genetics Branch*, localizado no *National Genome Research Institute* (NHGRI) do *National Institutes of Health* (NIH) na cidade de Bethesda, Estados Unidos. Durante o período de permanência no exterior, a aluna recebeu bolsa de estudos concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) através do Programa Ciências sem Fronteiras.

Dedico esta tese ao Tiago, meu marido e melhor amigo, e aos meus pais. Eles sempre acreditaram no meu potencial e me ensinaram o que a vida tem de melhor, o amor.

AGRADECIMENTOS

À minha orientadora Profa. Dra. Maria Luiza Saraiva Pereira, por sempre ter acreditado no meu potencial, pelo incentivo na busca por novos desafios, pelos ensinamentos e trocas de experiências, pela confiança e, principalmente, pela amizade. A profa. foi fundamental em todas as etapas deste trabalho, sempre proporcionando o apoio e o suporte necessário para que eu pudesse concluir cada uma das diferentes etapas. É notável o meu crescimento científico e profissional no decorrer desses anos de convivência. Agradeço imensamente todo seu carinho e dedicação.

À minha orientadora do doutorado-sanduíche, Profa. Dra. Ellen Sidransky, pela oportunidade de realizar parte do meu doutorado em seu laboratório, pelo aprendizado proporcionado, pela confiança e pelo carinho.

Ao Prof. Dr. Scott Martin por ter me acolhido em seu laboratório para realização de parte dos experimentos do doutorado-sanduíche, pelo apoio e suporte científico, por confiar e acreditar no meu potencial como cientista.

À Dra. Wendy Westbroek pela imensa amizade, pelos ensinamentos, pelo incentivo e por sempre acreditar no meu trabalho. O seu apoio foi de fundamental importância para que o período do doutorado-sanduíche fosse menos doloroso longe da minha família e resultasse em excelente produtividade científica.

Aos colegas dos laboratórios da Profa. Dra. Ellen Sidransky e do Prof. Dr. Scott Martin pelo apoio científico, pelos ensinamentos, pela amizade e pelo carinho.

Aos colegas do Laboratório de Identificação Genética pela amizade, pela motivação, pelo companheirismo, pelo apoio, pelas trocas de ideias e experiências.

Aos colegas do Centro de Pesquisa Experimental e do Serviço de Genética Médica do HCPA pelo auxílio, pelo apoio e pela amizade.

À Sílvia e ao Luciano por serem muito solícitos, atenciosos e por estarem sempre dispostos a ajudar.

Às amigas, Vanessa, Mirela, Carolina e Márcia que me acompanharam no decorrer dessa caminhada dando suporte, apoio e conselhos.

À minha amiga-irmã Mariana que está sempre ao meu lado (mesmo quando estávamos em países diferentes). Agradeço-a pela força, pelos conselhos, pela parceria e pela amizade.

Aos meus pais, Marli e Mauro, pelo amor incondicional e por estarem sempre ao meu lado torcendo pela minha felicidade e pelo meu sucesso. Mesmo sofrendo com a distância, eles me apoiaram e me incentivaram a ir em busca dos meus sonhos, acreditando fielmente que eu voltaria para perto deles cheia de experiências de vida e de conhecimentos. O constante estímulo e apoio emocional foram fundamentais para que eu concluísse mais esta etapa da minha trajetória profissional.

Aos meus irmãos, pelo carinho e pelo apoio.

Ao meu marido Tiago, pela confiança, pelo amor, pela compreensão, pela amizade e pela força nos momentos difíceis. O seu apoio e incentivo foram fundamentais para que eu suportasse a saudade, o medo do desconhecido e os desafios de morar em um país diferente, com outro idioma e sozinha. Com certeza, sem o seu suporte eu não teria conseguido concluir mais esta etapa.

Aos pacientes e seus familiares por proverem amostras biológicas para este estudo.

Ao CNPq, a CAPES e ao FIPE-HCPA pelo apoio financeiro.

A Deus por estar ao meu lado, por me dar forças e coragem para superar os obstáculos do dia a dia, sempre guiando e iluminando os meus passos.

A todos, que de alguma forma, contribuíram para a realização e conclusão deste trabalho.

Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é decidir.

Cora Coralina

SUMÁRIO

LISTA DAS PRINCIPAIS ABREVIATURAS

A	adenina
α-Sin	α-sinucleína
C	citosina
CCL18/PARC	*Chemokine* (*C-C motif*) *Ligand* 18/*Pulmonary and Activation-RegulatedCchemokine*
cDNA	ácido desoxirribonucleico complementar
DCL	demência com corpos de Lewy
DG	doença de Gaucher
DMJ/SCA3	doença de Machado-Joseph/ataxia espinocerebelar tipo 3
DNA	ácido desoxirribonucleico
DP	doença de Parkinson
G	guanina
GBA1	gene que codifica a enzima glicocerebrosidase
GC	glicocerebrosídeo
GCase	enzima glicocerebrosidase
kb	kilobases
kDa	kilodalton
LIMP-2	proteína lisossomal integral de membrana do tipo 2 (*Lysosomal Integral Membrane Protein type 2*)
miRNA	microRNA
mRNA	ácido ribonucleico mensageiro
OMIM	*Online Mendelian Inheritance in Man*
pb	pares de bases
PCR	reação em cadeia da polimerase

pre-miRNA	microRNA precursor
pri-miRNA	microRNA primário
pSap	prosaposina
PSAP	gene que codifica a prosaposina
PSGBA	gene que codifica o pseudogene da glicocerebrosidase
QT	quitotriosidase
RE	retículo endoplasmático
RISC	complexo de silenciamento induzido por RNA (*RNA-Induced Silencing Complex*)
RNA	ácido ribonucleico
RNAse	RNA polimerase
Sap-C	saposina C
SCARB2	*Scavenger Receptor Class B, member 2*
SNC	sistema nervoso central
SNP	polimorfismo de nucleotídeo único (*Single Nucleotide Polymorphism*)
T	timina
TRE	terapia de reposição enzimática
TRS	terapia de redução de substrato
3'UTR	região 3' não-traduzida (*3'untranslated region*)
5'UTR	região 5' não-traduzida (*5'untranslated region*)

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RESUMO

A doença de Gaucher (DG) é uma doença genética rara, de herança autossômica recessiva, causada pela deficiência da enzima glicocerebrosidase (GCase) devido a mutações no gene *GBA1*. Embora mais de 400 mutações já tenham sido identificadas no gene *GBA1*, a correlação entre o genótipo e o fenótipo permanece bastante limitada. Outros fatores parecem influenciar o fenótipo dos pacientes com DG. Além disso, a presença de mutações no *GBA1* tem sido associada a outras patologias como, por exemplo, o parkinsonismo. O objetivo principal deste trabalho foi investigar os efeitos de alterações no gene *GBA1* e em genes modificadores do fenótipo da DG e de patologias associadas à proteína GCase. Em um primeiro momento, a sequência completa do gene *GBA1* foi analisada em amostras de pacientes com a doença de Machado-Joseph/ataxia espinocerebelar tipo 3 (MJD/SCA3), com e sem sintomas parkinsonianos e de indivíduos controles. Três alterações de sequência (K(-27)R, E326K e T369M) foram identificadas no gene *GBA1*, as quais estavam presentes apenas em pacientes com MJD/SCA3 que apresentavam sintomas parkinsonianos (p = 0,03). Os resultados obtidos sugerem que mutações no gene *GBA1* podem estar agindo como modificador do fenótipo da MJD/SCA3, desempenhando um papel secundário. Além disso, sete polimorfismos de nucleotídeo único (SNPs) foram analisados em três genes candidatos a modificadores do fenótipo da DG, sendo eles: rs6532244 e rs6825004 no gene *SCARB2*; rs2070968, rs7869 e rs2854992 no gene *PSAP*; rs7008465 e rs11136424 no gene *CLN8*. Não houve diferença nas frequências alélicas destes SNPs entre os grupos de pacientes com DG e controles. Em relação a frequência genotípica, o genótipo A/A do rs6532244 (gene *SCARB2*) mostrou-se mais frequente em pacientes (p = 0,019), especialmente em pacientes com genótipo L444P/L444P no gene *GBA1*. A presença do alelo G no rs7008465 (gene *CLN8*) foi relacionada ao genótipo N370S/N370S em comparação aos pacientes com genótipos diferente (p = 0,027). Na última etapa do presente trabalho, a influência de microRNAs (miRNAs) sobre a atividade da GCase foi avaliada assim como o mecanismo pelo qual esta modulação estaria ocorrendo. Após o *screening* com 875 miRNAs diferentes, identificamos miRNAs que exerciam função regulatória sobre a atividade da GCase. Em especial, dois miRNAs específicos (miR-195-5p e miR-16-5p) influenciaram positivamente a GCase, provocando um aumento na atividade da enzima, na expressão do gene *GBA1* e nos níveis proteicos.

Por outro lado, um outro miRNA (miR-127-5p) desempenhou uma regulação negativa, causando alteração na atividade da GCase de forma indireta devido a modulação da expressão de *SCARB2* e, consequentemente, da redução dos níveis de LIMP-2. Considerando as diferentes etapas deste trabalho e os resultados obtidos, propomos que a enzima GCase tem um papel central envolvendo vários aspectos, tais como a influência sobre o fenótipo de outras patologias e ação desencadeada por outros genes e pequenas moléculas sobre a atividade da enzima. Mutações no gene *GBA1* causam deficiência da GCase, que pode estar relacionada não só com a DG, mas também ao aparecimento de sintomas parkinsonianos em MJD/SCA3. Além disso, outros fatores podem estar influenciando a atividade dessa enzima, dentre os quais destacamos: a alteração em genes modificadores como *SCARB2* e *CLN8* através da alteração das proteínas codificadas e a indução da modulação tanto positiva como negativa mediada por miRNAs. A ampliação da compreensão da fisiopatologia da DG e de rotas metabólicas influenciadas pela GCase poderão possibilitar o estabelecimento de melhores correlações genótipo-fenótipo e o desenvolvimento de novas abordagens terapêuticas.

ABSTRACT

Gaucher disease (GD) is a rare genetic disease inherited as an autosomal recessive trait, caused by deficiency of the glucocerebrosidase (GCase) due to mutations in the *GBA1* gene. Although more than 400 mutations have already been identified in this gene, the correlation between genotype and phenotype remains limited. Other factors appear to influence the phenotype of patients. Moreover, the presence of *GBA1* mutations has also been related to other diseases such as parkinsonism. In this study, the main goal was to investigate the effects caused by alterations in the *GBA1* gene and in modifier genes to the GD phenotype and to pathologies associated to GCase protein. At first, we analyzed the complete sequence of *GBA1* gene in samples obtained from patients with Machado-Joseph/ataxia spinocerebellar type 3 disease (MJD/SCA3) with and without parkinsonism, and control subjects. Three sequence alterations (K(-27)R, E326K, and T369M) were identified in the *GBA1* gene, which were present only in MJD/SCA3 patients with parkinsonian symptoms ($p = 0.03$). The results suggest that mutations in the *GBA1* may act as modifier of MJD/SCA3 phenotype, playing a minor role. In addition, seven single nucleotide polymorphisms (SNPs) from three modifier candidate genes of GD phenotype (*SCARB2* gene - rs6532244 and rs6825004; *PSAP* gene - rs2070968, rs7869, and rs2854992; *CLN8* gene - rs7008465 and rs11136424) were evaluated. No difference was found involving the allelic frequencies of these SNPs between GD patients and controls. The genotype A/A of rs6532244 (*SCARB2* gene) was found to be the most frequent in GD patients ($p = 0.019$), especially those sharing L444P/L444P genotype in *GBA1* gene. Presence of the G allele at rs7008465 (*CLN8* gene) was related to the genotype N370S/N370S compared to patients with different genotypes ($p = 0.027$). In the last part of the current work, we investigated whether microRNAs (miRNAs) could influence GCase activity and by which mechanism. Following the screening with 875 different miRNAs, we identified miRNAs that up- or down-regulated GCase activity. In particular, two specific miRNAs (miR-195-5p and miR-16-5p) influenced GCase by acting as up-regulators, leading to an increase in its activity, *GBA1* expression, and protein levels. On the other hand, another miRNA (miR-127-5p) played a down-regulation function, resulting in GCase activity alteration via an indirect effect due to the modulation of *SCARB2* expression and LIMP-2 reduction. Considering all steps addressed in this work and based

on obtained results, we propose that GCase enzyme plays a central role involving many aspects, such as the influence on the phenotype of other pathologies and the action triggered by other genes and small molecules on GCase activity. Mutations in the *GBA1* gene cause GCase deficiency, which may be not only related to GD, but also to the parkinsonism in MJD/SCA3. In addition, other factors may be influencing GCase activity, such as: alteration in modifier genes such as *SCARB2* and *CLN8* that may lead to change in their encoded proteins, and induction of up- or down-regulation by miRNAs. The growing understanding on GD pathophysiology and metabolic pathways influenced by GCase may enable the establishment of genotype-phenotype correlations and the development of new therapeutic approaches.

1. INTRODUÇÃO

As doenças lisossômicas constituem um grupo de mais de 50 patologias que envolvem vias de degradação associadas aos lisossomos (Staretz-Chacham *et al.*, 2009). Essas doenças ocorrem devido à deficiência de determinada hidrolase lisossômica (ocasionando o acúmulo de substrato específico), cofatores, proteínas ativadoras ou proteínas transportadoras envolvidas no processamento da enzima em questão (Zhang *et al.*, 2009). As doenças lisossômicas são doenças raras, sendo a incidência estimada em 1 em cada 7500 nascidos vivos na população em geral (Cox & Cachon-Gonzalez, 2012).

1.1. Doença de Gaucher

A doença de Gaucher (DG) é um erro inato do metabolismo dos glicoesfingolipídeos, herdada de forma autossômica recessiva, causada pela deficiência da enzima lisossômica glicocerebrosidase (β-glicosidase ácida; GCase; E.C. 3.2.1.45) devido a mutações no gene que codifica esta enzima (*GBA1*) (Grabowski *et al.*, 2013). A DG é a doença lisossômica de depósito mais comum, com uma frequência estimada em 1:40.000-60.000 indivíduos na população em geral, mas atinge 1:850 indivíduos na população de judeus Ashkenazi (Beutler *et al.*, 1993; Grabowski *et al.*, 2013). A DG é caracterizada pelo acúmulo progressivo do substrato glicocerebrosídeo (glicosilceramida; GC) no interior dos lisossomos das células do sistema retículo-endotelial (Grabowski *et al.*, 2013). Estas células repletas de lipídeos acumulam predominantemente no fígado, baço e medula óssea. A DG tem atraído cada vez mais a atenção de médicos e de pesquisadores pois suas formas de tratamento servem como paradigma no tratamento de doenças semelhantes (Rosenbloom & Weinreb, 2013).

1.1.1. Breve histórico

Em 1882, o médico francês Phillipe Charles Ernest Gaucher descreveu a DG pela primeira vez (Gaucher, 1882). Ele relatou o caso de uma mulher de 32 anos de idade com baço extremamente aumentado, no qual observou a presença de células incomuns, as quais

eram repletas de lipídeos. Naquele momento, Gaucher referiu-se a esta patologia como um epitelioma primário do baço. Após este primeiro relato, outros casos semelhantes foram sendo descritos, levando à identificação da natureza sistêmica da doença e de sua variabilidade quanto aos sinais e sintomas. A partir de 1885, essa patologia passou a ser conhecida como doença de Gaucher. Em 1904, Brill e colaboradores sugeriram que a doença era geneticamente herdada e demonstraram que o fígado, os linfonodos e os ossos eram acometidos (Brill *et al.*, 1904). Em 1927, Oberling e Woringer relataram o primeiro caso infantil envolvendo danos ao sistema nervoso central (SNC) (Oberling & Woringer, 1927). Em 1934, Aghion identificou GC como sendo o principal substrato acumulado nos órgãos de pacientes com DG (Aghion, 1934). A forma juvenil da doença foi descrita na Suécia no ano de 1959. As bases metabólicas dessa doença rara somente foram descobertas em 1965, quando Brady e colaboradores demonstraram que a degradação de GC era mediada por uma enzima, a GCase (Patrick, 1965; Brady *et al.*, 1966). Subsequentemente, GCase foi identificada como sendo uma enzima lisossômica, propiciando a classificação da DG como membro da grande família de doenças lisossômicas de depósito (Weinreb *et al.*, 1968).

1.1.2. Fisiopatogênese e biomarcadores

A DG é causada pela deficiência total ou parcial da enzima GCase. Esta enzima faz parte da rota de degradação dos glicoesfingolipídeos, que são componentes essenciais da membrana de células eucarióticas. Estas moléculas são internalizadas por endocitose e, consequentemente, degradadas no interior dos lisossomos (Futerman & van Meer, 2004). O principal substrato a ser metabolizado pela GCase é o GC. Esta molécula está distribuída em pequenas quantidades nos tecidos, sendo um metabólito intermediário da síntese e da degradação de glicoesfingolipídeos complexos, tais como gangliosídeos e globosídeos (Grabowski *et al.*, 2013). GC é um dos produtos produzido pelo *turnover* de células sanguíneas senescentes, dentre os quais leucócitos, eritrócitos e plaquetas (Grabowski, 2012). A GCase catalisa a reação de hidrólise da ligação β-glicosídica do GC no interior dos lisossomos das células da linhagem monócito-macrófago, produzindo glicose e ceramida (Figura 1) (Grabowski *et al.*, 2013).

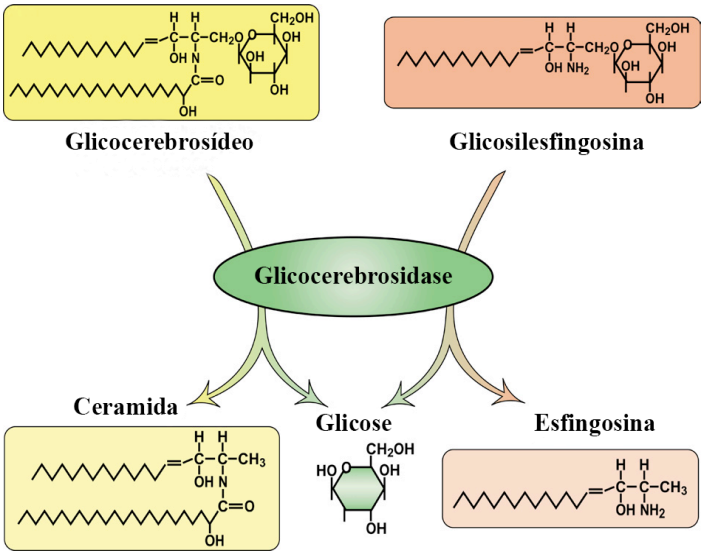

Figura 1. Reações de degradação catalisadas pela enzima GCase (adaptado de Sidransky, 2004).

Embora produzido em menor quantidade e não detectado em tecidos normais, a glicosilesfingosina também é um importante substrato da GCase (Figura 1). A glicosilesfingosina é a forma deacilada do GC, sendo identificada em níveis aumentados no cérebro de pacientes com a forma neuronopática da DG, sugerindo uma contribuição na patogênese do SNC (Orvisky *et al.*, 2002).

O GC não é degradado corretamente em decorrência da deficiência enzimática e acumula no interior das células. Consequentemente, macrófagos repletos de glicolipídeos são formados, originando as características células de Gaucher. Estas células apresentam núcleo excêntrico, citoplasma com estrias e aspecto geral de papel de seda amassado (Grabowski *et al.*, 2013). As células de Gaucher estão distribuídas por todo o corpo e são responsáveis pelo mau funcionamento de órgãos e tecidos nos quais encontram-se densamente acumuladas. Um grande número dessas células é encontrado no fígado, no baço e na medula óssea, acarretando sintomas como hepatomegalia, esplenomegalia, complicações hematológicas e ósseas. Embora menos frequentes, problemas no SNC, no coração, nos rins e na pele também podem estar presentes (Harmanci & Bayraktar, 2008).

Os níveis plasmático, hepático, renal e cerebral de GC encontram-se aumentados de 2 a 100 vezes em indivíduos com DG, mas tais níveis não estão diretamente correlacionados com o tipo clínico ou com a gravidade da doença. Níveis aumentados de GC estão presentes no cérebro de pacientes com a forma neuronopática da doença.

Contudo, a distribuição, a quantidade e o tipo de glicolipídeo acumulado varia conforme a forma clínica apresentada pelos pacientes (Grabowski *et al.*, 2013).

Os macrófagos de indivíduos com DG encontram-se ativados, resultando na liberação em excesso de citocinas e de outras moléculas no plasma desses pacientes. A enzima quitotriosidase e a quimiocina CCL18/PARC estão entre as moléculas liberadas pelos macrófagos ativados, sendo consideradas como biomarcadores da doença.

A enzima quitotriosidase (quitinase humana; QT; E.C. 3.2.1.14) é uma glicosil hidrolase sintetizada e liberada por macrófagos ativados. As células de Gaucher secretam QT no plasma e a atividade dessa enzima está geralmente aumentada em pacientes com DG, podendo atingir níveis 600 vezes maiores do que os valores normais (Hollak *et al.*, 1994; Wajner *et al.*, 2007; Harmanci & Bayraktar, 2008). Os níveis plasmáticos de QT não estão relacionados à gravidade da doença, mas podem estar relacionados à quantidade de GC armazenada nas células de Gaucher e à eficácia do tratamento. Os níveis plasmáticos de QT tendem a diminuir consideravelmente após o início do tratamento (Bodamer & Hung, 2010). Embora seja considerado um bom marcador bioquímico, a presença de níveis aumentados de QT não é exclusivo de pacientes com DG. Pacientes com outras doenças lisossômicas, tal como a doença de Niemman-Pick, também podem apresentar níveis bastante elevados de QT (Guo *et al.*, 1995; Wajner *et al.*, 2004). Além disso, aproximadamente 6% dos pacientes com DG e de indivíduos da população em geral apresentam deficiência de QT devido à duplicação de 24 pares de base (pb) no éxon 10 do gene que codifica esta enzima (gene *CHIT1*) em ambos alelos. Indivíduos homozigotos para esta duplicação são perfeitamente saudáveis e não estão associados a nenhuma condição patológica relacionada a esta deficiência (Boot *et al.*, 1998; Wajner *et al.*, 2004).

Outra proteína descrita como biomarcador da DG é a quimiocina CCL18/PARC (*Chemokine* (*C-C motif*) *Ligand* 18/*Pulmonary and Activation-Regulated Chemokine*). O principal papel biológico desta proteína é mediar a quimiotaxia dos leucócitos. Assim como a QT, a CCL18/PARC é produzida e liberada pelas células de Gaucher, resultando em níveis aumentados (10 a 40 vezes) no plasma de pacientes com DG. Esta proteína também não está relacionada a um dado sintoma, mas está diretamente correlacionada com a quantidade de GC acumulada no interior dos macrófagos e com a resposta ao tratamento (Boot *et al.*, 2004; Deegan *et al.*, 2005). O uso de CCL18/PARC como marcador

bioquímico de DG é de especial interesse em casos de pacientes homozigotos para duplicação no gene *CHIT1* e que, consequentemente, tem deficiência de QT (Boot *et al.*, 2004; Aerts *et al.*, 2005).

Além dos biomarcadores QT e CCL18/PARC, a glicosilesfingosina tem sido estudada como potente marcador da DG. A avaliação dos níveis de glicosilesfingosina no plasma de pacientes com DG, de pacientes com outras doenças lisossômicas e de indivíduos normais revelou que esta molécula encontra-se com níveis aumentados especificamente em pacientes com DG. Além disso, a glicosilesfingosina demonstrou estar correlacionada com o genótipo (no gene *GBA1*) do paciente e ser um reflexo do progresso e da melhora dos sintomas após o início do tratamento da DG (Dekker *et al.*, 2011; Rolfs *et al.*, 2013). Entretanto, mais estudos envolvendo uma população maior de pacientes ainda são necessários para validar estes achados.

1.1.3. Aspectos genéticos

O gene *GBA1* está localizado no *locus* 1q21, compreende 7,6 kb de DNA genômico e é dividido em 11 éxons (Figura 2) (Horowitz *et al.*, 1989). O RNA mensageiro (mRNA) transcrito a partir do *GBA1* tem aproximadamente 2 kb e apresenta dois códons ATG de iniciação da tradução, sendo ambos utilizados na produção da enzima funcional. A importância biológica destes dois sítios de iniciação permanece desconhecida (Grabowski *et al.*, 2013). Dependendo do ATG utilizado para o início da tradução, a proteína produzida terá 19 ou 39 resíduos de aminoácidos na sequência do peptídeo-sinal. Porém, uma única GCase matura, contendo 497 aminoácidos e 56 kDa de peso molecular, é gerada após o seu processamento (Hruska *et al.*, 2008; Grabowski *et al.*, 2013). Os níveis de expressão do mRNA produzido a partir do *GBA1* varia consideravelmente entre os diversos tipos celulares e não tem correlação direta com os níveis de atividade enzimática da GCase (Grabowski & Horowitz, 1997; Hruska *et al.*, 2008).

GBA1 possui um pseudogene (*PSGBA*) de aproximadamente 5 kb, que apresenta 96% de homologia de sequência com o gene funcional. *PSGBA* está localizado a 16 kb da porção 3' terminal do gene *GBA1* (Horowitz *et al.*, 1989; Hruska *et al.*, 2008). O elevado grau de homologia entre gene e pseudogene deve ser levado em consideração no momento

da investigação de mutações em pacientes com DG, visto que algumas das mutações encontradas em pacientes estão presentes na sequência do pseudogene (Hruska *et al.*, 2008; Blech-Hermoni *et al.*, 2010; Sidransky, 2012).

Até o momento, 412 mutações já foram identificadas no gene *GBA1* (www.hgmd.cf.ac.uk), dentre as quais estão incluídas mutações de ponto, inserções, deleções, alterações em sítios de *splicing*, rearranjos e alelos complexos (Figura 2) (Hruska *et al.*, 2008; Grabowski *et al.*, 2013). A presença de mutações no gene *GBA1* pode resultar na alteração da estabilidade e/ou da capacidade catalítica da GCase (Grabowski *et al.*, 2013).

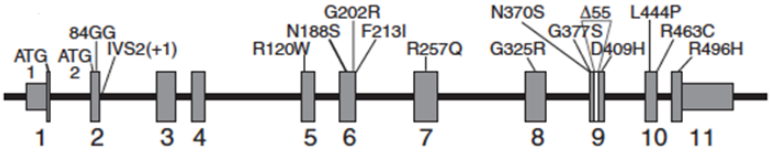

Figura 2. Representação esquemática do gene *GBA1* e localização de algumas das alterações de sequência já identificadas (adaptado de Hruska *et al.*, 2008).

A distribuição das mutações no gene *GBA1* varia em diferentes populações (Bodamer & Hung, 2010). As três mutações mais frequentes em pacientes com DG são N370S, L444P e 84GG (Figura 2) (Grabowski *et al.*, 2013). A mutação N370S (g.6728A>G; c.1226A>G) é caracterizada pela transição de uma adenina (A) para uma guanina (G) no éxon 9 do gene *GBA1*, causando a substituição do aminoácido asparagina (N) por serina (S) na posição 370 da proteína madura. A frequência desta alteração é de 53% em indivíduos com DG, sendo a mutação mais comumente encontrada tanto na população de judeus Ashkenazi como na população em geral (não-Ashkenazi) (Charrow *et al.*, 2000; Grabowski *et al.*, 2013). A mutação L444P (g.7319T>C; c.1448T>C) é a segunda mais frequente no gene *GBA1*, estando presente em 18% dos pacientes. É a alteração mais comum na população Norrbottniana do norte da Suécia (Grabowski *et al.*, 2013). Esta variação de sequência ocorre devido à substituição de uma timina (T) para uma citosina (C) no éxon 10 do gene. Consequentemente, há mudança do aminoácido leucina (L) por prolina (P) na posição 444 da proteína madura. A posição homóloga na sequência do *PSGBA* é ocupada por C, da mesma forma como acontece com a mutação L444P que produz a DG (Charrow *et al.*, 2000; Grabowski *et al.*, 2013). Por fim, a mutação 84GG está presente em 7% dos casos e resulta na alteração do quadro de leitura devido à inserção

de um G no nucleotídeo 84 do DNA complementar (cDNA). Em decorrência dessa inserção, não há produção de proteína (Beutler *et al.*, 1991; Charrow *et al.*, 2000; Grabowski *et al.*, 2013).

As mutações N370S, L444P e 84GG representam aproximadamente 95% dos alelos causadores de doença em judeus Ashkenazi, mas representam apenas entre 50 e 60% dos alelos mutantes em indivíduos não-Ashkenazi (Beutler *et al.*, 1991; Emre *et al.*, 2008). No Brasil, a frequência das mutações mais comuns no gene *GBA1* é semelhante àquela descrita nas demais populações (não-Ashkenazi). As três mutações mais frequentes no nosso país são N370S, L444P e G377S (éxon 9; g.6748G>A; c.1246G>A) (Rozenberg *et al.*, 2006; Siebert *et al.*, 2013).

1.1.4. Manifestações clínicas e classificação em subtipos

A DG é classificada em três tipos clínicos principais tendo como base a ausência (tipo 1) ou a presença e a gravidade dos sintomas neurológicos (tipos 2 e 3) (Grabowski *et al.*, 2013). Dentre as características clínicas apresentadas pelos pacientes de todas as formas da doença, bem como sua incidência, destacam-se: hepatomegalia (63%), esplenomegalia (85%), anemia (34%), trombocitopenia (68%), osteopenia (55%), fraturas (7%), crises ósseas (7%), dores ósseas (33%) e retardo no crescimento (36%) (Charrow *et al.*, 2000; Rosenbloom & Weinreb, 2013).

A forma não-neuronopática da doença, também conhecida como DG tipo 1 (OMIM 230800), é o tipo mais comum observado nos pacientes e representa mais de 90% dos casos (Beutler & Grabowski, 2001; Grabowski *et al.*, 2013; Rosenbloom & Weinreb, 2013). Essa forma é caracterizada por extensa variabilidade na progressão e na gravidade da doença, variando desde indivíduos assintomáticos até pacientes com grave comprometimento de órgãos específicos. O início das manifestações clínicas pode ocorrer em qualquer idade, inclusive na vida adulta. O aparecimento precoce dos sinais clínicos geralmente está associado a um grave e rápido comprometimento dos órgãos. A sobrevida do paciente pode ser normal dependendo da gravidade das complicações (Grabowski, 2004). A DG tipo 1 tem sido associada ao maior risco de desenvolvimento de certos cânceres, tais como mieloma múltiplo, carcinoma hepatocelular, linfoma não-Hodgkin,

melanoma e câncer no pâncreas (Zimran *et al.*, 2005; de Fost *et al.*, 2006; Ayto & Hughes, 2013; Mistry *et al.*, 2013; Weinreb & Lee, 2013).

Os tipos 2 e 3 da DG comprometem o funcionamento do SNC e são denominadas formas neuronopáticas aguda e crônica, respectivamente. A DG tipo 2 (OMIM 230900) é caracterizada pelo aparecimento precoce das manifestações clínicas sistêmicas e do envolvimento do SNC, resultando em óbito nos primeiros dois anos de vida devido à rápida e devastadora progressão da doença (Sidransky, 2004; Grabowski *et al.*, 2013). O tipo 3 (OMIM 231000) é a forma intermediária da DG, pois compromete as funções do SNC de maneira mais lenta e gradual (Sidransky, 2012; Grabowski *et al.*, 2013). Por definição, o tipo 3 inclui todos os pacientes com algum grau de comprometimento neurológico, mas que sobreviveram aos primeiros anos de vida (Sidransky, 2012). Epilepsia mioclônica, ataxia, atraso no desenvolvimento, prejuízo do intelecto, dificuldade de aprendizagem, além dos sintomas motores e viscerais, são algumas das manifestações clínicas apresentadas pelos pacientes com os tipos 2 e 3 da doença (Sidransky, 2004; Gupta *et al.*, 2011; Sidransky, 2012; Sidransky & Lopez, 2012).

Em muitos casos, a classificação dos pacientes em um determinado tipo clínico considerando apenas o genótipo e os sintomas apresentados por estes indivíduos é muito difícil. Por esta razão, a DG tem sido descrita como um contínuo de fenótipos ao invés da categorização em tipos clínicos (Figura 3).

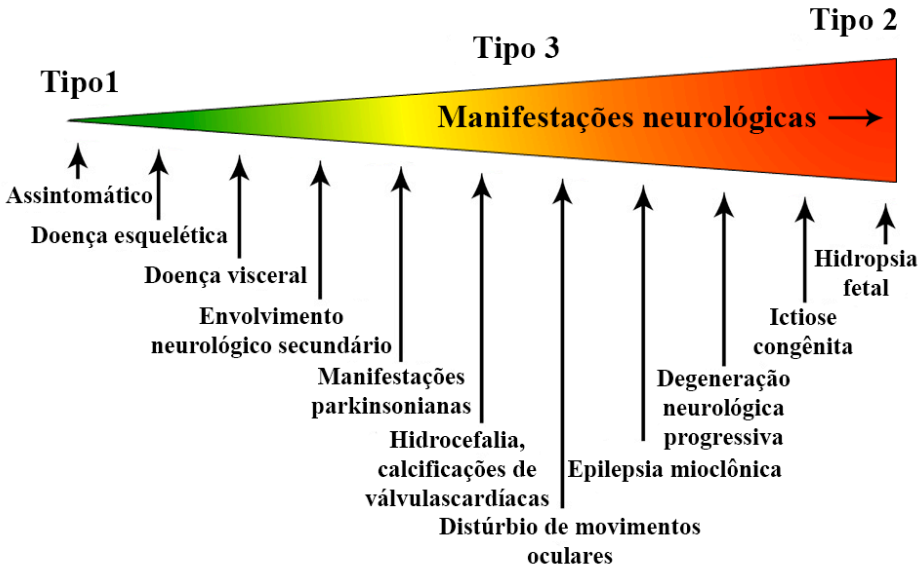

Figura 3. Classificação clínica da DG como um contínuo de fenótipos (adaptado de Sidransky, 2004).

1.1.5. Variabilidade fenotípica e correlação genótipo-fenótipo

A correlação entre o genótipo molecular e o fenótipo clínico permanece bastante limitada e incompleta, embora um grande número de mutações e polimorfismos já tenha sido identificado no gene *GBA1* (Goker-Alpan *et al.*, 2005; Hruska *et al.*, 2008; Sidransky, 2012). A variabilidade fenotípica existente é significativa. Pacientes com mesmo genótipo podem apresentar variação tanto na gravidade quanto no tipo de manifestações clínicas da doença, inclusive entre irmãos e gêmeos idênticos (Lachmann *et al.*, 2004; Sidransky, 2004; Zhao *et al.*, 2005; Biegstraaten *et al.*, 2011; Sidransky, 2012). Por outro lado, pacientes com diferentes genótipos, mas que apresentam mesmo tipo e intensidade de sintomas clínicos, também são observados (Hruska *et al.*, 2008). Além disso, não há uma correlação fidedigna entre a quantidade de substrato acumulada e/ou níveis de atividade enzimática residual com o fenótipo dos pacientes (Sidransky, 2012). Entretanto, algumas generalizações da associação genótipo-fenótipo podem ser feitas. Uma delas é a presença da mutação comum N370S e o tipo 1 da doença. Esta mutação parece estar exclusivamente associada aos pacientes sem danos neurológicos ou como sendo uma mutação

"neuroprotetora". Outro exemplo é a presença da mutação L444P em ambos alelos e o desenvolvimento de formas neuronopáticas da DG (Sidransky, 2012).

Apesar da DG ser uma doença monogênica, esta doença não é necessariamente simples de ser estudada e outros fatores como genes modificadores, proteínas transportadoras, substratos alternativos e fatores epigenéticos possivelmente estão influenciando a gravidade, o desenvolvimento e o fenótipo dos pacientes envolvidos (Figura 4) (Sidransky, 2004; Hruska *et al.*, 2008).

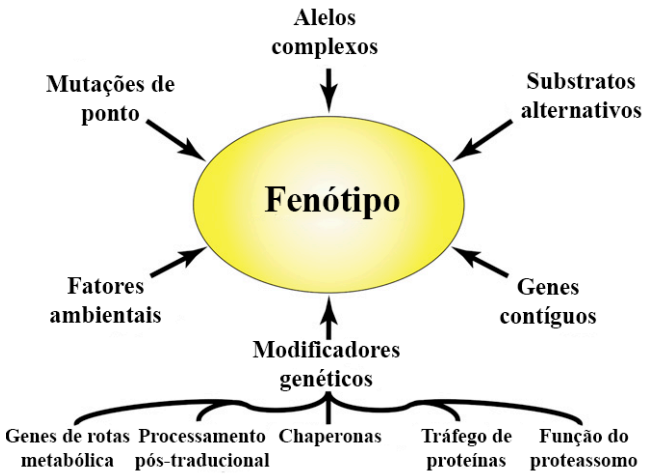

Figura 4. Fatores que contribuem para a variabilidade fenotípica observada em pacientes com DG (adaptado de Sidransky, 2004).

1.1.6. Diagnóstico

O diagnóstico da DG não deve ser baseado exclusivamente na avaliação clínica do paciente. Isto porque outras doenças lisossômicas de depósito podem apresentar sintomas semelhantes à DG, o que dificulta o estabelecimento preciso do diagnóstico. O diagnóstico laboratorial padrão é feito através da medida da atividade da GCase em células nucleadas, geralmente leucócitos e/ou fibroblastos. Apesar da diferença entre os protocolos utilizados na análise enzimática, a grande maioria utiliza um substrato sintético específico (4-metilumbeliferil-β-D-glicopiranosídeo) e detergentes a fim de solubilizar e estabilizar a enzima em questão (Grabowski *et al.*, 2013). Os valores de referência variam conforme o laboratório e a técnica utilizada. Contudo, a dosagem da atividade da GCase não nos permite classificar os pacientes em tipos clínicos e não nos permite distinguir indivíduos

heterozigotos de indivíduos normais. Desta forma, a análise molecular do gene *GBA1*, juntamente com os dados fenotípicos disponíveis, podem auxiliar na classificação do paciente em determinada forma clínica, ou pelo menos, possibilitar a distinção entre as formas neuronopáticas e não-neuronopática (Grabowski *et al.*, 2013).

A análise molecular do *GBA1* é complementar ao diagnóstico bioquímico, sendo muito importante para identificação dos alelos causadores da DG e para detecção de indivíduos portadores. De maneira geral, a análise molecular é iniciada pela busca de mutações comuns no gene, tais como as mutações N370S e L444P (Grabowski *et al.*, 2013). Caso o genótipo do paciente ainda esteja incompleto ou indefinido, pode-se realizar o sequenciamento completo do gene.

1.1.7. Tratamento

Durante muito tempo, o tratamento da DG foi baseado apenas em medidas paliativas para o manejo dos sintomas, não havendo um tratamento específico que estivesse disponível. A esplenectomia era amplamente utilizada para reduzir o atraso no crescimento, as citopenias e a compressão abdominal (Cassinerio *et al.*, 2014).

Em 1991, o tratamento da DG tornou-se uma realidade após o início da utilização da terapia de reposição enzimática (TRE). Desde então, a TRE é o tratamento de escolha da doença e atua por meio da redução da quantidade de substrato acumulada nos lisossomos através da administração de enzima recombinante exógena a fim de reestabelecer a degradação de GC (Figura 5) (Grabowski, 2008). O tratamento utilizando GCase recombinante tem como objetivo melhorar os sintomas causados pela doença, impedir alterações ósseas irreversíveis e proporcionar uma melhor qualidade de vida aos pacientes com DG (Pastores *et al.*, 2004; Harmanci & Bayraktar, 2008). A TRE teve início com o uso da GCase purificada de placenta humana (alglucerase), a qual foi substituída pela forma recombinante da enzima (imiglucerase) (Beck, 2010). Atualmente, existem 3 formas comercialmente disponíveis de TRE, sendo elas: imiglucerase, velaglucerase alfa e taliglucerase (Rosenbloom & Weinreb, 2013). As diferentes apresentações de TRE variam pelo modo como são produzidas e por apresentarem resíduos glicosilados que possibilitam a sua captação via receptores de manose-6-fosfato.

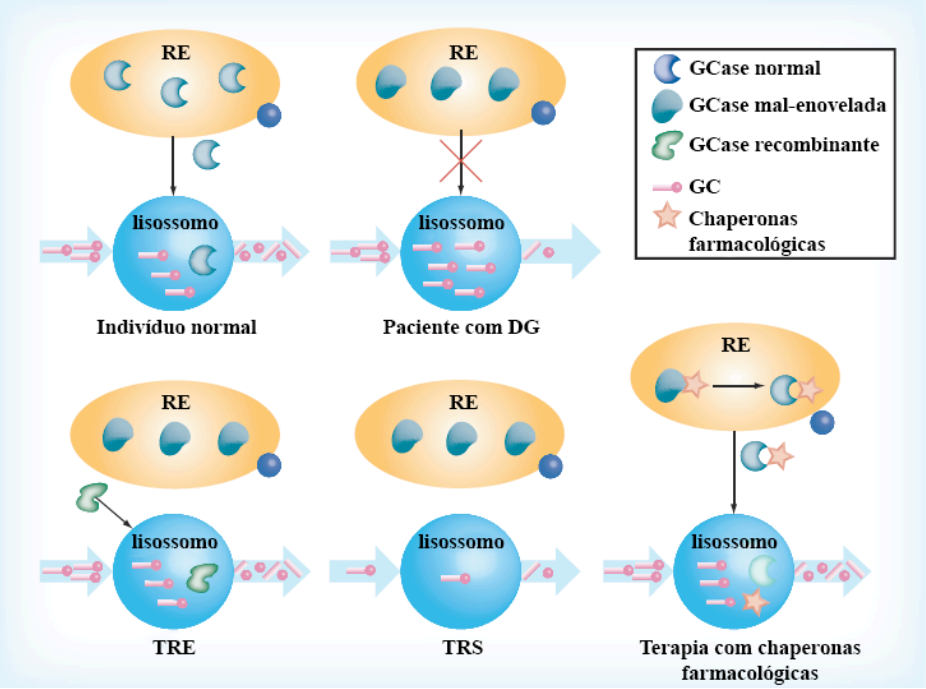

Figura 5. Opções terapêuticas para o tratamento da DG (adaptado de Trapero & Llebaria, 2013).

A GCase recombinante utilizada na TRE é incapaz de permear a barreira hematoencefálica, sendo então mais efetiva no tratamento dos pacientes com o tipo 1 (Cassinerio *et al.*, 2014). O tratamento é capaz de aliviar os sintomas sistêmicos dos pacientes com DG tipos 2 e 3 e, às vezes, pode levar à estabilização das manifestações neurológicas por um determinado período de tempo (Prows *et al.*, 1997; Schiffmann *et al.*, 1997). A dose a ser empregada é específica para cada paciente, levando em consideração a idade do indivíduo, a progressão da doença e a gravidade dos sintomas (Martins *et al.*, 2009; Cassinerio *et al.*, 2014). Embora seja um tratamento bastante eficaz no alívio dos sintomas sistêmicos, a TRE tem algumas desvantagens, das quais destacam-se: alto custo (de 100 a 200 mil dólares/ano por paciente), administração intravenosa a cada 14 dias, incapacidade de atravessar a barreira hematoencefálica (devido ao tamanho da molécula), eficácia e distribuição da enzima não uniformes em todos tecidos e aumento da produção de anticorpos contra a enzima recombinante (Parenti, 2009; de Souza *et al.*, 2010; Rosenbloom & Weinreb, 2013; Trapero & Llebaria, 2013).

Uma segunda opção de tratamento é a terapia de redução de substrato (TRS). Esta forma de tratamento atua indiretamente, pois ao invés de repor a enzima deficiente, reduz a produção do substrato GC pelo uso de um inibidor da enzima glicosilceramida sintase.

Dessa maneira, a primeira etapa da síntese dos glicoesfingolipídeos estará inibida, resultando em menor produção de GC e, consequentemente, em redução do acúmulo no interior dos macrófagos (Figura 5) (Trapero & Llebaria, 2013). Além disso, a molécula inibidora pode atuar como chaperona, de modo que estabiliza a GCase mutante quando administrada em baixas concentrações (Alfonso *et al.*, 2005). A TRS visa atenuar a taxa de síntese do substrato GC para alcançar um equilíbrio com os níveis enzimáticos reduzidos encontrados nos pacientes com DG (Rosenbloom & Weinreb, 2013; Cassinerio *et al.*, 2014). Um dos inibidores comercialmente disponíveis é o miglustate (N-butildeoxinojirimicina). A TRS é indicada como tratamento de escolha em pacientes com DG tipo 1 que apresentam contraindicação ou intolerância à GCase recombinante (Grabowski, 2008; Rosenbloom & Weinreb, 2013). Algumas das vantagens da utilização de TRS são a administração via oral (maior conveniência ao paciente), a habilidade de atravessar a barreira hematoencefálica (molécula pequena) e a ausência de reações imunológicas. Entretanto, os efeitos adversos são mais significativos quando comparados à TRE, dentre os quais podemos citar: diarreia, perda de peso moderada, tremores e parestesia (Rosenbloom & Weinreb, 2013; Cassinerio *et al.*, 2014).

O uso de chaperonas farmacológicas é uma abordagem terapêutica promissora tanto para DG como para outras doenças genéticas. No caso específico das doenças lisossômicas, as pequenas moléculas chaperonas formam um complexo estável com a enzima mutante em pH neutro do retículo endoplasmático (RE), impedindo a degradação e, consequentemente, aumentando a estabilidade da enzima em questão. Em meio ao excesso de GC e ao pH ácido dos lisossomos, este complexo enzima-chaperona é desfeito e a enzima encontra-se livre para desempenhar a sua função catalítica (Parenti, 2009; Suzuki, 2013; Trapero & Llebaria, 2013). Após ser liberada, a molécula chaperona é secretada para o exterior da célula, podendo ser eliminada ou utilizada na estabilização de outra enzima mutante (Figura 5) (Trapero & Llebaria, 2013). O emprego de chaperonas farmacológicas tem algumas desvantagens, dentre elas: a necessidade da enzima mutante apresentar pelo menos atividade residual e uma chaperona específica pode não ser igualmente efetiva para mais de uma forma mutante (seu efeito pode ser mutação-específica) (Trapero & Llebaria, 2013). Por outro lado, as chaperonas tem como vantagens o fato de se distribuírem uniformemente entre os tecidos e serem capaz de atravessar a barreira hematoencefálica, o que justificaria a sua utilização no tratamento das formas

neuronopáticas da doença (Parenti, 2009). Sendo assim, a combinação de TRE ou TRS com chaperonas farmacológicas pode ser uma estratégia terapêutica muito vantajosa em comparação ao uso dessas pequenas moléculas na forma de monoterapia.

1.2. Modificadores genéticos

A DG é conhecida pela sua ampla variabilidade fenotípica, apesar de algumas correlações entre genótipo e fenótipo terem sido estabelecidas. Diversas vezes, a presença de uma mutação específica no gene *GBA1* não é suficiente para explicar as divergências fenotípicas (Scriver, 2002; Sidransky, 2004; Goker-Alpan *et al.*, 2005). Genes modificadores, genes contíguos, alelos complexos, proteínas transportadoras, proteínas ativadoras, substratos alternativos, fatores epigenéticos e ambientais, de alguma maneira, parecem influenciar o aparecimento e a intensidade das manifestações clínicas apresentadas pelos pacientes (Sidransky, 2004; Hruska *et al.*, 2008). Por exemplo, genes modificadores contribuindo para o fenótipo de pacientes com Fibrose Cística estão descritos na literatura (Scriver & Waters, 1999; Dipple & McCabe, 2000; Cutting, 2010). Em relação à DG, os modificadores podem estar envolvidos em etapas metabólicas, em genes que codificam proteínas ativadoras (como a saposina C), em alterações vinculadas ao transporte (através de LIMP-2), processamento ou degradação da GCase (Figura 4) (Sidransky, 2004). O estudo aprofundado de genes modificadores é extremamente importante, pois permite ampliar as bases de conhecimento envolvidas na DG, o desenvolvimento de novas formas de tratamento e o estabelecimento de novas correlações genótipo-fenótipo.

1.2.1. *SCARB2*

Embora a grande maioria das enzimas lisossômicas alcance o seu destino final via receptores de manose-6-fosfato, a GCase é direcionada aos lisossomos por uma rota independente (Coutinho *et al.*, 2012). Em 2007, a proteína LIMP-2 (proteína lisossomal integral de membrana do tipo 2/*Lysosomal Integral Membrane Protein type 2*), a qual é codificada pelo gene *SCARB2* (*Scavenger Receptor Class B, member 2*) (4q13-21), foi

identificada como responsável pela ligação e direcionamento da enzima GCase aos lisossomos (Reczek *et al.*, 2007). A interação entre GCase e LIMP-2 é dependente do pH, sendo favorecida no pH neutro do RE e interrompida ao alcançar o pH ácido no interior dos lisossomos (Zachos *et al.*, 2012). Ao atingir o seu local de ação, a enzima GCase é liberada do seu receptor LIMP-2 e, consequentemente, torna-se disponível para degradação do seu substrato GC (Reczek *et al.*, 2007; Blanz *et al.*, 2010; Zachos *et al.*, 2012).

O LIMP-2 é uma proteína expressa constitutivamente, sendo uma das mais abundantes presente na membrana dos lisossomos (Eskelinen *et al.*, 2003). A análise de mutações e de superexpressão do gene *SCARB2* sugere que LIMP-2 é de fundamental importância na biogênese e na manutenção tanto de endossomos tardios como de lisossomos, assim como, na fusão entre lisossomos e autofagossomos (Kuronita *et al.*, 2002; Eskelinen *et al.*, 2003; Gleich *et al.*, 2013).

A presença de mutações no gene *SCARB2* está associada à síndrome de insuficiência renal com crises mioclônica. Esta síndrome é herdada de forma autossômica recessiva, sendo caracterizada por dano renal, epilepsia mioclônica progressiva e ataxia (Berkovic *et al.*, 2008; Blanz *et al.*, 2010). Mutações no *SCARB2* causam deficiência da proteína LIMP-2, levam a sua retenção no interior do RE e influenciam negativamente a atividade da GCase por impedir a translocação da enzima para os lisossomos (Balreira *et al.*, 2008; Blanz *et al.*, 2010). Em 2011, um estudo envolvendo dois irmãos com genótipo idêntico no *GBA1* e sintomas clínicos bastante discordantes, descreveu *SCARB2* como sendo um gene modificador da DG. A mutação E471G foi identificada em um dos alelos do paciente que apresentava sintomas muito graves. Além de reduzir a quantidade de LIMP-2 disponível, detectou-se a diminuição dos níveis de GCase, da sua atividade, assim como, a secreção da enzima para o espaço extracelular (Velayati *et al.*, 2011). A presença de LIMP-2 é de fundamental importância para o correto funcionamento da GCase, particularmente em pacientes com DG. Segundo Velayati e colaboradores, uma mutação no gene *SCARB2* pode ter sido responsável pela conversão de um paciente com DG tipo 1 em tipo 3 (Velayati *et al.*, 2011).

Sendo assim, *SCARB2* é um fortíssimo candidato a modificador do fenótipo da DG visto que alterações nesse gene resultam em prejuízo do transporte da GCase aos lisossomos, na redução dos níveis proteicos e da atividade enzimática, no acúmulo de GC,

na liberação da GCase para fora da célula e/ou na degradação da enzima via proteassomo (Balreira *et al.*, 2008; Blanz *et al.*, 2010; Velayati *et al.*, 2011).

1.2.2. *PSAP*

As saposinas são um grupo constituído por quatro pequenas glicoproteínas, denominadas saposinas A, B, C e D. Estas proteínas atuam como ativadoras enzimáticas em múltiplos estágios da rota de degradação dos glicoesfingolipídeos e de membranas lisossômicas (Kishimoto *et al.*, 1992; Vaccaro *et al.*, 1999). As saposinas são geradas a partir da clivagem proteolítica da proteína precursora, a prosaposina (pSap), que acontece nos lisossomos e é mediada pela catepsina D (Hiraiwa *et al.*, 1997). A pSap é codificada pelo gene *PSAP*, localizado no *locus* 10q21 (Furst & Sandhoff, 1992). Cada uma das saposinas resultantes contém aproximadamente 80 aminoácidos, contendo seis resíduos de cisteína conservados em sua estrutura. Estes resíduos formam três pontes dissulfídicas, as quais contribuem para manutenção da estrutura terciária, resistência ao calor e desempenho de suas funções (Tamargo *et al.*, 2012).

Assim como LIMP-2 é essencial para o correto direcionamento da GCase aos lisossomos, a proteína saposina C (Sap-C) é crucial para a atividade da enzima devido ao seu papel como ativadora (Vaccaro *et al.*, 2010; Grabowski *et al.*, 2013). A importância da Sap-C para a GCase é bem consolidada na literatura, embora o mecanismo pelo qual essa ativação ocorra permaneça incerto. Um dos mecanismos propostos e mais bem aceitos sugere que a Sap-C induz alterações na membrana, intermediando a exposição de GC e, por fim, facilitando a interação da GCase com o seu substrato (Figura 6) (Alattia *et al.*, 2007; Tamargo *et al.*, 2012). Evidências experimentais sugerem que Sap-C interage com a membrana lipídica, de modo a solubilizar o substrato GC, tornando-o acessível à hidrólise pela GCase (Alattia *et al.*, 2006; 2007). Além de seu papel como ativadora enzimática, Sap-C atua protegendo a GCase da degradação proteolítica (Sun *et al.*, 2003).

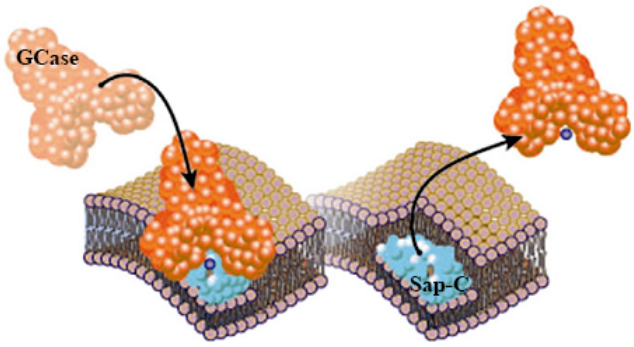

Figura 6. Mecanismo de ativação da GCase pela Sap-C (adaptado de Tamargo *et al.*, 2012).

Até o momento, seis pacientes com deficiência de Sap-C foram descritos na literatura. Indivíduos com mutações nos resíduos de cisteína do domínio de *PSAP* que codifica Sap-C apresentam fenótipo muito parecido ao tipo 3 da DG, enquanto que alterações em outras regiões deste domínio acarretam características clínicas que assemelham-se ao tipo 1 (Tylki-Szymanska *et al.*, 2007; Sun *et al.*, 2010; Vaccaro *et al.*, 2010). Dessa forma, existe uma correlação entre o tipo e a localização da mutação com a natureza do fenótipo Gaucher-*like*. Apesar de pacientes com deficiência conjunta de GCase e de Sap-C ainda não terem sido identificados, Sap-C é um potencial candidato a modificador da DG. Isto porque alterações na sua expressão podem influenciar os sintomas clínicos e a intensidade dos mesmos (Tamargo *et al.*, 2012).

1.3. Glicocerebrosidase e parkinsonismo

O aparecimento de sintomas parkinsonianos em pacientes com DG tipo 1 foi descrito pela primeira vez em 1996 (Neudorfer *et al.*, 1996). Subsequentemente, outros estudos foram publicados na literatura envolvendo poucos indivíduos e/ou pequenos grupos que apresentavam estas mesmas características (Neudorfer *et al.*, 1996; Machaczka *et al.*, 1999; Tayebi *et al.*, 2001; Bembi *et al.*, 2003; Varkonyi *et al.*, 2003). Estes pacientes com DG demonstraram diferenças na extensão das manifestações parkinsonianas, na idade de início dos sintomas e na resposta ao tratamento (Sidransky, 2012). Além disso, o número de familiares com parkinsonismo nestas famílias parece estar aumentado em

comparação a população em geral (Goker-Alpan *et al.*, 2004; Halperin *et al.*, 2006). Além disso, a análise de 57 amostras cerebrais oriundas de pacientes diagnosticados com doença de Parkinson revelou que 21% destas eram portadoras de mutações no gene *GBA1* (Lwin *et al.*, 2004). Todos estes resultados sugeriam que mesmo indivíduos heterozigotos para mutações no *GBA1* tinham maior chance de desenvolver manifestações parkinsonianas (tremor em repouso, bradicinesia, rigidez e instabilidade postural). Os estudos experimentais que se seguiram identificaram variações quanto a valores de frequência, dependendo do tipo de metodologia empregada e do grupo étnico analisado, mas a grande maioria dos resultados mostrou um aumento da frequência de mutações no gene *GBA1* entre pacientes com parkinsonismo quando comparados a indivíduos controles. Os achados foram semelhantes em judeus Ashkenazi e em não-judeus (Sidransky, 2012). Contudo, a confirmação dos achados aconteceu apenas em 2009 após a publicação de um estudo multicêntrico envolvendo 16 centros de pesquisa espalhados pelo mundo todo. Neste estudo, um total de 5691 pacientes com DP e 4898 controles foram analisados. A análise dos dados revelou que pacientes com DP tem 5.43 mais chance de serem portadores de uma mutação no gene *GBA1* em relação ao grupo controle (Sidransky *et al.*, 2009). Dessa forma, a presença de mutações no *GBA1* foi amplamente consolidada na literatura como o maior fator de risco para o desenvolvimento de parkinsonismo. A presença de mutações no gene *GBA1* está associada ao aparecimento precoce das manifestações parkinsonianas, isto significa uma antecipação média de 5 anos em relação a indivíduos sem as mutações (Sidransky *et al.*, 2009).

Além do parkinsonismo, mutações no *GBA1* também são frequentemente encontradas em pacientes apresentando demência com corpos de Lewy (DCL) (Goker-Alpan *et al.*, 2006; Clark *et al.*, 2009). Indivíduos com mutações no *GBA1* têm idade de diagnóstico de DCL precoce em relação à DCL sem a presença de mutações (Nalls *et al.*, 2013). A DP e a DCL são sinucleinopatias, um grupo de doenças com características parkinsonianas caracterizado pela presença de corpos de Lewy. Os corpos de Lewy são agregados insolúveis formados pela oligomerização da proteína α-sinucleína (α-Sin) em diferentes regiões do cérebro (substância nigra, córtex e hipocampo) (Westbroek *et al.*, 2011; Puschmann *et al.*, 2012). O gene *SNCA* codifica a proteína α-Sin e mutações, assim como, duplicações nesse gene estão associadas à DP (Kasten & Klein, 2013). A proteína α-Sin é altamente expressa no cérebro, e mostra-se envolvida na regulação de vesículas

sinápticas e na liberação de neurotransmissores, embora sua exata função ainda precise ser elucidada (Westbroek *et al.*, 2011).

O mecanismo envolvido na interação entre a GCase e a α-Sin permanece desconhecido, mas acredita-se na existência de uma relação recíproca entre estas duas proteínas (Figura 7). Quando há alteração na homeostase da α-Sin devido ao prejuízo no seu enovelamento, alteração da degradação associada ao RE ou autofagia, ocorre um aumento nos níveis de α-Sin. Isso leva à inibição da translocação da GCase para os lisossomos. Consequentemente, menos enzima estará disponível para hidrólise do substrato, que acumula gradualmente, induzindo a oligomerização da α-Sin nos lisossomos. Em decorrência dessa disfunção lisossômica, o *turnover* da α-Sin por meio de processos autofágicos torna-se prejudicado, havendo estímulo da formação de agregados proteicos no citoplasma. Estes agregados, por sua vez, também inibem o endereçamento da GCase aos lisossomos (Mazzulli *et al.*, 2011). Análises biofísicas indicam que a interação direta entre GCase e α-Sin é favorecida em pH ácido e que α-Sin inibe a atividade enzimática da GCase de maneira quantidade-dependente (Yap *et al.*, 2011; Yap *et al.*, 2013). A GCase com atividade parcial interfere na degradação proteica que acontece nos lisossomos, promove o acúmulo de α-Sin e aumenta a neurotoxicidade mediada pela α-Sin (Mazzulli *et al.*, 2011). Entretanto, a grande maioria dos pacientes com DG não desenvolve sintomas parkinsonianos. A presença de mutações no gene *GBA1* e a deficiência enzimática, por si só, não são suficientes para causar a DP ou DCL. O componente central desse modelo de interação recíproca é o lisossomo, por ser a organela responsável pela degradação de outras organelas (como as mitocôndrias), proteínas e lipídeos (Dehay *et al.*, 2013). Muitas evidências indicam que a disfunção lisossômica contribui na neuropatologia do parkinsonismo e que agregados de α-Sin podem prejudicar a ocorrência de autofagia e de outras funções relacionadas aos lisossomos (Winslow *et al.*, 2010; Tofaris, 2012).

Devido à relação entre DG tipo 1 e o aparecimento de sintomas parkinsonianos, a classificação desta forma como não-neuronopática deve ser reconsiderada. Cada vez mais, deve-se avaliar os pacientes considerando os fenótipos como um contínuo (Sidransky, 2004; Sidransky *et al.*, 2009; Nalls *et al.*, 2013).

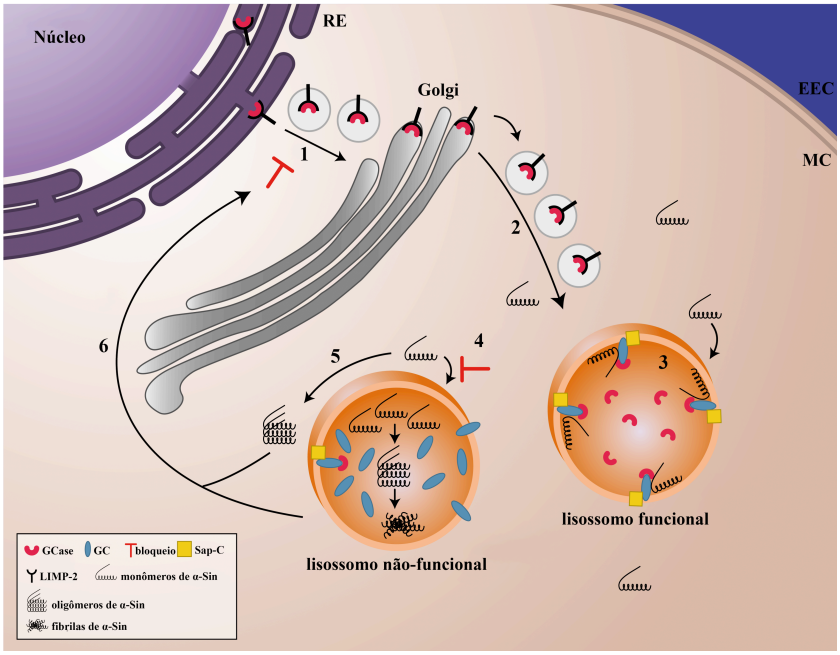

Figura 7. Interação recíproca existente entre GCase e α-Sin. (1 e 2) A GCase é transportada do RE para os lisossomos através da sua ligação à proteína LIMP-2. (3) A enzima interage com seu substrato GC e com monômeros de α-Sin, facilitando a degradação de ambos no interior dos lisossomos. (4) Em situações em que há disfunção lisossômica pelo acúmulo de GC e/ou formação de agregados de α-Sin, haverá prejuízo dos processos autofágicos e α-Sin acumulará no citoplasma. (5) Os monômeros solúveis de α-Sin irão formar oligômeros. (6) Estes oligômeros de α-Sin impedirão que a GCase seja direcionada do RE aos lisossomos. RE = retículo endoplasmático, MC = membrana citoplasmática e EEC = espaço extracelular (adaptado de Siebert *et al.*, 2014).

1.4. MicroRNAs

MicroRNAs (miRNAs) são uma classe de pequenos RNAs de fita simples, endógenos, não-codificantes e conservados evolutivamente, que regulam a expressão gênica em etapas posteriores à transcrição. Estas pequenas moléculas de aproximadamente 22 nucleotídeos de extensão ligam-se em sequências de mRNA-alvo por complementaridade e interferem na maquinaria da tradução, impedindo ou alterando a produção de proteínas (Bhaskaran & Mohan, 2013). A regulação da expressão gênica ocorre através da ligação do miRNA a regiões 3' não-traduzidas (3'UTRs; 3'-*untranslated regions*), sequências codificantes ou 5' não-traduzidas (5'UTRs; 5'-*untranslated regions*)

de mRNAs, levando à inibição da tradução ou à degradação do mRNA específico (Bartel, 2004).

1.4.1. Descoberta

O primeiro miRNA, denominado lin-4, foi identificado em *Caenorhabditis elegans* (*C. elegans*) (Lee *et al.*, 1993). Esforços conjuntos de dois grupos de pesquisa revelaram que lin-4 controla uma etapa específica do desenvolvimento em *C. elegans*, a qual envolve a progressão da primeira para a segunda fase larval. Lin-4 regula negativamente um gene codificador de proteína, denominado *lin-14* (Lee *et al.*, 1993; Wightman *et al.*, 1993). O gene *lin-4* não codifica proteína, mas é transcrito em dois pequenos RNAs de aproximadamente 21 e 61 nucleotídeos cada (Lee *et al.*, 1993). O RNA maior forma uma estrutura em forma de grampo e serve como precursor do RNA menor. O RNA de 21 nucleotídeos de *lin-4* possui complementaridade imperfeita a múltiplos sítios na região 3'UTR de *lin-14*. Após a ligação de lin-4, a tradução de *lin-14* é inibida sem causar alteração nos seus níveis de expressão (Lee *et al.*, 1993; Wightman *et al.*, 1993).

Um segundo miRNA, conhecido como let-7, foi identificado em *C. elegans* e também mostrou-se envolvido na regulação do desenvolvimento. Diferentemente de lin-4, let-7 regula a transição do último estágio larval para o estágio adulto (Reinhart *et al.*, 2000). *Let-7* tem sequência e padrão de expressão altamente conservados durante a fase de desenvolvimento em muitos organismos, inclusive em humanos (Pasquinelli *et al.*, 2000). Assim como lin-4, let-7 atua através da ligação à região 3'UTR dos genes *lin-41* e *hbl-1* (*lin-57*), ocasionando a inibição da tradução destes genes (He & Hannon, 2004).

Após a descoberta dos primeiros miRNAs, diversos grupos de pesquisa construíram e sequenciaram bibliotecas de pequenos RNAs obtidos de *C. elegans*, *Drosophila* e mamíferos, confirmando que lin-4 e let-7 são, de fato, membros de uma classe abundante de pequenos RNAs endógenos que exercem funções regulatórias (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001). A busca incessante por novos membros resultou na descoberta de múltiplos miRNAs em diferentes espécies. Em 2002, o primeiro banco de dados de miRNAs (miRBase; http://www.mirbase.org) foi criado. A importância deste banco de dados é notória por conter todas as sequências de miRNA já descritas, além

de reunir outras informações relevantes, tais como: evidências experimentais, anotação de sequência, coordenadas genômicas, nomenclatura, predição de mRNAs-alvo e literatura relacionada. Segundo a atualização mais recente do miRBase (*release* 20; Junho de 2013), este banco de dados tem o registro de 24521 precursores de miRNAs, que expressam 30424 miRNAs maduros em 206 espécies distintas (Kozomara & Griffiths-Jones, 2014). Embora um grande número de miRNAs já tenha sido identificado, a relevância biológica da grande maioria deles permanece desconhecida, necessitando estudos de validação funcional (Bhaskaran & Mohan, 2013).

1.4.2. Biogênese

Os genes que codificam miRNAs são evolutivamente conservados e podem estar localizados em íntrons de genes codificadores de proteínas, em íntrons ou em éxons de genes não-codificadores ou, até mesmo, em regiões intergênicas (Rodriguez *et al.*, 2004; Lee, 2013).

Os miRNAs maduros são gerados através de múltiplas etapas que envolvem tanto o núcleo como o citoplasma. O primeiro passo da biogênese dos miRNAs é a transcrição pela RNA polimerase (RNAse) II em longos transcritos primários, conhecidos como pri-miRNAs. Assim como os mRNAs, os pri-miRNAs tem cap 5'-metil-guanosina, cauda poli(A) e variam de centenas a milhares de nucleotídeos de extensão (Cai *et al.*, 2004; Chang & Mendell, 2007). Uma molécula representativa de pri-miRNA em animais exibe uma estrutura secundária complexa, podendo abranger vários miRNAs em forma de grampo, sendo que cada um deles é formado por uma haste dupla fita, uma alça com bases não complementares e sequências flanqueadoras (Bartel, 2004).

Os pri-miRNAs dobram-se para formar estruturas em forma de grampo, as quais contêm entre 60 e 80 nucleotídeos. Ainda no núcleo, estas estruturas são excisadas do pri-miRNA por um microprocessador complexo, composto pela RNAse III Drosha e pelo seu cofator DGCR8 (*DiGeorge syndrome critical region in gene 8*) (Lee *et al.*, 2003). As estruturas em forma de grampo excisadas são denominadas de miRNAs precursores (pre-miRNAs). Os pre-miRNAs têm aproximadamente 22 nucleotídeos pareados, um grupamento fosfato na extremidade 5' e 2 nucleotídeos não-pareados na extremidade 3',

que são essenciais para o reconhecimento dos pre-miRNAs pela exportina-5 (He & Hannon, 2004). A proteína exportina-5 é uma proteína de exportação nuclear que utiliza Ran-GTP como cofator a fim de realizar o transporte ativo dos pre-miRNAs do núcleo para o citoplasma (Lund *et al.*, 2004). Uma vez que estão no citoplasma, os pre-miRNAs são processados por outra RNAse III, a Dicer, juntamente com a proteína TRBP (proteína Tar de ligação ao RNA) sendo responsáveis pela remoção da alça ou *loop* terminal da estrutura em forma de grampo e, subsequente, liberação de um miRNA dupla-fita de aproximadamente 22 nucleotídeos (miRNA/miRNA*) (Hutvagner *et al.*, 2001). Este duplex de miRNA é separado em fitas simples por ação de uma helicase e a fita com maior instabilidade na região 5' atuará como fita-guia (Bhaskaran & Mohan, 2013). A fita-guia será preservada, constituindo o miRNA maduro, enquanto que a fita complementar será rapidamente direcionada à degradação. O miRNA maduro é então incorporado em um complexo multiproteico, conhecido como complexo de silenciamento induzido por RNA (*RNA-induced silencing complex*; RISC) e, assim, permanece associado de maneira estável (Chang & Mendell, 2007). Este complexo direcionará o miRNA maduro até a região 3'UTR do mRNA-alvo, possibilitando a regulação gênica através de dois mecanismos pós-transcricionais (Figura 8).

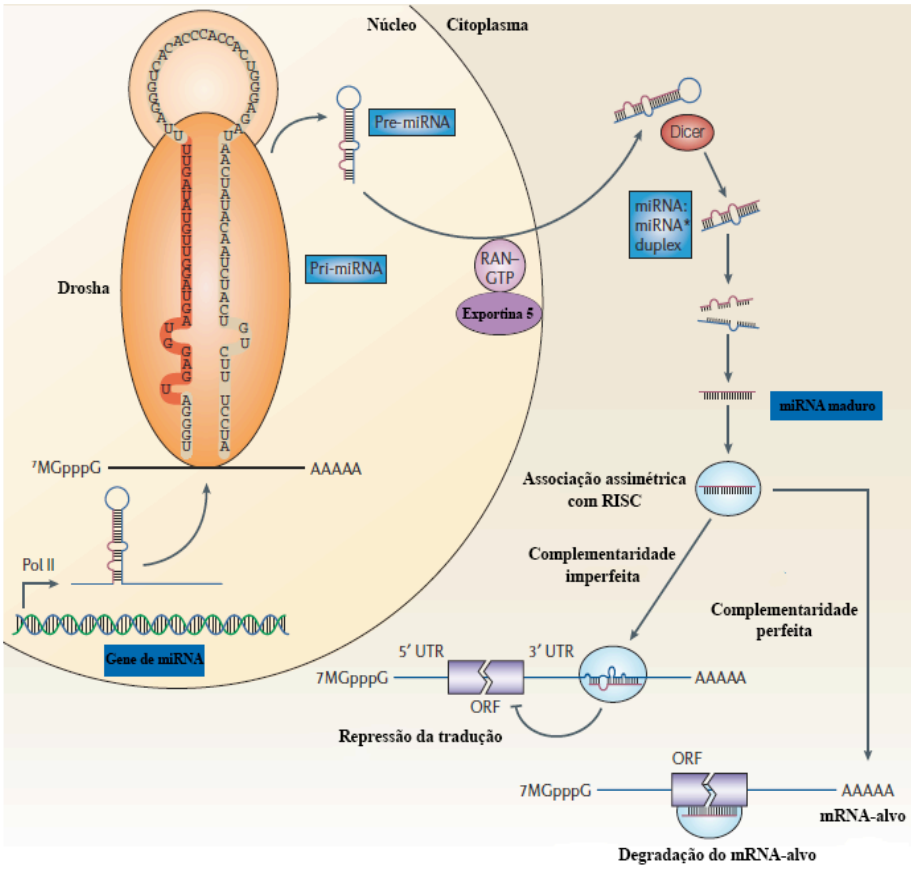

Figure 8. Biogênese de miRNAs e mecanismos de regulação gênica (adaptado de Esquela-Kerscher & Slack, 2006).

1.4.3. Mecanismos de ação ou regulação por miRNAs

Uma vez que o miRNA maduro associa-se ao RISC, a regulação gênica exercida pelos miRNAs pode acontecer por meio de dois mecanismos: degradação do mRNA-alvo ou repressão da tradução. A escolha de qual mecanismo direcionará o silenciamento depende do grau de complementaridade existente entre o miRNA e seu mRNA-alvo (Bartel, 2004).

A degradação do mRNA-alvo ocorre devido ao perfeito ou quase perfeito pareamento existente entre o miRNA e o seu mRNA-alvo, permitindo que RISC realize a clivagem endonucleolítica do mRNA em questão (Figura 8). A complementaridade de sequência se estende por toda a região transcrita do gene alvo, ao invés de ficar limitada a região 3'UTR (He & Hannon, 2004). Após a clivagem do mRNA, o miRNA maduro permanece intacto e pode guiar o reconhecimento e a degradação de outros mRNAs

(Bartel, 2004). Em plantas, a maioria dos miRNAs exerce sua função regulatória por meio desse mecanismo de ação (Hake, 2003).

Diferentemente do que acontece em plantas, miRNAs em *C. elegans*, *Drosophila melanogaster* e vertebrados geralmente reconhecem um ou mais sítios de imperfeita complementaridade na região 3'UTR dos mRNAs-alvo. RISC não é capaz de degradar o mRNA, ao invés disso, inibe a tradução. A repressão pode ocorrer tanto no início como durante o processo de tradução. O pareamento imperfeito também pode ocasionar a redução da abundância do mRNA-alvo, embora isto provavelmente seja fruto do aumento do *turnover* do mRNA do que pela clivagem mediada pelo RISC (Chang & Mendell, 2007). Devido à complementaridade imperfeita, um determinado miRNA pode se ligar e regular mais de um mRNA, ao mesmo tempo que muitos miRNAs diferentes podem se ligar e controlar cooperativamente um único mRNA-alvo (Kim, 2005). A inibição da tradução é o principal mecanismo de ação dos miRNAs em mamíferos (Bartel, 2004).

Os nucleotídeos 2 a 8 da extremidade 5' do miRNA maduro (conhecida como sequência *seed*) são extremamente conservados na maioria dos miRNAs homólogos, mostrando-se cruciais para estabilidade e ligação ao RISC (Figura 9) (Esquela-Kerscher & Slack, 2006). O grau de complementaridade entre esta região e o mRNA-alvo é bastante importante para o desempenho da função biológica do miRNA, visto que determina o mRNA ao qual determinado miRNA irá se ligar (Bartel, 2004; Chang & Mendell, 2007).

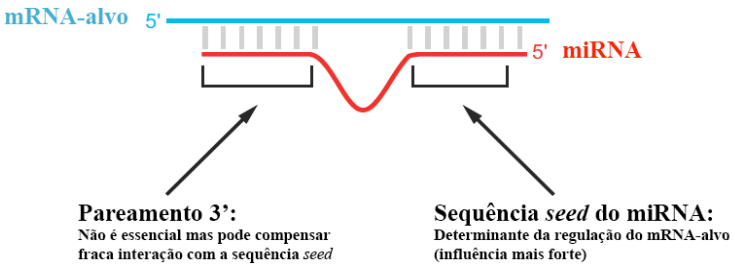

Figura 9. Sequência *seed* do miRNA pareada com seu mRNA-alvo (adaptado de Chang & Mendell, 2007).

Por muito tempo, pensou-se que miRNAs interagiam apenas com a região 3'UTR dos mRNAs-alvo. Entretanto, descobriu-se que os miRNAs são capazes de se associar em qualquer região dos mRNAs-alvo, dentre elas: 5'UTR, 3'UTR e região codificante (Lytle *et al.*, 2007; Tay *et al.*, 2008; Almeida *et al.*, 2011). Além dos dois mecanismos de silenciamento acima descritos, miRNAs também são capazes de induzir a expressão gênica

devido à ligação a regiões promotoras e de ativar a tradução de mRNAs-alvo (Vasudevan *et al.*, 2007; Place *et al.*, 2008). Então, dependendo da situação, miRNAs específicos podem atuar tanto como repressores como ativadores (Vasudevan *et al.*, 2007).

1.4.4. Diversidade de funções biológicas

Estes pequenos RNAs não-codificantes têm sido relacionados a muitos processos biológicos, tanto normais como patológicos, desde que os primeiros miRNAs lin-4 e let-7 foram identificados e da descoberta do seu envolvimento no desenvolvimento larval de *C. elegans*. Em humanos, mais de 60% dos genes codificadores de proteínas sofrem pressão seletiva a fim de manter o pareamento com miRNAs. Isso evidencia o quão difícil é identificar um processo biológico que não esteja sob influência de miRNAs (Bartel, 2009; Friedman *et al.*, 2009).

Os miRNAs estão envolvidos em uma diversidade de processos fisiológicos, dentre os quais podemos citar: proliferação e diferenciação de células-tronco hematopoiéticas (Bhaskaran & Mohan, 2013); desenvolvimento e diferenciação do coração (Zhao *et al.*, 2005), da pele e do sistema nervoso (Ebert & Sharp, 2012; Bhaskaran & Mohan, 2013); sinalização e ciclo celular (Bueno & Malumbres, 2011; Ichimura *et al.*, 2011); modulação da inflamação e da resposta imune (Chaudhuri *et al.*, 2011; Shaham *et al.*, 2012; Rebane & Akdis, 2013); regulação da secreção de insulina (Poy *et al.*, 2004); controle do metabolismo lipídico (Esau *et al.*, 2004; Esau *et al.*, 2006); apoptose (Walker & Harland, 2009; Adlakha & Saini, 2011) e autofagia (Zhu *et al.*, 2009; Frankel *et al.*, 2011).

Além da participação em funções normais do organismo, anormalidades nos níveis de expressão de determinados miRNAs ou no desempenho de suas funções podem influenciar o fenótipo de algumas doenças humanas (Chang & Mendell, 2007). A alteração da expressão de miRNAs específicos pode estar relacionada a doenças infecciosas, autoimunes, metabólicas e genéticas (Lewis & Jopling, 2010; Junker *et al.*, 2011; Contreras & Rao, 2012; Bhaskaran & Mohan, 2013).

Além disso, os miRNAs estão amplamente associados a muitos tipos de cânceres. Diversos estudos demonstram o envolvimento de miRNAs em diferentes etapas do processo de formação do tumor, tanto desempenhando a função de oncogenes como de

supressores de tumor. MiRNAs também desempenham importante papel como biomarcadores, auxiliando o diagnóstico e o prognóstico de muitos tipos de câncer (Rachagani *et al.*, 2010; Farazi *et al.*, 2013).

1.5. Justificativa

A doença de Gaucher é uma doença genética rara e de causa conhecida, mas influenciada por outros fatores (genéticos, epigenéticos e ambientais) ainda não identificados. Embora diversas mutações no gene *GBA1* já tenham sido descritas como causadoras da doença, a associação entre o tipo de mutação e as características clínicas apresentadas pelos pacientes é bastante limitada e incompleta. Poucas extrapolações e predições quanto ao fenótipo podem ser feitas com base exclusivamente no genótipo do gene *GBA1* do paciente, dificultando ainda mais o prognóstico dos mesmos. Sendo assim, analisar polimorfismos em genes candidatos a modificadores do fenótipo da DG pode auxiliar na identificação de correlações genótipo-fenótipo até então desconhecidas. A escolha de genes candidatos utilizando dados relativos à importância ou influência de determinada proteína sobre a GCase é um excelente referencial.

Diversos estudos abordando a presença de mutações no gene *GBA1* como fator de risco para o desenvolvimento de parkinsonismo estão descritos na literatura. Estas manifestações parkinsonianas estão associadas à DP, à DCL, à DG tipo 1 e a muitas outras doenças. Dessa forma, estudos adicionais são necessários para avaliar se mutações no *GBA1* também estão frequentemente associadas ao aparecimento de sintomas parkinsonianos observados em outras patologias, como por exemplo, no caso da doença de Machado-Joseph/ataxia espinocerebelar tipo 3.

Além disso, o importante papel desempenhado por miRNAs em diferentes processos biológicos e patológicos estimulou o interesse pela relação existente entre a GCase e os miRNAs. A identificação de miRNAs que exerçam funções regulatórias sobre a enzima, tanto direta como indiretamente, pode ser de extrema relevância para aumentar as bases do conhecimento da DG e para possibilitar o desenvolvimento de novos alvos terapêuticos.

A presente tese de doutorado buscou abordar, de forma abrangente, diversos aspectos relacionados à proteína GCase e seu papel como modificador de outras patologias, bem como, de outros genes e fatores que podem estar contribuindo de alguma maneira no fenótipo da DG.

2. OBJETIVOS

2.1. Objetivo geral

Investigar os efeitos de alterações no gene *GBA1* e em genes modificadores do fenótipo da doença de Gaucher e de patologias associadas à proteína glicocerebrosidase.

2.2. Objetivos específicos

2.2.1. Analisar a sequência completa do gene *GBA1* e avaliar a correlação entre mutações no gene *GBA1* e sintomas parkinsonianos associados à doença de Machado-Joseph/ataxia espinocerebelar tipo 3 com e sem manifestações parkinsonianas;

2.2.2. Analisar polimorfismos gênicos em três genes (*SCARB2*, *PSAP* e *CLN8*) candidatos a modificadores do fenótipo da doença de Gaucher em pacientes brasileiros com genótipo *GBA1* definido;

2.2.3. Avaliar o genótipo dos polimorfismos nos genes *SCARB2*, *PSAP* e *CLN8* em relação ao tipo de mutação no *GBA1* no grupo de pacientes com doença de Gaucher;

2.2.4. Investigar a influência de microRNAs na atividade da enzima glicocerebrosidase a fim de identificar modificadores;

2.2.5. Selecionar microRNAs que exerçam funções regulatórias sobre a glicocerebrosidase e elucidar o possível mecanismo de ação.

3. RESULTADOS

Os resultados deste trabalho serão apresentados na forma de três artigos científicos, sendo que cada um deles faz parte de um Capítulo.

3.1. Capítulo I

ARTIGO 1

Glucocerebrosidase gene variants in parkinsonian patients with Machado Joseph/spinocerebellar ataxia 3

Artigo científico publicado no periódico *Parkinsonism and Related Disorders*,

doi: 10.1016/j.parkreldis.2011.09.024.

Parkinsonism and Related Disorders 18 (2012) 185–190

Contents lists available at SciVerse ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

Glucocerebrosidase gene variants in parkinsonian patients with Machado Joseph/spinocerebellar ataxia 3

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ARTICLE INFO

Article history:
Received 27 June 2011
Received in revised form
19 September 2011
Accepted 28 September 2011

Keywords:
Glucocerebrosidase gene
Gaucher disease
Machado Joseph disease
Parkinson disease
Spinocerebellar ataxia 3

ABSTRACT

Machado-Joseph disease/spinocerebellar ataxia type 3 (MJD/SCA3) may rarely presents a parkinsonian phenotype. Considering that mutations in the glucocerebrosidase (GBA) gene have been associated with Parkinson disease, we investigated whether these would be more prevalent in MJD/SCA3 patients with parkinsonian manifestations than in those without them.

Methods: MJD/SCA3 patients with parkinsonian features were identified and compared to relatives and to a MJD/SCA3 control group with no such features. The *GBA* gene was sequenced and, in a subset of patients and in normal volunteers, GBA enzyme activity was measured.

Results: We have identified nine index MJD/SCA3 patients with parkinsonian manifestations. Overall, *GBA* sequence variations were found in 3/9 MJD/SCA3 index cases with parkinsonian manifestations (33%) and in 0/40 MJD/SCA3 controls without parkinsonism ($p = 0.03$, Fisher exact test). The *GBA* sequence variations found were p.K(-27)R, p.E326K, and p.T369M. The latter two sequence variations were also found in two symptomatic relatives with no parkinsonian manifestations. A MJD/SCA3 relative belonging to the first positive pedigree and carrier of the p.K(-27)R mutation also presented parkinsonian manifestations. GBA activity in MJD/SCA3 patients was similar to those found in the normal control group.

Conclusion: Sequence variations at the *GBA* gene may play a role as a minor, modifying gene of MJD/SCA3 phenotype. This hypothetical role was not related to changes in GBA activity in peripheral leukocytes.

1. Introduction

One of the most prevalent autosomal dominant cerebellar ataxias, Machado-Joseph disease (also known as spinocerebellar ataxia type 3) (MJD/SCA3) is related to an expansion of a trinucleotide (CAG) tract in the *ATXN3* gene, above the threshold of about 54–61 repeats [1,2]. There are four typical phenotypes of MJD/SCA3. According to each main subtype, the ataxic manifestations are combined with other neurological findings: pyramidal and extrapyramidal signs with an early age at onset in type 1; moderate pyramidal signs in type 2; anterior horn cell symptoms and peripheral neuropathy with a later age at onset in type 3 [3]; and parkinsonian features in the rare type 4 [4–7].

Mutations in the glucocerebrosidase (*GBA*) gene have been recently identified as associated with Parkinson disease (PD). PD has been detected in a few patients with Gaucher disease, the inherited homozygous deficiency of GBA [8,9]; whereas, the much more common heterozygote state for *GBA* mutations has been

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doi:10.1016/j.parkreldis.2011.09.024

Table 1
Description of individual characteristics of the twelve type 4 MJD/SCA3 patients, and comparison with the MJD/SCA3 control group.

	Patient (family)	GBA sequence variations in heterozygous state	ATXN3 (CAG)n	Gender	Age at onset	Disease duration	Parkinsonian were the initial symptoms?	Parkinsonian manifestations					Other neurological findings						
								Tremor at rest	Rigidity	Brady-kinesia	Postural instability	on Ldopa	Ataxia	Ophthalmo paresis	Nystagmus	Dysphagia	Pyramidal findings	Fasciculations and/or amyotrophy	Sensitive losses
Type 4 MJD/ SCA3 patients	**1 * (O)**	**p.T369M exon 8**	23/70	m	35	5	+	+	+	+	+	+	+					+		
	2 ** (C)	–	27/71	m	38	5	+	+	+	+	+	+								
	3 *** (C)		21/70	m	39	10	+	+	+	+	+	+	+					+		+
	4 (F)	–	23/72	m	38	3	+	+	+	+	+	+	+							
	5 (G)	**p.E326K exon 8**	14/78	m	27	18	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6 (A)	–	14/75	f	30	10	+	+	+	+	+	+	+	+	+			+		
	7 (B)	–	29/75	m	33	5		+	+	+	+	+	+		+			+		
	8 (E)	–	23/80	f	28	13		+	+	+	+	+	+	+	+	+	+	+	+	+
	9 (E)		25/68	m	50	22		+	+	+	+		+	+	+				+	+
	10 (H)	**p.K(-27)R exon 2**	27/75	m	45	3			+	+	+		+	+	+					
	11 (H)		23/78	f	36	2			+	+	+	+	+	+		+	+			
	12 (I)	–	23/74	m	54	10		+	+		+		+	+	+				+	+
	Total	33%					50%	83%	100%	91%	100%	75%	91%	58%	58%	25%	58%	33%	41%	
MJD/SCA3 patients without parkinsonian features	Relatives with GBA heterozygous genotypes: 2 patients	**p.T369M exon 8**	30/69	f	52	14							+	+	+	+	+			+
		p.E326K exon 8	14/75	m	25	29							+	+			+			+
	GBA normal homozygotes: 40 unrelated index cases and 4 belonging to families G and H	–	#		#	#		0%	4%	4%		0%	100%	42%	89%	63%	82%	25%	56%	

Families O and C: Porto Alegre; A and B: Azores; E, F, G, H and I: Campinas. Legend: these patients have been previously reported by Socal et al. 2009b as: * patient family O IV1, ** patient family C III4, *** patient family C III3; # see Table 1.

repeatedly associated with PD in case control studies from different countries [10,11].

With this scenario, we raised the hypothesis that possible unidentified mutations and sequence variations in the *GBA* gene were also present in these MJD/SCA3 patients, acting as modifier factor that could predispose patients with MJD to parkinsonism.

2. Methods

Two groups of MJD/SCA3 patients were formed: one with PD or predominantly parkinsonian phenotype ("type 4 MJD"), and the other with any of the usual ataxic phenotypes ("non-type 4 MJD").

We included patients with MJD/SCA3 and at least three of the four criteria for PD (tremor, hypokinesia, rigidity and postural instability) [12] as "type 4 MJD". This group included two unrelated MJD patients from Porto Alegre, Brazil, and one brother, all of whom have been previously described [13,14], plus seven additional unrelated patients with distinct geographical origins: two patients from Ponta Delgada, Azorean Islands, Portugal, and five patients from Campinas, Brazil, giving a total of nine unrelated families and twelve patients with "type 4 MJD". "Non-type 4 MJD" patients were available from some of these nine families as well as from other MJD/SCA3 families from the large MJD cohort followed at Porto Alegre, Brazil [15,16]. Their clinical characteristics and molecular data are depicted in Table 1. The study was approved by the local Ethics Committee, and all patients had given previous consent for additional studies in their clinical data and DNA samples, which have been stored in the three institutes of origin (Porto Alegre, Campinas, and Ponta Delgada).

Peripheral blood was collected, and genomic DNA was isolated from leukocytes [17]. Fluorescence-based assay (Quant-iT – Invitrogen) was used for DNA quantitation. Evaluation of the (CAG)n tract in the *ATXN3* gene was performed as previously described [18]. *GBA* mutations were defined by DNA sequencing of the whole coding region, using as template the long-range PCR product as reported elsewhere [19]. In a subset of patients, the GBA enzyme activity was estimated in leukocytes as previously described [20].

Median values between groups were analyzed using Mann–Whitney's *U*-test since one variable, the CAG repeats in the normal alleles, did not show normal distribution on One-Sample Kolmogorov–Smirnov test. Qualitative variables were tested using chi-square and Fisher's exact tests. All tests were two-tailed; p values less than 0.05 were considered statistically significant. Statistical analyses were performed using PASW Statistics 18 for Windows.

3. Results

Clinical characteristics and molecular data of patients are described in Tables 1 and 2. All type 4 patients presented predominantly with parkinsonism; other neurological findings were also present in all but one patient (case 2C, Table 1). Since there were three pairs of siblings, the following molecular findings will be described in relation to the nine index cases – cases 1O, 2C, 4F, 5G, 6A, 7B, 8E, 10H, and 12I.

Three heterozygotes for *GBA* sequence variations were found among the nine index cases. Two sequence variations are considered polymorphisms, p.T369M (case 1O) and p.E326K (case 5G), whereas the third one is an atypical mutation, p.K(-27)R (case 10H) [21–23]. This mutation, previously described in a Brazilian Gaucher patient [23], is characterized by A to G nucleotide change at position 38 of cDNA (c.38A > G). The amino acid change is located at the leader sequence of GBA, which is removed from the mature protein. As a result, it is predicted that the residue produced by this mutant sequence will not reach its final cellular destination, the lysosome. The three parkinsonian MJD/SCA3 patients, heterozygous for *GBA* sequence variations, did not present any clinical peculiarity when compared to the other six parkinsonian index cases (Table 1).

In the same three pedigrees (O, G and H), six "non-type 4 MJD" individuals were identified and included in the molecular evaluation. In pedigrees carrying p.T369M and p.E326K (families O and G), one of these "non-type 4 MJD" individuals was also a carrier of a *GBA* sequence variation. In pedigree carrying the mutation p.K(-27)R (family H), a double heterozygote – for MJD/SCA3 and for GBA – presented only ataxic manifestations in the first year of disease duration. In the follow-up visit of the second year of her disease, parkinsonian features such as bradikynesia, expressionless facies, postural instability and rigidity were also present. She was numbered as 11(H) in Table 1 (Fig. 1).

Another forty index "non-type 4 MJD" cases, unrelated to each other and to these previous families and with clinical and (CAG)n information, were also evaluated (Tables 1 and 2). Age at onset, disease duration, and CAG at expanded and at normal *ATXN3* alleles were similar to those found in "type 4 MJD" patients (Table 2). Several neurological manifestations were defined either as present or absent, and none of them differed between groups (Table 1). Heterozygotes for *GBA* were more frequent in the "type 4 MJD" than in the "non-type 4 MJD" index cases (3/9 versus 0/40, $p = 0.03$, Fisher's exact test; Table 2).

GBA activities in leukocytes were measured in 27 MJD/SCA3 patients (including 2 "type 4 MJD" patients) and were compared to activities of 35 healthy adult volunteers. Median ± sem values of GBA activities were not different between groups – 12 ± 1.1 in MJD/SCA3 group and 13.5 ± 0.9 in control group, (ns, Mann–Whitney's *U*-test). Number of individuals with GBA activities below 10 nmol/h/mg/protein was also similar between groups in 6/25 (ns, chi-square) (Fig. 2).

4. Discussion

A heterozygous state in the *GBA* gene was found in three out of nine MJD/SCA3 pedigrees presenting individuals with parkinsonian manifestations. In contrast, in the other 40 index cases with no such symptoms, no *GBA* heterozygotes were detected. In pedigrees with positive findings in GBA locus, parkinsonian symptoms were present in 4 out of 6 (or 66%) *GBA* heterozygotes (Fig. 1). These preliminary results may suggest that the *GBA* gene plays a role as a minor, non-obligate, modifying gene of the MJD/SCA3 phenotype.

Table 2
General characteristics of a case series of twelve patients with type 4 MJD/SCA3 versus a control group of forty MJD/SCA3 patients without type 4 phenotype.

	Type 4 MJD/SCA3	No Type 4 MJD/SCA3	*P*	
Number of families (patients)	9 (12)	40 unrelated index cases	–	–
Geographical origin of the families	2 from Porto Alegre, Brazil 5 from Campinas, Brazil 2 from São Miguel, Azores	Porto Alegre, Brazil	–	–
Males (all)	9 (12)	24 (40)	0.09	Chi-square
Age at onset median (95% CI)	38 (32–44)	36 (33–39)	0.38	Mann–Whitney's *U*-test
Disease duration median (95% CI)	9 (5–14)	13 (11–14)	0.07	
Small CAG median (95% CI)	23 (19–26)	23 (21–24)	0.64	
Range	14–29	14–32		
Large CAG median (95% CI)	73 (71–76)	75 (74–76)	0.17	
Range	68–80	69–81		
Heterozygotes at the GBA gene (only one index-case per family)	**3/9**	**0/40**	**0.03**	Fisher exact test
All cases evaluated.	**4/10**	**2/46**	**0.001**	χ^2

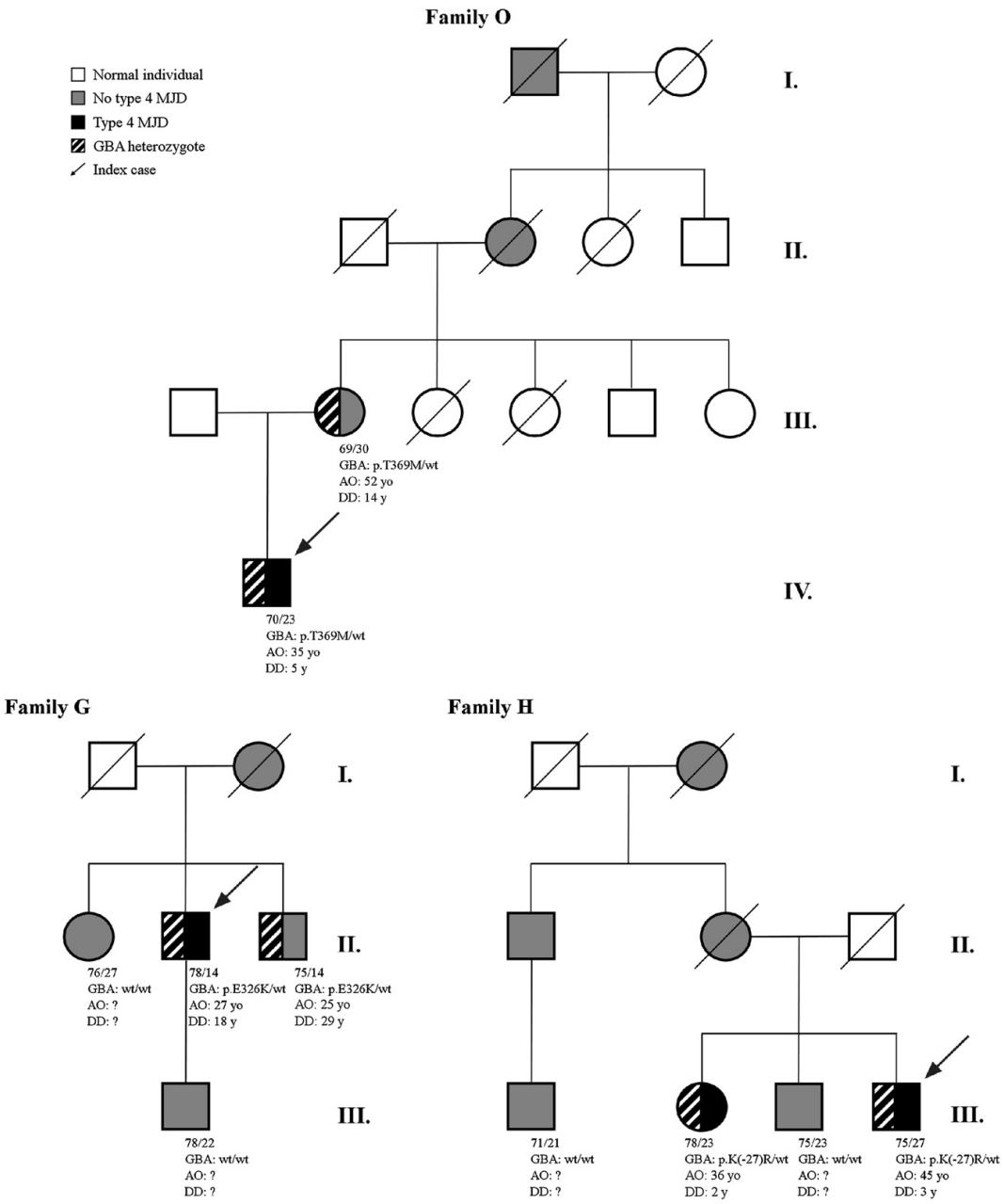

Fig. 1. MJD/SCA3 pedigrees with *GBA* sequence alterations.

Both *GBA* and *ATXN3* mutations were independently associated with parkinsonian manifestations [4–7,10,11], and ataxin-3 was recently related to parkin, a protein directly associated with the pathogenesis of PD [24]. Results presented here may suggest that an additive effect of both mutant *GBA* and *ATXN3*, or epistasis, could be acting.

Ataxin-3 is a transcriptional co-repressor as well as a deubiquitinating (DUB) enzyme that functions in cellular pathways that regulate protein homeostasis [25]. Recent evidence showed that ataxin-3 is the first DUB partner for parkin [24].

GBA is a lysosomal enzyme that catalyzes the breakdown of the glucosylceramide. As a result of GBA deficiency, there is

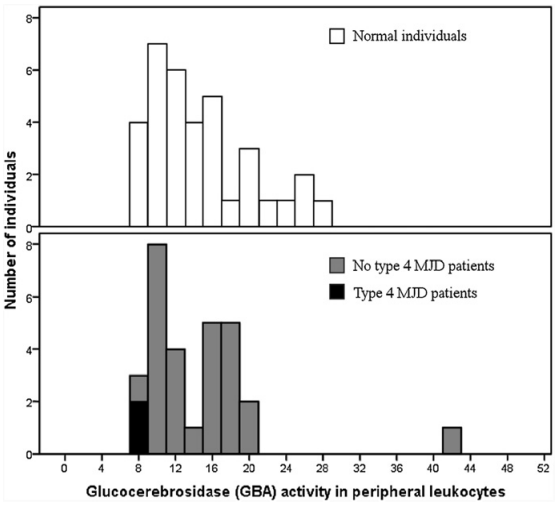

Fig. 2. Glucocerebrosidase activity in peripheral leukocytes of normal individuals and MJD/SCA3 patients.

intracellular storage of glucosylceramide within cells of mononuclear phagocyte origin, giving rise to hepatosplenomegaly, pancytopenia, and bone marrow infiltration. No neuron storage has been detected even in the neuronopathic forms of Gaucher disease [26]. Since GBA is a lysosomal enzyme, heterozygote mutations might interfere with lysosomal function or with receptor binding of other proteins at the lysosomal membrane. Lysosomes are fundamental to autophagy, and autophagy has been implicated in clearing mutant ataxin-3 [27], alpha-synuclein [28], and now parkin [25]. On the other hand, a hypothetical effect of the CAG tract expansion in *ATXN3* over the GBA enzymatic function seems to be less probable, given our results of GBA activities in MJD/SCA3 and control groups.

Previous publications related to "type 4 MJD/SCA3" to shorter expanded CAG repeats [5–7]. Our results were different from those former reports (Table 2).

We are aware that in two pedigrees the sequence variations found have been more commonly described as polymorphisms – p.T369M and p.E326K. Given the present debate about the effect of functional polymorphisms and even of synonymous variations in human diseases [29], we believe that it would be wise to keep in mind possible associations between these variants and phenotypes until functional neutrality is determined. Moreover both p.E326K and p.T369M have been shown to be significantly associated with PD in a recent large-scale screening for GBA variants in European patients with PD, supporting some possible functional effect [30].

Finally, taking into account fewness cases of type 4 MJD/SCA3 patients worldwide, data present are relevant to be reported in order to call other groups attention to our conclusion and to drive replication studies that are undoubtedly needed. We therefore believe in the plausibility of our working hypothesis, which supports further research into the overlap between parkinsonism, heterozygotes for *GBA* mutations and MJD/SCA3.

Acknowledgements

We are grateful to patients who agreed to participate in this study. This study was supported by CNPq, CAPES, FAPERGS, FAPESP, INAGEMP, and FIPE-HCPA. França Jr M is supported by a Post-doctoral fellowship from FAPESP. Bettencourt C is supported by a Post-doctoral fellowship from FCT [SFRH/BPD/63121/2009]. Siebert M, Rieder CRM, Lopes-Cendes I, Saraiva-Pereira ML, and Jardim LB were supported by CNPq.

Author roles: (1. Research project: A. Conception, B. Organization, C. Execution; 2. Statistical Analysis: A. Design, B. Execution, C. Review and Critique; 3. Manuscript: A. Writing of the first draft, B. Review and Critique). Siebert: 1B, 1C (molecular studies), 2C, 3B. Donis: 1C (clinical evaluations and data registry), 3B. Socal: 1C (clinical evaluations), 3B. Rieder: 1C (clinical evaluations), 3B. Emmel: 1C (molecular studies), 3B. Vairo: 1C (biochemical studies and data registry), 3B. Michelin-Tirelli: 1C (biochemical studies), 3B. França: 1C (clinical studies and data registry), 3B. D'Abreu: 1C (clinical studies and data registry), 3B. Bettencourt: 1C (data registry), 3B. Lima: 1C (clinical studies and data registry), 3B. Lopes Cendes: 1C (data registry), 2A, 2C, 3B. Saraiva-Pereira: 1B, 1C (molecular studies), 3B. Jardim: 1A, 1B, 2A, 2B, 2C, 3A, 3B.

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3.2. Capítulo II

ARTIGO 2

Evaluation of single nucleotide polymorphisms in *SCARB2*, *PSAP*, and *CLN8* genes in a group of Brazilian patients with Gaucher disease

Artigo científico a ser submetido para publicação no periódico *Molecular Genetics and Metabolism*.

Evaluation of single nucleotide polymorphisms in *SCARB2, PSAP*, *and CLN8* genes in a group of Brazilian patients with Gaucher disease

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Abstract

Genotype-phenotype correlations are always hard to be established even when considering Gaucher disease (GD), a monogenic and rare lysosomal storage disorder. GD is caused by deficiency of glucocerebrosidase (GCase) enzyme due to pathogenic mutations in the GBA1 gene. We investigated single nucleotide polymorphisms (SNPs) in three candidates to modifier genes (SCARB2, PSAP, and CLN8) in a group of Brazilian GD patients. Seven different SNPs were chosen using the HapMap project (rs6532244 and rs6825004 - SCARB2; rs2070968, rs7869, and rs2854992 - PSAP; rs7008465 and rs11136424 - CLN8) and analyzed by real-time PCR with fluorescence-based probes. One hundred and thirty-four unrelated GD patients and 100 healthy individuals were included in this study. A significant difference was found in rs6532244 (SCARB2 gene) between patients and controls ($p = 0.019$), being the A/A genotype more frequently found in patients than in controls (OR = 2.46; 95% CI = 1.22-4.93). Additionally, G/G and G/T genotypes of rs7008465 in CLN8 were linked to N370S/N370S patients ($p = 0.027$). The G allele in patients homozygous for N370S mutation may be associated to a protective effect, corroborating with a previous study. The results showed in the present study confirmed that SCARB2 and CLN8 are potent candidates as modifiers of GD phenotype, although coding and regulatory regions of these genes should be considered in future studies to validate our findings.

Keywords: Gaucher disease, SCARB2, PSAP, CLN8, Brazilian patients

1. Introduction

Genotype-phenotype correlations are always hard to be established, even when considering monogenic diseases due to single-gene defect, being an evident challenge for researchers and physicians [1-4]. The phenotypic heterogeneity can be linked to the nature of disease-causing mutation; however, this rule is not relevant in many cases [5]. Both severity and clinical symptoms discrepancies are commonly seen in patients who share the same genotypes, highlighting that other factors such as genetic background, epigenetics, modifier genes, and environment may contribute to the alteration of disease progression [3-12].

Gaucher disease (GD), the most common lysosomal storage disorder, is caused by deficiency of the glucocerebrosidase enzyme (GCase, E.C. 3.2.1.45) due to mutations in the *GBA1* gene [13]. This rare genetic disease affects approximately 1 in 40,000-60,000 live births in the general population, although it has an increased frequency in Ashkenazi Jews (1:850 live births) [13, 14]. GCase is responsible for degradation of glucocerebroside (GC) into glucose and ceramide inside lysosomes [13]. Undegraded or partially degraded GC is found in macrophages of GD patients, and accumulates throughout the body, leading to clinical manifestations mainly in the liver, spleen, bone marrow, and lungs. Several symptoms are commonly found in GD patients, such as hepatosplenomegaly, thrombocytopenia, anemia, and bone damage [15]. GD is generally classified into three clinical variants according to the absence (type 1; the most common form) or presence and severity of central nervous system damage (types 2 and 3) [13]. Currently, GD is being considered as a continuum spectra of phenotypes based on the limitations in divide many patients into the classic three types [4].

Up to date, more than 400 different mutations have been identified in the *GBA1* gene associated to GD, with variable individual frequencies depending on the studied population. Two mutations, N370S (c.1226A>G) and L444P (c.1448T>C), are by far the most common variations encountered in GD patients worldwide [16]. Few genes have been described as potential modifiers of GD phenotypes, including *SCARB2*, *PSAP*, and *CLN8*. *SCARB2* encodes the ubiquitously expressed lysosomal integral membrane protein type 2 (LIMP-2), which is essential for GCase sorting to lysosomes [17]. Altered LIMP-2 leads to

mislocalization and secretion of GCase, resulting in decreased amounts of the enzyme reaching the lysosomes to degrade its substrate [12]. *PSAP* gene encodes 4 different saposins, including saposin C (SapC) that is an activator for GCase [18]. *CLN8* gene was identified as a candidate to GD modifier by a genome-wide association study (GWAS). Although the physiological relevance of CLN8 protein remains to be determined, a previous work has demonstrated that this protein may act as a protective agent against lipid storage in N370S/N370S GD patients [19].

Based on these considerations, the aim of this work was to evaluate the association of *SCARB2*, *PSAP*, and *CLN8* genes in a group of Brazilian GD patients through single nucleotide polymorphisms (SNPs). We evaluated whether specific polymorphisms can be associated to common *GBA1* genotypes (N370S/N370S, L444P/L444P or N370S/L444P) as well as analyzed frequency differences between groups of patients and controls. The identification of GD modifiers is crucial to improve our knowledge about distinct elements that may be related to disease variability.

2. Materials and methods

2.1. Subjects

One hundred and thirty-four Brazilian GD patients, 68 males (50.7%) and 66 females (49.3%), were included in this study. These patients were from different regions of Brazil that were referred to our service in order to establish the diagnosis. The age at diagnosis varied from 8 days to 80 years old. The inclusion criteria were low GCase activity detected in leukocytes and/or fibroblasts [20], and both disease-causing alleles were characterized using different approaches as previously described [21]. Three prevalent *GBA1* genotypes (N370S/N370S, L444P/L444P, and N370S/L444P) in GD population were chosen for further analyses. Eighty-five out of 134 patients were included in these analyses, being N370S/N370S genotype found in 15 patients, L444P/L444P found in 11, and N370S/L444P identified in 59 of them. The remaining 49 GD patients carry rare mutations; among them few individuals share the same genotype while the others carry a unique one. Additionally, one hundred unrelated healthy Brazilian individuals (50% males

and 50% females) were also included in this study as the control group. This study was approved by the Institutional Research and Ethics Committee.

Genomic DNA was isolated from leukocytes using a modified salting out method based on Miller and co-workers [22].

2.2. SNP selection and genotyping

A total of seven SNPs were selected using data from the International HapMap project (www.hapmap.ncbi.nlm.nih.gov), and comprised *SCARB2* variants rs6532244C>A and rs6825004C>G, *PSAP* variants rs2070968A>T, rs7869C>T, and rs2854992A>G, and *CLN8* variants rs7008465G>T and rs11136424G>A. Only SNPs with a minor allele frequency (MAF) ≥ 0.05 were considered, and these SNPs were included due to the fact that they are tag SNPs for the specific gene or a representative ones.

Genotyping was performed using validated TaqMan SNP Genotyping Assays (Applied Biosystems) specific for each polymorphism, containing two primers for amplification of each target region and two probes for fluorescence-based allele detection.

PCR reactions were carried out in a total volume of 12 μL containing 5 ng of genomic DNA, 0.3 μL of specific 40X TaqMan assays, and 6 μL of 2X TaqMan Genotyping Master Mix (Applied Biosystems), and reaction was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Positive and negative controls were included in all experiments. Amplification conditions were used according to manufacturer's instructions. Allelic discrimination results were analyzed by the SDS software v.1.2.1 (Applied Biosystems).

2.3. Statistical analysis

Allelic and genotypic frequencies of each SNP in patients and controls were compared using chi-square or Fisher's exact tests when a certain genotype had a frequency lower than 5%. All SNPs were tested for Hardy-Weinberg equilibrium (HWE). Associations between SNPs and GD were estimated using odds ratios (ORs), and 95% confidence intervals (CIs). The specific contribution of each SNP to the phenotypic outcome was assessed by co-dominant (every genotype gives a different and non-additive

risk), dominant (major homozygous allele versus heterozygous + minor homozygous allele), recessive (minor homozygous allele versus heterozygous + major homozygous allele), and over-dominant (heterozygous versus pool of both homozygous alleles) multiple inheritance models. Akaike's information criteria (AIC) were used to determine the best-fit model for each SNP. Statistical analyses were performed using SPSS software v.17, and SNPStats (available at http://bioinfo.iconcologia.net/SNPstats) [23]. Results were considered statistically significant when $p < 0.05$.

3. Results

SNPs were genotyped in all individuals but 3% in variant rs7869. Allelic and genotypic frequencies are summarized in Table 1. All seven polymorphisms investigated were in HWE in both GD patients and controls ($p > 0.01$; Supplemental Table 1). Allelic frequencies showed no statistical difference between groups (Table 1). Data generated by this work were compared to data from European population available on the 1000genomes database (http://www.1000genomes.org/), and no difference was observed between groups ($p > 0.01$; Supplemental Table 2), except rs6532244 that showed significant difference when compared to the patients' group [24].

Genotypic frequency of variant rs6532244 (*SCARB2* gene) has shown to be statistically different between patients and controls ($p = 0.019$), when a co-dominant model of inheritance was assumed. Furthermore, assuming a recessive model of inheritance, which seems to be more adequate for this case, the difference between groups becomes even stronger ($p = 0.008$; Supplemental Table 3). Specifically, the A/A genotype showed an OR = 2.46 (95% CI = 1.22-4.93) in GD patients. Genotypic frequencies between patients and controls for other SNPs did not show any statistical significance.

We also investigated whether the association of SNPs included in this study with the three prevalent *GBA1* genotypes (N370S/N370S, L444P/L444P, and N370S/L444P). First, a group consisting of 15 patients carrying N370S/N370S genotype and 59 patients with N370S/L444P (n = 74) was compared to the control group. No statistical difference was found between groups (Supplemental Table 4). However, when comparing

N370S/N370S patients (n = 15) to controls we have demonstrated that T/T genotype in the rs2070968 was found only among patients (p = 0.041; Supplemental Table 5).

Second, another group including L444P/L444P (n = 11) and N370S/L444P (n = 59) patients (in total, n = 70) was organized to evaluate if one or two L444P alleles would be related to results observed in the SNP analysis. The A/A genotype of rs6532244 (*SCARB2* gene) was linked to the group of patients (p = 0.021; Supplemental Table 6). This association was still present when we compared only L444P/L444P patients (n = 11) to controls (p = 0.016; Supplemental Table 7).

Thereafter, N370S/L444P patients (n = 59) were compared to controls in order to analyze if both mutations were having an effect on the SNP's genotypes (Supplemental Table 8). We have found that patients sharing N370S/L444P genotype did not alter SNP results compared to the control group.

At last, we also compared patients homozygous for N370S or for L444P and patients who do not carry any of these *GBA1* mutations. While no statistical difference was observed related to the L444P/L444P patients (data not shown), three out of seven SNPs showed difference between groups involving N370S mutation, which are: rs6825004, rs2854992, and rs7008465 (Supplemental Table 9). The G/G genotype of variant rs6825004 (*SCARB2* gene) was found only in GD patients with no N370S in either alleles (p = 0.042). In the *PSAP* gene, A/G and A/A genotypes in rs2854992 were more common among N370S/N370S patients (p = 0.027). The polymorphism rs7008465 (*CLN8* gene) showed an association between the G/G and G/T genotypes and N370S/N370S patients (p = 0.027). The remaining SNPs did not demonstrate any difference between groups (data not shown).

Results in Supplemental Tables 4 to 9 are displayed considering the best model of inheritance for the specific SNP based on the lower AIC value compared to other models.

4. Discussion

Genotype-phenotype correlations elucidated in GD patients to date are still in need of further investigation due to clinical heterogeneity observed in these patients. Modifier

genes would be a reasonable explanation for inter-patient phenotypic variability. In the present study, we evaluated seven polymorphisms in three different modifier candidates of GD phenotypes (*SCARB2*, *PSAP*, and *CLN8*) in Brazilian GD patients and controls.

We demonstrated here a significant association between an intronic *SCARB2* SNP (rs6532244) and GD, with the A/A genotype for this variant being more frequent in patients than controls. This association was stronger in L444P/L444P genotypes after the stratification of patients by genotype. Patients carrying L444P mutation in both alleles have almost no GCase activity, and present a very severe phenotype involving neurological symptoms [25]. The A/A genotype in L444P/L444P patients may result in a less efficient LIMP-2 protein, disturbing even more whatever remaining GCase still exists inside the cells and would be able to reach the lysosome. As stated previously, a defect in the *SCARB2* gene would alter the GCase transport from the endoplasmic reticulum (ER) to lysosomes by impairment of LIMP-2 [17]. Presence of a non-synonymous mutation in *SCARB2* in one allele in a patient with GD has been shown to be responsible for reduced levels of LIMP-2, leading to secretion of GCase to the extracellular compartment and a more severe GD phenotype when compared to the other sibling [12].

The *PSAP* gene is a natural modifier candidate considering its function as precursor of an important protein for GCase activation [18], even though it remains unclear the mechanism by which SapC interacts with GCase and GC. Despite of three variants in the *PSAP* gene were chosen for investigation, no significant difference were shown between patients and controls. This finding might be related to the fact that these variants included here might not be directly associated to GD function and limits our remarks considering that protein domains interactions remain unknown.

We have also evaluated two variants in the *CLN8* gene, following its identification as a modifier by GWAS study. No statistical difference was demonstrated between patients and controls in the work presented here. However, we were able to identify a significant difference between N370S/N370S GD patients and those with a different *GBA1* genotype, considering the rs7008465 variant. In this case, G allele was more commonly found among patients sharing N370S mutation in both alleles, and is possibly associated to the protective effect against neurological damage as suggested by Zhang and co-workers. Although the physiological function of this protein is not fully established, it is thought to be involved in

lipid synthesis, transport of vesicles and membranes, autophagy, mitophagy, and apoptosis [26].

In summary, the associations found in the present study raise novel insight into the role of these candidate genes as modifiers of GD phenotypes. We are aware of the limitations related to the size of our sample population, and results presented here need to be validated in a large number of subjects to exclude the possibility of "spurious" association. However, the assembly of a large group of patients with a rare disorder is a hard task, and the relationship between a specific SNP and a given phenotype is even harder to be identified. It remains possible that other SNPs located nearby the ones investigated here could be the factual susceptibility variants. Furthermore, we postulate that coding and regulatory regions of *SCARB2*, and *CLN8* genes may play a role as GD modifiers and should be considered in future studies. Moreover, measurements of mRNA expression and protein levels of each gene may also be important for better understanding the role of these modifier candidates in the phenotype of GD.

Conflict of interest

There are no conflicts of interest to report.

Acknowledgments

This research was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Fundo de Incentivo à Pesquisa e Evento do Hospital de Clínicas de Porto Alegre (FIPE-HCPA). MS, GVF, and MLSP were supported by CNPq, Brazil.

Appendix A. Supplementary data

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Table 1. Allelic and genotypic frequencies of seven SNPs in candidate genes as phenotype modifiers in GD patients and controls, considering a co-dominant model of inheritance.

Gene	SNP ID	Allele/ Genotype	Group frequency (n)		OR (95% CI)	*p* value
			Patients (134)	Controls (100)		
SCARB2	rs6532244	C	0.53 (142)	0.59 (119)		
		A	0.47 (126)	0.41 (81)		0.160
		C/C	0.33 (44)	0.32 (32)	1.00	
		A/C	0.40 (54)	0.55 (55)	0.71 (0.40-1.29)	
		A/A	0.27 (36)	0.13 (13)	2.01 (0.92-4.40)	**0.019**
	rs6825004	C	0.66 (177)	0.68 (135)		
		G	0.34 (91)	0.32 (65)		0.741
		C/C	0.47 (63)	0.45 (45)	1.00	
		C/G	0.38 (51)	0.45 (45)	0.81 (0.47-1.41)	
		G/G	0.15 (20)	0.10 (10)	1.43 (0.61-3.34)	0.404
PSAP	rs2070968	A	0.85 (227)	0.87 (174)		
		T	0.15 (41)	0.13 (26)		0.482
		A/A	0.72 (97)	0.74 (74)	1.00	
		A/T	0.25 (33)	0.26 (26)	0.97 (0.53-1.76)	
		T/T	0.03 (04)	0 (0)	NA (0.00-NA)	0.263
	rs7869[a]	C	0.72 (189)	0.71 (141)		
		T	0.28 (73)	0.29 (59)		0.699
		C/C	0.50 (66)	0.48 (48)	1.00	
		C/T	0.44 (57)	0.45 (45)	0.92 (0.54-1.58)	
		T/T	0.06 (08)	0.07 (07)	0.83 (0.28-2.45)	0.923
	rs2854992	G	0.70 (187)	0.69 (139)		
		A	0.30 (81)	0.31 (61)		0.949
		G/G	0.50 (67)	0.51 (51)	1.00	
		A/G	0.39 (53)	0.37 (37)	1.09 (0.63-1.90)	
		A/A	0.11 (14)	0.12 (12)	0.89 (0.38-2.08)	0.878
CLN8	rs7008465	T	0.76 (203)	0.77 (154)		
		G	0.24 (65)	0.23 (46)		0.752
		T/T	0.57 (77)	0.59 (59)	1.00	
		G/T	0.37 (49)	0.36 (36)	1.04 (0.60-1.80)	
		G/G	0.06 (08)	0.05 (05)	1.23 (0.38-3.94)	0.937

rs11136424	A	0.65 (175)	0.64 (128)		
	G	0.35 (93)	0.36 (72)		0.771
	A/A	0.43 (58)	0.40 (40)	1.00	
	A/G	0.44 (59)	0.48 (48)	0.85 (0.49-1.48)	
	G/G	0.13 (17)	0.12 (12)	0.98 (0.42-2.27)	0.831

OR: *odds ratio*; NA: not applicable; [a]131 patients were considered for frequency calculations.

Supplemental Table 1. Results of Hardy-Weinberg equilibrium (HWE) based on each individual group (patients and controls) and based on samples assembled in a unique group (total subjects).

SNP ID	HWE *p* value		
	Patient	Control	Total subjects
rs6532244	0.03	0.21	0.43
rs6825004	0.08	1.00	0.24
rs2070968	0.51	0.36	1.00
rs7869	0.51	0.48	0.26
rs2854992	0.54	0.24	0.17
rs7008465	1.00	1.00	1.00
rs11136424	0.71	0.83	1.00

Supplemental Table 2. Allelic frequencies of SNPs in *SCARB2*, *PSAP*, and *CLN8* between patients/controls and 1000genomes database.

Gene	SNP ID	Allele	Group frequency (n)			*p* value	
			Patients (134)	Controls (100)	1000genomes[a]	Patients	Controls
SCARB2	rs6532244	C	0.53 (142)	0.59 (119)	0.67 (508)	<0.0001	0.046
		A	0.47 (126)	0.41 (81)	0.33 (250)		
	rs6825004	C	0.66 (177)	0.68 (135)	0.71 (540)	0.111	0.303
		G	0.34 (91)	0.32 (65)	0.29 (218)		
PSAP	rs2070968	A	0.85 (227)	0.87 (174)	0.80 (609)	0.114	0.030
		T	0.15 (41)	0.13 (26)	0.20 (149)		
	rs7869[b]	C	0.72 (189)	0.71 (141)	0.72 (548)	1.000	0.617
		T	0.28 (73)	0.29 (59)	0.28 (210)		
	rs2854992	G	0.70 (187)	0.69 (139)	0.76 (577)	0.041	0.055
		A	0.30 (81)	0.31 (61)	0.24 (181)		
CLN8	rs7008465	T	0.76 (203)	0.77 (154)	0.79 (596)	0.330	0.617
		G	0.24 (65)	0.23 (46)	0.21 (162)		
	rs11136424	A	0.65 (175)	0.64 (128)	0.70 (534)	0.117	0.079
		G	0.35 (93)	0.36 (72)	0.30 (224)		

[a]Data obtained from 1000genomes database (http://www.1000genomes.org/) based on European population; [b]131 patients were considered for frequency calculations.

Supplemental Table 3. Genotypic frequencies and association analyses between GD patients and controls and SNPs in *SCARB2*, *PSAP*, and *CLN8*, considering different models of inheritance.

Gene	SNP ID	Genotype	Group frequency (n)		OR (95% CI)	Model of inheritance	*p* value	AIC
			Patients (134)	Controls (100)				
SCARB2	rs6532244	C/C	0.33 (44)	0.32 (32)	1.00	Co-dominant	**0.019**	317.2
		A/C	0.40 (54)	0.55 (55)	0.71 (0.40-1.29)			
		A/A	0.27 (36)	0.13 (13)	2.01 (0.92-4.40)			
		C/C	0.33 (44)	0.32 (32)	1.00	Dominant	0.887	323.4
		A/C+A/A	0.67 (90)	0.68 (68)	0.96 (0.55-1.67)			
		C/C+A/C	0.73 (98)	0.87 (87)	1.00	Recessive	**0.008**	316.5
		A/A	0.27 (36)	0.13 (13)	2.46 (1.22-4.93)			
		C/C+A/A	0.60 (80)	0.45 (45)	1.00	Overdominant	**0.026**	318.5
		A/C	0.40 (54)	0.55 (55)	0.55 (0.33-0.93)			
	rs6825004	C/C	0.47 (63)	0.45 (45)	1.00	Co-dominant	0.404	323.6
		C/G	0.38 (51)	0.45 (45)	0.81 (0.47-1.41)			
		G/G	0.15 (20)	0.10 (10)	1.43 (0.61-3.34)			
		C/C	0.47 (63)	0.45 (45)	1.00	Dominant	0.764	323.3
		C/G+G/G	0.53 (71)	0.55 (55)	0.92 (0.55-1.55)			
		C/C+C/G	0.85 (114)	0.90 (90)	1.00	Recessive	0.265	322.2
		G/G	0.15 (20)	0.10 (10)	1.58 (0.70-3.54)			
		C/C+G/G	0.62 (83)	0.55 (55)	1.00	Overdominant	0.286	322.3
		C/G	0.38 (51)	0.45 (45)	0.75 (0.44-1.27)			

PSAP	rs2070968	A/A	0.72 (97)	0.74 (74)	1.00	Co-dominant	0.263	320.9
		A/T	0.25 (33)	0.26 (26)	0.97 (0.53-1.76)			
		T/T	0.03 (04)	0 (0)	NA (0.00-NA)			
		A/A	0.72 (97)	0.74 (74)	1.00	Dominant	0.777	323.4
		A/T+T/T	0.28 (37)	0.26 (26)	1.09 (0.60-1.95)			
		A/A+A/T	0.97 (130)	1.00 (100)	1.00	Recessive	0.138	318.9
		T/T	0.03 (04)	0 (0)	NA (0.00-NA)			
		A/A+T/T	0.75 (101)	0.74 (74)	1.00	Overdominant	0.806	323.3
		A/T	0.25 (33)	0.26 (26)	0.93 (0.51-1.69)			
	rs7869[b]	C/C	0.50 (66)	0.48 (48)	1.00	Co-dominant	0.923	321.9
		C/T	0.44 (57)	0.45 (45)	0.92 (0.54-1.58)			
		T/T	0.06 (08)	0.07 (07)	0.83 (0.28-2.45)			
		C/C	0.51 (66)	0.48 (48)	1.00	Dominant	0.718	319.9
		C/T+T/T	0.49 (65)	0.52 (52)	0.91 (0.54-1.53)			
		C/C+C/T	0.94 (123)	0.93 (93)	1.00	Recessive	0.791	320.0
		T/T	0.06 (08)	0.07 (07)	0.86 (0.30-2.47)			
		C/C+T/T	0.56 (74)	0.55 (55)	1.00	Overdominant	0.823	320.0
		C/T	0.44 (57)	0.45 (45)	0.94 (0.56-1.59)			
	rs2854992	G/G	0.50 (67)	0.51 (51)	1.00	Co-dominant	0.878	325.2
		A/G	0.39 (53)	0.37 (37)	1.09 (0.63-1.90)			
		A/A	0.11 (14)	0.12 (12)	0.89 (0.38-2.08)			
		G/G	0.50 (67)	0.51 (51)	1.00	Dominant	0.887	323.4
		A/G+A/A	0.50 (67)	0.49 (49)	1.04 (0.62-1.75)			

		G/G+A/G	0.90 (120)	0.88 (88)	1.00	Recessive	0.708	323.3
		A/A	0.10 (14)	0.12 (12)	0.86 (0.38-1.94)			
		G/G+A/A	0.61 (81)	0.63 (63)	1.00	Overdominant	0.689	323.3
		A/G	0.39 (53)	0.37 (37)	1.11 (0.65-1.90)			
CLN8	rs7008465	T/T	0.57 (77)	0.59 (59)	1.00	Co-dominant	0.937	325.3
		G/T	0.37 (49)	0.36 (36)	1.04 (0.60-1.80)			
		G/G	0.06 (08)	0.05 (05)	1.23 (0.38-3.94)			
		T/T	0.58 (77)	0.59 (59)	1.00	Dominant	0.806	323.4
		G/T+G/G	0.42 (57)	0.41 (41)	1.07 (0.63-1.80)			
		T/T+G/T	0.94 (126)	0.95 (95)	1.00	Recessive	0.751	323.3
		G/G	0.06 (08)	0.05 (05)	1.21 (0.38-3.80)			
		T/T+G/G	0.63 (85)	0.64 (64)	1.00	Overdominant	0.920	323.4
		G/T	0.37 (49)	0.36 (36)	1.02 (0.60-1.76)			
	rs11136424	A/A	0.43 (58)	0.40 (40)	1.00	Co-dominant	0.831	325.1
		A/G	0.44 (59)	0.48 (48)	0.85 (0.49-1.48)			
		G/G	0.13 (17)	0.12 (12)	0.98 (0.42-2.27)			
		A/A	0.43 (58)	0.40 (40)	1.00	Dominant	0.617	323.2
		A/G+G/G	0.57 (76)	0.60 (60)	0.87 (0.52-1.48)			
		A/A+A/G	0.87 (117)	0.88 (88)	1.00	Recessive	0.887	323.4
		G/G	0.13 (17)	0.12 (12)	1.07 (0.48-2.35)			
		A/A+G/G	0.56 (75)	0.52 (52)	1.00	Overdominant	0.548	323.1
		A/G	0.44 (59)	0.48 (48)	0.85 (0.51-1.43)			

OR: *odds ratio*; AIC: Akaike's information criteria; [b]131 patients were considered for frequency calculations; values are shown in bold when $p < 0.05$.

Supplemental Table 4. Association analyses between GD patients carrying at least one N370S allele and controls compared to the SNP's genotypes in *SCARB2*, *PSAP*, and *CLN8*, considering the best model of inheritance for each SNP.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			N370S group (74)	Controls (100)		
SCARB2	rs6532244	C/C+A/C	0.76 (56)	0.87 (87)	Recessive	0.055
		A/A	0.24 (18)	0.13 (13)		
	rs6825004	C/C+C/G	0.89 (66)	0.90 (90)	Recessive	0.708
		G/G	0.11 (06)	0.10 (10)		
PSAP	rs2070968	A/A+A/T	0.96 (71)	1.00 (100)	Recessive	0.075
		T/T	0.04 (03)	0 (0)		
	rs7869[a]	C/C+T/T	0.51 (37)	0.55 (55)	Overdominant	0.639
		C/T	0.47 (35)	0.45 (45)		
	rs2854992	G/G+A/A	0.54 (40)	0.63 (63)	Overdominant	0.235
		A/G	0.46 (34)	0.37 (37)		
CLN8	rs7008465	T/T	0.50 (37)	0.59 (59)	Dominant	0.238
		G/T+G/G	0.50 (37)	0.41 (41)		
	rs11136424	A/A+G/G	0.54 (40)	0.52 (52)	Overdominant	0.791
		A/G	0.46 (34)	0.48 (48)		

[a]72 patients were considered for frequency calculations.

Supplemental Table 5. Association analyses between N370S/N370S patients and controls compared to the SNP's genotypes in *SCARB2*, *PSAP*, and *CLN8* considering the best model of inheritance for each SNP.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			N370S/N370S patients (15)	Controls (100)		
SCARB2	rs6532244	C/C+A/A	0.67 (10)	0.45 (45)	Overdominant	0.118
		A/C	0.33 (05)	0.55 (55)		
	rs6825004	C/C+C/G	1.00 (15)	0.90 (90)	Recessive	0.355
		G/G	0 (0)	0.10 (10)		
PSAP	rs2070968	A/A	0.86 (13)	0.74 (74)	Co-dominant	**0.041**
		A/T	0.7 (01)	0.26 (26)		
		T/T	0.7 (01)	0 (0)		
	rs7869[a]	C/C+T/T	0.77 (11)	0.55 (55)	Overdominant	0.147
		C/T	0.21 (03)	0.45 (45)		
	rs2854992	G/G	0.33 (05)	0.51 (51)	Dominant	0.202
		A/G+A/A	0.67 (10)	0.49 (49)		
CLN8	rs7008465	T/T	0.33 (05)	0.59 (59)	Dominant	0.062
		G/T+G/G	0.67 (10)	0.41 (41)		
	rs11136424	A/A+A/G	0.73 (11)	0.88 (88)	Recessive	0.220
		G/G	0.27 (04)	0.12 (12)		

Values are shown in bold when $p < 0.05$; [a]14 patients were considered for frequency calculations.

Supplemental Table 6. Association analyses between GD patients carrying at least one L444P allele and controls compared to SNP's genotypes in *SCARB2*, *PSAP*, and *CLN8*, considering the best model of inheritance for each SNP.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			L444P group (70)	Controls (100)		
SCARB2	rs6532244	C/C+A/C	0.73 (51)	0.87 (87)	Recessive	**0.021**
		A/A	0.27 (19)	0.13 (13)		
	rs6825004	C/C+C/G	0.86 (60)	0.90 (90)	Recessive	0.393
		G/G	0.14 (10)	0.10 (10)		
PSAP	rs2070968	A/A+A/T	0.97 (68)	1.00 (100)	Recessive	0.168
		T/T	0.03 (02)	0 (0)		
	rs7869[a]	C/C+T/T	0.46 (32)	0.55 (55)	Overdominant	0.269
		C/T	0.54 (37)	0.45 (45)		
	rs2854992	G/G+A/G	0.93 (65)	0.88 (88)	Recessive	0.299
		A/A	0.07 (05)	0.12 (12)		
CLN8	rs7008465	T/T+G/G	0.60 (42)	0.64 (64)	Overdominant	0.597
		G/T	0.40 (28)	0.36 (36)		
	rs11136424	A/A	0.44 (31)	0.40 (40)	Dominant	0.578
		A/G+G/G	0.56 (39)	0.60 (60)		

Values are shown in bold when $p < 0.05$; [a]69 patients were considered for frequency calculations.

Supplemental Table 7. Association analyses between L444P/L444P patients and controls compared to SNP's genotypes in *SCARB2*, *PSAP*, and *CLN8* considering the best model of inheritance for each SNP.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			L444P/L444P patients (11)	Controls (100)		
SCARB2	rs6532244	C/C+A/C	0.55 (06)	0.87 (87)	Recessive	**0.016**
		A/A	0.45 (05)	0.13 (13)		
	rs6825004	C/C+G/G	0.82 (09)	0.55 (55)	Overdominant	0.114
		C/G	0.18 (02)	0.45 (45)		
PSAP	rs2070968	A/A	0.73 (08)	0.74 (74)	Co-dominant	0.999
		A/T	0.27 (03)	0.26 (26)		
		T/T	0 (0)	0 (0)		
	rs7869[a]	C/C+C/T	0.91 (10)	0.93 (93)	Recessive	0.999
		T/T	0.09 (01)	0.07 (07)		
	rs2854992	G/G+A/A	0.55 (06)	0.63 (63)	Overdominant	0.745
		A/G	0.45 (05)	0.37 (37)		
CLN8	rs7008465	T/T+G/G	0.73 (08)	0.64 (64)	Overdominant	0.744
		G/T	0.27 (03)	0.36 (36)		
	rs11136424	A/A+A/G	0.73 (08)	0.88 (88)	Recessive	0.168
		G/G	0.27 (03)	0.12 (12)		

Values are shoen in bold when $p < 0.05$; [a]11 patients were considered for frequency calculations.

Supplemental Table 8. Association analyses between N370S/L444P GD patients and controls compared to SNP's genotypes in *SCARB2*, *PSAP*, and *CLN8* considering the best model of inheritance for each SNP.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			N370S/L444P patients (59)	Controls (100)		
SCARB2	rs6532244	C/C+A/C	0.76 (45)	0.87 (87)	Recessive	0.082
		A/A	0.24 (14)	0.13 (13)		
	rs6825004	C/C+C/G	0.86 (51)	0.90 (90)	Recessive	0.512
		G/G	0.14 (08)	0.10 (10)		
PSAP	rs2070968	A/A+A/T	0.97 (57)	1.00 (100)	Recessive	0.136
		T/T	0.03 (02)	0 (0)		
	rs7869[a]	C/C+T/T	0.45 (26)	0.55 (55)	Overdominant	0.218
		C/T	0.55 (32)	0.45 (45)		
	rs2854992	G/G+A/G	0.93 (55)	0.88 (88)	Recessive	0.415
		A/A	0.07 (04)	0.12 (12)		
CLN8	rs7008465	T/T+G/G	0.58 (34)	0.64 (64)	Overdominant	0.424
		G/T	0.43 (25)	0.36 (36)		
	rs11136424	A/A+A/G	0.91 (54)	0.88 (88)	Recessive	0.488
		G/G	0.09 (05)	0.12 (12)		

[a]58 patients were considered for frequency calculations.

Supplemental Table 9. Genotypic frequencies of rs6825004, rs2854992, and rs7008465 in GD patients that are homozygous for N370S mutation or who do not carry the N370S allele.

Gene	SNP ID	Genotype	Group frequency (n)		Model of inheritance	*p* value
			N370S/N370S (15)	No N370S alleles (31)		
SCARB2	rs6825004	C/C+C/G	1.00 (15)	0.71 (22)	Recessive	**0.042**
		G/G	0 (0)	0.29 (09)		
PSAP	rs2854992	G/G	0.33 (05)	0.68 (21)	Dominant	**0.027**
		A/G+A/A	0.67 (10)	0.32 (10)		
CLN8	rs7008465	T/T	0.33 (05)	0.68 (21)	Dominant	**0.027**
		G/T+G/G	0.67 (10)	0.32 (10)		

Values are shown in bold when $p < 0.05$.

3.3. Capítulo III

ARTIGO 3

Identification of miRNAs that modulate glucocerebrosidase activity in Gaucher disease cells

Artigo científico a ser submetido para publicação no periódico *Nucleic Acids Research*.

Identification of miRNAs that modulate glucocerebrosidase activity in Gaucher disease cells

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Abstract

Gaucher disease is an autosomal recessive disorder caused by deficiency of the enzyme glucocerebrosidase. Although it is a monogenic disease, there is vast phenotypic heterogeneity, even among patients with the same genotype. MicroRNAs (miRNAs) are a family of small non-coding RNAs involved in many biological processes and diseases. To determine whether miRNAs can affect glucocerebrosidase activity and/or expression, we performed a screen of a miRNA mimic panel consisting of 875 different miRNAs. The screen was performed using Gaucher fibroblasts, and glucocerebrosidase activity was used as the initial outcome parameter. We found several miRNAs that either up- or down-regulated glucocerebrosidase activity. In follow-up assays, we confirmed that one specific miRNA (miR-127-5p) down-regulated both glucocerebrosidase activity and protein levels by down-regulation of LIMP-2, the receptor involved in proper trafficking of glucocerebrosidase from the endoplasmic reticulum to the lysosome. A conditioned media assay demonstrated that cells treated with this miRNA secreted glucocerebrosidase into the extracellular environment, supporting impaired LIMP-2 function. Two other miRNAs identified, miR-16-5p and miR-195-5p, found to up-regulate glucocerebrosidase activity by greater than 40% and to enhance expression and protein levels of the enzyme. In conclusion, we show that miRNAs alter glucocerebrosidase activity in patient cells, and may serve to modify disease manifestations in patients sharing the same genotype. Our data suggest that miRNAs may be acting as modifiers in Gaucher disease.

Keywords: glucocerebrosidase, Gaucher disease, microRNAs, modifiers.

INTRODUCTION

Gaucher disease (GD) is the most common lysosomal storage disorder worldwide. Overall, the disease frequency has been estimated as between 1:40,000 and 1:60,000 individuals in the general population, while it has a higher frequency in the Ashkenazi Jewish population (1:850 individuals) (1,2). GD, an autosomal recessive disorder, has been clinically subdivided according to severity of symptoms and involvement of the Central Nervous System (CNS) (1). GD type 1 (also known as non-neuronopathic, OMIM #230800) is by far the most common form of GD. Patients present clinical manifestations at variable ages of onset, ranging from asymptomatic individuals to children with significant hepatosplenomegaly, anemia, thrombocytopenia, and bone disease. Type 2 (acute neuronopathic, OMIM #230900) is the most severe form of GD, leading to a very short life expectancy (less than 2 years) due to devastating and rapidly progressive neurological impairment. The intermediate form of GD is type 3 (chronic neuronopathic, OMIM #2301000). Although type 3 patients also have CNS involvement, they develop these symptoms later in life, and the progression of the disease is less aggressive. Generally, all three GD types are characterized by hepatosplenomegaly, anemia, thrombocytopenia, and bone involvement (1,3).

Glucocerebrosidase (GCase, E.C. 3.2.1.45) is the lysosomal enzyme responsible for the degradation of glucocerebroside (GC) into glucose and ceramide. This enzyme is deficient or absent in patients with GD, resulting in accumulation of GC within lysosomes of macrophages, contributing to the clinical manifestations of this disease (1). GCase is encoded by the *GBA1* gene on chromosome 1q21 (4). Approximately 400 different disease-causing mutations have been identified throughout the 11 exons of *GBA1*. Some are more common in specific ethnicities of patients with GD; for instance, N370S (c.1226A>G) in Ashkenazi Jews (5). Although there is some correlation between genotype and phenotype in GD, often mutations in *GBA1* cannot be used to predict a patient's clinical symptoms. Studies have demonstrated that patients with the same genotype even twins and sibling pairs, can have differences in disease severity and response to treatment (6,7). While GD has been considered a simple monogenic disorder, this paradigm is being challenged due to the vast phenotype heterogeneity, as well as the variable therapeutic

responsiveness. Thus, additional factors are likely involved in GD, such as epigenetic elements and modifier genes (8). To date, a few well-defined modifier genes have been identified that modulate and regulate GCase protein levels or activity. Two important modifiers are Saposin C (SapC), an activator of GCase encoded by the *PSAP* gene (9), and Lysosomal Integral Membrane Protein type 2 (LIMP-2), encoded by *SCARB2*, a receptor responsible for the sorting of GCase to the lysosomes (10). However, these modifiers do not fully explain all of the divergent clinical findings encountered in patients sharing the same genotype. For this reason, it is necessary to identify other pathways that may affect GCase function in patients with GD in order to better understand disease pathogenesis and to identify targets for future therapeutic approaches.

One class of small non-coding RNAs, microRNAs (miRNAs), play a very important role as regulators of gene expression in plants, animals and viruses. Mature miRNA duplexes are approximately 22 nucleotides in length and bind to their messenger RNA (mRNA) targets primarily in the 3' untranslated region (UTR) through critical nucleotides 2-8 at the 5' end of the miRNA sequence (seed sequence). miRNAs mediate gene silencing post-transcriptionally by mRNA degradation and/or repression of translation (11). Hundreds of genes and corresponding pathways can be potentially regulated by a unique miRNA, and numerous miRNAs can control a specific mRNA (12). miRNAs are involved in many biological processes including the stress response (13), inflammation (14), heart diseases (11), autophagy (15), apoptosis, cancer (12), and neurological diseases such as Alzheimer and Parkinson diseases (16). Because of the diverse functional roles of miRNAs in many processes and diseases, these small RNAs are being explored as disease-related biomarkers and are being considered in a variety of therapeutic applications (either as miRNA antagonists or miRNA mimics).

The limited genotype-phenotype correlations in GD, as well as the remarkable importance of miRNAs in a wide range of biological processes, prompted us to investigate whether miRNAs could affect GCase expression and/or activity. To address this question, we conducted miRNA mimic screening to identify miRNAs that regulate GCase activity in N370S/N370S Gaucher fibroblasts. We found that GCase activity can be altered by select miRNA mimics, either by down- or up-regulation of *GBA1* mRNA levels, GCase protein levels and/or by affecting other proteins related to GCase, such as LIMP-2.

MATERIAL AND METHODS

Cell lines and cell culture

Human wild type (WT) primary fibroblasts and N370S/N370S Gaucher fibroblasts were used in this study. Cells were grown in monolayers and maintained using growth medium (Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Life Technologies)), supplemented with 10% Fetal Bovine Serum (FBS; Life Technologies) and antibiotic-antimycotic (Life Technologies) at 37ºC in a humidified 5% CO_2 incubator.

miRNA mimic screening

Fibroblast cells were screened with the Human miRNA Mimic Library (Qiagen) consisting of 875 different miRNAs (Sanger 13.0). Primary screening was performed in duplicate both for GCase activity and viability assays in 384-well plates. Sixteen wells containing Silencer Negative Control #2 ("NegCtrl"; Ambion) were included on all plates for data normalization. GBA siRNA (Qiagen) and AllStars Hs Cell Death ("Killer"; Qiagen) were included as positive assay/transfection controls for GCase and viability screens, respectively. For transfection, 0.8 pmol of each miRNA or siRNA was initially spotted to the corresponding wells followed by the addition of 20 µL of serum-free DMEM containing 0.15 µL of Lipofectamine RNAiMax (Life Technologies) per well. Plates were incubated at room temperature for 45 min. Following incubation, $1.2x10^3$ cells in 20 µL of DMEM supplemented with 20% FBS (containing no antibiotic-antimycotic) were added in each well. Transfected cells were incubated at 37ºC in 5% CO_2 for 72 hours. After assaying, sample well data was normalized to the median value of the NegCtrl wells on respective assay plates. The average was taken for each replicate, and a Z-score was then calculated for each mimic by using the mean and standard deviation of each screen respectively. All screening data can be found in Supplementary Table S1. Hit selection focused on those mimics exhibiting a Z-score of > +/- 2 in N370S/N370S Gaucher fibroblasts. A small edge effect was noted that resulted in a ~15% increase in GCase signal from edge wells. Accordingly, a simple heuristic test was applied to correct the data in those wells by scaling for this increase and avoid the selection of false positives arising from this edge effect. Viability data was used to filter out mimics that also caused an

appreciable increase or decrease in viable cells (+/- 1 standard deviation from the screen mean).

GCase activity assay

GCase screening was conducted in black clear bottom 384-well plates (Corning 3712). Seventy-two hours after transfection, the medium was removed, and the cells were washed once with 1X phosphate buffered saline (PBS; Life Technologies). Twenty microliters of assay buffer consisting of 3 mM 4-methylumbelliferyl-β-D-glucopyranoside substrate (Sigma), 0.2 M acetate buffer at pH 4, 1X PBS, and EDTA-free protease inhibitors (Roche) were added to the wells. Plates were incubated at 37ºC for 70 min. The reaction was stopped by adding 20 μL of stop solution (1 M NaOH (Sigma) and 1 M Glycine (Sigma)) to each well. Fluorescence was read at 365 nm excitation and 440 nm emission λ length using an EnVision Plate Reader (PerkinElmer). The median signal from > 80 wells without cells was used as background. Background was subtracted from the values for all wells prior to normalization.

Cell viability measurement

For each experiment, a separate set of 384-well plates (solid white bottom, Corning 3570) prepared as the enzyme activity assay plates, was assayed for cell viability using the CellTiter-Glo Luminescent Cell Viability kit (Promega). Twenty microliters of the luminescent reagent was added directly to the wells, incubated at room temperature for 20 min and measured in an EnVision Plate Reader according to the manufacturer's instructions.

RNA extraction, cDNA synthesis and relative expression analysis

Based on the primary screening data, candidate miRNAs were selected and subjected to follow-up experiments in 96-well plates. Two pmol of each miRNA or siRNA was added to the corresponding wells, followed by the addition of 50 μL of serum-free DMEM with 0.375 μL of Lipofectamine RNAiMax. After a 45 min incubation at room temperature, $3x10^3$ cells in 50 μL of DMEM supplemented with 20% FBS were added to

each well. Transfected cells were incubated at 37ºC in 5% CO_2 for 72 hours. Total RNA was harvested from cells using a MagMax-96 Total RNA Isolation kit (Life Technologies) according to manufacturer's instructions. Complementary DNA (cDNA) was generated from 9 μL of total RNA using the High Capacity RNA-to-cDNA kit (Life Technologies) following the manufacturer's protocol.

A TaqMan probe-based assay (Life Technologies) was used to evaluate the relative expression levels of *GBA1* (Hs00164683_m1), *SCARB2* (Hs01072100_m1), *PSAP* (Hs01551096_m1), and *GAPDH* as a housekeeping gene (4352934E). Real-time PCR mixes were prepared based on the manufacturer's instructions and run in a 7900HT Fast Real-time PCR System (Life Technologies). Eight biologic replicates were performed in duplicate for each experiment. The results were normalized using *GAPDH* expression levels and the $2^{-\Delta\Delta Ct}$ method (17). *p*-values and 95% confidence intervals were calculated using RT^2 Profiler PCR Array Data Analysis v3.5 (Qiagen).

SDS-PAGE, Western blotting and conditioned media analysis

Cells were transfected with miRNAs or control siRNAs in 6-well plates for protein analysis. Sixty pmol of each miRNA/siRNA was added to the corresponding wells, followed by the addition of 1.5 mL of serum-free DMEM containing 9.4 μL of Lipofectamine RNAiMax. After 45 min of incubation at room temperature, $1x10^5$ cells in 1.5 mL of DMEM supplemented with 20% FBS were added to each well. Plates were incubated in 5% CO_2 at 37ºC for 72 hours.

For protein analysis, the media was aspirated and cells were rinsed three times with 1X PBS. Cells were lysed and scrapped in 50 mM Citrate 175 mM KH_2PO_4 with 0.01% Tween-20 at pH 5.9. Cell lysates were sonicated for 1x10 sec at 50% amplitude and centrifuged at 4ºC for 10 min at 10000 rcf. Protein levels were measured using a BCA assay (BioRad). Ten micrograms of each protein sample was separated using NuPage Novex 4-12% Bis-Tris gel (Life Technologies) by SDS-PAGE at 125V. Samples were transferred on PVDF membrane (Life Technologies) using iBlot (Life Technologies), followed by blocking 1 hour at room temperature in 1X PBS containing 5% of fat-free milk and 0.5% Tween-20. This was followed by incubation in blocking solution with R386 GCase antibody (a custom-made antibody), LIMP II antibody (D4, sc-55571, Santa Cruz

Biotechnology), and GAPDH antibody (ab9385, Abcam) overnight at 4ºC. The membranes were rinsed with blocking solution three times for 10 min each, followed by incubation with blocking solution containing 1:3000 horseradish peroxidase (HRP)-conjugated secondary antibody (KPL) for 1 hour at room temperature. HRP-probed immunoblots were developed using enhanced chemiluminescence (GE Healthcare). Experiments evaluating protein level were done in triplicates.

For assessment of GCase secretion into the extracellular media, WT and N370S/N370S Gaucher fibroblasts were transfected with specific miRNA (miR-127-5p) or with control siRNAs in 6-well plates as described above. The same cell density was used ($1x10^5$ cells per well) for both cell lines, and the experiment was performed in duplicate. Seventy-two hours after transfection, the media was removed and each well was washed three times with 1X PBS and two times with serum-free DMEM. Two mL of serum-free DMEM (conditioned media) was added to each well and incubated at 37ºC in 5% CO_2. Twenty-four hours later, conditioned media was collected and combined from two different wells containing the same miRNA/siRNA. Collected media was filtered using a 0.22 μM filter (Millipore) and transferred to a Pierce Concentrators 9K MWCO (Thermo Scientific). The media was concentrated by centrifugation at 2500 rcf at 4ºC until it reaches a 500 μL of volume. A total of 30 μL of the concentrated media was loaded on a NuPAGE 4-12% Bis-Tris 1.5 mm gel, transferred to a membrane and incubated with GCase antibody as described above.

SCARB2 **luciferase assay**

The full-length of the *SCARB2* 3'UTR covering two predicted target sites of miR-127-5p was cloned into the pMirTarget vector (Origene). For luciferase assays, HEK293 cells ($2.4x10^3$ cells/well) were transfected with 0.8 pmol of miR-127-5p or NegCtrl siRNA using 0.05 μL of Lipofectamine RNAiMax in a 384-well plate. After 48 hours of transfection, the cells were further transfected with 25 ng of the firefly reporter construct (pMir-SCARB2-3'UTR) or pMirTarget empty vector (control plasmid) using 0.05 μL of TransIT-LT1 transfection reagent (MirusBio). Twenty-four hours later, the luciferase assay was evaluated using the ONE-Glo Luciferase assay reagent (Promega) and measured in an EnVision Plate Reader.

Statistical analysis

Analysis was performed using GraphPad Prism 5.0 and Microsoft Excel software.

RESULTS

miRNA screening, hit selection, and reconfirmation

In the present study, miRNA mimic screening (Sanger miRBase 13.0) was performed in WT and N370S/N370S Gaucher fibroblasts to evaluate the effects of introducing different miRNAs in increased abundance on GCase activity. Primary screening was performed in duplicate, and after 72 hours of incubation GCase activity was evaluated. GCase enzyme activity was chosen as the outcome parameter, while cell viability measurement in corresponding plates identified toxic miRNAs. A summary of the entire workflow is shown in Figure 1, and all screening data can be found in the Supplementary Table S1.

For both WT and N370S/N370S Gaucher fibroblasts, the control samples on the assay plates used were consistent, independent of cell type, assay (activity or viability), and plate analyzed (Figure 2). Replicates for GCase enzyme activity and for the viability assay showed consistent and reproducible results, both for the WT (Figure 3A and B) and N370S/N370S (Figure 3D and E) lines. When we compared GCase activity and viability, the correlation was not strong in either cell type, indicating that the observed miRNA effects were not strictly related to cell toxicity or number (Figure 3C and F). Moreover, combining the primary screening data of GCase activity or viability obtained using WT and N370S/N370S cells (Figure 3G and H), indicated that the results were reproducible, regardless of cell type used.

Based on the primary screening data, we selected 13 miRNAs that up-regulated and 8 that down-regulated GCase activity. Selected candidates exhibited a Z-score of at least +/- 2 in N370S/N370S cells and did not affect the viability by more than 1 standard deviation. To confirm our findings, we performed follow-up testing in 384- (Figure 4A and B) and 96-well (Figure 4C and D) plates under the same conditions as the primary screen. Our results were reproducible regardless of the cell number or reagents used. From the 21 miRNAs chosen for reconfirmation (Figure 4), 8 of the most active, including 5 miRNAs that up-regulated (miR-195-5p, miR-16-5p, miR-765, miR-493-5p, and miR-1243) and 3 that down-regulated (miR-127-5p, miR-19a-5p, and miR-1262) GCase activity, were selected for further studies. As an initial test, the 8 mimics were shown to be active in a

different N370S/N370S Gaucher fibroblast line from that used for the primary screen (Figure 5).

Analysis of *GBA1*, *SCARB2*, and *PSAP* expression levels in N370S/N370S cells

We hypothesized that a decrease or increase of the GCase activity by specific miRNAs could be due to changes in the mRNA expression levels of *GBA1* or other genes that regulate *GBA1* expression. Gene expression was measured by real-time PCR based on the $2^{-\Delta\Delta Ct}$ method. To identify alterations in *GBA1* levels, we compared *GBA1* expression in N370S/N370S Gaucher cells transfected with selected miRNAs (miR-195-5p, miR-16-5p, miR-765, miR-493-5p, miR-1243, miR-127-5p, miR-19a-5p, and miR-1262) to cells transfected with NegCtrl siRNA. As expected, cells transfected with GBA siRNA showed significant reduction of *GBA1* expression when compared to cells treated with NegCtrl siRNA ($p < 0.001$; Figure 6A). Surprisingly, none of the three GCase enzyme activity down-regulators (miR-127-5p, miR-19a-5p and miR-1262) significantly affected *GBA1* expression ($p > 0.001$). On the other hand, two (miR-195-5p and miR-16-5p) of the five up-regulators showed a significant increase in *GBA1* expression compared to control (> 2-fold, $p < 0.001$). Moreover, both miRNAs increased GCase protein levels by approximately 40% compared to NegCtrl siRNA (Figure 7A and Supplementary Figure S1).

Since the miRNA down-regulators did not have any effect on *GBA1* expression, we hypothesized that these miRNAs might act on the expression levels of known modifiers of GCase enzyme activity such as *SCARB2* and *PSAP*. In N370S/N370S fibroblasts, *SCARB2* expression was significantly down-regulated by miR-127-5p, miR-19a-5p, and miR-1262. ($p < 0.001$; Figure 6B) with miR-127-5p and miR-1262 causing stronger reduction compared to miR-19a-5p. Moreover, miR-127-5p led to a strong reduction in LIMP-2 protein levels (Figure 7A). There was no statistical significant difference in *PSAP* after miRNA transfection compared to control. The only exception was cells transfected with GBA siRNA that showed a greater than 100% increase in *PSAP* expression (Figure 6C). This result is in agreement with other studies showing a compensatory increase in *PSAP* in cells with GCase deficiency (9).

miR-127-5p treatment results in the secretion of GCase

It is known that LIMP-2 is the receptor for the trafficking of GCase to the lysosome. Studies on *SCARB2* knockout mice, as well as patient fibroblasts carrying mutations in *SCARB2* showed no change in *GBA1* mRNA levels, but decreased GCase activity and protein levels due to GCase missorting and its subsequent secretion into the extracellular environment (10,18). This prompted an evaluation of the effect of miR-127-5p on GCase secretion into the cellular environment. WT and N370S/N370S Gaucher fibroblasts were treated with miR-127-5p and NegCtrl siRNA, and the conditioned media was collected. After successive steps of filtration and protein concentration by centrifugation, we analyzed GCase protein levels by Western blot, loading equal volumes of lysed cells to ensure that conditioned media was collected from an equal amount of cells for each condition, we loaded the same volume of lysed cells. The conditioned media analysis was performed on two different N370S/N370S Gaucher fibroblast lines; the line used in the primary screen (Figure 7) and another used for confirmation. Both Gaucher lines treated with miR-127-5p showed reduced GCase protein levels in cell lysates compared to cells treated with NegCtrl siRNA (approximately 50% of reduction; Figure 7A and C, Supplementary Figure S2), while the corresponding conditioned media samples revealed up-regulation of secreted GCase enzyme (Figure 7B). As expected, while N370S/N370S fibroblasts showed reduced expression of GCase compared to WT, cells without any siRNA ("Cells only") or with NegCtrl siRNA showed similar results (Supplementary Figure S2B). N370S/N370S fibroblasts treated with miR-127-5p showed a reduced amount GCase compared to NegCtrl siRNA (Supplementary Figure S2B). As shown in both Figure 7B and Supplementary Figure S2A, the reduction of GCase appears to occur as a result of secretion of GCase into the extracellular environment. This is not seen in WT cells and N370S/N370S cells treated without/with NegCtrl siRNA (Supplementary Figure S2A). These results suggest that expression of miR-127-5p down-regulates GCase activity and protein levels by down-regulating *SCARB2* expression.

***SCARB2* is a direct target of miR-127-5p**

Next, we wanted to evaluate whether *SCARB2* is a direct target of miR-127-5p. *SCARB2* contains two predicted miR-127-5p target sites at its 3'UTR region (data not

shown). To analyze whether miR-127-5p directly interacts with these predicted sites, we subcloned the *SCARB2* 3'UTR region into a luciferase reporter vector. HEK293 cells were transfected with the resulting pMir-*SCARB2* 3'UTR or with the control plasmid. Twenty-four hours after transfection, cells were treated with miR-127-5p or NegCtrl siRNA. As expected, miR-127-5p had no effect on the luciferase activity of the control plasmid. In contrast, HEK293 cells transfected with pMir-*SCARB2* 3'UTR showed significant reduction of the reporter activity after transfection with miR-127-5p compared to cells treated with NegCtrl siRNA ($p < 0.001$; Figure 8). These data indicate that miR-127-5p directly targets *SCARB2*.

L444P/L444P Gaucher fibroblasts is also affected by miRNAs

To check if our miRNA candidates could also affect GCase activity in cells with a different *GBA1* genotype, we transfected L444P/L444P Gaucher fibroblasts with miR-127-5p, miR-16-5p, and miR-195-5p. As shown in Figure 9, miR-127-5p reduced LIMP-2 levels in the L444P/L444P line. In general, this particular genotype results in extremely low GCase activity and protein amount, due to ER retention and subsequent proteasomal degradation of mutant GCase (19). Therefore, GCase levels in L444P/L444P fibroblasts are very low (Figure 9). Both up-regulators, miR-16-5p and miR-195-5p increased GCase protein levels in L444P/L444P fibroblasts. We could not detect significant down-regulation of GCase protein levels with miR-127-5p treatment, which is very likely due to very low GCase protein levels in this particular cell line.

DISCUSSION

Understanding the biological relevance of miRNAs to human disease depends primarily on the identification of the targets involved. In the present study, we conducted a miRNA mimic screen to identify any miRNA sequences that modulate GCase enzymatic activity in N370S/N370S Gaucher fibroblasts. The screening identified a number of mimics that up-regulated or down-regulated GCase activity without a correlative effect on cell viability. Many of the hits were reconfirmed in independent tests in the same and an additional Gaucher fibroblast line; several mimics increased GCase activity by over 1.4-fold, which is thought to be of potential clinical significance (20,21). Notably, a number of enhancers shared the same hexamer miRNA seed sequence (AGCAGC) including members of the miR-15/16/195/424/497 family. Further studies using two of these family members (miR-16-5p and miR-195-5p) revealed increased levels of both *GBA1* transcript and GCase protein. Although the mechanism underlying the up-regulation is unclear, miRNAs can enhance transcript and protein levels through a variety of means including interactions with promoter regions (RNA activation (RNAa), reviewed in (22,23)), stabilization of mRNA by blocking destabilizing proteins (24), and through the down-regulation of proteins that negatively regulate transcription factors. For example, miR-16 can enhance NF-kB driven expression of inflammatory response genes through the down-regulation of SMRT (25). Additional studies are needed to further elucidate how members of the miR-15/16/195/424/497 family act to up-regulate *GBA1* transcript levels.

In addition to up-regulation, a number of the miRNA mimics decreased GCase activity. Since miRNAs typically down-regulate target transcripts through interactions with their 3'UTR, we investigated potential binding sites in *SCARB2* and *PSAP*, two known modifiers of GCase activity. In silico target prediction for the top three down-regulating mimics did not identify binding sites (26). However, closer inspection revealed strong seed matches in the *SCARB2* 3'UTR for both miR-127-5p and miR-19a-5p. Real-time PCR analysis indicated that both miR-127-5p and miR-19a-5p down-regulated *SCARB2*, and a *SCARB2* 3'UTR luciferase reporter assay confirmed direct targeting of *SCARB2* by miR-127-5p. Levels of LIMP-2 protein, encoded by the *SCARB2* gene, were down-regulated in fibroblasts treated with miR-127-5p. LIMP-2 is critical for the proper trafficking of GCase

from the ER to lysosomes. Defects in LIMP-2 result in the extracellular secretion of GCase. Accordingly, we found that treatment with miR-127-5p led to increased levels of GCase in the supernatant, supporting its impact on LIMP-2 function.

Furthermore, our results indicate that the effects of miRNA treatment (e.g. miR-127-5p, miR-16-5p, and miR-195-5p) are not exclusive to cells with genotype N370S/N370S. Reproducible findings were observed in L444P/L444P Gaucher fibroblasts. The fact that miRNAs might interact in the same way in cells with different *GBA1* genotypes highlights the importance and relevance of understanding its mechanism in GD.

The identification of miRNA sequences that modulate GCase activity improves our understanding of its regulation, and may aid in the identification of additional protein targets and potential therapeutic strategies. In fact, mimic sequences themselves may prove useful, and certain miRNA mimics are being studied in the clinic (27,28). Some of the identified candidates may not be optimal starting points in this regard. Notably, miR-15/16/195/424/497 family members have been shown to regulate key cell cycle genes and also to induce apoptosis (29-31), which may be attractive for targeting cancer, but perhaps not for other diseases. However, the mechanism underlying the enhancement of GCase activity is unclear for a number of the other mimics (e.g., miR-765 and miR-1243). It will be important to further explore their effect on activity, both with regard to GCase and other genes/pathways throughout the cell.

Taken together, our data demonstrates that GCase activity might be affected by specific miRNAs. Our data strongly suggests an important role for miR-127-5p in the modulation of GCase activity via regulation of the LIMP-2 receptor. .Furthemore, miR-16-5p and miR-195-5p up-regulate GCase activity through an unknown pathway that merits further investigation. This is particularly relevant as miRNAs might prove to be potential therapeutic targets. Further studies are needed to validate the role of specific miRNAs or miRNA families in cellular pathways related to GD pathogenesis and treatment.

SUPPLEMENTARY DATA

Supplementary data will be available at NAR Online.

FUNDING

This work was supported by the Intramural Research Programs of National Human Genome Research Institute, RNAi Screening Facility of National Center for Advancing Translational Sciences, and National Institutes of Health. M.S. received support by the Brazilian Federal Agency for Support and Evaluation of Graduate Education - CAPES and Fulbright Brazil.

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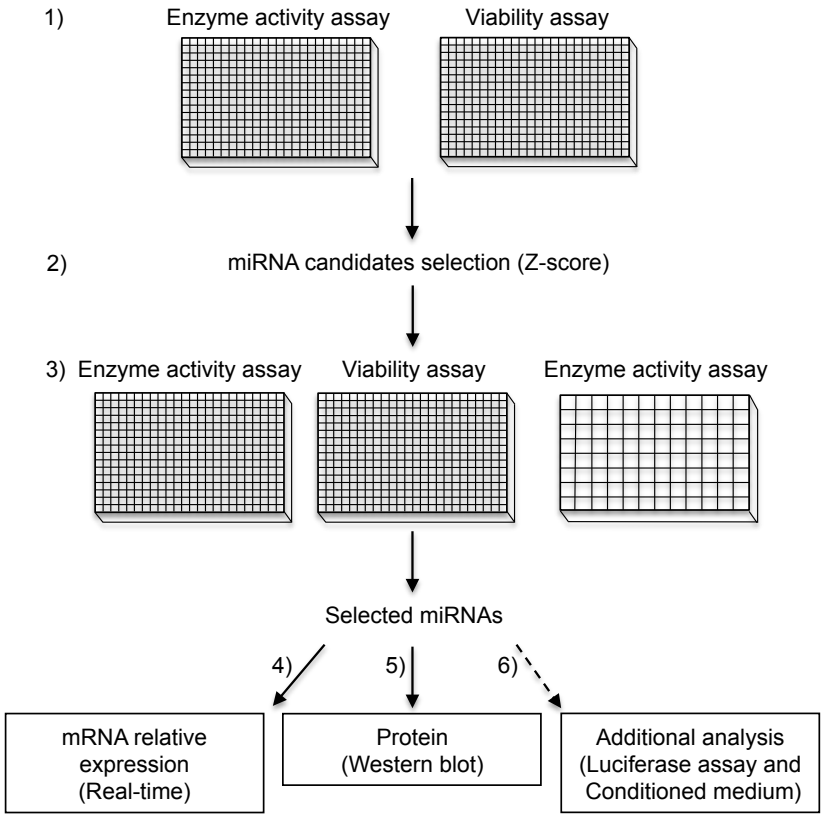


Figure 1. Experimental workflow of miRNA mimic screening and follow-up experiments. (1) The primary miRNA screen was performed in duplicate, assaying both GCase activity and cell viability assays in WT and N370S/N370S Gaucher fibroblasts. (2) miRNA candidates were chosen based on the GCase Z-score with consideration of their effect on viability. (3) Results were confirmed by retesting selected miRNAs in both 384- and 96-well plates. After the confirmation step, the top 5 miRNAs that up-regulated and 3 miRNAs that down-regulated GCase activity were selected for additional studies. (4) The mRNA relative expression of *GBA1*, *SCARB2*, and *PSAP* genes was evaluated by real-time PCR. (5) Changes in protein levels were investigated by Western blot. (6) Additional studies were performed on miRNA candidates identified in the previous step.

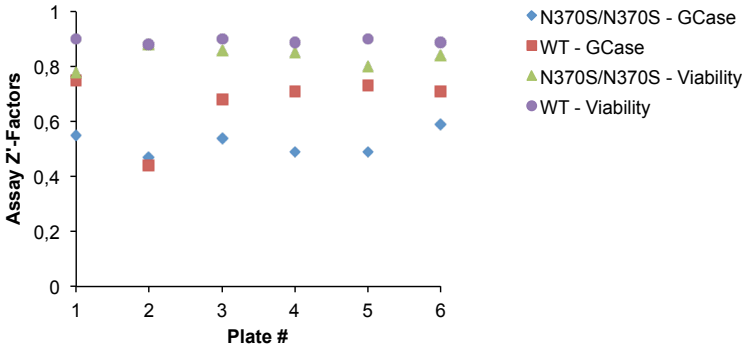


Figure 2. Performance of control siRNAs used during the primary miRNA screen using both WT and N370S/N370S Gaucher fibroblasts.

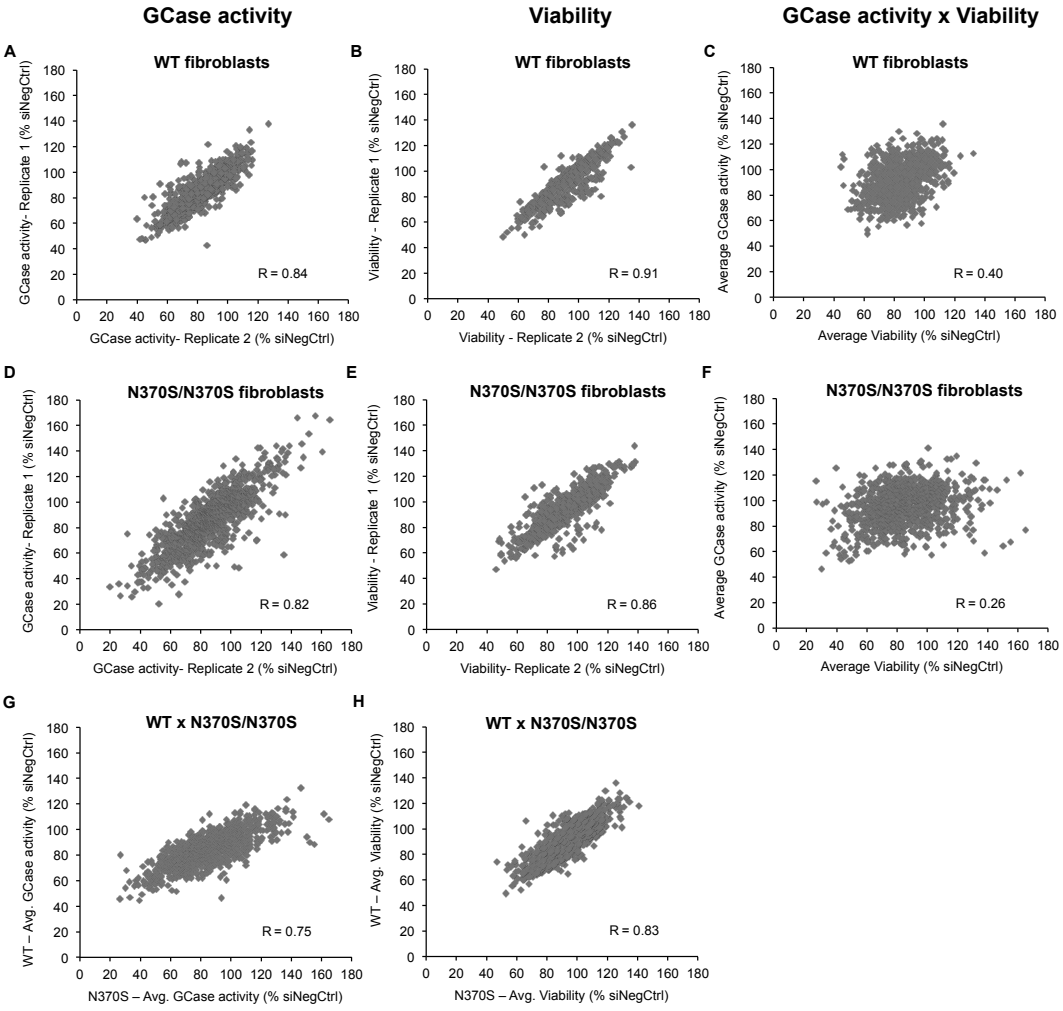


Figure 3. Primary screening data showed consistent results between replicates and different cell lines. Replicates of GCase activity (A and D) and viability (B and E) signal measured in WT and N370S/N370S cells, respectively. Correlation between GCase activity and viability in WT (C) and N370S/N370S (F) fibroblasts showed that the signal could be influenced by cell viability. Comparison of the results of enzyme activity (G) and viability (H) in both cell types.

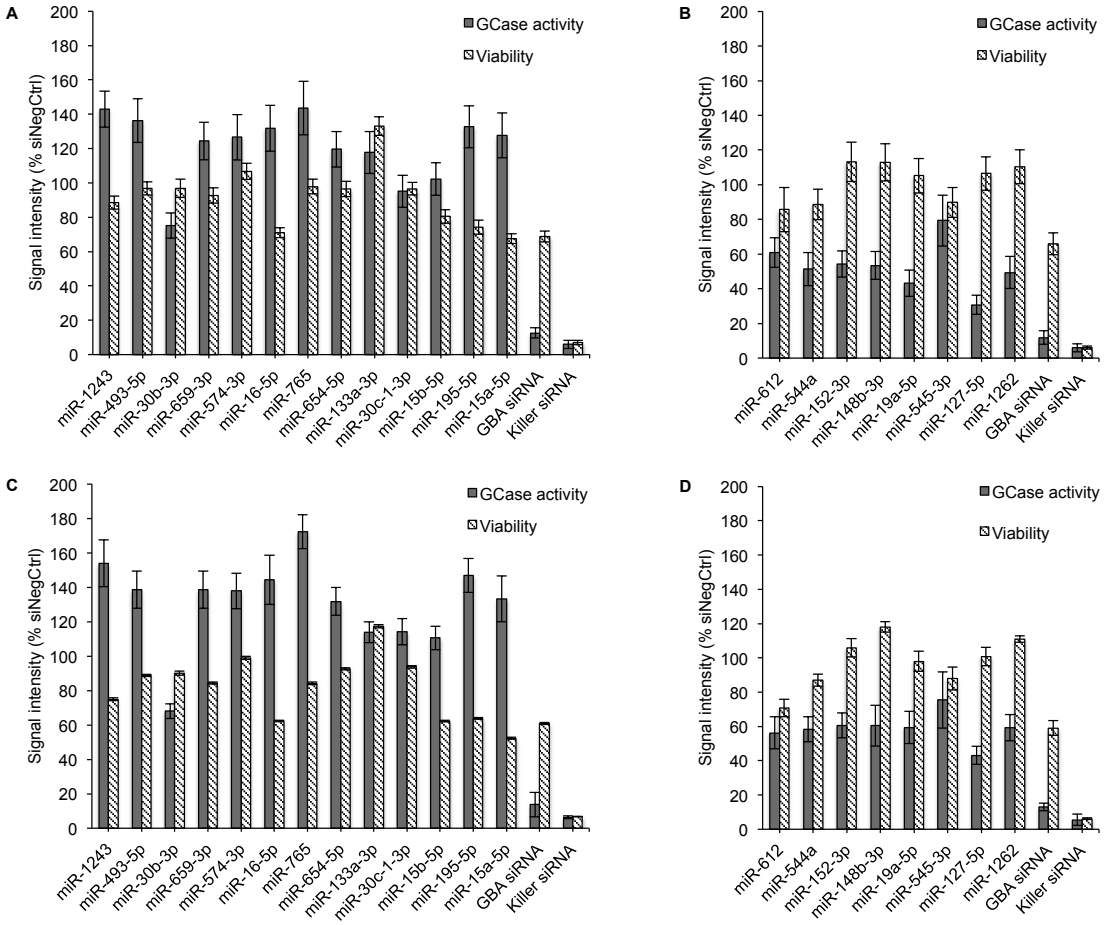


Figure 4. Experiments confirming the top miRNA hits using N370S/N370S Gaucher fibroblasts. Selected miRNA up-regulators (A) and down-regulators (B) of GCase activity were plated in a 384-well format. The same experiment was also performed in 96-well plates (C and D). Results were consistent between 384- and 96-well plates. Data is presented with the average and standard deviation for replicate wells (384-well n=16, 96-well n=8).

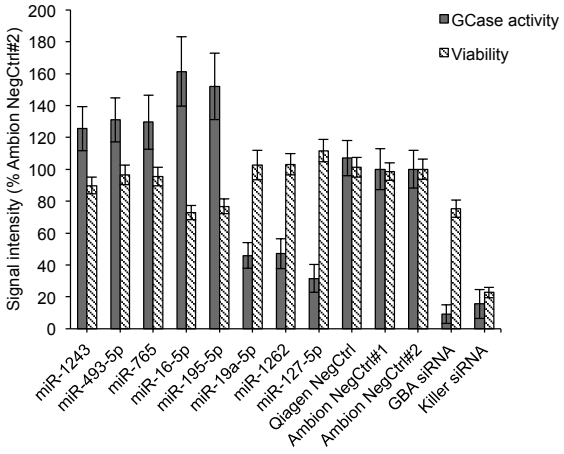


Figure 5. Additional N370S/N370S Gaucher lines were used to confirm the effect of the 8 best active miRNA candidates on GCase activity and viability. In addition to the NegCtrl (Ambion NegCtrl#2) used for normalization, the experiment had two other controls, Ambion NegCtrl#1 and Qiagen NegCtrl. Results represent the average value along with the standard deviation for replicate wells. The experiment was performed in a 384-well plate (16 replicates for each siRNA/miRNA).

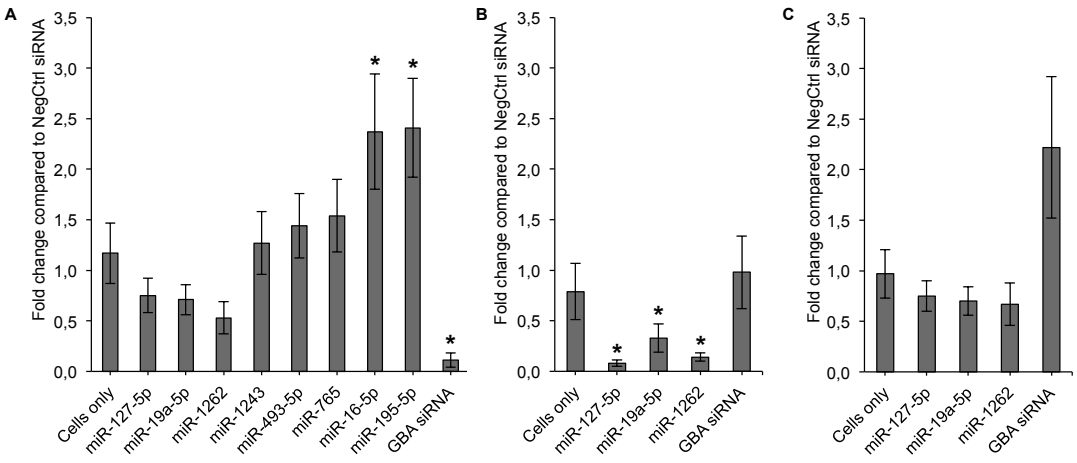


Figure 6. mRNA expression profiles evaluated in N370S/N370S fibroblasts after 72 hours of transfection with selected miRNAs that down- and up-regulated GCase activity. *GBA1* (A), *SCARB2* (B) and *PSAP* (C) levels were analyzed by real-time PCR. Error bars represent 95% of confidence intervals (CI). *Indicates *p*-value < 0.001 compared to NegCtrl siRNA.

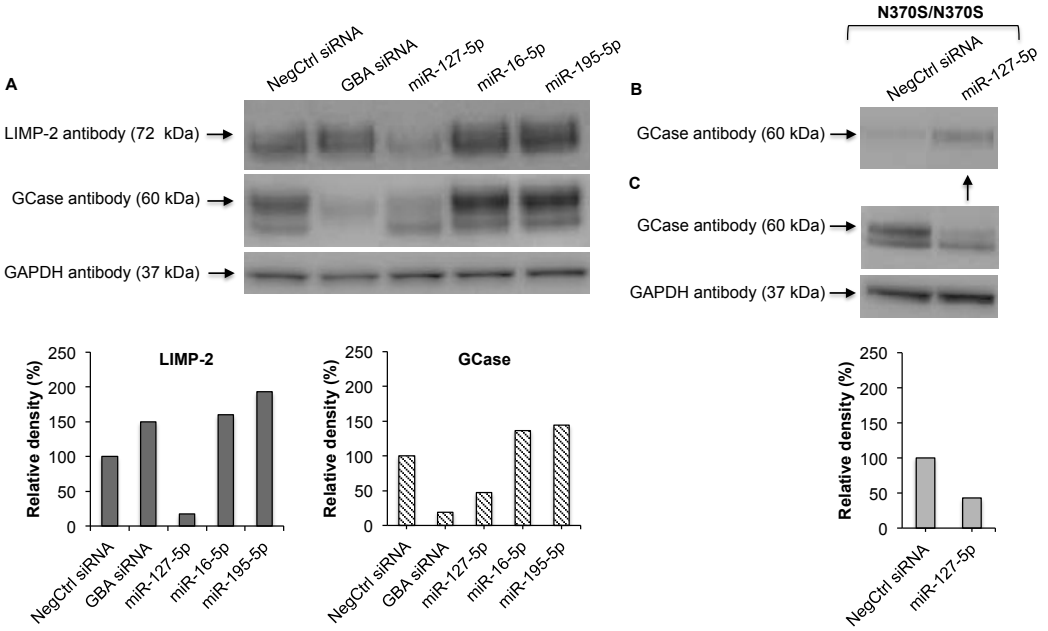


Figure 7. Analyses of GCase and LIMP-2 levels and GCase secretion into the extracellular environment. (A) Western blot shows a reduced amount of GCase after transfection with GBA siRNA and miR-127-5p compared to the NegCtrl. Conversely, GCase amount is increased in cells treated with miR-16-5p and miR-195-5p. Clearly, LIMP-2 levels are decreased by miR-127-5p. The lower graphs indicate the percentage of relative density of each band normalized to the corresponding GAPDH band. (B) Evaluation of conditioned media shows GCase secretion (arrow) into the extracellular environment after transfection with miR-127-5p in comparison to cells treated with NegCtrl siRNA. (C) Corresponding cell lysates from the conditioned media experiment show reduced GCase protein in N370S/N370S cells transfected with miR-127-5p. The bottom graph represents the intensity of each band normalized with the corresponding GAPDH band. For the relative density calculations in both (A) and (C), NegCtrl was considered as 100%.

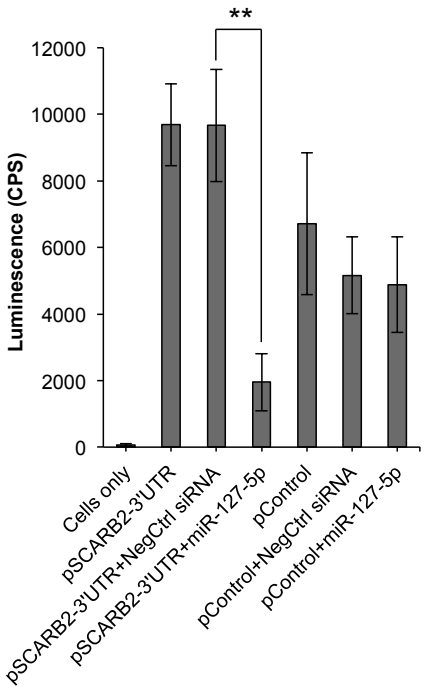


Figure 8. miR-127-5p directly interacts with the *SCARB2* 3'UTR. HEK293 cells were transfected with the *SCARB2* 3'UTR-luciferase plasmid or with the control plasmid (empty plasmid) along with the miR-127-5p or NegCtrl siRNA. Luciferase activity was measured; histograms show mean values for each condition normalized with background. Cells without plasmid or siRNA/miRNA ("Cells only") were used as background for normalization. Error bars represent 95% of confidence intervals (CI). *Indicates *p*-value < 0.001 compared to NegCtrl siRNA. CPS: counts per second.

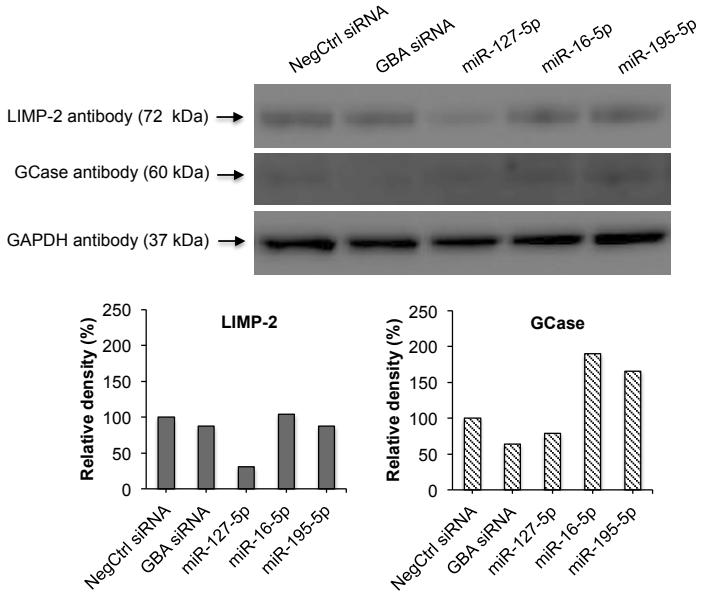
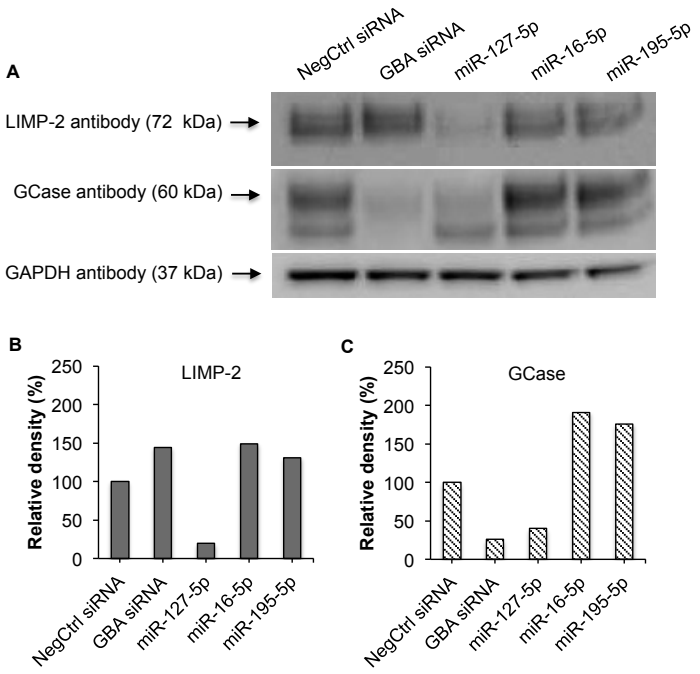
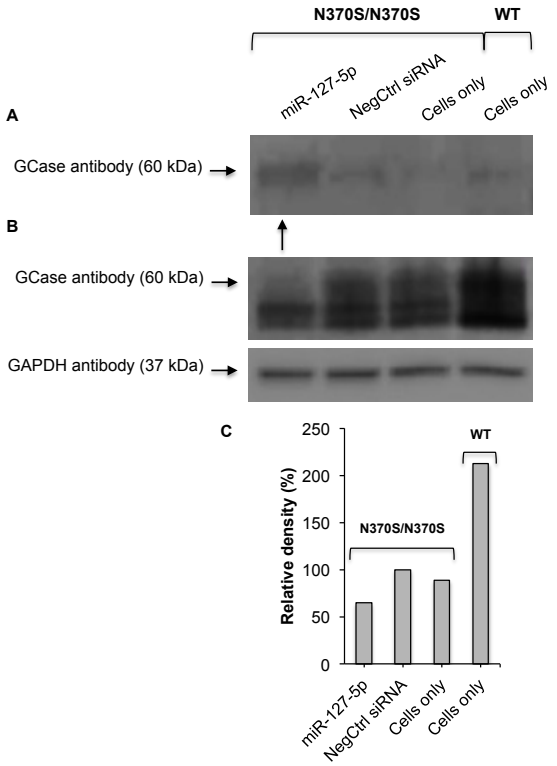


Figure 9. GCase and LIMP-2 are affected by specific miRNAs in L444P/L444P Gaucher fibroblasts. (A) LIMP-2 levels are reduced after miR-127-5p treatment compared to the NegCtrl. In contrast, GCase is increased in cells treated with miR-16-5p and miR-195-5p. The bottom graphs represent relative density of each band normalized to the corresponding GAPDH band. NegCtrl was considered as 100%.



Supplementary Figure S1. Confirmation of the effect of miR-127-5p, miR-16-5p, and miR-195-5p on GCase and LIMP-2 proteins in N370S/N370S Gaucher fibroblasts. (A) GCase levels were down-regulated after treatment with GBA siRNA and miR-127-5p. Both miR-16-5p and miR-195-5p induced an increase in GCase, while miR-127-5p caused a reduction of LIMP-2 levels. Graphs indicate the relative density (%) of each band normalized to the corresponding GAPDH band.



Supplementary Figure S2. GCase secretion is observed after miR-127-5p treatment in a second N370S/N370S fibroblast line. (A) Evaluation of conditioned media shows up-regulation of GCase (indicated by the arrow) after transfection with miR-127-5p in comparison to conditioned media derived from cells treated with NegCtrl siRNA, N370S/N370S or WT without siRNA/miRNA. (B) Corresponding cell lysates from the conditioned media experiment show down-regulation of GCase in N370S/N370S fibroblasts transfected with miR-127-5p. (C) Intensity of each band is measured and normalized to the corresponding GAPDH band. The NegCtrl siRNA was considered 100% in the relative density calculation.

4. DISCUSSÃO

Este estudo enfatiza três aspectos relacionados à enzima GCase, sendo eles: o efeito de alterações no gene *GBA1* como modificador do fenótipo de pacientes com a doença de Machado-Joseph/ataxia espinocerebelar tipo 3, o efeito de polimorfismos em genes candidatos (*SCARB2*, *PSAP* e *CLN8*) a modificadores do fenótipo da DG e a influência de miRNAs sobre a atividade da enzima GCase.

A doença de Machado-Joseph, também conhecida como ataxia espinocerebelar tipo 3 (MJD/SCA3), é uma doença neurodegenerativa, de herança autossômica dominante, causada por expansões trinucleotídicas (CAG) no gene *ATXN3*. Indivíduos normais possuem de 12 a 41 repetições, enquanto que indivíduos com MJD/SCA3 têm entre 61 e 87 repetições nos alelos afetados (Kawaguchi *et al.*, 1994; Nóbrega & Almeida, 2012). As características clínicas dos pacientes com MJD/SCA3 podem variar significativamente, inclusive entre indivíduos da mesma família (Paulson, 2012). A MJD/SCA3 é dividida em quatro tipos clínicos conforme as manifestações atáxicas e outros sintomas neurológicos apresentados pelos pacientes. O tipo 4 é a forma mais rara da doença, sendo caracterizado pela presença de manifestações parkinsonianas (Nóbrega & Almeida, 2012). De uma maneira geral, o principal sintoma clínico da MJD/SCA3 é a presença de ataxia progressiva devido à disfunção cerebelar e do tronco cerebral. A idade de início dos sintomas varia consideravelmente na MJD/SCA3, estendendo-se de 5 a 70 anos de idade. Esta variabilidade na idade de início está associada ao tamanho da expansão nucleotídica no alelo afetado. A presença de um maior número de repetições CAG no gene *ATXN3* está geralmente associada ao aparecimento precoce dos sintomas da doença (Paulson, 2012).

No primeiro capítulo deste estudo foi avaliado se variações de sequência no gene *GBA1* seriam mais frequentes em pacientes com MJD/SCA3 tipo 4 (pacientes com sintomas parkinsonianos) em comparação a pacientes não-tipo 4 e indivíduos controles. A hipótese levantada foi a de que alterações no *GBA1* poderiam estar agindo como fator de risco para o desenvolvimento de parkinsonismo também em pacientes com MJD/SCA3. Após a análise completa do gene *GBA1*, variações de sequência neste gene foram identificadas apenas nos pacientes com MJD/SCA3 tipo 4.

Os lisossomos são organelas essenciais para o *turnover* de proteínas através da autofagia, sendo este um mecanismo essencial para a homestase neuronal (Nixon, 2013; Yang *et al.*, 2013). A existência de distúrbios nos processos autofágicos está diretamente associada ao aparecimento de determinadas doenças neurodegenerativas, tais como DP e MJD/SCA3. Estudos científicos evidenciam que uma redução da atividade da GCase devido à presença de mutações no gene *GBA1*, mesmo em indivíduos heterozigotos, pode alterar o funcionamento dos lisossomos ou a ligação de proteínas aos seus respectivos receptores na membrana lisossômica. A ataxina-3, a α-Sin e a parkina são algumas das proteínas afetadas em casos de disfunção envolvendo os lisossomos (Cuervo *et al.*, 2004; Soong & Paulson, 2007; Menzies *et al.*, 2010).

A ataxina-3 é codificada pelo gene *ATXN3* e tem importante papel no sistema proteassomo dependente de ubiquitina, pois atua como uma enzima deubiquitinase. A ataxina-3 interage diretamente com a proteína parkina, uma E3 ligase que adiciona moléculas de ubiquitina a proteínas direcionadas para degradação via proteassomo. Dessa forma, ataxina-3 e parkina possuem mecanismo de ação opostos. Em pacientes com MJD/SCA3, a presença de ataxina-3 mutante estimula a degradação de parkina via autofagia, devido a remoção dos resíduos de ubiquitina e ao impedimento da auto-ubiquitinação exercida pela parkina. Consequentemente, há redução dos níveis de parkina embora o mecanismo pelo qual isso ocorra permaneça incerto. A presença de ataxina-3 mutante induz a formação de agregados, que por sua vez podem interagir com parkina, levando a degradação destes agregados por autofagia (Durcan & Fon, 2013).

Dessa forma, a GCase e a ataxina-3 estão associadas independentemente a manifestações parkinsonianas. Um mecanismo recíproco entre as proteínas GCase e α-Sin foi sugerido na literatura (Mazzulli *et al.*, 2011), enquanto que a relação da ataxina-3 com o parkinsonismo parece ocorrer pela interação direta com a proteína parkina. Portanto, um mecanismo de inter-relação entre a GCase, o aparecimento de sintomas parkinsonianos e uma possível influência no fenótipo de pacientes com MJD/SCA3 é sugerido com base nos resultados obtidos no presente trabalho.

O mecanismo de inter-relação proposto envolve as seguintes etapas: 1) alterações no gene *GBA1* levam a redução na atividade da GCase; 2) essas variações de sequência ocasionam uma GCase mal-enovelada ou instável, levando a sua retenção no RE e

subsequente degradação via proteassomo; 3) diminuição nos níveis de GCase no interior dos lisossomos e acúmulo do substrato GC não-degradado; 4) esse acúmulo estimula a formação de oligômeros e fibrilas de α-Sin, além de impedir a entrada de α-Sin nos lisossomos via receptor LAMP-2A; 5) o acúmulo de α-Sin no citosol leva à formação de oligômeros e agregados; 6) estes agregados de α-Sin agravam ainda mais a disfunção do lisossomo por inibirem o transporte da GCase remanescente para os lisossomos, resultando em maior acúmulo de GC; 7) a formação de agregados de ataxina-3 mutante e parkina acumulam no citosol devido à disfunção lisossômica provocada pela alteração da GCase; 8) os agregados insolúveis de α-Sin e de ataxina-3 mutante causam toxicidade aos neurônios. Em suma, sugerimos que a presença de mutações no gene *GBA1* esteja associada ao aparecimento de sintomas parkinsonianos em pacientes com MJD/SCA3 por agravar ainda mais o mal funcionamento dos lisossomos ocasionado pelo acúmulo de agregados de ataxina-3 mutante. Mutações no *GBA1* poderiam estar relacionadas ao aparecimento precoce de sintomas parkinsonianos em pacientes com MJD/SCA3 de modo que os classificariam como tipo 4. Com base nos resultados obtidos e nos dados disponíveis na literatura, sugerimos que a inter-relação entre *GBA1*, parkinsonismo e MJD/SCA3 é plausível, embora estudos adicionais envolvendo um número maior de pacientes com MJD/SCA3 tipo 4 sejam necessários para confirmar a nossa hipótese.

Além de avaliar a presença de mutações no gene *GBA1* como um fator de modificação do fenótipo da MJD/SCA3, o segundo capítulo deste trabalho envolveu a análise de 7 SNPs em três genes candidatos (*SCARB2*, *PSAP* e *CLN8*) a modificadores do fenótipo da DG. A escolha dos genes candidatos foi baseada na função exercida pelas proteínas codificadas e/ou em dados da literatura. O gene *SCARB2* codifica o receptor LIMP-2, que é essencial para o transporte da GCase do RE em direção aos lisossomos (Reczek *et al.*, 2007). O gene *PSAP* codifica 4 saposinas diferentes, dentre elas a Sap-C. A Sap-C é uma proteína importante por ser ativadora da GCase (Tamargo *et al.*, 2012). Por fim, o gene *CLN8* (*Ceroid-Lipofuscinosis Neuronal* 8) codifica a proteína CLN8, cuja função permanece desconhecida. O gene *CLN8* foi identificado como um gene candidato a modificador do fenótipo da DG em um estudo de GWAS (*genome-wide association study*), possivelmente agindo como um neuroprotetor associado ao genótipo N370S/N370S em pacientes com DG (Zhang *et al.*, 2012).

Após a análise dos polimorfismos em pacientes com DG e em indivíduos controles, nenhuma diferença entre as frequências alélicas nestes grupos foi identificada. Em relação a frequência genotípica, o genótipo A/A do rs6532244 (gene *SCARB2*) foi mais frequente em pacientes com DG, especialmente em pacientes com genótipo L444P/L444P. A estratificação dos pacientes de acordo com seu genótipo no gene *GBA1* nos permitiu identificar algumas associações interessantes, como a de que o alelo G no rs7008465 (gene *CLN8*) tem frequência aumentada em pacientes homozigotos para mutação N370S em comparação a pacientes sem N370S em seu genótipo. A comparação entre estes dois grupos de pacientes também revelou diferença estatística nos polimorfismos rs6825004 (gene *SCARB2*) e rs2854992 (gene *PSAP*).

Os resultados obtidos apontam para a realização de abordagens adicionais para aumentarmos a compreensão da relação entre genes modificadores e a DG: análise de *tag* SNPs abrangendo toda a extensão dos genes *SCARB2*, *PSAP* e *CLN8*; inclusão de um maior número de pacientes na amostra; realização de estudos funcionais envolvendo análise da expressão gênica, avaliação de níveis proteicos da GCase e das proteínas-alvo, assim como, silenciamento destes genes utilizando siRNAs específicos.

Além de analisar genes candidatos a modificadores do fenótipo, o terceiro capítulo teve como objetivo principal a identificação de miRNAs específicos que modulam a atividade da GCase. O estudo de miRNAs como moléculas modificadoras da DG foi motivado pela envolvimento destes pequenos RNAs não-codificantes em diversos processos biológicos e patológicos, assim como, pela escassez de dados na literatura abrangendo tal relação. O efeito de 875 miRNAs distintos foi avaliado, tanto em células N370S/N370S de paciente com DG como em células normais (*GBA1* não-mutado), quanto à influência na atividade da GCase. Diversos miRNAs foram identificados como possíveis moduladores da GCase e três deles (miR-127-5p, miR-195-5p e miR-16-5p) escolhidos para um estudo mais aprofundado.

miR-127-5p regula negativamente a atividade da GCase por ação indireta via receptor LIMP-2. Este miRNA reduz a expressão gênica de *SCARB2*, ocasionando diminuição dos níveis de LIMP-2 disponíveis para efetuar o transporte adequado da GCase do RE aos lisossomos. Dessa forma, a GCase tem níveis de secreção aumentada para o espaço extracelular e, consequentemente, níveis reduzidos da enzima alcançam o interior

dos lisossomos. O resultado obtido está de acordo com estudos anteriores em que a deficiência em LIMP-2 acarretou a liberação da enzima para o espaço extracelular e o agravamento dos sintomas no paciente portador de mutação no gene *SCARB2* (Velayati *et al.*, 2011).

Regulação positiva de miR-195-5p e miR-16-5p sobre a atividade da GCase foi observado, levando ao aumento da expressão de *GBA1* e, consequentemente, dos níveis de GCase. Estes miRNAs podem estar interagindo com a região promotora do *GBA1*, estabilizando o mRNA transcrito ou reduzindo os níveis de proteínas que afetam negativamente fatores de transcrição do *GBA1*. Entretanto, o mecanismo envolvido nessa regulação permanece desconhecido. A elucidação dos mecanismos envolvidos nessa regulação positiva da GCase é importante, pois poderá propiciar o desenvolvimento de novas terapias para DG e aumentar o entendimento de fatores envolvidos na regulação desta doença genética rara.

Com base nos dados obtidos nesta tese de doutorado, propomos que a enzima GCase tem um papel central envolvendo vários aspectos (Figura 10). Mutações no gene *GBA1* causam deficiência parcial ou absoluta da GCase. Essa deficiência está relacionada não só com a DG, mas também com o aparecimento de sintomas parkinsonianos na MJD/SCA3, na DP e na DCL. Além disso, outros fatores podem estar influenciando a atividade dessa enzima, dentre eles: alteração em genes modificadores como *SCARB2* e *CLN8* por afetarem as proteínas-alvo codificadas; indução de modulação positiva ou negativa por miRNAs específicos, resultando na regulação direta ou indireta da GCase.

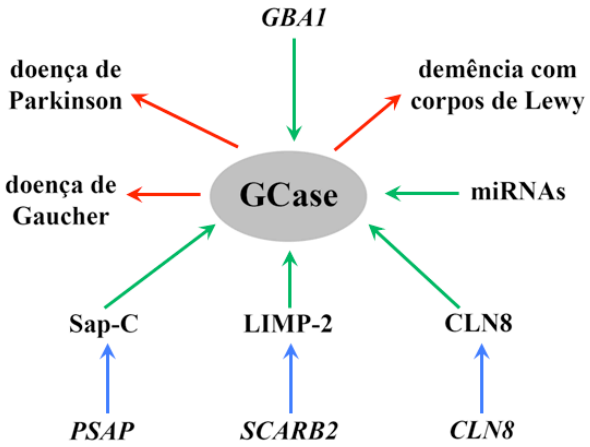


Figura 10. Envolvimento da GCase como fator central envolvendo diversas vias.

Por fim, os resultados apresentados nesse trabalho corroboram que a DG, apesar de ser uma doença monogênica, não é uma doença simples. Diversos fatores parecem estar envolvidos na patologia da mesma, assim como, em doenças influenciadas pela alteração dos níveis da GCase. A realização de estudos funcionais são essenciais para melhorar a abordagem de alguns aspectos identificados nesse trabalho, os quais irão contribuir para o melhor entendimento da patogênese da DG e das rotas metabólicas influenciadas pela GCase a fim de estabelecer novas correlações genótipo-fenótipo e proporcionar fundamentação para o desenvolvimento de novas abordagens terapêuticas.

5. CONCLUSÕES

- Mutações no gene *GBA1* são mais frequentes em pacientes com MJD/SCA3 tipo 4 (com presença de sintomas parkinsonianos) do que em pacientes MJD/SCA3 não-tipo 4 e indivíduos controles;
- Mutações no gene *GBA1* podem ser fator de risco para o desenvolvimento de parkinsonismo em MJD/SCA3;
- Não houve diferença estatisticamente significativa nas frequências alélicas e genotípicas dos polimorfismos nos genes *PSAP* e *CLN8* estudados neste trabalho entre os grupos de pacientes e controles;
- O genótipo A/A no rs6532244, localizado no gene *SCARB2*, mostrou-se mais frequente em pacientes com DG do que em controles, especialmente em pacientes com genótipo L444P/L444P;
- O alelo G no rs7008465 (gene *CLN8*) parece estar mais associado ao genótipo N370S/N370S no gene *GBA1* em pacientes com DG;
- miRNAs influenciam a enzima GCase, sendo que alguns atuam como reguladores positivos e outros como reguladores negativos;
- O miR-127-5p atua indiretamente na regulação da GCase por reduzir os níveis do receptor LIMP-2. Consequentemente, a enzima é secretada para fora da célula;
- Os miRNAs miR-195-5p e miR-16-5p regulam a atividade da GCase por meio da indução transcricional do gene *GBA1*. Entretanto, o mecanismo envolvido nesse processo regulatório permanece desconhecido;
- Os miRNAs miR-127-5p, miR-195-5p e miR-16-5p exercem função regulatória direta ou indireta sobre a atividade da GCase. Estes miRNAs podem ser estudados futuramente como biomarcadores de patogenicidade, de gravidade da DG e como base para o desenvolvimento de novos alvos terapêuticos.

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ANEXO I

Brain Advance Access published February 14, 2014

doi:10.1093/brain/awu002 Brain 2014: Page 1 of 19 | 1

BRAIN
A JOURNAL OF NEUROLOGY

REVIEW ARTICLE

Glucocerebrosidase is shaking up the synucleinopathies

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The lysosomal enzyme glucocerebrosidase, encoded by the glucocerebrosidase gene, is involved in the breakdown of glucocerebroside into glucose and ceramide. Lysosomal build-up of the substrate glucocerebroside occurs in cells of the reticulo-endothelial system in patients with Gaucher disease, a rare lysosomal storage disorder caused by the recessively inherited deficiency of glucocerebrosidase. Gaucher disease has a broad clinical phenotypic spectrum, divided into non-neuronopathic and neuronopathic forms. Like many monogenic diseases, the correlation between clinical manifestations and molecular genotype is not straightforward. There is now a well-established clinical association between mutations in the glucocerebrosidase gene and the development of more prevalent multifactorial disorders including Parkinson's disease and other synucleinopathies. In this review we discuss recent studies advancing our understanding of the cellular relationship between glucocerebrosidase and α-synuclein, the potential impact of established and emerging therapeutics for Gaucher disease for the treatment of the synucleinopathies, and the role of lysosomal pathways in the pathogenesis of these neurodegenerative disorders.

Keywords: glucocerebrosidase; α-synuclein; Gaucher disease; Parkinson's disease; lysosome

Abbreviations: CBE = conduritol-B-epoxide; ERAD = endoplasmic reticulum-associated degradation; GBA1 = glucocerebrosidase; mTOR = mammalian target of rapamycin; PERK = RNA-activated protein kinase-like endoplasmic reticulum kinase

Introduction

The enzyme glucocerebrosidase (EC 3.2.1.45) breaks down the glycolipid glucocerebroside into glucose and ceramide inside lysosomes (Beutler and Grabowski, 2001). Glucocerebrosidase is encoded by the glucocerebrosidase (*GBA1*) gene, which is located on chromosome 1q21 spanning 7.6 kb and includes 11 exons. A highly homologous pseudogene is located 16 kb downstream and presents challenges for the molecular analysis of *GBA1* (Horowitz *et al.*, 1989; Winfield *et al.*, 1997). Mutations in *GBA1* cause glucocerebrosidase deficiency and the subsequent accumulation of the undegraded substrate glucocerebroside inside the lysosomes of cells composing the reticulo-endothelial system. This accumulation of glucocerebroside substrate results in Gaucher disease (OMIM #606463), a rare pan-ethnic autosomal recessive lysosomal storage disorder (Beutler and Grabowski, 2001). The main cell biological function of macrophages is phagocytosis-mediated breakdown of senescent cells such as erythrocytes, which have

Received September 27, 2013. Revised November 22, 2013. Accepted November 25, 2013.

glucocerebroside-rich membranes. Gaucher disease macrophages that have accumulated glucocerebroside appear engorged and are often referred to as 'Gaucher cells'. Gaucher cells primarily populate the spleen, liver and bone marrow, resulting in inflammation and organomegaly (Lachmann *et al.*, 2004; Sidransky, 2012). Although Gaucher disease is rare, it is the most common lysosomal storage disorder with an estimated frequency of 1:40 000–60 000 live births in the general population and an exceptionally high prevalence in the Ashkenazi Jewish population (1:850 individuals) (Beutler *et al.*, 1993; Beutler and Grabowski, 2001). To date, over 300 *GBA1* pathogenic mutations and polymorphisms have been reported (Hruska *et al.*, 2008), but correlations between the clinical phenotype and molecular genotype remain limited (Goker-Alpan *et al.*, 2005; Hruska *et al.*, 2008; Sidransky, 2012). Indeed, studies have shown that patients with identical genotypes can have vastly different clinical manifestations and severity, even between siblings and twins (Lachmann *et al.*, 2004; Sidransky, 2004; Biegstraaten *et al.*, 2011), whereas patients with different molecular genotypes can share similar clinical phenotypes (Hruska *et al.*, 2008). Furthermore, there is not a strong correlation between the amount of accumulated substrate and/or residual glucocerebrosidase enzyme activity and clinical manifestations in patients (Sidransky, 2012). This suggests that Gaucher disease is not a 'simple' monogenic disorder, and that additional factors such as epigenetics and/or genetic modifiers may contribute to disease development and phenotype (Koprivica *et al.*, 2000; Sidransky, 2004; Goker-Alpan *et al.*, 2005).

Gaucher disease is classified into three different types, based on the absence (type 1) or the presence and severity of neurological manifestations (types 2 and 3). Non-neuronopathic type 1 Gaucher disease (OMIM #230800) is the most common form and accounts for >90% of cases in the USA and Europe (Beutler and Grabowski, 2001; Cherin *et al.*, 2010). Clinical manifestations include enlarged liver and spleen, bone complications, anaemia and thrombocytopaenia (Pastores *et al.*, 2000; Beutler and Grabowski, 2001). The extent of symptoms is highly variable and many affected individuals will never reach medical attention. One common *GBA1* mutation, N370S, seems to be exclusively associated with type 1 Gaucher disease, although other mutations can also be seen in patients with Gaucher disease type 1. It has been reported that Gaucher disease type 1 is associated with an increased risk of certain malignancies such as multiple myeloma, hepatocellular carcinoma, non-Hodgkin's lymphoma, malignant melanoma and pancreatic cancer (Zimran *et al.*, 2005; de Fost *et al.*, 2006; Ayto and Hughes, 2013; Mistry *et al.*, 2013; Weinreb and Lee, 2013). Acute neuronopathic or type 2 Gaucher disease (OMIM #230900) is the rarest and most severe form of the disease (Beutler and Grabowski, 2001) with rapidly progressing neurological deterioration, resulting in death within the first years of life (Beutler and Grabowski, 2001; Sidransky, 2004). The onset and progression of neurological symptoms in chronic neuronopathic Gaucher disease type 3 (OMIM #2301000) is later and slower compared with Gaucher disease type 2 (Beutler and Grabowski, 2001; Sidransky, 2012). Clinical manifestations can include myoclonic epilepsy and ataxia, developmental delay, intellectual deterioration, and learning disabilities, in addition to skeletal and visceral involvement (Sidransky, 2004, 2012; Gupta *et al.*, 2011; Sidransky and Lopez, 2012). Patients with type 3 Gaucher disease develop a specific oculomotor abnormality consisting of the slowing or looping of the horizontal saccades. It is often difficult to classify patients as a specific type of Gaucher disease because of the broad range of manifestations encountered (Sidransky, 2004). Thus it may be more appropriate to view Gaucher disease-associated phenotypes as a continuum because of the broad range of associated manifestations observed (Sidransky, 2004, 2012).

The classification of type 1 as the non-neuronopathic form of Gaucher disease has recently been questioned because of its association with synucleinopathies including Parkinson's disease and dementia with Lewy bodies (Sidransky *et al.*, 2009; Nalls *et al.*, 2013). The synucleinopathies include different disorders with parkinsonian features characterized pathologically by the presence of Lewy body inclusions, composed of aggregates of the small unstructured protein α-synuclein (Puschmann *et al.*, 2012). The association between *GBA1* mutations and parkinsonism was first established based on longitudinal clinical studies, in which it was observed that some patients with Gaucher disease developed parkinsonism (Tayebi *et al.*, 2001, 2003; Bembi *et al.*, 2003). It was then recognized that Parkinson's disease was more frequent in first-degree relatives of patients with Gaucher disease. Studies in specific cohorts of patients with Parkinson's disease and associated Lewy body disorders indicated these patients had an increased frequency of *GBA1* mutations compared with control groups (Goker-Alpan *et al.*, 2004; Lwin *et al.*, 2004; Eblan *et al.*, 2006; Ziegler *et al.*, 2007). In 2009, a large international collaborative group including 16 participating centres performed molecular analysis of *GBA1* on >5000 DNA samples from patients with Parkinson's disease and an equal number of controls including subjects with different ethnicities. The resulting odds ratio (OR) of 5.43 clearly demonstrated a strong association between *GBA1* mutations and Parkinson's disease. Moreover, subjects with *GBA1* mutations had an earlier onset of Parkinson's disease symptoms and more frequent cognitive changes (Sidransky *et al.*, 2009). Interestingly, genome-wide association studies initially failed to identify *GBA1* as a genetic risk factor for parkinsonism, but more recent genome-wide association studies have identified specific *GBA1* single nucleotide polymorphisms (Pankratz *et al.*, 2009; Satake *et al.*, 2009; Simon-Sanchez *et al.*, 2009). Since then, this association persisted and has been reproduced in multiple cohorts around the world (Dos Santos *et al.*, 2010; Mao *et al.*, 2010; Huang *et al.*, 2011; Lesage *et al.*, 2011*a*, *b*; Moraitou *et al.*, 2011; Noreau *et al.*, 2011; Anheim *et al.*, 2012; Choi *et al.*, 2012; Emelyanov *et al.*, 2012; Tsuang *et al.*, 2012; Wang *et al.*, 2012; Becker *et al.*, 2013; Kumar *et al.*, 2013). It is now widely accepted that the frequency of *GBA1* mutations in subjects with Parkinson's disease from varied ethnicities is greater than any other genetic risk factor for Parkinson's disease, once common risk variants of low effect are excluded. Recently, this association was expanded to dementia with Lewy bodies, with the identification of *GBA1* mutations in 3.5% to 23% of subjects in genotyping studies of various independent cohorts (Goker-Alpan *et al.*, 2006; Mata *et al.*, 2008). Another large international multicentre study of *GBA1* mutations in dementia with Lewy bodies was undertaken. Eleven centres contributed a total of 721 cases with dementia with Lewy bodies and 151 cases

of Parkinson's disease with dementia, which were compared with 1962 control subjects, matched for age, sex and ethnicity. A significant association between *GBA1* mutations and dementia with Lewy bodies, as well as Parkinson's disease with dementia, was established, with odds ratios of 8.28 and 6.48, respectively. Similar to Parkinson's disease, the age of diagnosis of dementia with Lewy bodies in patients with *GBA1* mutations was younger compared to dementia with Lewy bodies without *GBA1* mutations (Nalls *et al.*, 2013). These *GBA1* studies establish its involvement in several synucleinopathies, although *GBA1* mutations are not seen with multiple system atrophy, an α-synucleinopathy with α-synuclein inclusions mainly in glial oligodendrocytes (Spillantini *et al.*, 1998; Beyer and Ariza, 2007; Segarane *et al.*, 2009; Jamrozik *et al.*, 2010; Srulijes *et al.*, 2013; Sun *et al.*, 2013*a*).

The major pathological characteristics of Parkinson's disease and dementia with Lewy bodies are the presence of insoluble oligomeric and fibrillar α-synuclein-positive inclusions known as Lewy bodies and Lewy neurites in neurons in the substantia nigra, cerebral cortex, and hippocampus and the selective loss of dopaminergic neurons in the midbrain (Puschmann *et al.*, 2012). Aggregation of α-synuclein seems to correlate with the onset and progression of synucleinopathies, and its direct role in disease manifestation is clear from the association of familial Parkinson's disease with mutations, duplications, and triplications in the α-synuclein gene (Fearnley and Lees, 1991; Puschmann, 2013). Monomeric α-synuclein is a small 14 kDa protein that is highly expressed in the brain, where it is likely involved in the regulation of synaptic vesicle clustering and the release of neurotransmitters through interaction with lipids and members of the soluble NSF Attachment Protein Receptor complex assembly machinery (Abeliovich *et al.*, 2000; Cabin *et al.*, 2002; Burre *et al.*, 2010; Garcia-Reitbock *et al.*, 2010; Ito *et al.*, 2012; Diao *et al.*, 2013), but its exact biological function remains elusive. Monomers, fibrils, and aggregates of α-synuclein can undergo transmission between cells, a process that may be facilitated by small 50–100 nm vesicles called exosomes that are released into the extracellular environment upon exocytosis of multivesicular bodies (Desplats *et al.*, 2009; Alvarez-Erviti *et al.*, 2011; Hansen *et al.*, 2011; Russo *et al.*, 2012; Chang *et al.*, 2013). The neuropathology in Parkinson's disease with *GBA1* mutations is similar to other synucleinopathies without *GBA1* mutations; α-synuclein-positive Lewy bodies are found in the brains of patients with Parkinson's disease and dementia with Lewy bodies with *GBA1* mutations (Neumann *et al.*, 2009; Goker-Alpan *et al.*, 2010). The exact molecular mechanisms involved in the interaction between glucocerebrosidase and α-synuclein remain unresolved, but experimental data indicate that there is a reciprocal relationship between glucocerebrosidase activity and α-synuclein. When the delicate balance of α-synuclein homeostasis is disturbed, possibly by impairment of essential protein turnover pathways such as the unfolded protein response, endoplasmic reticulum-associated degradation and autophagy, cell stress or environmental factors, an increase in α-synuclein levels will inhibit translocation of glucocerebrosidase from the endoplasmic reticulum to the lysosome. In turn, less lysosomal glucocerebrosidase leads to a gradual increase in glucocerebroside substrate and subsequent oligomerization and accumulation of α-synuclein in the lysosomes. Eventually, the lysosomes become dysfunctional, and autophagy-mediated α-synuclein turnover is impaired, leading to α-synuclein aggregates in the cytoplasm, which, in turn, inhibit trafficking from the endoplasmic reticulum to the lysosome. This positive feedback loop of dysfunctional glucocerebrosidase trafficking, impaired lysosomal function, and progressive α-synuclein accumulation will eventually cause neurodegeneration (Mazzulli *et al.*, 2011) (Fig. 1). A recent *in vivo* observation of reduced enzyme activity and protein levels of glucocerebrosidase in the substantia nigra of brains from patients with Parkinson's disease without *GBA1* mutations supports this reciprocal relationship, and expands our understanding of the key function of glucocerebrosidase in the pathology of synucleinopathies (Gegg *et al.*, 2012).

As most subjects with Gaucher disease never develop Parkinson's disease, *GBA1* mutations and a reduction in glucocerebrosidase enzyme activity alone cannot be the cause of Parkinson's disease or dementia with Lewy bodies. As Parkinson's disease and dementia with Lewy bodies are disorders associated with ageing, it is likely that cellular processes impacted during the ageing process are linked with Parkinson's disease pathogenesis. Indeed, it has been reported that ageing is associated with the diminished function of tightly regulated protein and organelle homeostasis pathways such as autophagy-lysosomal function (Cook *et al.*, 2012), endoplasmic reticulum stress (Lu *et al.*, 2014), and mitochondrial stress (Lu *et al.*, 2014; Venkataraman *et al.*, 2013). The critical balance between α-synuclein proteostasis and optimal function of the proteasome and/or autophagy-lysosome-mediated breakdown may become compromised during ageing. Mutant, absent, or downregulated glucocerebrosidase could contribute to this scenario by further compromising these cellular pathways. In this review, we will discuss new insights into the reciprocal relationship between glucocerebrosidase and α-synuclein, the implications of deficient α-synuclein breakdown in key quality control and turnover pathways, modifiers of *GBA1* that might contribute to the development of Parkinson's disease, Gaucher disease therapeutics and their implications for the treatment of synucleinopathies, and the expansion of Parkinson's disease risk factors to enzymes involved in other lysosomal storage disorders.

Cellular role of glucocerebrosidase in the neuropathology of synucleinophathies

The reciprocal relationship between glucocerebrosidase, α-synuclein and lysosomal impairment

The involvement of glucocerebrosidase in the pathogenesis of the synucleinopathies is still not completely understood. Initially, arguments were made for either a gain-of-function or loss-of-function model. The gain-of-function model implies that the aberrant enzyme is directly involved in α-synuclein

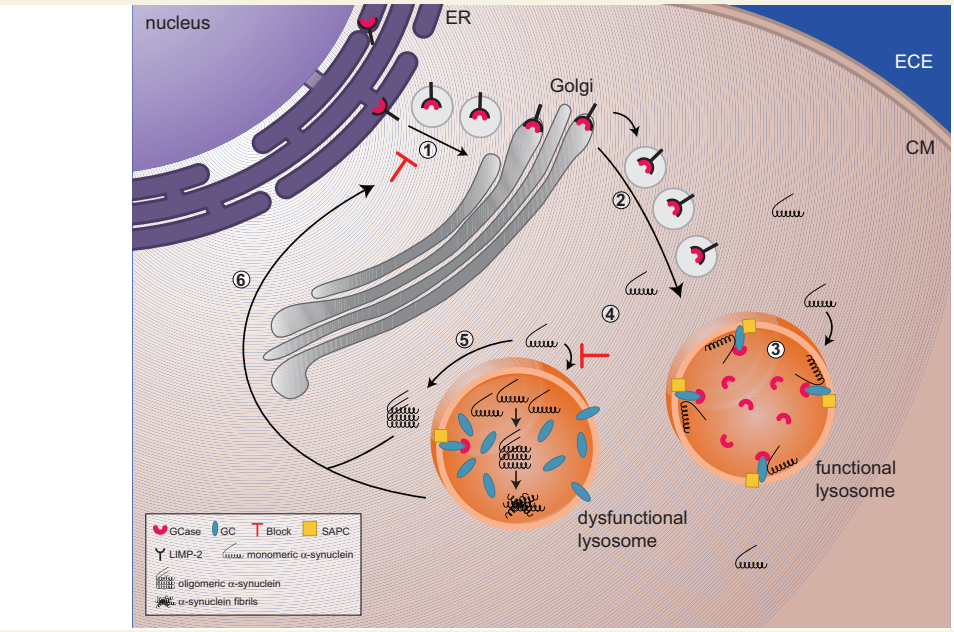

Figure 1 The reciprocal relationship between glucocerebrosidase and α-synuclein. (1 and 2) Glucocerebrosidase (GCase) is sorted via the endoplasmic reticulum and Golgi to the lysosomes. (3) In lysosomes, glucocerebrosidase interacts with its substrate glucocerebroside (GC) as well as monomers of α-synuclein, facilitating the breakdown of both at acidic pH. (4) Decreased levels of glucocerebrosidase will result in a slowdown of α-synuclein degradation and a gradual build up of glucocerebroside substrate, with the eventual formation of α-synuclein oligomers and fibrils. (4) Impaired lysosomes, as a result of build up of substrate and/or α-synuclein oligomers and fibrils, will show impaired chaperone-mediated autophagy and autophagosome fusion, which implies that α-synuclein cannot undergo autophagy-mediated degradation, resulting in an increased accumulation of α-synuclein in the cytoplasm. (5) These soluble monomers will eventually assemble in oligomers and will block trafficking of glucocerebrosidase from the endoplasmic reticulum (ER) to the Golgi.
ECE = extracellular environment; CM = Cell Membrane; SAPC = saposin C.

aggregation through a biochemical interaction with α-synuclein or interference with α-synuclein homeostasis pathways such as the unfolded protein response and autophagy. Evidence for gain-of-function was provided in a comprehensive study by Cullen *et al*. (2011) where it was shown that transient over-expression of different *GBA1* mutant constructs in MES23.5 and PC12 cells expressing wild-type α-synuclein promoted accumulation of α-synuclein in a time- and dose-dependent manner that was independent of glucocerebrosidase enzyme activity status. Furthermore, an *in vivo* mouse model with mutant *Gba* ($Gba^{D409V/D409V}$), demonstrated age-dependent accumulation of α-synuclein. However, the contribution of reduced enzyme activity, which was 20% of control, to the α-synuclein accumulation process could not be ruled out in this model (Cullen *et al.*, 2011). In the loss-of-function model, the progressive build up of glucocerebroside substrate in lysosomes may directly promote α-synuclein aggregation, or have an indirect effect because of altered lysosomal pH and/or diminished function of lysosomal and autophagy-mediated breakdown pathways. Studies on cell and mouse models, as well as patient samples, have provided evidence for both models (Westbroek *et al.*, 2011). In the context of these two models, the E326K mutation deserves special mention as it has long been the subject of controversy. Independent studies have established that E326K is associated with Parkinson's disease (Horowitz *et al.*, 2011; Pankratz *et al.*, 2012; Duran *et al.*, 2013), but this mutation is believed to be non-pathogenic (Park *et al.*, 2002; Liou and Grabowski, 2012) with only a significant effect on enzyme activity when found in combination with other pathogenic *GBA1* mutations (Montfort *et al.*, 2004; Horowitz *et al.*, 2011; Liou and Grabowski, 2012; Malini *et al.*, 2013). This suggests that in the case of E326K, reduced glucocerebrosidase activity might not be the principal factor contributing to the development of Parkinson's disease.

Several experimental observations have expanded on the model of a reciprocal relationship as the basis for a mechanistic link between glucocerebrosidase and α-synuclein. The effect of manipulation of glucocerebrosidase enzyme activity or expression levels on α-synuclein accumulation was demonstrated in mice and neuroblastoma cells treated with the glucocerebrosidase inhibitor conduritol-B-epoxide (CBE), which showed significant accumulation of α-synuclein protein, but no change in messenger RNA levels (Manning-Bog *et al.*, 2009; Cleeter *et al.*, 2013). However, α-synuclein accumulation or compromised lysosomal function could not be observed in long-term CBE-treated

SH-SY5Y neuroblastoma cells or primary rat cortical neurons, indicating that deficient glucocerebrosidase activity alone does not alter α-synuclein in these cell models (Dermentzaki *et al.*, 2013). Interestingly, primary mouse neurons silenced for *GBA1* expression showed enhanced α-synuclein accumulation that was not observed in the neuroblastoma cell line (Mazzulli *et al.*, 2011; Cleeter *et al.*, 2013). Cullen and co-workers showed that over-expression of wild-type *GBA1* in HEK293 cells expressing A53T α-synuclein and PC12 cells expressing wild-type α-synuclein induced down-regulation of α-synuclein levels. However, this observation appeared to be cell-model specific, since this was not observed with transiently transfected wild-type *GBA1* in MES23.5 cells expressing wild-type α-synuclein (Cullen *et al.*, 2011). *In vivo* studies by Sardi *et al*. (2011, 2013) showed that adenovirus-mediated expression of wild-type glucocerebrosidase in the CNS of mice with features of neuronopathic Gaucher disease corrected substrate accumulation, cognitive impairment, and α-synuclein aggregation in the brain, and in transgenic mice over-expressing A53T α-synuclein without *GBA1* mutations, adenovirus-mediated expression of wild-type glucocerebrosidase reduced α-synuclein levels (Sardi *et al.*, 2011, 2013). Additionally, increasing α-synuclein levels downregulated glucocerebrosidase activity and protein levels in several *in vitro* and *in vivo* models of Parkinson's disease. Biophysical studies indicate that at acidic pH, there is a direct interaction between wild-type α-synuclein and glucocerebrosidase that is speculated to be beneficial for α-synuclein turnover in the lysosome. Additional biophysical evaluations indicate that α-synuclein inhibits glucocerebrosidase activity in a dose-dependent manner (Yap *et al.*, 2011, 2013). In cells, increased levels of α-synuclein caused a reduction in wild-type glucocerebrosidase activity, and to a lesser extent, glucocerebrosidase protein levels (Mazzulli *et al.*, 2011; Gegg *et al.*, 2012). Studies performed in a limited number of post-mortem brain samples from subjects with sporadic Parkinson's disease without *GBA1* mutations, show a significant decrease in glucocerebrosidase activity and protein levels in the substantia nigra and cerebellum (Gegg *et al.*, 2012). The pivotal experimental evidence regarding the reciprocal relationship between glucocerebrosidase and α-synuclein came from an elaborate and comprehensive study by Mazzulli and co-workers (2011), where it was shown that partial loss of glucocerebrosidase activity in primary mouse neurons, or human neuronal cultures derived from induced pluripotent stem cells, interfered with protein degradation in the lysosome, promoted accumulation of α-synuclein, and enhanced α-synuclein-mediated neurotoxicity. *In vitro*, glucocerebroside substrate was shown to promote stabilization of α-synuclein oligomers and aggregation. Over-expression of α-synuclein inhibited endoplasmic reticulum-to-Golgi trafficking of glucocerebrosidase, presumably by inhibiting formation of soluble NSF attachment protein receptor complexes (Cooper *et al.*, 2006; Thayanidhi *et al.*, 2010), which resulted in the downregulation of lysosome-resident glucocerebrosidase. Analyses of brain samples indicated that soluble α-synuclein oligomers were increased in both subjects with neuronopathic Gaucher disease and subjects with Parkinson's disease with *GBA1* mutations; however, brain samples from subjects with Parkinson's disease without *GBA1* mutations were not included in this study (Mazzulli *et al.*, 2011). This is in contrast with a study where α-synuclein solubility was measured in brain samples from patients with Gaucher disease as well as patients with synucleinopathies with and without *GBA1* mutations, demonstrating the presence of α-synuclein aggregates exclusively in the brains from subjects known to have synucleinopathies (Choi *et al.*, 2011). It should be noted that both studies used different methods for brain lysate preparation and analysis. An *in vivo* study on induced pluripotent stem cells-derived cortical neurons from a patient with the A53T mutation in α-synuclein showed an increase in the ratio of endoplasmic reticulum-resident to post-endoplasmic reticulum glucocerebrosidase compared to isogenic gene-edited wild-type cortical neurons. This was also observed in brain samples from a subject with A53T α-synuclein and in cortex samples from patients with sporadic Parkinson's disease. These observations suggest that glucocerebrosidase trafficking from the endoplasmic reticulum to the lysosomes was significantly reduced in A53T cells as well as Parkinson's disease brain samples (Chung *et al.*, 2013). Thus, a majority of the studies described provide evidence supporting a reciprocal relationship model between glucocerebrosidase and α-synuclein (Fig. 1). The central player in this reciprocal relationship model is the lysosome, which is the main organelle responsible for the degradation of proteins, lipids and organelles such as defective mitochondria (Dehay *et al.*, 2013). Lysosome-mediated degradation of α-synuclein occurs through both macroautophagy and chaperone-mediated autophagy. Proteins destined for chaperone-mediated autophagy, such as α-synuclein, form a complex with heat shock cognate 70, which is targeted to the lysosomal membrane where it interacts with lysosomal associated membrane protein 2A, and undergoes translocation to the lysosome, followed by degradation (Arias and Cuervo, 2011); impaired chaperone-mediated autophagy has been reported in Parkinson's disease brain samples (Alvarez-Erviti *et al.*, 2010). In macroautophagy, double-membraned autophagosomes carry engulfed proteins, lipids and organelles destined for breakdown to lysosomes. This occurs via the formation of the autophagolysosome, resulting from the fusion of autophagosomes with lysosomes (Yang and Klionsky, 2010). Accumulating evidence indicates that lysosomal impairment contributes to the neuropathology of Parkinson's disease (Tofaris, 2012) and in turn, α-synuclein aggregation can impair autophagy and lysosomal function (Winslow *et al.*, 2010). A well-illustrated example of the implications of lysosomal dysfunction in Parkinson's disease is the *PARK9* gene, which encodes the ATP13A2 protein, a lysosomal P-type ATPase. Mutations in *PARK9* have been shown to cause severe lysosomal impairment and accumulation of α-synuclein, and are associated with Kufor-Rakeb syndrome, a rare genetic form of parkinsonism (Schultheis *et al.*, 2013). Autophagy and lysosomal dysfunction have been reported in models and in tissue samples from subjects with *GBA1*-associated Parkinson's disease. In primary cortical wild-type mouse neurons infected with lentiviral α-synuclein and *GBA1* short hairpin RNA, resulting in 50% reduction in glucocerebrosidase protein levels, lysosomal protein turnover capacity was impaired, and enlarged lysosomal-associated membrane protein 1-positive structures accumulated. These results were validated in dopaminergic neurons generated from induced pluripotent stem cells derived from Gaucher disease fibroblasts (Mazzulli *et al.*, 2011). Inhibiting glucocerebrosidase with CBE or

partial *GBA1* silencing in SHSY-5Y neuroblastoma cells did not change the lysosomal content or the levels of LC3-II, which corresponds to the number of accumulated autophagosomes (Cleeter *et al.*, 2013). Dermentzaki *et al.* (2013) saw a slight but significant increase in LC3-II levels in SHSY-5Y cells treated with CBE for 100 days. Similar observations were made in brain samples from subjects with both sporadic Parkinson's disease and *GBA1*-associated Parkinson's disease (Gegg *et al.*, 2012). In a *Gba*$^{-/-}$ mouse model representative of neuronopathic type 2 Gaucher disease, impairment of autophagy, mitophagy and also the proteasome-mediated degradation pathway were observed in midbrain neurons and astrocytes. This resulted in the accumulation of fragmented mitochondria and α-synuclein. Although this model does not represent Parkinson's disease, it should be noted that α-synuclein pathology was present. This finding clearly illustrates that dysfunction in the reciprocal balance between glucocerebrosidase and α-synuclein can lead to neurodegeneration (Osellame *et al.*, 2013).

The unfolded protein response

Chaperones, lectins and foldases all assist with the protein folding process, but it is believed that about one-third of all newly synthesized proteins are still damaged or misfolded (Schubert *et al.*, 2000). To maintain balanced cell proteostasis, aberrant proteins are turned over via endoplasmic reticulum-associated degradation (ERAD) through ubiquitination by ubiquitin ligases and subsequent 26S proteasome-mediated degradation (Kaufman, 2002; Goldberg, 2003). In the case of persistent accumulation, the unfolded protein response is activated through three endoplasmic reticulum transmembrane sensor proteins: activating transcription factor 6 (ATF6), type I transmembrane protein kinase and endoribonuclease (IRE1), and RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), which are negatively regulated by binding of BiP, a member of the heat shock protein 70 protein family. In the presence of excessive unfolded proteins, BiP is recruited to the endoplasmic reticulum lumen, where it activates IRE1, ATF6 and PERK by its dissociation. The three sensors initiate various signalling pathways, which include restoration of endoplasmic reticulum homeostasis by the regulation of the endoplasmic reticulum folding load recovery process through induction of ERAD and protein folding chaperones, attenuation of protein translation, and, if the restoration of endoplasmic reticulum homeostasis fails, cell death (Germain, 2008; Chakrabarti *et al.*, 2011; Brodsky, 2012; Sano and Reed, 2013).

Several recent studies indicate that both ERAD and the unfolded protein response play crucial roles in the cellular pathology of Parkinson's disease. Experimental cell and mouse models and studies in post-mortem Parkinson's disease brain samples indicate that the first stages of α-synuclein accumulation and aggregation lead to induction of the unfolded protein response, with upregulation of unfolded protein response markers in dopaminergic neurons and eventual neurodegeneration (Bellucci *et al.*, 2011; Colla *et al.*, 2012*a*, *b*; Gorbatyuk *et al.*, 2012; Hoozemans *et al.*, 2012).

The role of glucocerebrosidase in endoplasmic reticulum stress and neurodegeneration has only recently begun to emerge. Under normal conditions, newly synthesized glucocerebrosidase is correctly folded in the endoplasmic reticulum and is then translocated to the lysosomes. Aberrant glucocerebrosidase fails to fold correctly, is arrested in the endoplasmic reticulum and is redirected to undergo polyubiquitination, followed by degradation. Studies on fibroblasts derived from patients with Gaucher disease have established that glucocerebrosidase mutants undergo polyubiquitination and proteasomal degradation via ERAD (Ron and Horowitz, 2005; Bendikov-Bar *et al.*, 2011; Bendikov-Bar and Horowitz, 2012). Heat shock protein 90β was identified as the key chaperone for the redirection of aberrant glucocerebrosidase for breakdown via ERAD (Lu *et al.*, 2010, 2011; Yang *et al.*, 2013). Several E3-ubiquitin ligases such as ITCH, c-Cbl, and parkin recognize mutant glucocerebrosidase as their substrate for polyubiquitination (Lu *et al.*, 2010; Ron *et al.*, 2010; Maor *et al.*, 2013). As a result of ERAD, lysosomal levels of glucocerebrosidase are significantly decreased, resulting in the accumulation of glucocerebroside substrate. It was suggested that the severity of Gaucher disease symptoms might correlate with the degree of endoplasmic reticulum retention of mutant glucocerebrosidase (Ron and Horowitz, 2005; Bendikov-Bar *et al.*, 2011; Bendikov-Bar and Horowitz, 2012). Persistent accumulation of aberrant proteins can activate the unfolded protein response, which has been reported in human fibroblasts derived from patients with Gaucher disease and carriers of *GBA1* mutations (Brady *et al.*, 1974; Mu *et al.*, 2008; Wei *et al.*, 2008; Lee *et al.*, 2011; Maor *et al.*, 2013). Fly models corresponding to *GBA1* carriers and transgenic flies expressing human N370S or L444P glucocerebrosidase mutants both showed unfolded protein response activation and both the development of a locomotion impairment reminiscent of parkinsonian features and an early death (Maor *et al.*, 2013). Activation of the unfolded protein response in Gaucher disease and carriers of *GBA1* mutations support a previous report demonstrating that the interaction of mutant glucocerebrosidase with the E3-ubiquitin ligase parkin leads to K48-mediated polyubiquitination of glucocerebrosidase, before breakdown in the proteasome. It was proposed that this interaction might impair interactions with other, potentially neurotoxic, parkin substrates, and that interference with their breakdown would result in endoplasmic reticulum stress, followed by unfolded protein response activation and the subsequent elevation of ERAD. This, in turn, would lead to further accumulation of neurotoxic substrates, resulting in cell death (Ron *et al.*, 2010). However, a study performed on parkin-deficient fibroblasts provided evidence that parkin is not a crucial E3-ubiquitin ligase for glucocerebrosidase (McNeill *et al.*, 2013). Not every Gaucher disease model demonstrates unfolded protein response activation. CBE-treated cultured primary mouse neurons and astrocytes show no changes in expression of common unfolded protein response makers (Farfel-Becker *et al.*, 2009). The same was observed for select brain regions of the two mouse models representative of neuronopathic Gaucher disease; the *Gba*$^{-/-}$ knock-out (Tybulewicz *et al.*, 1992) and the conditional *Gba*$^{-/-}$ knock-out, which is restricted to neural and glial progenitor cells (Enquist *et al.*, 2007) as well as the *Gba*$^{L444P/L444P}$mouse, a model with partial enzyme deficiency and no neurological involvement (Mizukami *et al.*, 2002; Farfel-Becker *et al.*, 2009). Cullen *et al.* (2011) failed to detect activation of the unfolded protein response in MES23.5 cells expressing wild-type α-synuclein that were transiently transfected with mutant *GBA1* constructs.

Finally, an increase in unfolded protein response markers was seen in post-mortem brain samples from subjects with sporadic Parkinson's disease with and without *GBA1* mutations (Gegg *et al.*, 2012). ERAD of aberrant glucocerebrosidase protein, or endoplasmic reticulum retention of wild-type glucocerebrosidase as a result of α-synuclein mediated blocking of trafficking, might contribute to the increased unfolded protein response. This remains speculative because increased unfolded protein response could also be induced by other pathways associated with Parkinson's disease, such as a malfunction in Ca^{2+} release, metabolic stress, inflammation, and mitochondrial oxidative stress (Doyle *et al.*, 2011; Wang and Kaufman, 2012). The exact relationship between glucocerebrosidase, α-synuclein accumulation, and the role of the unfolded protein response remains to be fully determined.

Mitochondrial dysfunction

Growing evidence indicates that mitochondrial turnover, dysfunction, and oxidative stress play key roles in the development and progression of Parkinson's disease. Indeed, mutations in three genes (*PINK1*, *PARK2* and *PARK7*) involved in these pathways cause familial Parkinson's disease (Puschmann, 2013). The expression, activity and mitochondrial localization of the DJ-1 protein, which is encoded by the *PARK7* gene, are regulated by oxidative stress. DJ-1 is a neuroprotective protein that protects cells from oxidative stress-induced death by positively regulating pathways such as mitophagy. High levels of oxidation or genetic mutations can inactivate DJ-1, which induces impairments in complex I activity and subsequent reactive oxygen species production, reduced membrane potential and fragmented mitochondrial morphology (Ariga *et al.*, 2013). In healthy cells, mitochondrial turnover is regulated by PTEN-induced putative kinase 1 (PINK1) and the E3-ubiquitin ligase parkin in a process called mitophagy, which is the autophagy of damaged mitochondria. In functional mitochondria, PINK1 is translocated from the outer membrane to the inner membrane for degradation, while in dysfunctional mitochondria with a reduced membrane potential, PINK1 will accumulate on the outer mitochondrial membrane and serve as a receptor for recruitment of parkin (Jin *et al.*, 2010; Lazarou *et al.*, 2012). Parkin-mediated polyubiquitination of mitochondrial proteins recruits p62, which induces the aggregation of damaged mitochondria (Narendra *et al.*, 2010). Failure of the turnover of accumulated damaged mitochondria by mitophagy leads to cell death.

Evidence for mitochondrial involvement in *GBA1*-associated Parkinson's disease has now emerged (Gegg *et al.*, 2012; Cleeter *et al.*, 2013; Osellame *et al.*, 2013). To mimic glucocerebrosidase deficiency, Cleeter and co-workers (2013) treated the human neuroblastoma SHSY-5Y cell line with the glucocerebrosidase suicide inhibitor CBE. Long-term treatment with CBE resulted in fragmentation of mitochondria, significant progressive decline in mitochondrial membrane potential, reduction of ATP synthesis and an increase in reactive oxygen species production. Although α-synuclein levels increased by ~50%, surprisingly, the major routes for protein degradation and turnover such as the ERAD and the autophagy-lysosomal pathways were not affected. Although a stable partial *GBA1* knockdown of 62% of enzyme activity confirmed the decline in mitochondrial membrane potential and increase in oxidative stress, it failed to show significant accumulation of α-synuclein (Cleeter *et al.*, 2013). Another study performed on cultured primary midbrain neurons and astrocytes derived from a mouse model representative of acute neuronopathic Gaucher disease, revealed severe impairments in autophagy and ubiquitin-proteasome-mediated protein breakdown pathways, resulting in the accumulation of insoluble α-synuclein and ubiquitinated proteins. Mitochondrial dysfunction, due to defective mitochondrial complex I, led to increased reactive oxygen species production. Fragmented and dysfunctional mitochondria were not turned over by mitophagy because of a failure of recruitment of parkin to the mitochondrial membrane (Osellame *et al.*, 2013). Both studies provide evidence for a link between mitochondrial function and the turnover and inhibition or absence of glucocerebrosidase. These studies indicate that there are similarities in cellular pathophysiological mechanisms in both type 2 Gaucher disease and Parkinson's disease with regard to protein and organelle turnover pathways and energy homeostasis. To date, no studies using relevant neuronal cell and animal models or brain samples representing Parkinson's disease with *GBA1* mutations have addressed mitophagy and energy homeostasis. However, a study on SHSY-5Y cells silenced for *PINK1*, a cell model for familial Parkinson's disease, showed significant reduction of glucocerebrosidase protein levels and activity that could be rescued by exogenous expression of wild-type *PINK1* (Gegg *et al.*, 2012).

Regulators of *GBA1* and/or glucocerebrosidase expression and their implications in the synucleinopathies

It is now clear that Gaucher disease encompasses a broad spectrum of clinical phenotypes, with limited correlation between genotype and phenotype (Sidransky, 2004, 2012). This suggests the involvement of modifier genes that can interact with the disease-causing allele and influence its phenotypic expression (Goker-Alpan *et al.*, 2005). Several potential modifiers for Gaucher disease have been proposed (Latham *et al.*, 1990; Winfield *et al.*, 1997; Montfort *et al.*, 2004). Although mutations in *GBA1* are a common risk factor for the development of Parkinson's disease, only a fraction of patients with Gaucher disease and carriers for *GBA1* mutations develop Parkinson's disease (Sidransky *et al.*, 2009). This leads us to speculate that potential disease modifiers in this process might serve as additional risk factors that, in combination with *GBA1* mutations, might favour the development and progression of synucleinopathies.

SCARB2/LIMP2: a genetic modifier of *GBA1*

Although the majority of lysosomal enzymes reach their destination via the mannose-6-phosphate receptor pathway, a subset

gets sorted through mannose-6-phosphate receptor-independent pathways (Coutinho *et al.*, 2012). One of these enzymes is glucocerebrosidase, which reaches the lysosome via lysosomal integral membrane protein type 2 (LIMP-2) mediated trafficking (Reczek *et al.*, 2007) (Fig. 2). LIMP-2, encoded by the scavenger receptor class B member 2 (*SCARB2*) gene located on chromosome 4q13-21, belongs to the CD36 family of scavenger receptor proteins (Fujita *et al.*, 1991; Febbraio *et al.*, 2001; Berkovic *et al.*, 2008). LIMP-2, which is ubiquitously expressed, is one of the most abundant transmembrane proteins in the lysosomal membrane (Eskelinen *et al.*, 2003); mutation and over-expression studies suggest that it plays a role in the biogenesis and maintenance of late endosomes and lysosomes (Kuronita *et al.*, 2002; Eskelinen *et al.*, 2003), as well as in the fusion between lysosomes and autophagosomes (Gleich *et al.*, 2013).

It was not until 2007 that LIMP-2 was identified as the receptor required for the trafficking of glucocerebrosidase to the lysosomes (Reczek *et al.*, 2007). Protein interaction studies showed a pH-dependent interaction between LIMP-2 and glucocerebrosidase, which was regulated by the pH-sensor amino acid histidine 171 (Zachos *et al.*, 2012). This direct interaction between glucocerebrosidase and LIMP-2 is initiated at neutral pH within the endoplasmic reticulum, and is disrupted upon reaching the acidic lysosome (Reczek *et al.*, 2007; Blanz *et al.*, 2010; Zachos *et al.*, 2012). Studies of *SCARB2* knock-out mice showed *GBA1* messenger RNA levels were not affected, but there was a decrease in glucocerebrosidase activity and protein levels along with lysosomal accumulation of glucocerebroside (Reczek *et al.*, 2007). Conditioned media taken from *SCARB2* knock-out cells in culture demonstrate that glucocerebrosidase is secreted into the extracellular environment as a result of impaired trafficking of glucocerebrosidase (Reczek *et al.*, 2007; Velayati *et al.*, 2011). Mutations in *SCARB2* are associated with action myoclonus-renal failure syndrome (OMIM #254900), an autosomal recessive disorder characterized by renal pathology, progressive myoclonus epilepsy and ataxia (Berkovic *et al.*, 2008; Blanz *et al.*, 2010). Studies on four different mutant *SCARB2* lines showed that the mutant proteins are retained in the endoplasmic reticulum and affect glucocerebrosidase activity in the lysosomes by binding to glucocerebrosidase in the endoplasmic reticulum and preventing its translocation to the lysosomes (Balreira *et al.*, 2008; Blanz *et al.*, 2010). *SCARB2* was recently identified as a genetic modifier for *GBA1* in a study of a unique pair of siblings who had discordant Gaucher disease phenotypes but identical genotypes. One sib suffered myoclonic seizures and sequencing of his *SCARB2* gene revealed a novel heterozygous c.1412A>G (p.Glu471Gly) mutation in one allele, which was absent from the brother. Expression studies in fibroblasts from this patient revealed significant downregulation of LIMP-2 and glucocerebrosidase protein levels, as well as glucocerebrosidase enzyme activity. Secretion of mature glucocerebrosidase into the extracellular environment was observed (Velayati *et al.*, 2011). As LIMP-2 is crucial for the correct trafficking of glucocerebrosidase, and LIMP-2 malfunction can lead to a reduction in glucocerebrosidase levels and activity, it is tempting to speculate a role for LIMP-2 in the development of Parkinson's disease. *SCARB2* mutations and Parkinson's disease could be related through the modulation of glucocerebrosidase protein levels and activity in the cell. LIMP-2 deficiency could lead to glucocerebrosidase secretion instead of proper delivery to the lysosome, which could result in accumulation of glucocerebroside substrate, alterations in lysosomal function, and aggregation of proteins such as α-synuclein inside the lysosomes. It was demonstrated in a cell model over-expressing α-synuclein that less glucocerebrosidase was bound to LIMP-2, which indicates less translocation of glucocerebrosidase to the lysosome (Gegg *et al.*, 2012).

It still remains unclear how a variation near or inside *SCARB2* could be associated with Parkinson's disease (Hopfner *et al.*, 2013). Recent genetic-based evidence has suggested an association between *SCARB2* and Parkinson's disease. Genome-wide association studies identified an association between rs6812193, a single nucleotide polymorphism located upstream of the *SCARB2* gene, and Parkinson's disease (OR = 0.84) in a population of European ancestry (Do *et al.*, 2011). The single nucleotide polymorphism is located in an intron of *FAM47E*, a gene encoding a protein of unknown function (Do *et al.*, 2011). This association was confirmed by the International Parkinson's Disease Genomics Consortium (2011) in a two-stage meta-analysis, and further supported by an independent genotyping study of 984 patients with Parkinson's disease and 1014 controls of German/Austrian descent (Hopfner *et al.*, 2013). However, the association was not seen in a Chinese study of 449 patients with Parkinson's disease and 452 controls (Chen *et al.*, 2012; Hopfner *et al.*, 2013). A candidate gene screen, performed on 347 subjects with sporadic Parkinson's disease and 329 controls from Greece, revealed an additional single nucleotide polymorphism, rs6825004, located within intron 2 of *SCARB2*, that appeared to be associated with Parkinson's disease (OR = 0.68). However, the authors recognized the limitations in their study because of the small sample size (Michelakakis *et al.*, 2012). In another small study, the presence of rs6812193 and/or rs6825004 single nucleotide polymorphisms and corresponding *SCARB2* and LIMP-2 expression levels were assessed in 15 lymphocyte and leucocyte samples derived from individuals without Parkinson's disease. There was no indication that the *SCARB2* single nucleotide polymorphism genotypes described were associated with the modulation of *SCARB2* messenger RNA and LIMP-2 protein expression levels (Maniwang *et al.*, 2013).

Saposin C: an activator of glucocerebrosidase

Mature saposin C (SAPC) is a glucocerebrosidase enzyme activator in lysosomes and is essential in the hydrolysis of glucocerebroside (Beutler and Grabowski, 2001; Sidransky, 2004; Vaccaro *et al.*, 2010), but the mechanism for this activation is not fully understood. Saposin A, B, C, and D are small homologous glycoproteins with six cysteine residues forming disulphide bridges. The bridges are crucial for saposin C function (Tamargo *et al.*, 2012). Saposin proteins are generated through proteolytic cathepsin D-mediated cleavage of its precursor prosaposin (Hiraiwa *et al.*, 1997; Yuan and Morales, 2011). Biophysical experimental evidence indicates that saposin C-mediated extraction and solubilization of

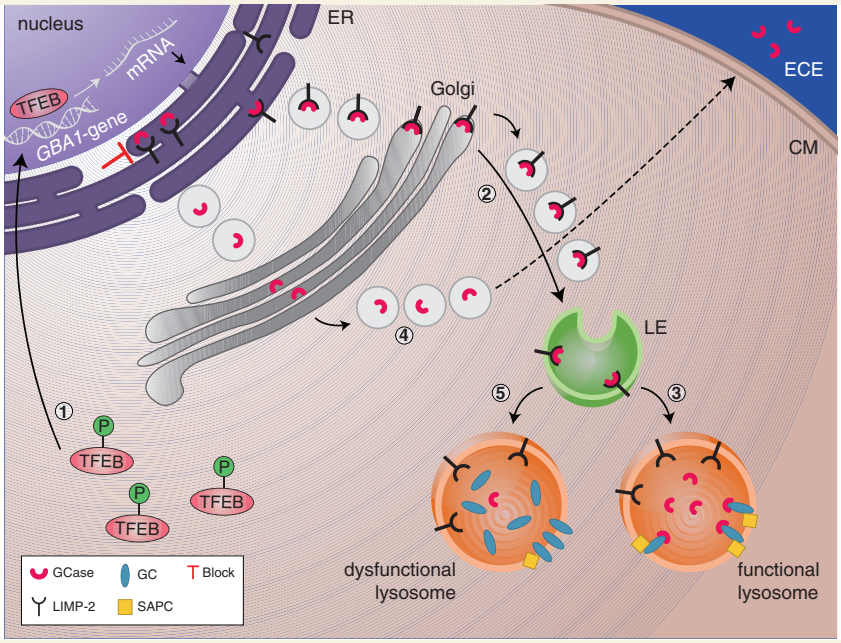

Figure 2 Regulators of *GBA1* expression and glucocerebrosidase activity and trafficking. (1) Phosphorylated TFEB is located in the cytoplasm. Under starvation conditions, dephosphorylated TFEB translocates to the nucleus where it regulates the transcription of genes involved in the CLEAR network, including *GBA1*. (2) *GBA1* messenger RNA is translated into glucocerebrosidase. The interaction with its receptor LIMP-2 facilitates translocation of glucocerebrosidase to the lysosomes via the endoplasmic reticulum (ER), Golgi and late endosomes (LE). (3) In the lysosomes, glucocerebrosidase dissociates from LIMP-2. The glucocerebrosidase activator, saposin C (SAPC), lifts glucocerebroside from the lysosomal membrane and/or membranes within the lysosome and makes it available for glucocerebrosidase-mediated breakdown. (4) When LIMP-2 is deficient or absent, the glucocerebrosidase enzyme cannot be correctly sorted to the lysosomes. As a consequence, glucocerebrosidase is secreted into the extracellular environment (ECE). (5) Saposin C deficiency leads to the impairment of glucocerebroside degradation since the substrate is not available to glucocerebrosidase and subsequently accumulates inside the lysosomes. CM = Cell Membrane.

glucocerebroside exposes the lipid substrate to glucocerebrosidase for subsequent hydrolysis (Alattia *et al.*, 2006, 2007) (Fig. 2). An additional role for saposin C is the protection of glucocerebrosidase against proteolytic breakdown, which is demonstrated by a significant reduction in levels of glucocerebrosidase protein and enzyme activity in saposin C-deficient cells (Sun *et al.*, 2003, 2010). Because of its essential role as a glucocerebrosidase activator, saposin C could be a modifier gene for Gaucher disease and potentially Parkinson's disease. Indeed, crossbreeding studies with a mouse model of saposin C, created by a knock-in mutation in exon 11 of the prosaposin gene, and the V394L homozygous Gaucher mouse (Xu *et al.*, 2003; Hruska *et al.*, 2008; Sun *et al.*, 2010), revealed that the combined deficiencies exacerbate the Gaucher disease phenotype, with progressive neurological complications resulting in early death, greater glucocerebrosidase activity reduction, significant defects in glucocerebroside 18:0 species breakdown in the brain, and increased storage of the substrates glucocerebroside and glucosylsphingosine (Sun *et al.*, 2013*b*). This model confirmed that saposin C could act as a disease modifier for Gaucher disease. Only six patients with saposin C deficiency have been described in the literature and a correlation was observed between the type of mutation and the nature of their Gaucher-like phenotype. Patients with mutations in the crucial cysteine residues in the saposin C domain of prosaposin had a clinical phenotype similar to Gaucher disease type 3, whereas those with other mutations resembled non-neuronopathic type 1 Gaucher disease (Christomanou *et al.*, 1986, 1989; Schnabel *et al.*, 1991; Rafi *et al.*, 1993; Diaz-Font *et al.*, 2005; Tylki-Szymanska *et al.*, 2007, 2011; Vaccaro *et al.*, 2010). Complete deficiency of prosaposin and consequently all saposins, resulted in a severe fatal neurological infantile sphingolipidosis (Hulkova *et al.*, 2001). As patients with both saposin C and glucocerebrosidase deficiencies have never been identified, it is difficult to assess the role of saposin C as a modifier gene in human samples. Interestingly, patient fibroblasts with cysteine saposin C mutations showed an accumulation of autophagosomes, which was believed to be caused by reduced protein levels and enzymatic activity of both cathepsin B and D (Tatti *et al.*, 2011, 2012, 2013). Exogenous over-expression of both cathepsins restored autolysosomal degradation (Tatti *et al.*, 2013). This secondary effect of

saposin C deficiency is of great interest as malfunctions in the autophagy clearance pathway and their role in the development of Parkinson's disease are well documented (Lim and Zhang, 2013), as is the role of cathepsin D in proteolytic breakdown of α-synuclein (Cullen *et al.*, 2009). Although saposin C can act as a modifier gene for Gaucher disease in a mouse model (Sun *et al.*, 2010), appropriate saposin C expression studies on samples from patients with Gaucher disease with discordant phenotypes are currently lacking. Also, considering the recent observations of reduced wild-type glucocerebrosidase activity in Parkinson's disease models and patient samples, it is possible that altered saposin C levels in patients with Parkinson's disease (with or without *GBA1* mutations) could be a crucial determinant in the development of synucleinopathies.

TFEB: a regulator of *GBA1*/ glucocerebrosidase expression

A majority of the genes involved in both lysosomal function and biogenesis are part of the coordinated lysosomal expression and regulation network. Expression of gene members of this network is positively regulated by the basic helix-loop-helix leucine zipper transcription factor EB (TFEB), which binds to the GTCACGTGAC motif element within their promoter region (Sardiello *et al.*, 2009). Work by Ballabio and colleagues established that TFEB is part of a signalling pathway by which lysosomes self-regulate. Indeed, experimental data in *Drosophila* S2, human HEK293T cells, and a cell-free system support an 'inside-out' model in which accumulated amino acids inside the lysosome initiate signalling through the v-ATPase-Ragulator protein complex to Rag-GTPases, which, in turn, recruit mammalian target of rapamycin (mTOR) to the surface of the lysosomes (Sancak *et al.*, 2008, 2010; Zoncu *et al.*, 2011). TFEB interacts with mTOR on the lysosomal surface, where phosphorylation of multiple serine residues by mTOR prevents TFEB translocation to the nucleus (Settembre and Ballabio, 2011; Settembre *et al.*, 2012; Martina and Puertollano, 2013). Cell starvation, which includes amino acid depletion within the lysosome, results in inhibition of the 'inside-out' signalling pathway, and eventual mTOR release from the lysosome surface. TFEB is no longer phosphorylated, and translocates to the nucleus, where it activates transcription of the coordinated lysosomal expression and regulation network genes and autoregulates its own expression through a feedback loop (Settembre *et al.*, 2012, 2013). In addition to its role in lysosomal function and biogenesis, TFEB is also a key player in lipid metabolism (Settembre *et al.*, 2013), autophagosome formation and autophagosome-lysosome fusion (Settembre and Ballabio, 2011), and Ca^{2+}-mediated lysosomal exocytosis, which can positively affect cellular substrate clearance in select lysosomal storage disorders, including Batten disease, Pompe disease, neuronal ceroid lipofuscinoses, multiple sulphatase deficiency, and mucopolysaccharidosis type IIIA (Medina *et al.*, 2011).

TFEB over-expression and silencing studies in HeLa cells showed that TFEB positively regulated *GBA1* messenger RNA expression (Fig. 2). Additionally, chromatin immunoprecipitation analysis confirmed that *GBA1* is a direct target of TFEB (Sardiello *et al.*, 2009).

The TFEB field is still in its infancy and very few studies on its role in neurodegeneration are available. One study showed that adenovirus-mediated over-expression of human α-synuclein in the midbrain of rats induced TFEB retention in the cytoplasm, blockage of lysosomal function, accumulation of α-synuclein in autophagosomes, and progressive build-up of α-synuclein oligomers. Co-immunoprecipitation experiments showed an interaction between α-synuclein and TFEB, suggestive of a role for α-synuclein in cytoplasmic sequestration of TFEB. These observations were confirmed in nigral dopaminergic neurons of post-mortem Parkinson's disease midbrains. In the α-synuclein rat model, both over-expression of TFEB or activation through pharmacological inhibition of mTOR resulted in a block in the progression of α-synuclein-mediated neurodegeneration. This study puts TFEB on the map as a key player in Parkinson's disease (Decressac *et al.*, 2013). Recently, reduced wild-type glucocerebrosidase protein levels were observed in samples from patients with synucleinopathies (Balducci *et al.*, 2007; Parnetti *et al.*, 2009; Gegg *et al.*, 2012). It is possible that this could be because of α-synuclein-induced TFEB retention in the cytoplasm with consequently lower transcription of *GBA1* messenger RNA. Currently, TFEB is the only known transcription factor for *GBA1,* but a study of the promoter and regulatory regions of *GBA1* revealed several conserved transcription factor-binding sites resulting in altered *GBA1* expression levels when mutated. This suggests that these regions might be involved in transcriptional regulation of *GBA1* and potentially contribute to the complex phenotypic diversity observed in Gaucher disease including the development of Parkinson's disease (Blech-Hermoni *et al.*, 2010).

Therapeutics for Gaucher disease may have promise for the treatment of the synucleinopathies

As previously mentioned in this review, studies performed on cell free systems, cell and animal models, and patient samples have demonstrated that knockdown of *GBA1* expression, the introduction of *GBA1* mutations, inhibition by CBE, or treatment with glucocerebroside substrate all enhance accumulation and/or oligomerization of α-synuclein (Manning-Bog *et al.*, 2009; Cullen *et al.*, 2011; Mazzulli *et al.*, 2011; Sardi *et al.*, 2011, 2013; Gegg *et al.*, 2012; Cleeter *et al.*, 2013; Osellame *et al.*, 2013). On the other hand, upregulation of α-synuclein levels decrease glucocerebrosidase protein and activity levels in cell-free systems, cell and mouse models, and post-mortem brains of Parkinson's disease patients with and without *GBA1* mutations (Mazzulli *et al.*, 2011; Sardi *et al.*, 2011, 2013; Yap *et al.*, 2011, 2013; Gegg *et al.*, 2012). This reciprocal relationship between glucocerebrosidase activity and α-synuclein levels has generated great interest in the potential role of Gaucher disease therapeutics for the treatment of the synucleinopathies (Sardi *et al.*, 2013; Schapira and Gegg, 2013). Therapies for Gaucher disease, which are targeted towards augmenting glucocerebrosidase activity or decreasing glucocerebroside

storage, could prove to be promising strategies for modulating α-synuclein proteostasis and its subsequent aggregation and oligomerization. This rational was supported by experimental evidence showing that viral-mediated infection into the central nervous system of a mouse model with *GBA1* mutations representing neuronopathic Gaucher disease and a transgenic mouse model overexpressing A53T α-synuclein without *GBA1* mutations significantly reduced α-synuclein levels (Sardi *et al*., 2013). This set a paradigm for augmentation of glucocerebrosidase activity as a beneficial therapeutic strategy for halting disease progression in patients with Parkinson's disease, both with and without *GBA1* mutations, and even preventing the onset of Parkinson's disease in healthy individuals. In this review, we address FDA-approved and 'under development' therapeutics for Gaucher disease and their potential implications for treatment of the synucleinopathies.

The first available FDA-approved therapy for Gaucher disease was enzyme replacement therapy, which was developed at the National Institutes of Health. Patients with Gaucher disease type 1 received intravenous infusion of exogenous enzyme, which improved haematologic and visceral manifestations and reduced glucocerebroside levels (Brady *et al*., 1974; Barton *et al*., 1991). Currently, three different recombinant enzymes are commercially available, imiglucerase, taliglucerase alfa, and velaglucerase alfa. Although each of the enzymes differ in their cell system production and glycosylation pattern, the function and biodistribution of all three enzymes are comparable (Tekoah *et al*., 2013) (Fig. 3). As intravenous enzyme replacement therapy does not cross the blood–brain barrier it does not ameliorate neurological manifestations and would not be suitable for treatment of Parkinson's disease neuropathology (Erikson, 2001; Beck, 2007). In fact, patients with Gaucher disease undergoing enzyme replacement therapy have still gone on to develop Parkinson's disease.

The accumulation of glucocerebroside in the lysosome can impact α-synuclein breakdown and oligomerization (Mazzulli *et al*., 2011), which suggests that therapeutic reduction of excessive glucocerebroside substrate could potentially be beneficial for Parkinson's disease. Therapeutic inhibition of the enzyme glucosylceramide synthase, which catalyzes the synthesis of glucocerebroside, attenuates glucocerebroside production and has been used as a form of substrate reduction therapy. Treatment of patients with Gaucher disease with two glucosylceramide synthase inhibitors, miglustat (*N*-butyldeoxynojirimycin) and eliglustat tartrate, resulted in visceral and hematopoietic improvement but failed to impact neurological manifestations (Lukina *et al*., 2010). Recently, a screening effort of novel compounds identified the compound GZ 161, which successfully reduced both glucocerebroside and glucosylsphingosine accumulation in the brain of the K14 acute neuronopathic Gaucher disease mouse model and significantly increased their lifespan (Cabrera-Salazar *et al*., 2012).

Another approach gaining much momentum in the field of lysosomal storage disorders is pharmacological chaperone therapy. The proper folding process of glucocerebrosidase takes place in the endoplasmic reticulum by direct interaction with endogenous cellular chaperones such as heat shock protein 90 and heat shock protein 70 (Lu *et al*., 2011). Studies have demonstrated that several disease-causing glucocerebrosidase mutants are misfolded and do not pass the ERAD quality control system, which leads to early proteasome-mediated degradation (Ron and Horowitz, 2005; Ron *et al*., 2010; Bendikov-Bar *et al*., 2011; Bendikov-Bar and Horowitz, 2012; Maor *et al*., 2013*a*, *b*). Therapy with pharmacological chaperones, which specifically bind to the newly synthesized mutant enzyme, can prevent premature ERAD and promote trafficking to the lysosome, where most mutant glucocerebrosidase proteins can exert sufficient residual enzyme activity for the breakdown of accumulated lysosomal glucocerebroside (Lieberman *et al*., 2009; Bendikov-Bar *et al*., 2013) (Fig. 3). The drawback of such a therapeutic approach is that translation of mutated glucocerebrosidase and an intact chaperone-binding site are required. Treatment will not be effective in the case of null alleles, large deletions, or mutations affecting the chaperone-binding site. Many of the pharmacological chaperones are inhibitors of glucocerebrosidase that bind to its active site. Ambroxol is a pH-dependent mixed inhibitor of glucocerebrosidase that was identified by screening of an FDA-approved drug library (Maegawa *et al*., 2009); it is a potent chaperone for the translocation of mutant glucocerebrosidase to lysosomes (Bendikov-Bar *et al*., 2011, 2013; Babajani *et al*., 2012; Luan *et al*., 2013). One limited pilot study conducted in a small group of patients with Gaucher disease indicated amelioration of clinical symptoms after ambroxol treatment (Zimran *et al*., 2013), but its efficacy in relevant Parkinson's disease models has not yet been evaluated. Another glucocerebrosidase inhibitor, Isofagomine, showed great efficacy in cell and mouse models of Gaucher disease, resulting in increased glucocerebrosidase protein levels and enzyme activity, reduction in levels of glucocerebroside and glucosylsphingosine, delayed neurological manifestations, and increased life span (Khanna *et al*., 2010; Sun *et al*., 2011, 2012), but improvement in clinical symptoms were not observed in a phase 2 clinical trial (Zimran, 2011). In a cell model for Parkinson's disease consisting of PC12 cells over-expressing α-synuclein and transfected with wild-type or mutant glucocerebrosidase, the efficacy of isofagomine treatment on α-synuclein levels was non-significant (Cullen *et al*., 2011); its efficacy in relevant *in vivo* models of Parkinson's disease remains to be investigated. Clinical development of these inhibitory chaperones has major obstacles as both drug dosage and the length of treatment have to be carefully optimized for high endoplasmic reticulum to lysosome chaperone activity, yet minimal lysosomal enzyme inhibition. This can be circumvented by using pharmacological chaperones that both facilitate the lysosomal translocation and residual activity of mutant glucocerebrosidase without enzyme inhibition. Recent efforts have identified promising activators that increase translocation and enzyme activity of mutant glucocerebrosidase in fibroblasts (Goldin *et al*., 2012; Patnaik *et al*., 2012).

As ERAD is a major player in the premature degradation of many glucocerebrosidase mutants, targeting proteins that regulate the proteostasis of mutated glucocerebrosidase could serve as an alternative therapy. Recently, histone deacetylase inhibitors were identified as modulators of heat shock protein 90-dependent degradation of mutated glucocerebrosidase by inhibiting the deacetylation of heat shock protein 90. Treatment resulted in increased glucocerebrosidase protein levels and enzyme activity in Gaucher disease fibroblasts (Lu *et al*., 2011; Yang *et al*., 2013). Future development of histone deacetylase inhibitors

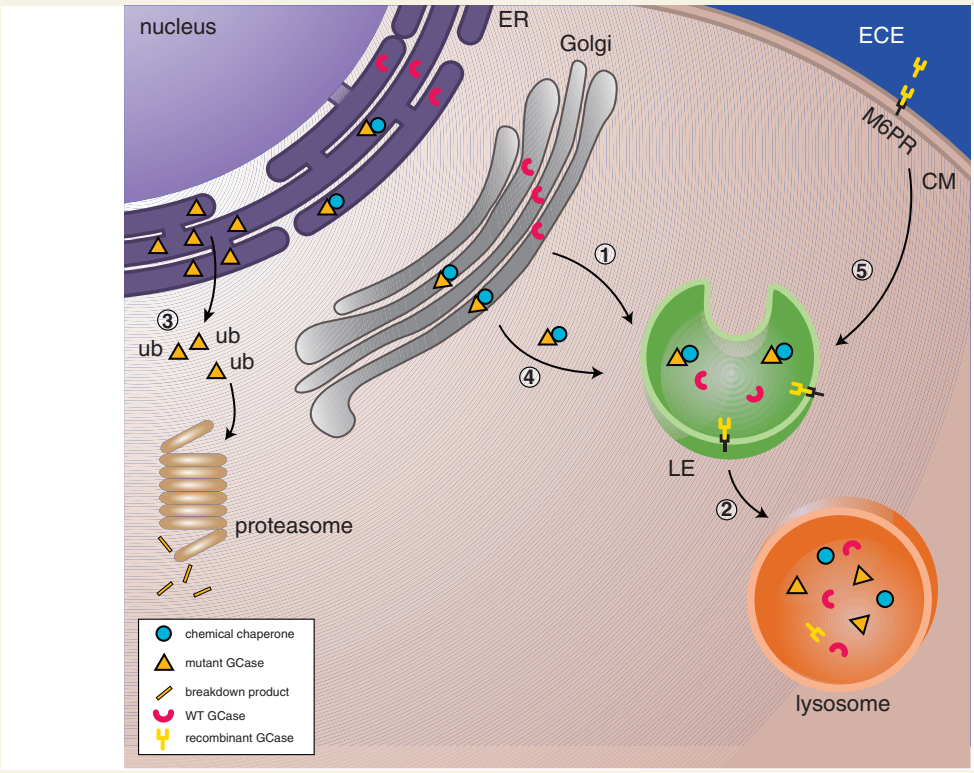

Figure 3 Therapeutic strategies to enhance glucocerebrosidase. (1 and 2) In healthy cells, wild-type glucocerebrosidase (WT GCase) is sorted to the lysosome via the endoplasmic reticulum, Golgi, and late endosomes (LE) where it will degrade its substrate glucocerebroside. (3) Mutant glucocerebrosidase is misfolded in the endoplasmic reticulum, becomes polyubiquitinated (ub) and undergoes proteasome-mediated degradation. (4) Pharmacological chaperones can stabilize mutant glucocerebrosidase and facilitate translocation to lysosomes. (5) In enzyme replacement therapy, recombinant glucocerebrosidase enzyme is delivered into the cells via the mannose-6-phosphate receptor and trafficked through the late endosomes to the lysosomes where it is able to degrade substrate. CM = Cell Membrane.

for therapeutics might prove challenging, as the target of this therapy remains broad rather than glucocerebrosidase-specific. Additionally, the exact molecular mechanism of regulation of proteostasis by histone deacetylase inhibitors remains unclear.

Other lysosomal storage disorders and the development of the synucleinopathies

There are >50 different lysosomal storage disorders. There have been several individual case reports of patients and carriers with specific lysosomal storage disorders that suggest that there may be neuropathological findings suggestive of Parkinson's disease, with reports of α-synuclein accumulation and inclusions, and the loss of neurons in the substantia nigra. This indicates that it might be worthwhile to expand research into defects in the cellular pathways that link synucleinopathies with changes in glucocerebrosidase protein amount and activity to a variety of enzymes involved in lysosomal storage disorders (reviewed by Shachar *et al.*, 2011). Recently, molecular studies screening for mutations for genes involved in specific lysosomal storage disorders shed new light on the association with synucleinopathies.

Niemann-Pick disease is a lysosomal storage disorder with heterogeneous clinical features and severity. Types A (OMIM #257200) and B (OMIM #607616) are both associated with a deficiency of the acid sphingomyelinase enzyme, which is encoded by the sphingomyelin phosphodiesterase gene, and catalyzes the breakdown of sphingomyelin into ceramide and phosphorylcholine in lysosomes. Acid sphingomyelinase deficiency results in sphingomyelin accumulation in phagocytic cells and neurons resulting in clinical symptoms such as failure to thrive, hepatosplenomegaly and progressive neurodegeneration (Vanier, 2013). Two recent independent reports identified variations in the sphingomyelin phosphodiesterase gene as risk factors for Parkinson's disease. Gan-Or and colleagues (2013) identified the founder mutation to be associated with Parkinson's disease with an odds ratio of

9.4 in two Ashkenazi Jewish Parkinson's disease patient cohorts consisting of 938 patients (Gan-Or *et al.*, 2013). Another variant (p.R591C) increasing the risk for Parkinson's disease was identified from a cohort of 1004 patients of Chinese ancestry (Foo *et al.*, 2013).

Sanfilippo syndrome B or Mucopolysaccharidosis type III B (OMIM #252920) is an autosomal recessive lysosomal storage disorder caused by mutations in the α-N-acetylglucosaminidase gene leading to the accumulation of heparan sulphate in lysosomes. Clinical symptoms range from mild to severe and include progressive neurodegeneration, skeletal changes, and behavioural problems (Chinen *et al.*, 2005). Allelic analysis of two single nucleotide polymorphisms in α-*N*-acetylglucosaminidase on DNA samples of 926 patients with Parkinson's disease and 2308 control subjects showed an association between rs2071046 and an increased risk for developing Parkinson's disease (Winder-Rhodes *et al.*, 2012).

Wu *et al*. (2008) measured enzyme activity of 11 lysosomal hydrolases in peripheral blood leucocytes of 38 patients with sporadic Parkinson's disease and 258 controls. Only the activity of alpha-galactosidase A was significantly reduced. Deficiency in alpha-galactosidase A causes the X-linked lysosomal storage disorder Fabry disease (OMIM#301500), which is characterized by lysosomal storage of globotriaosylceramides and glycosphingolipids (Desnick *et al.*, 2001). Interestingly, in this study glucocerebrosidase did not show significant reduction in enzyme activity in peripheral blood leucocytes of patients with Parkinson's disease compared to controls (Wu *et al.*, 2008). A molecular follow-up study identified no differences in the frequency of single nucleotide polymorphisms in the promoter and exonic regions of the alpha-galactosidase gene in patients with sporadic Parkinson's disease and healthy control subjects (Wu *et al.*, 2011).

Concluding remarks

Recent insights into the relationship between glucocerebrosidase (wild-type and mutant) and α-synuclein in the synucleinopathies have shed new light on the cellular mechanism of α-synuclein pathology in Parkinson's disease and dementia with Lewy bodies. As only a minority of patients with Gaucher disease, *GBA1* mutation carries, or individuals in the overall population develop synucleinopathies, it is apparent that the delicate balance between α-synuclein proteostasis and glucocerebrosidase enzyme activity must be affected by other modifiers manipulating glucocerebrosidase or α-synuclein levels. It will be of interest to investigate some of the potential modifiers in cell and animal models, as well as in patient samples. This new understanding of balancing α-synuclein proteostasis by correcting glucocerebrosidase enzyme levels holds novel possibilities for the future treatment of parkinsonism. Glucocerebrosidase-specific pharmacological chaperones, especially activators that cross the blood–brain barrier, will be of great interest in this endeavour. Finally, limited research on other lysosomal storage disorders suggests that mutations in other lysosomal enzymes may similarly play a role as risk factors for the synucleinopathies, but it remains to be seen whether the activity of these enzymes also affect α-synuclein proteostasis. This story clearly illustrates how studies into the pathogenesis and therapy of a rare genetic disorder can lead to advances impacting the multitudes of patients with common complex diseases like Parkinson's disease worldwide.

Acknowledgements

The authors would like to thank Julia Fekecs for assistance with drafting of the figures and Dr. Elma Aflaki and Ashley Gonzalez for critical comments and careful reading of the manuscript.

Funding

This work was supported by the Intramural Research Programs of the National Human Genome Research Institute and the National Institutes of Health. M.S. received support by the Brazilian Federal Agency for Support and Evaluation of Graduate Education – CAPES and Fulbright Brazil.

Highlight

Siebert *et al*. review recent discoveries regarding the molecular link between the lysosomal enzyme glucocerebrosidase and the synucleinopathies. Current research utilizing cell, animal, and patient samples indicate a reciprocal relationship between glucocerebrosidase and α-synuclein. Therapeutic strategies for Gaucher disease may have implications for treatment of synucleinopathies.

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ANEXO II

Gene 523 (2013) 114–115

Contents lists available at SciVerse ScienceDirect

Gene

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Letter to the Editor

Screening of high-risk Gaucher disease patients using dried blood spots techniques

To the editor:

After reading with great interest the short communication by Goldim et al. [1], regarding the utilization of miniaturized dried blood spots for screening of Gaucher Disease (GD), we would like to report an interesting case that we have been working on.

In the Reference Center for the Treatment of GD from state of Rio Grande do Sul, Brazil, we are presently following 45 GD patients (42 GD type I and 3 GD type III). Every patient with suspicion of presenting GD in our state is sent to us in order to be evaluated, and if the diagnosis is confirmed, we are responsible for his/her treatment and follow-up [2].

Two years ago, a 32 year old male was referred to our center to receive enzyme replacement therapy (ERT), which had already been prescribed by the local physician. The diagnosis of GD had been based on the levels of glucocerebrosidase (GCase) activity found both in a miniaturized dried blood spot (DBS) (0.56 nmol/h/mL, RV = 2.19–16.8) and in leukocytes (4.6 nmol/h/mg prot, RV = 10–45); the chitotriosidase (CTH) activity in plasma was normal (Laboratory A, Table 1). The patient was adopted soon after his birth and had a history of neonatal hepatitis, liver cirrhosis and a possible cognitive decline. Besides that, he presented pulmonary hypertension, chronic anemia, and mild epistaxis and gingival bleeding. Femural MRI showed no bone marrow infiltration and bone pain was denied. Blood counts were: hemoglobin: 9.7 g/dL, leukocytes: 2820/μL, platelets: 63,000/μL. Pulmonary X-ray was normal. A previous bone marrow biopsy (BMB) showed hypocellularity. At physical examination, he presented with obesity (weight = 100.8 kg; height =162.5 cm; BMI = 38.1 kg/m^2), strabismus, short phalanges and splenomegaly.

Since the clinical picture was not typical of GD, we decided to re-evaluate the patient. The previous BMB was revised by our pathologist and no Gaucher cells were found. Serum total cholesterol levels were 210 mg/dL (HDL = 26, LDL = 131) and triglyceride levels were 264 mg/dL. Blood counts showed anemia (hemoglobin 9.2 g/dL), leukopenia (leukocytes: 2000/μL) and thrombocytopenia (platelets: 58,000/μL). The enzymatic activities of the GCase and sphingomyelinase in DBS (non-miniaturized technique) and in leukocytes, as well as the CTH activity in plasma found in our laboratory (Laboratory B) are normal (Table 1). As he presented mild cholesterol abnormalities and liver cirrhosis, lipase acid deficiency was suspected but its activity was within the normal range.

As the new results did not confirm the previous diagnosis of GD, patient's DBS were sent to be analyzed by laboratories C and D, both reference laboratories for the diagnosis of GD and located at different distances from our center (Table 1), but the results were both inconclusive. At the same time, the Filipin staining test was found to be normal and the sequencing of the *GBA* and *SCARB2* genes, that codify for GCase and LIMP-2, did not reveal any alteration. *CHIT1* gene analysis, by PCR amplification, did not show the duplication of 24 bp in the exon X associated to CTH deficiency. Unfortunately the patient passed away during our investigation without a correct diagnosis being reached. Although unlikely, the diagnosis of GD by SAPC deficiency could not be completely ruled out.

We brought up this case as a reminder that the clinical picture is sovereign on a laboratory analysis. GD diagnosis could be an odyssey since the CTH activity, which may be a helpful biomarker for the diagnosis, could be misinterpreted due to polymorphisms that cause its deficiency (partial or total) and are present in up to 27% of the European population [3]. So, patients who present low GCase activity but normal CTH activity, such as our patient, should be investigated through ancillary tests (e.g., DNA analysis) in order to have their diagnosis confirmed, especially if they are asymptomatic or if their clinical picture is not typical or suggestive of GD.

Besides that, GCase assays in DBS are associated to some pitfalls that could lead to a mistake in the diagnosis. For instance, Goldim et al. [1] found reduced GCase and increased CTH activities in 94/274 DBS samples from GD suspected patients; a new and adequate blood sample was available for 67/94 patients, who were so retested through the "gold-standard" tests (GCase activity in leukocyte and CTH in plasma) — the diagnosis of GD was confirmed in only 37/67 patients, which led us to a false-positive rate of 44.7%. The high-rate of false-positive results in DBS samples could be related to the stability of the enzymes assessed, to the non-optimal storage conditions of DBS [4], or even to the assay used (miniaturized technique). In our patient, GCase values found at our laboratory – through a non-miniaturized assay in DBS [5] – were, as expected from his clinical and DNA findings, normal (although within the low normal range); on the other hand, DBS assays performed at laboratories C and D suggested that the samples were not adequate since the control enzymes also showed low activities (Table 1).

Finally, we point out that ERT or substrate reduction therapy should be prescribed only to oligosymptomatic/symptomatic patients whose diagnosis of GD had been confirmed by gold-standard methods such as the measurement of GCase activity in leukocytes or fibroblasts and/or *GBA* gene analysis. DBS samples could be helpful as diagnostic tools if they are used not only for enzymatic assays but also as a source of DNA for *GBA* and *CHIT1* analysis.

Conflict of interest

There is no conflict of interest.

Acknowledgments

We would like to thank the patient and his family and Prof Roberto Giugliani, Prof Michael Beck and Prof. Vânia D'Almeida for their helpful comments along the evaluation of this case.

Funding

AB is supported by a fellowship from Fundação Portuguesa para a Ciência e Tecnologia (SFRH/BPD/64074/2009). *SCARB2* gene analysis

http://dx.doi.org/10.1016/j.gene.2013.03.106

Table 1
Gaucher disease — results of the investigation performed at different laboratories in the patient reported.

Laboratory	Distance to our center	Glucocerebrosidase activity (DBS) nmol/h/mL	Glucocerebrosidase activity (leukocytes) nmol/h/mg prot	Glucocerebrosidase activity (fibroblasts) nmol/h/mg prot	β-galactosidase activity	Chitotriosidase activity nmol/h/mL	Other investigations
A	0 km	0.56 (RV = 2.2–17) *Miniaturized technique*	4.7 (RV = 10–45)	–	–	Plasma: 76 (RV = 8.8–132) nmol/h/mL	
B	0 km						
Sept/2010		–	11 (RV = 10–45)		Leukocytes: 154 (RV = 78–280)[a]	Plasma: 31 (RV = 8.8–132)	Sphingomyelinase in leukocytes : 1.1 (RV = 0.74–4.9)[a]
Dec/2010		–	12 (RV = 10–45)		Leukocytes: 200 (RV = 78–280)[a]	Plasma: 28 (RV = 8.8–132)	Sphingomyelinase in leukocytes : 1.6 (RV = 0.74–4.9)[a]
May/2011		2.2 (RV = 2.2–17) *Technique according to Civallero et al., 2006* [5]	–	189 (RV = 257–668)	DBS:22 nmol/h/mL (RV = 35–126) Fibroblasts: 450 (RV = 394–1440)[a]	DBS: 9.2 (RV = <44)	Filipin test — negative Acid lipase: normal
C May/2011	1200 km	0.6 (RV = 2.1–8.8) *Technique according to Civallero et al., 2006* [5]	–	–	DBS:4.2 nmol/h/mL (RV = 12–36)	–	IDUA DBS 3.0 (RV = 2.5–17)
D April/2011	10,000 km	0.0 (RV = 200–2000) pmol/spot/20 h *Tandem mass spectrometry*		–	DBS:0.32 nmol/spot/20 h (RV = 0.5–3.2)	–	Sphingomyelinase DBS: 62 (RV = 200–3500) pmol/spot/20 h

DBS: dried blood spot, RV: normal range value, IDUA: iduronate sulfatase activity.
[a] nmol/g/mg prot.

was performed with funding from Fundação Portuguesa para a Ciência e Tecnologia (PTDC/SAU-GMG/105344/2008).

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ANEXO III

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Título: Efeitos de alterações no gene da glicocerebrosidase e de genes modificadores do fenótipo da doença de Gaucher e do fenótipo de patologias associadas à proteína glicocerebrosidase.

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Bolsista: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

3. ESTÁGIOS E BOLSAS

2003-2004 Bolsista PIBIC/CNPq de Iniciação científica no Laboratório de Biologia Celular Vegetal do Centro de Biotecnologia da UFRGS. Orientador: Prof. Dr. Giancarlo Pasquali. Resumo das atividades: Construção de vetores plasmidiais para o envio de proteínas recombinantes ao cloroplasto de cana-de-açúcar.

2005-2007 Bolsista BIC/PROPESQ de Iniciação científica no Laboratório de Genética Molecular do Serviço de Genética Médica (SGM) do Hospital de Clínicas de Porto Alegre (HCPA). Orientador: Profa. Dra. Maria Luiza Saraiva Pereira. Resumo das atividades: Identificação de mutações raras no gene da glicocerebrosidase em pacientes com doença de Gaucher.

2008-2010 Bolsista CNPq de Mestrado.

2010-2014 Bolsista CNPq de Doutorado

2012-2013 Bolsista CAPES de Doutorado-Sanduíche.

4. PRÊMIOS

2011 Third prize in the Basic Science category, 10th International Symposium on Lysosomal Storage Diseases.

5. EXPERIÊNCIA PROFISSIONAL

Tem experiência na área de genética humana, biologia molecular e celular, com ênfase em diagnóstico molecular de doenças genéticas, identificação de polimorfismos, PCR convencional, PCR *real time*, expressão gênica, sequenciamento de DNA, clonagem, cultura de células, *western blot* e *high-throughput screening* utilizando siRNAs e microRNAs.

6. ARTIGOS COMPLETOS PUBLICADOS

1. BUENO, D., **SIEBERT, M.** Contribuição de grupos operacionais no fortalecimento da Atenção Primária à Saúde. *Revista de APS (Online)*, 11: 468-473, 2008.

2. **SIEBERT, M.**, DONIS, K.C., SOCAL, M., RIEDER, C.R.M., EMMEL, V.E., VAIRO, F., MICHELIN-TIRELLI, K., FRANÇA, M., D'ABREU, A.C., BETTENCOURT, C., LIMA, M., LOPES-CENDES, I., SARAIVA-PEREIRA, M.L., JARDIM, L.B. Glucocerebrosidase gene variants in parkinsonian patients with Machado Joseph/spinocerebellar ataxia 3. *Parkinsonism & Related Disorders*, 18: 185-190, 2012.

3. VAIRO, F., NETTO, C., TIRELLI, K.M., **SIEBERT, M.**, BURIN, M., SARAIVA-PEREIRA, M.L., BALREIRA, A., SÁ MIRANDA, M.C., LUKACS, Z., SCHWARTZ, I.V.D. Screening of high-risk Gaucher disease patients using dried blood spots techniques. *Gene (Amsterdam)*, 523(1): 114-115, 2013.

4. **SIEBERT, M.**, BOCK, H., MICHELIN-TIRELLI, K., COELHO, J.C., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Novel mutations in the glucocerebrosidase gene of Brazilian patients with Gaucher disease. *JIMD reports*, 9: 7-16, 2013.

5. DAL'MASO, V.B., MALLMANN, L., **SIEBERT, M.**, SIMON, L., SARAIVA-PEREIRA, M.L., DALCIN, P.T.R. Contribuição da análise molecular do gene regulador da condutância transmembrana na fibrose cística na investigação diagnóstica de pacientes com suspeita de fibrose cística leve ou doença atípica. *Jornal Brasileiro de Pneumologia (Impresso)*, 39: 181-189, 2013.

6. **SIEBERT, M.**, SIDRANSKY, E., WESTBROEK, W. Glucocerebrosidase is shaking up the synucleinopathies. *Brain*, 137(Pt 5): 1304-1322, 2014.

7. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

1. **SIEBERT, M.**, BOGDAWA, H.M., PASQUALI, G., HENRIQUES, J.A.P. Construção de vetor plasmidial para o envio de proteínas aos cloroplastos de cana-de-açúcar. In: XV Salão de Iniciação Científica e XII Feira de Iniciação Científica, 2003, Porto Alegre.

2. **SIEBERT, M.**, BOGDAWA, H.M., CARDONE, J., HENRIQUES, J.A.P., PASQUALI, G. Construção de vetores plasmidiais para o transporte de proteínas recombinantes aos plastídeos. In: XVI Salão de Iniciação Científica, XIII Feira de Iniciação Científica, 2004, Porto Alegre.

3. **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Diagnóstico laboratorial de deleções do gene *SMN1* em pacientes com Atrofia Muscular Espinhal. In: XVII Salão de

Iniciação Científica e XIV Feira de Iniciação Científica, 2005, Porto Alegre.

4. **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Diagnóstico laboratorial de Atrofia Muscular Espinhal: detecção das deleções do gene *SMN1*. In: XVII Congresso Brasileiro de Genética Clínica, 2005, Curitiba.

5. **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Diagnóstico laboratorial de Atrofia Muscular Espinhal: detecção das deleções do gene *SMN1*. In: 25ª Semana Científica do Hospital de Clínicas de Porto Alegre, 12º Congresso de Pesquisa e Desenvolvimento em Saúde do Mercosul, 2005, Porto Alegre.

6. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Protocolo de identificação de mutações raras no gene da glicocerebrosidase em pacientes com doença de Gaucher. In: XVIII Salão de Iniciação Científica, XV Feira de Iniciação Científica e I Salão UFRGS Jovem, 2006, Porto Alegre.

7. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Identificação de alterações no gene da glicocerebrosidase em pacientes com doença de Gaucher. In: 26ª Semana Científica do Hospital de Clínicas de Porto Alegre, 5ª Reunião da Rede Nacional de Pesquisa Clínica em Hospitais de Ensino, 13° Congresso de Pesquisa e Desenvolvimento em Saúde do Mercosul, 2006, Porto Alegre.

8. GODINHO, F.M.S., **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Caracterização molecular de pacientes com Atrofia Muscular Espinhal - resultados preliminares. In: XVIII Salão de Iniciação Científica, XV Feira de Iniciação Científica, I Salão UFRGS Jovem, 2006, Porto Alegre.

9. GODINHO, F.M.S., **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Análise molecular de pacientes com suspeita clínica de Atrofia Muscular Espinhal In: 26ª Semana Científica do Hospital de Clínicas de Porto Alegre, 5ª Reunião da Rede Nacional de Pesquisa Clínica em Hospitais de Ensino, 13° Congresso de Pesquisa e Desenvolvimento em Saúde do Mercosul, 2006, Porto Alegre.

10. SARAIVA-PEREIRA, M.L., BOCK, H., CUNHA, G.R., EMMEL, V.E., FURTADO, G.V., GHENO, T.C., GIUGLIANI, R., GODINHO, F.M.S., KIEHL, M.F., LEMOS, H., RODRIGUES, G.F., SANTA-RITA, T., **SIEBERT, M.**, VIRGENS, M.Y.F. Laboratório de Identificação Genética In: XVI Encontro de Geneticistas do Rio Grande do Sul, 2008, Porto Alegre.

11. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO,

J.C., SARAIVA-PEREIRA, M.L. Identificação de alterações no gene da glicocerebrosidase em pacientes com doença de Gaucher. In: XX Congresso Brasileiro de Genética Médica, 2008, Gramado.

12. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Identificação de alterações no gene da glicocerebrosidase em pacientes com doença de Gaucher. In: 28ª Semana Científica do Hospital de Clínicas de Porto Alegre, 15º Congresso de Pesquisa e Desenvolvimento em Saúde do Mercosul, 2008, Porto Alegre.

13. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Doença de Gaucher: identificação de mutações raras no gene da glicocerebrosidase em pacientes brasileiros In: 54° Congresso Brasileiro de Genética, 2008, Salvador.

14. GODINHO, F.M.S., BOCK, H., **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Atrofia Muscular Espinhal: diagnóstico molecular de pacientes com deleção no gene SMN1. In: XX Congresso Brasileiro de Genética Médica, 2008, Gramado.

15. GODINHO, F.M.S., BOCK, H., **SIEBERT, M.**, VEIT, T.D., SARAIVA-PEREIRA, M.L. Atrofia Muscular Espinhal: diagnóstico molecular de pacientes com deleção do gene SMN1. In: 28ª Semana Científica do Hospital de Clínicas de Porto Alegre, 15° Congresso de Pesquisa e Desenvolvimento em Saúde do Mercosul, 2008, Porto Alegre.

16. **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Mutation Analysis of 12 Gaucher Disease Patients: Identification of Rare and Novel Mutations. In: XXXVIII Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq), 2009, Águas de Lindóia.

17. **SIEBERT, M.**, SARAIVA-PEREIRA, M.L., SOCAL, M., MICHELIN, K., BOCK, H., RIEDER, C.R.M., JARDIM, L.B. Intermediate activities of beta-glucosidase screened new GBA mutations carriers among Parkinson patients. In: 9th International Symposium on Lysosomal Storage Diseases, 2009, Frankfurt.

18. SILVA, E.Z., **SIEBERT, M.**, BOCK, H., MICHELIN, K., PIRES, R.F., GIUGLIANI, R., COELHO, J.C., SARAIVA-PEREIRA, M.L. Análise Molecular de 12 Pacientes com Doença de Gaucher: Identificação de Mutações Raras In: XXI Salão de Iniciação Científica, XVIII Feira de Iniciação Científica, IV Salão UFRGS Jovem, 2009, Porto Alegre.

19. **SIEBERT, M.**, BOCK, H., POL-FACHIN, L., GIUGLIANI, R., VERLI, H.,

SARAIVA-PEREIRA, M.L. Structural Analysis of Glucocerebrosidase and Molecular Analysis of the GBA Gene In: XXXIX Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society, 2010, Foz do Iguaçu.

20. VIRGENS, M.Y.F., **SIEBERT, M.**, POL-FACHIN, L., BOCK, H., BURIN, M.G., VERLI, H., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Caracterização genotípica de pacientes brasileiros com Leucodistrofia Metacromática e dinâmica estrutural da Arilsulfatase A In: 30ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2010, Porto Alegre.

21. VIRGENS, M.Y.F., **SIEBERT, M.**, BOCK, H., BURIN, M.G., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Caracterização genotípica de pacientes brasileiros com Leucodistrofia Metacromática In: 56º Congresso Brasileiro de Genética, 2010, Guarujá.

22. **SIEBERT, M.**, BOCK, H., MICHELIN-TIRELLI, K., COELHO, J.C., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Análise molecular em pacientes com doença de Gaucher: uma abordagem abrangente para identificação de alelos mutantes In: 30ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2010, Porto Alegre.

23. **SIEBERT, M.**, DONIS, K.C., SOCAL, M., RIEDER, C.R.M., EMMEL, V.E., VAIRO, F., MICHELIN-TIRELLI, K., FRANCA JR, M., D'ABREU, A.C., BETTENCOURT, C., LIMA, M., LOPES-CENDES, I., SARAIVA-PEREIRA, M.L., JARDIM, L.B. The *GBA* heterozygous state may convert the ataxic SCA3 phenotype towards a parkinsonian syndrome In: 10th International Symposium on Lysosomal Storage Diseases, 2011, Madri.

24. **SIEBERT, M.**, BOCK, H., MICHELIN-TIRELLI, K., COELHO, J.C., SCHWARTZ, I.V., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Spectrum of mutations in the glucocerebrosidase gene of a cohort of Brazilian patients with Gaucher disease In: Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, 2011, Genebra.

25. **SIEBERT, M.**, DONIS, K.C., SOCAL, M., RIEDER, C.R.M., EMMEL, V.E., VAIRO, F., MICHELIN-TIRELLI, K., FRANCA JR, M., D'ABREU, A.C., Bettencourt, C., LIMA, M., LOPES-CENDES, I., JARDIM, L.B., SARAIVA-PEREIRA, M.L. Mutations in the glucocerebrosidase gene of parkinsonian patients with Machado-Joseph disease In: XL Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society, 2011, Foz do Iguaçu.

26. CEOLATO, J.C., **SIEBERT, M.**, SCHWARTZ, I.V., SARAIVA-PEREIRA, M.L. Molecular analysis of phenylalanine hydroxylase gene of patients with Phenylketonuria In: XL Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society, 2011, Foz do Iguaçu.

27. BAMPI, G.B., **SIEBERT, M.**, BOCK, H., DALMASO, V., DALCIN, P., SARAIVA-PEREIRA, M.L. Identificação de variações de sequências em regiões reguladoras da expressão do gene CFTR In: 31ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2011, Porto Alegre.

28. **SIEBERT, M.**, VAIRO, F., BOCK, H., MICHELIN-TIRELLI, K., SCHWARTZ, I.V., SARAIVA-PEREIRA, M.L. Caracterização de uma mutação nova no gene da glicocerebrosidase em um paciente com doença de Gaucher In: 31ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2011, Porto Alegre.

29. CHERUBINI, P.A., TORRES, C.M., BRAGATTI, J.A., OLIVEIRA, M.A., SCHENKEL, L.C., **SIEBERT, M.**, BOCK, H., LEISTNER-SEGAL, S., SARAIVA-PEREIRA, M.L., BIACHIN, M.M. Associação entre alelos variantes do gene NTRK2 e epilepsia do lobo temporal In: 31ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2011, Porto Alegre.

30. POLETTO, E., **SIEBERT, M.**, FITARELLI-KIEHL, M., SANSEVERINO, M.T., SILVA, F.A., SARAIVA-PEREIRA, M.L. Aplicação da análise por HRM para identificação de variações de sequência no domínio regulatório do gene CFTR em pacientes com Fibrose Cística In: 31ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2011, Porto Alegre.

31. CONDE, E.M., **SIEBERT, M.**, STRADA, B.C., MATTE, U.S., SARAIVA-PEREIRA, M.L. Lipofuscinose Ceróide Neuronal tipo 3: identificação da deleção comum no gene CLN3 In: 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre.

32. **SIEBERT, M.**, BOCK, H., MICHELIN-TIRELLI, K., SCHWARTZ, I.V., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Investigação abrangente do gene GBA para genotipagem de pacientes com doença de Gaucher In: XXIV Congresso Brasileiro de Genética Médica, 2012, Porto Alegre.

33. **SIEBERT, M.**, BOCK, H., MICHELIN-TIRELLI, K., SCHWARTZ, I.V., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Investigação abrangente do gene GBA para genotipagem de pacientes com doença de Gaucher In: 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre.

34. POLETTO, E., **SIEBERT, M.**, FITARELLI-KIEHL, M., SANSEVERINO, M.T., SILVA, F.A., SARAIVA-PEREIRA, M.L. Identificação de variações de sequência no domínio regulatório do gene CFTR em pacientes com Fibrose Cística In: 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre.

35. POLETTO, E., **SIEBERT, M.**, FITARELLI-KIEHL, M., SANSEVERINO, M.T., SILVA, F.A., SARAIVA-PEREIRA, M.L. Identificação de variações de sequência no domínio regulatório do gene *CFTR* em pacientes com Fibrose Cística In: XXIV Congresso Brasileiro de Genética Médica, 2012, Porto Alegre.

36. BAMPI, G.B., POLETTO, E., **SIEBERT, M.**, BOCK, H., SILVA, F.A., SARAIVA-PEREIRA, M.L. Análise de polimorfismos extragênicos ao gene *CFTR* e sua associação à doença Pulmonar em pacientes com Fibrose Cística In: XXIV Congresso Brasileiro de Genética Médica, 2012, Porto Alegre.

37. BAMPI, G.B., **SIEBERT, M.**, POLETTO, E., BOCK, H., SANSEVERINO, M.T., SARAIVA-PEREIRA, M.L. Análise de Polimorfismos extragênicos ao gene *CFTR* e sua associação a doença pulmonar em pacientes com Fibrose Cística In: 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre.

38. VAIRO, F., NETTO, C., MICHELIN-TIRELLI, K., **SIEBERT, M.**, BURIN, M.G., SARAIVA-PEREIRA, M.L., BALREIRA, A., MIRANDA, M.C.S., BECK, M., D'ALMEIDA, V., SCHWARTZ, I.V. Pitfalls of the diagnosis of Gaucher disease using miniaturized dried blood spots assay In: 5º Congreso Latinoamericano de Enfermedades Lisosomales, 2013, Buenos Aires.

39. SCHNEIDER, J., POLETTO, E., **SIEBERT, M.**, SILVA, F.A., SANSEVERINO, M.T., SARAIVA-PEREIRA, M.L. Identificação de Mutações Frequentes no Gene CFTR em Pacientes com Suspeita Clínica de Fibrose Cística In: 33ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2013, Porto Alegre.

40. BAMPI, G.B., POLETTO, E., **SIEBERT, M.**, BOCK, H., SANSEVERINO, M.T., SILVA, F.A., SARAIVA-PEREIRA, M.L. Associação de Polimorfismos Extragênicos ao Gene CFTR com a Doença Pulmonar em Pacientes com Fibrose Cística In: 33ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2013, Porto Alegre.

41. **SIEBERT, M.**, FURTADO, G.V., SARAIVA-PEREIRA, M.L. Análise de Variantes Polimórficas em Genes Candidatos a Modificadores de Fenótipo da Doença de Gaucher In: 33ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2013, Porto Alegre.

42. CONDE, E.M., **SIEBERT, M.**, BURIN, M.G., JARDIM, L.B., GIUGLIANI, R., SARAIVA-PEREIRA, M.L. Anáise Molecular do Gene da Arilsulfatase A em Três Famílias com Suspeita Clínica de Leucodistrofia Metacromática In: 33ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2013, Porto Aegre.