UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

TESE DE DOUTORADO

ASPECTOS GENÉTICOS E BIOQUÍMICOS DA DOENÇA DE GAUCHER COM ÊNFASE NO GENE *GBA1* E NO METABOLISMO DA VITAMINA B_{12}

SUELEN PORTO BASGALUPP

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Tese apresentada como requisito parcial para obtenção de título de Doutora em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

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"Quem quer aprender a voar um dia deve primeiro aprender a ficar de pé, a andar, a correr, a saltar, a subir e a dançar: não se aprende a voar de repente!"

Friedrich Nietzsche

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SUMÁRIO

RESUMO

A doença de Gaucher (DG) é uma doença rara, herdada de forma autossômica recessiva, causada pela atividade reduzida da enzima lisossomal glicocerebrosidase (GCase) devido a mutações patogênicas no gene *GBA1*, o que leva ao acúmulo do substrato glicocerebrosídeo em macrófagos. A DG apresenta uma grande variabilidade fenotípica, sendo caracterizada principalmente por manifestações hematológicas, viscerais, ósseas e neurológicas. Embora cerca de 500 mutações já tenham sido descritas no gene *GBA1*, não há uma associação estabelecida entre mutações específicas e as características clínicas apresentadas pelos pacientes com DG. O acúmulo de glicocerebrosídeo pode estar afetando a função lisossômica, o que poderia levar a uma disfunção celular e a anormalidades clínicas, como a deficiência de vitamina B_{12} (B_{12}). **Objetivos:** Caracterizar o genótipo e o metabolismo da vitamina B_{12} em pacientes com DG. **Métodos:** Foram analisados, por *Multiplex ligation-dependent probe amplification* (MLPA), 33 pacientes com DG para identificar a presença de deleções e inserções no *GBA1* em pacientes que não apresentavam genótipo definido. Foram caracterizadas amostras de 72 pacientes com DG (n=40 da região Sul do Brasil, n=32 de outras regiões do país) utilizando o método de Sanger ou sequenciamento de nova geração e as frequências alélicas das mutações identificadas foram comparadas entre pacientes com DG de diferentes regiões do Brasil. Biomarcadores funcionais do metabolismo da B_{12} foram mensurados em fibroblastos de indivíduos saudáveis (n=3), pacientes com DG não tratados (tipo I=1; tipo II=1; tipo III=1) e um heterozigoto assintomático. A expressão de transcobalamina (TC) em células de indivíduos saudáveis e de pacientes com DG foi analisada por *Western blot*. Níveis de B_{12} total, holo-transcobalamina (holo-TC), homocisteína total (tHcy) e ácido metilmalônico (MMA) foram analisados retrospectivamente em 52 amostras de plasma de 10 pacientes brasileiros com DG tipo I acompanhados pelo Centro de Referência em DG do Sul do Brasil. **Resultados:** Foi identificada uma deleção em heterozigose de uma região do gene *GBA1* em uma paciente com DG que apresentava genótipo incompleto. Trinta e uma mutações foram identificadas em pacientes brasileiros com DG, sendo duas delas não descritas na literatura. Pacientes com DG pertencentes à região Sul do Brasil apresentaram perfil alélico diferente daqueles que pertenciam às demais regiões do país. Níveis de biomarcadores funcionais do *status* de B_{12} não diferiram entre amostras de células de pacientes com DG e de indivíduos saudáveis. A análise por *Western blot* a partir de lisados de células indicou a presença de conteúdo normal de TC em ambas as linhagens celulares. Nenhum paciente com DG tipo I apresentou níveis

reduzidos de B_{12} total em amostras de plasmas. A análise de quatro biomarcadores do *status* de B_{12} sugere que não há uma deficiência funcional desse micronutriente em pacientes com DG tipo I. **Conclusão:** A técnica de MLPA é uma metodologia complementar que pode ser utilizada para analisar a presença de deleções e de inserções em pacientes com DG que não apresentam genótipo definido. Em relação à caracterização genética de pacientes brasileiros com DG, o alelo mais frequente foi o N370S. O estudo sugere que existe um perfil alélico específico no *GBA1* entre pacientes com DG de diferentes regiões do Brasil, sendo que os alelos N370S, Rec*Nci*I e L444P foram mais representativos na região Sul do país. A investigação do metabolismo da B_{12} em pacientes com DG mostrou que as vias de transporte e de processamento da B_{12} estão preservadas em células de pacientes com DG. A análise de biomarcadores do *status* de B_{12} em amostras de plasmas mostrou que pacientes brasileiros com DG tipo I apresentam níveis normais de $B_{12,}$ o que difere de um estudo prévio que demonstrou uma alta prevalência de níveis reduzidos de B_{12} em pacientes com DG da população judeus Ashkenazi. Além disso, a análise de biomarcadores funcionais (tHcy e MMA) não indicou deficiência de B_{12} em pacientes com DG. Portanto, o acúmulo de glicocerebrosídeo nos lisossomos parece não afetar o processamento e o tráfego da vitamina B_{12}, não ocasionando uma deficiência funcional de B_{12} em pacientes com DG tipo I.

Palavras-chave: doença de Gaucher, mutações, metabolismo da B_{12}, biomarcadores da B_{12}, Holo-TC, MMA, tHcy, *status* de B_{12}.

ABSTRACT

Gaucher disease (GD) is a rare autosomal recessive disease caused by the reduced activity of the lysosomal enzyme glucocerebrosidase (GCase) due to pathogenic mutations in *GBA1* gene, which leads to an accumulation of its substrate, glucocerebroside, in the macrophages. GD presents a considerable phenotypic variation, and it is characterized mainly by hematological, visceral, skeletal and neurological manifestations. Although around 500 mutations have been described in the *GBA1*, there is no clear association between mutation and clinical features presented by patients with GD. The accumulation of glucocerebroside may be affecting the lysosomal function and leads to cellular dysfunction and clinical abnormalities, such as a vitamin B_{12} deficiency. **Objectives:** To characterize the genotype and vitamin B_{12} metabolism in GD patients. **Methods:** Thirty-three patients with GD were analyzed by Multiplex ligation-dependent probe amplification (MLPA) method to detect deletions or insertions in the *GBA1* in patients with uncharacterized alleles. Samples from 72 GD patients (n=40 from South Brazil, n=32 from other regions of Brazil) were analyzed by Sanger or next-generation sequencing, and allele frequencies were compared in GD from different regions of Brazil. Functional biomarkers of B_{12} metabolism were measured in fibroblasts from healthy individuals (n=3), GD untreated patients (type I=1; type II=1; type III=1) and an asymptomatic heterozygous. The expression of transcobalamin (TC) in cells from healthy individuals and GD patients was analyzed by Western blot. Total B_{12}, holo-transcobalamin (holo-TC), total homocysteine (tHcy) and methylmalonic acid (MMA) were prospectively measured in 52 plasma samples from 10 Brazilian patients with GD type I followed by Reference Center for GD from south of Brazil. **Results:** A deletion in a region of the *GBA1* gene was identified in one GD patient presenting incomplete genotype. Thirty-one mutations were identified in Brazilian patients with GD, two of them not described in the literature. GD patients from the southern region of Brazil had different allelic profile than those from other regions of the country. B_{12} biomarkers levels did not differ between cells from GD patients and healthy individuals. Western blot analysis of whole cell lysates indicated standard content of TC in both cell lines. No patient with GD type I presented low levels of total B_{12} in plasma samples. Four biomarkers of B_{12} status suggests that there is no functional deficiency of this micronutrient in GD type I patients. **Conclusion:** The MLPA technique is a complementary methodology that can be used to analyze the presence of deletions and insertions in patients with GD that do not have defined genotype. Regarding the genetic characterization of Brazilian GD patients, the most frequent allele was N370S. The

study suggests that there is a specific allele profile in *GBA1* among patients with GD from different regions of Brazil, whereas the N370S, Rec*Nci*I, and L444P alleles are more representative in the southern region of the country. The investigation of B_{12} metabolism in GD patients showed that transport and processing pathways of B_{12} are overall preserved in GD cells. Analysis of B_{12} status biomarkers in plasma samples showed that Brazilian GD type I patients present normal levels of B_{12}, in contrast with the previous study that demonstrated high prevalence of low B_{12} in untreated patients with GD from the Ashkenazi Jewish population. Furthermore, the analysis of functional biomarkers (tHcy and MMA) was not suggestive of B_{12} deficiency in GD patients. Therefore, glucocerebroside accumulation in lysosomes seems not affect the processing and trafficking of B_{12}, and it not causing a functional impairment of B_{12} in patients with GD type I.

Key Words: Gaucher disease, mutations, B_{12} metabolism, B_{12} biomarkers, Holo-TC, MMA, tHcy, B_{12} status.

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AdoCbl	Adenosilcobalamina
B_{12}	Vitamina B_{12}
BMMT	Betaína-homocisteína metiltransferase
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Cbl	Cobalamina
CCL18	Do inglês, *chemokine* (*C-C motif*) *ligand* 18
CG	Células de Gaucher
CHIT1	Gene que codifica a enzima quitotriosidase
CN	Cianocobalamina
CRDG-RS	Centro de referência em doença de Gaucher do Rio Grande do Sul
Co	Cobalto
DG	Doença de Gaucher
DL	Doença lisossômica
DMB	5,6-dimetilbenzimidazol
DNA	Ácido desoxirribonucleico
DP	Doença de Parkinson
Dup24	Duplicação de 24 pares de base
EIM	Erros inatos do metabolismo
FDA	Do inglês, *Food and Drug Administration*
FI	Fator intrínseco
GBA1	Gene que codifica a enzima glicocerebrosidase
GBA2	Gene que codifica a enzima glicocerebrosidase não-lisossomal
GBAP	Pseudogene da glicocerebrosidase
GC	Glicocerebrosídeo
GCase	Enzima glicocerebrosidase
GlcSph	Glicosilesfingosina
HC	Haptocorrina
HCC	Carcinoma hepatocelular/ *Hepatocellular carcinoma*
HCl	Ácido clorídrico
Hcy	Homocisteína
HCPA	Hospital de Clínicas de Porto Alegre
HGMD	Do inglês, *Human Gene Mutation Database*

Holo-TC	Holo-transcobalamina
Kb	Kilobase
LIMP-2	Do inglês, *Lysosome integral membrane protein type 2*
MCM	Metilmalonil-CoA mutase/ *Methylmalonyl-CoA mutase*
Met	Metionina
MeCbl	Metilcobalamina
MLPA	Do inglês, *Multiplex ligation-dependent probe amplification*
MM	Mieloma múltiplo
MMA	Ácido metilmalônico/ *Methylmalonic acid*
MS	Metionina sintase
NGS	Sequenciamento de nova geração/ *Next-generation sequencing*
NK	Do inglês, *Natural killer cells*
OMIM	Do inglês, *Online Mendelian Inheritance in Man*
PARC	Do inglês, *Pulmonary and Activation-Regulated Chemokine*
pb	Pares de bases
pH	Potencial hidrogeniônico
pSAP	Prosaposina
QT	Quitotriosidase
RE	Retículo endoplasmático
RNA	Ácido ribonucleico
RTC	Receptor de transcobalamina
Sap-C	Saposina C
SCARB2	Do inglês, *Scavenger receptor class B member 2*
SNC	Sistema nervoso central
Sph	Esfingosina
TC	Transcobalamina
TRE	Terapia de reposição enzimática
TRS	Terapia de redução de substrato

1. INTRODUÇÃO

1.1 Erros inatos do metabolismo

Os erros inatos do metabolismo (EIM) representam um grupo heterogêneo de defeitos genéticos que envolvem a síntese ou o catabolismo de moléculas dentro de uma via metabólica específica, os quais ocasionam alteração da síntese, degradação, processamento e/ou transporte de moléculas no organismo. De forma geral, os EIM são considerados raros quando analisados individualmente, mas numerosos quando em conjunto, podendo chegar a uma incidência de 1:2.500 indivíduos (BLAU et al., 2006). Atualmente, existem 1015 EIM conhecidamente causadores de alterações em vias metabólicas específicas já descritos na literatura (FERREIRA et al., 2018). Esse grupo de doenças representa em torno de 80% das doenças raras, embora sua prevalência tenha uma ampla variação em diferentes doenças (FERREIRA et al., 2018). A maioria dos EIM apresenta tratamento disponível, seja a partir do manejo dietético, administração enzimática, terapia gênica, entre outros (COLONETTI; ROESCH; SCHWARTZ, 2018; HARDING, 2017; KRUSZKA; REGIER, 2019; SHARMA; PRASAD, 2017; SHEMESH et al., 2013).

A maioria dos EIM envolve anormalidades em enzimas ou proteínas transportadoras e podem ser divididos em duas grandes categorias. A primeira inclui doenças que envolvem apenas um sistema funcional ou que afetam apenas um órgão ou sistema anatômico. Nessa categoria, os sintomas apresentados são uniformes e o diagnóstico correto geralmente é fácil de ser realizado. A segunda categoria inclui doenças nas quais o defeito bioquímico afeta uma via metabólica comum a diferentes células ou órgãos, ou o defeito está restrito a um órgão, mas dá origem a consequências humorais e sistêmicas. As doenças pertencentes a esta categoria apresentam uma grande diversidade de sintoma e, devido a sua heterogeneidade, os EIM são classificados em três grupos, os quais incluem: 1) doenças do metabolismo intermediário afetando pequenas moléculas; 2) doenças envolvendo principalmente o metabolismo energético e 3) doenças envolvendo moléculas complexas (BELLETTATO et al., 2018).

1.2 Doenças lisossômicas

Entre as doenças que envolvem moléculas complexas, estão as doenças lisossômicas (DLs), as quais são responsáveis pelo acúmulo intracelular de um substrato não degradável no interior dos lisossomos das células reticuloendoteliais. Consequentemente, há um comprometimento e prejuízo da função de diferentes órgãos e sistemas no corpo, causando

manifestações clínicas tais como hepatomegalia, disfunção neurológica e problemas ósseos (BELLETTATO et al., 2018; HOFFMANN; ZSCHOCKE; NYHAN, 2017).

As DLs compreendem um grupo heterogêneo de mais de 50 doenças causadas pela deficiência de uma enzima específica ou componente lisossomal (BELLETTATO et al., 2018; STARETZ-CHACHAM et al., 2009). Esse grupo de doenças tem uma incidência estimada de 1 a cada 7.500 nascidos vivos na população em geral (COX; CACHÓN-GONZÁLEZ, 2012). As DLs são causadas principalmente por mutações em genes que codificam enzimas lisossomais responsáveis pela degradação de macromoléculas como glicolipídeos, glicoproteínas e mucopolissacarídeos (BRAVO et al., 2017; MEIKLE et al., 1999), podendo também afetar a função de transportadores específicos que são necessários para exportação de moléculas degradadas nos lisossomos (FERREIRA; GAHL, 2017; STIRNEMANN et al., 2017). Muitas das DLs possuem tratamento específico disponível, podendo ser através de terapia de reposição enzimática, transplante de células-tronco hematopoiéticas, inibição da síntese de substrato, chaperonas farmacológicas, entre outras estratégias (PLATT; LACHMANN, 2009; SHEMESH et al., 2013). Quanto mais precoce o diagnóstico do paciente e o início do tratamento, a melhor intervenção terapêutica poderá prevenir danos irreversíveis ou minimizar significativamente algumas manifestações clínicas da doença (GABRIELLI et al., 2016; MCGILL et al., 2010).

Entre as DLs mais frequentes está a doença de Gaucher (DG), a qual envolve o acúmulo de um substrato não degradável (glicoesfingolipídeos) decorrente de uma deficiência enzimática causada por mutações no gene que codifica essa enzima. O acúmulo de glicoesfingolipídeos dentro dos lisossomos dos pacientes com DG pode prejudicar o adequado funcionamento destas organelas, o que pode afetar diferentes vias e processos que ocorrem nos lisossomos. Visto que a DG apresenta uma grande heterogeneidade fenotípica, estratégias como identificação das mutações causadoras da doença, bem como a investigação de possíveis fatores modificadores do fenótipo podem ampliar o conhecimento da fisiopatogênese desta, permitindo um melhor manejo, acompanhamento e tratamento das comorbidades apresentadas pelos pacientes.

2. REVISÃO DA LITERATURA

2.1 Estratégias para localizar e selecionar as informações

Esta revisão da literatura foi focada na DG, apontando suas características moleculares e bioquímicas, manifestações clínicas, bem como diagnóstico e tratamento. A procura de artigos científicos também teve como foco o metabolismo da vitamina B_{12}, abordando o *status* desse micronutriente, o uso de biomarcadores para determinação dos níveis de B_{12} e o diagnóstico da deficiência de B_{12}. A estratégia de busca envolveu as seguintes bases de dados: *PubMed*, *Web of Science*, *LILACS* e *Scielo*, no período de 1882 a 2019. Foram realizadas buscas através dos termos "*Gaucher disease*", "*lysosomal disease*", "*glucocerebrosidase*", "*B_{12} metabolism*", "*B_{12} status*", "*B_{12} biomarkers*", "*B_{12} deficiency*", "*holo-TC*", "MMA", "*tHcy*", e algumas combinações. A figura esquemática contendo as combinações utilizadas estão apresentadas na Figura 1. A escolha dos artigos levou em consideração a data de publicação, a relevância e o número de citações.

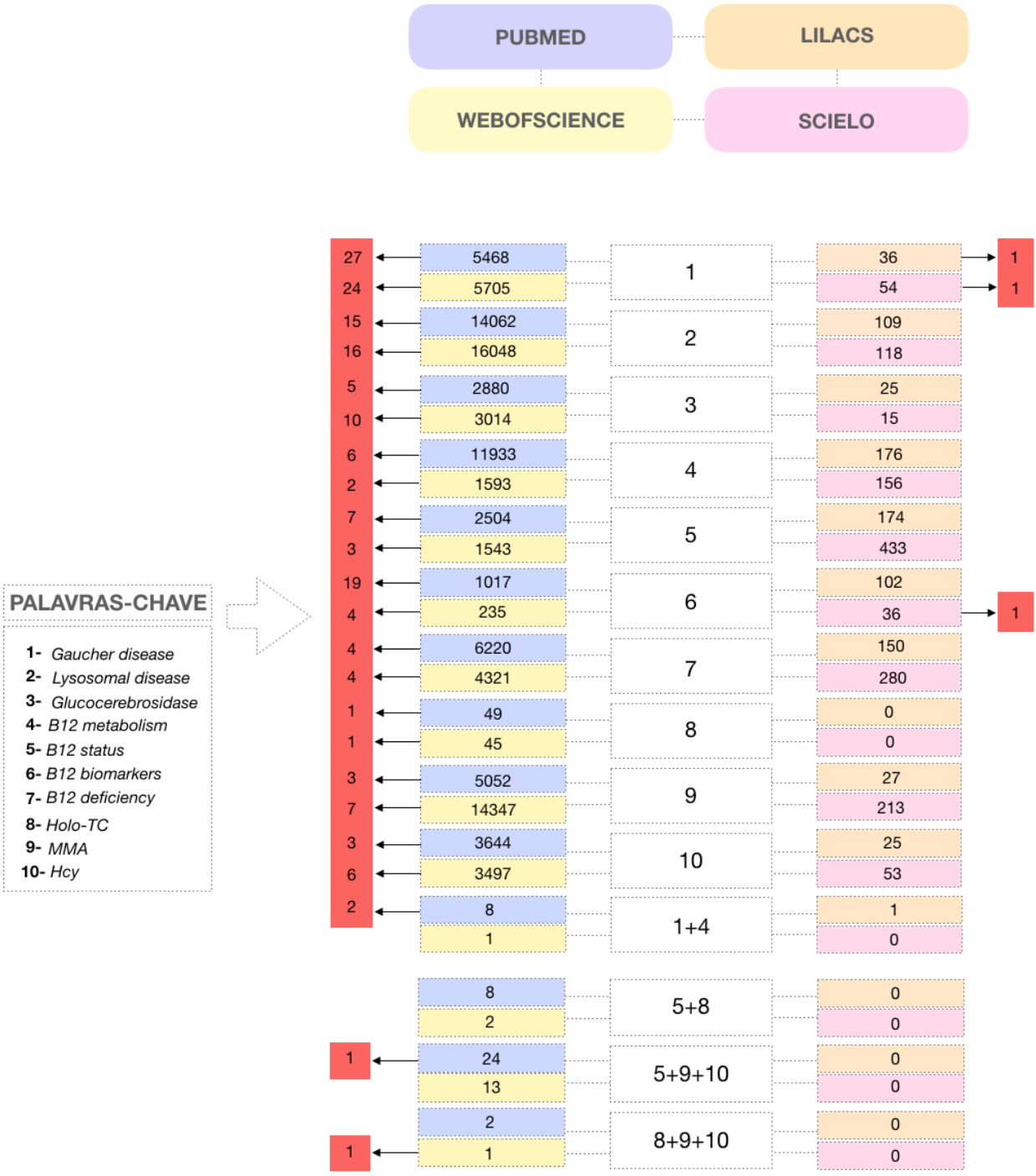

Figura 1. Estratégias para localizar e selecionar as informações. Caixas em vermelho indicam os artigos que foram incluídos na revisão. Este é o resultado da busca da combinação das palavras-chave. Fonte: Elaborado pela Autora (2019).

2.2 Doença de Gaucher

A doença de Gaucher (DG), uma das mais frequentes doenças lisossômicas, apresenta incidência estimada de 1 em cada 70.000 nascidos vivos na população geral, alcançando a frequência de 1 em cada 850 indivíduos na população de judeus Ashkenazi (BELLETTATO et al., 2018; BEUTLER et al., 1993). A DG é um EIM dos glicoesfingolipídeos, sendo herdada de forma autossômica recessiva. Esta doença é causada pela deficiência total ou parcial da atividade da enzima glicocerebrosidase (GCase; beta-glicosidade ácida (EC 3.2.1.45)) devido a mutações patogênicas bialélicas no gene *GBA1* (OMIM 606463, http://www.omim.org/entry/606463; VANIER; FROISSART, 2013), o qual codifica a enzima GCase (Figura 2). A GCase é responsável pela hidrólise do glicocerebrosídeo (GC) em glicose e ceramida nas células do sistema reticuloendotelial. Nos casos em que há um *déficit* enzimático, o substrato GC acumula-se no interior dos lisossomos, principalmente em macrófagos, resultando na formação das células de Gaucher (CG). Esse acúmulo de glicolipídeos nos macrófagos prejudica o adequado funcionamento de diferentes órgãos, principalmente fígado, baço e medula óssea (BEUTLER, 2006; HARMANCI; BAYRAKTAR, 2008a; HUGHES, 2009). Dessa forma, a DG é caracterizada como uma doença multissistêmica, apresentando grande heterogeneidade fenotípica, apesar de ser uma doença monogênica (NYHAN et al., 2012; PASTORES; HUGHES, 1993). Cabe destacar que existe uma forma extremamente rara da DG, a qual é causada por mutações bialélicas no gene *PSAP*. Esse gene codifica a enzima prosaposina (pSAP) que será processada a partir de uma clivagem proteolítica gerando a saposina C (Sap-C), a qual tem como função a ativação da GCase. Existem poucos casos descritos na literatura de pacientes com DG devido à deficiência de Sap-C (KANG et al., 2018; MOTTA et al., 2014; SCHNABEL; SCHRÖDER; SANDHOFF, 1991; TAMARGO et al., 2012; TYLKI-SZYMAŃSKA et al., 2007), sendo que esses pacientes geralmente apresentam manifestações clínicas semelhantes aos pacientes DG tipo III (STIRNEMANN et al., 2017).

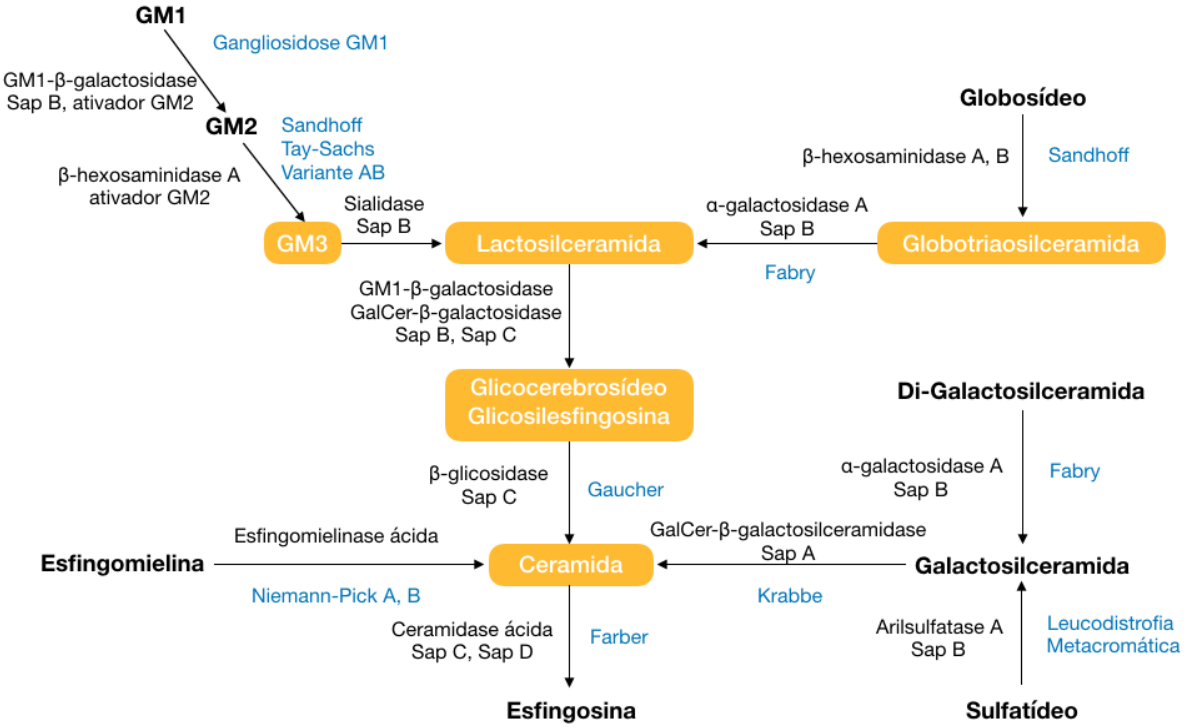

Figura 2. Rota de degradação dos glicoesfingolipídeos (Adaptado de SUN; ZHANG, 2013).

2.2.1 Breve histórico da doença

A DG foi a primeira doença lisossômica identificada, sendo descrita em 1882 por Phillipe CE Gaucher em sua tese de doutorado. Foi observado um aumento considerável do volume do baço de uma mulher de 32 anos, sendo reportada a presença de células repletas de lipídeos (GAUCHER, 1882). Novos casos foram sendo descritos ao longo dos anos possibilitando a identificação de uma heterogeneidade de manifestações clínicas da doença. Em 1934, o GC foi identificado como o principal substrato acumulado em diferentes órgãos de indivíduos com DG (AGHION, 1934). O mecanismo enzimático, entretanto, foi elucidado somente em 1965 por Roscoe Brady (BRADY; KANFER; SHAPIRO, 1965), quando foi demonstrado que a degradação do substrato era mediada pela enzima GCase (BRADY et al., 1966). Em 1989, o gene *GBA1* foi descrito, mostrando estar localizado no cromossomo 1 (HOROWITZ et al., 1989).

2.2.2 Fisiopatogênese

A deficiência total ou parcial da GCase, a qual atua no metabolismo dos glicoesfingolipídeos, ocorre devido a mutações no gene *GBA1*. O principal substrato a ser

metabolizado pela GCase é o GC a partir da via de hidrólise do substrato em ceramida e glicose (GRABOWSKI, 2013). Outra via em que a GCase atua é na formação de esfingosina (Sph) e ceramida a partir do substrato glicosilesfingosina (GlcSph), o qual constitui uma forma deacilada do GC, sendo potencialmente tóxico e geralmente apresenta níveis aumentados em pacientes com comprometimento neurológico, sugerindo uma importante contribuição na patogênese da forma neuronopática da DG (ORVISKY et al., 2002) (Figura 3). Como consequência, tal deficiência leva ao acúmulo principalmente do substrato GC nos lisossomos de macrófagos (SMITH; MULLIN; SCHAPIRA, 2017). Esses macrófagos ingurgitados de glicolipídeos são conhecidos como CG e são característicos da doença na análise histológica, sendo distribuídas por todo o corpo e comprometendo o funcionamento de diferentes órgãos, o que caracteriza a doença como multissistêmica (MARKUSZEWSKA-KUCZYNSKA et al., 2015).

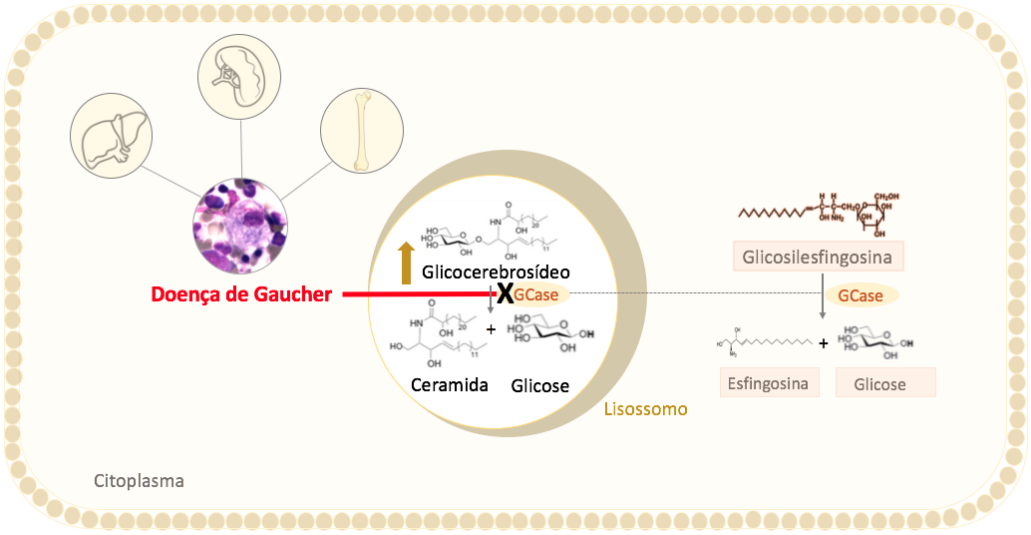

Figura 3. Reações de degradação catalisadas pela enzima GCase representando a DG e os principais órgãos afetados nessa doença (Autora, 2019).

2.2.3 Manifestações clínicas

As principais manifestações clínicas da DG incluem hepatoesplenomegalia, trombocitopenia, anemia, problemas ósseos e pulmonares, além de envolvimento neurológico nas formas neuronopáticas da doença (GRABOWSKI, 2012; HRUSKA et al., 2008). Os sintomas variam entre os pacientes, inclusive entre os que possuem o mesmo genótipo e/ou apresentam o mesmo nível de deficiência da atividade enzimática (BIEGSTRAATEN et al., 2011; STIRNEMANN et al., 2017). A DG é classificada em três principais categorias: DG

tipo I (sem acometimento neurológico) e DG tipos II e III (com envolvimento neurológico). (SIDRANSKY, 2004).

- DG tipo I: conhecida como forma não neuronopática (OMIM 230800), corresponde a mais de 90% dos casos de DG no mundo (GRABOWSKI, 2013; ZIMRAN et al., 2018). Essa forma é caracterizada por uma grande variabilidade na progressão e gravidade da doença, variando de indivíduos assintomáticos até pacientes com grave comprometimento de determinados órgãos, acomete crianças e adultos, podendo ocorrer o início dos sintomas da doença em qualquer idade. As manifestações clínicas mais comuns são hepatoesplenomegalia, anemia, trombocitopenia e problemas ósseos. As citopenias decorrem do sequestro esplênico e da infiltração da medula óssea, já o acúmulo de CG na medula óssea pode levar a diferentes doenças ósseas, incluindo a osteonecrose. Quando as manifestações clínicas aparecem precocemente, geralmente o quadro da doença está associado a um grave e rápido comprometimento dos órgãos afetados. A sobrevida do paciente pode ser normal dependendo da gravidade das complicações (GRABOWSKI, 2004). Pacientes com DG tipo I possuem risco aumentado de desenvolvimento de mieloma múltiplo (MM), carcinoma hepatocelular (HCC), linfoma, doença de Parkinson (DP) e leucemia (DAVIDSON et al., 2018; GEGG; SCHAPIRA, 2018; LANDGREN et al., 2007; MISTRY et al., 2013; ZIMRAN et al., 2005).
- DG tipo II: conhecida como forma neuronopática aguda (OMIM 230900), é o tipo mais raro da doença, representando em torno de 2% dos casos (STIRNEMANN et al., 2017). É caracterizado pelo aparecimento precoce das manifestações clínicas sistêmicas e do envolvimento do sistema nervoso central (SNC), resultando em óbito nos primeiros dois anos de vida devido à rápida progressão da doença (GRABOWSKI, 2013; SIDRANSKY, 2004). Nesse tipo de DG ocorre o comprometimento do cérebro, fígado, baço e pulmões, sendo a falência pulmonar uma das principais causas de falecimento dos pacientes (MIGNOT et al., 2006). Entre as principais manifestações clínicas da DG tipo II estão: atraso no desenvolvimento, problemas oculares como estrabismo, paralisia do olhar vertical, opistótono, espasticidade, hidropsia fetal, ictiose congênita, entre outras (GOKER-ALPAN et al., 2003; TAYEBI; STONE; SIDRANSKY, 1999).

- DG tipo III: conhecida como forma neuronopática crônica (OMIM 231000), corresponde à forma intermediária da DG, pois compromete as funções do SNC de maneira mais lenta e gradual em comparação à DG tipo II (GRABOWSKI, 2013; SIDRANSKY, 2012), representando em torno de 5% dos casos (STIRNEMANN et al., 2017). Essa forma da DG

inclui pacientes com manifestações sistêmicas e comprometimento neurológico que podem se manifestar ao longo do tempo, geralmente acompanhado de epilepsia, ataxia, paralisia do olhar vertical, além de demência. Além disso, pacientes com DG tipo III podem apresentar doença cardíaca valvular (HRUSKA et al., 2008).

Apesar da DG ser classificada em diferentes tipos, muitas vezes não é possível fazer uma distinção entre as diferentes formas da doença. Portanto, a DG pode ser considerada como um espectro de fenótipos, visto que ela apresenta uma heterogeneidade de manifestações clínicas, variando desde pacientes assintomáticos até pacientes com sintomas extremamente graves (SIDRANSKY, 2004) (Figura 4).

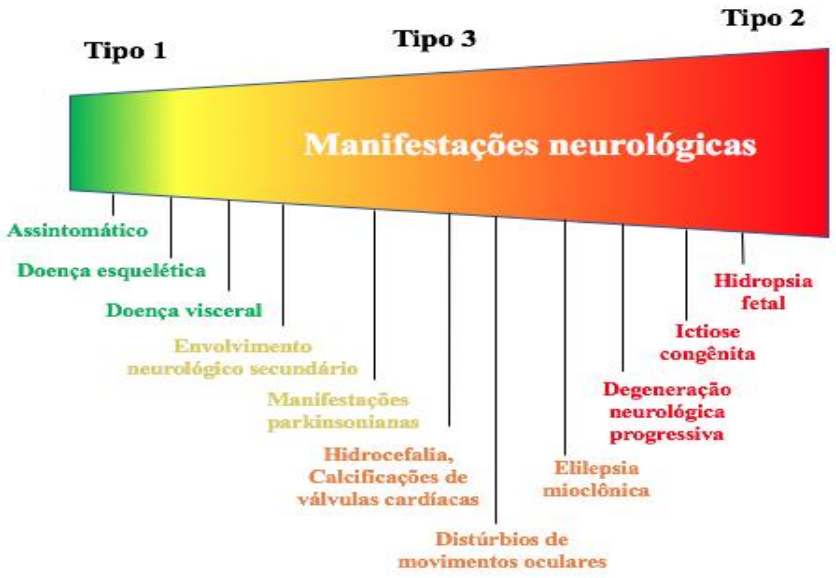

Figura 4. Classificação clínica da DG como um espectro de fenótipos (adaptado de SIDRANSKY, 2004).

2.2.4 Genética

O gene *GBA1* está localizado no *locus* 1q21, é dividido em 11 éxons e compreende 7,6 kb de DNA genômico (Figura 5). O *GBA1* possui um pseudogene, conhecido como *GBAP*, que apresenta 96% de homologia de sequência, representa 5,7 kb do genoma e está localizado a 16 kb do gene funcional (HOROWITZ et al., 1989; WINFIELD et al., 1997). O RNA mensageiro transcrito a partir do *GBA1* apresenta dois sítios de iniciação da tradução, sendo um localizado no éxon 1 e o outro no éxon 2. Ambos os sítios são eficientemente traduzidos e produzem proteínas funcionais. Enquanto o primeiro códon ATG utilizado para

o início da tradução produz uma proteína com 39 resíduos de aminoácidos na sequência do peptídeo sinal, o segundo ATG produz uma proteína com 19 resíduos de aminoácidos, sendo ambos processados para gerar uma GCase madura contendo 497 aminoácidos (HRUSKA et al., 2008). Dessa forma, existem duas nomenclaturas para designar as mutações. A forma recomendada pela *Human Genome Variation Society* (HGVS) é nomear de acordo com o aminoácido da metionina do primeiro sítio de iniciação da tradução, considerando a proteína de 39 resíduos de aminoácidos na sequência do peptídeo sinal. Porém, por muitos anos, as mutações no *GBA1* foram descritas considerando o primeiro aminoácido da proteína madura, sendo essa forma de nomenclatura mais conhecida e predominantemente utilizada nos estudos (GRABOWSKI, 2013; HRUSKA et al., 2008).

Já foram descritas na base de dados *The Human Gene Mutation Database* cerca de 500 variantes patogênicas no *GBA1* (HGMD, 2019; www.hgmd.cf.ac.uk), incluindo diferentes tipos de mutações, tais como: de ponto (*missense*, *nonsense* e sinônima), em sítios de *splicing*, mutações que alteram o quadro de leitura (*frameshift*), inserções e deleções, além de alelos complexos resultantes de eventos de recombinação ou conversão com o *GBAP*. A presença de variantes no *GBA1* pode resultar na alteração da estabilidade e/ou da capacidade catalítica da GCase (GRABOWSKI, 2013).

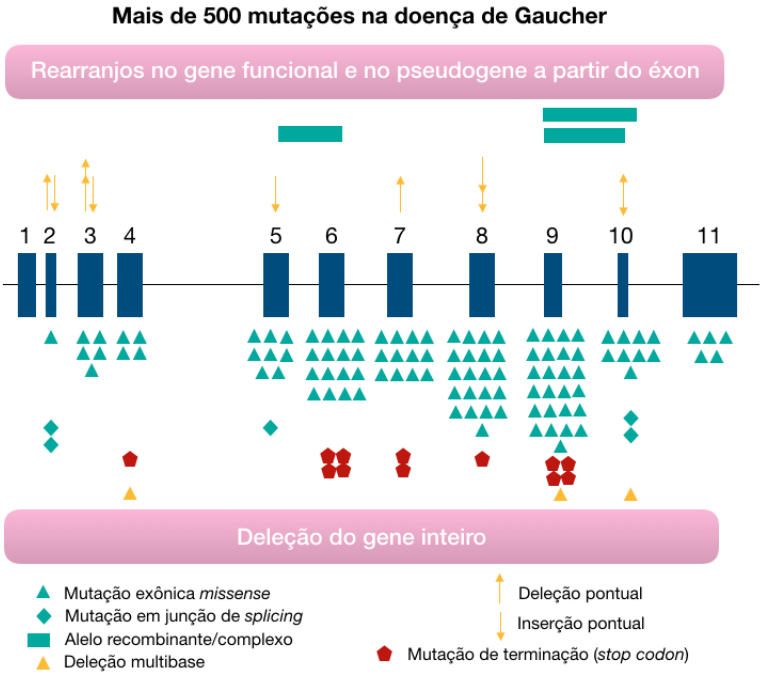

Figura 5. Estrutura do gene *GBA1* representando os tipos e as localizações das mutações mais comuns (Adaptado de VANIER; FROISSART, 2013).

A distribuição e a frequência das diferentes mutações variam conforme a população em estudo. De uma maneira geral, três dessas alterações são frequentemente encontradas na população de pacientes com DG, sendo elas: N370S (c.1226A>G; p.Asn409Ser), L444P (c.1448T>C; p.Leu483Pro) e 84GG (c.84dupG), sendo a N370S e a L444P as primeiras mutações descritas (GRABOWSKI, 2013; TSUJI et al., 1988, 1987). A frequência dessas duas mutações em pacientes brasileiros é semelhante a de outras populações, correspondendo a 50% e 25% dos alelos mutantes identificados, respectivamente. Além disso, a mutação G377S (c.1246G>A; p.Gly416Ser) é frequente no Brasil, representando a terceira variante mais frequente entre os pacientes com DG no nosso país (ROZENBERG et al., 2006). Em pacientes com DG de origem Ashkenazi, quatro mutações (N370S, L444P, 84GG e IVS2+1G>A) representam cerca de 90% dos alelos causadores da doença. Porém, essas mesmas mutações representam em torno de 50% dos alelos patogênicos em outras populações (HUGHES; PASTORES, 2010).

Eventos de recombinação entre *GBA1* e *GBAP* ocorrem com frequência, podendo dar origem aos alelos complexos. O alelo complexo mais comum é o Rec*Nci*I, o qual compreende 3 mutações (L444P, A456P e V460V) *in cis* no éxon 10 do *GBA1* (HRUSKA et al., 2008; ZAMPIERI et al., 2017). Visto que existe uma grande heterogeneidade de mutações no *GBA1*, é extremamente importante realizar a análise completa do gene, bem como de suas regiões de junção éxon-íntron, para definir o genótipo dos pacientes com DG. Além disso, diferentes técnicas de análise genética podem ser utilizadas para detecção de alelos recombinantes, além de deleções e/ou inserções no *GBA1* (BASGALUPP et al., 2018). É importante destacar que a análise molecular deve ser extremamente cuidadosa e específica para análise do gene funcional (*GBA1*), garantindo que a amplificação não ocorra no *GBAP*, evitando um resultado falso positivo.

2.2.5 Variabilidade fenotípica e correlação genótipo-fenótipo

Existe uma grande variabilidade fenotípica em pacientes com DG. Embora exista a predominância de determinadas variantes, como a N370S e a L444P, há uma intensa heterogeneidade alélica dessa doença, o que dificulta estabelecer uma associação genótipo-fenótipo. Pacientes com mesmo genótipo, inclusive irmãos gêmeos, podem apresentar diferentes fenótipos, complicações e respostas ao tratamento, da mesma forma que indivíduos com diferentes genótipos muitas vezes apresentam manifestações clínicas semelhantes (BIEGSTRAATEN et al., 2011; DAVIDSON et al., 2018; HRUSKA et al., 2008; MISTRY

et al., 2017). Portanto, não existe uma correlação direta entre determinadas mutações e manifestações clínicas da doença, nem entre a quantidade de substrato acumulado e/ou níveis de atividade da GCase e fenótipo dos pacientes (SIDRANSKY, 2012).

A mutação N370S geralmente tem um efeito menor sobre o fenótipo dos pacientes, sendo considerada neuroprotetora, pois protege os indivíduos de desenvolver a forma neuronopática da doença (HUANG; ZHANG; CHEN, 2015; PASTORES; HUGHES, 1993). Quando presente em homozigose, os pacientes apresentam um amplo espectro de sintomas clínicos, variando desde a forma assintomática até multissistêmica (HOROWITZ; ZIMRAN, 1994). No entanto, menos frequentemente, pacientes com essa mutação podem apresentar envolvimento neurológico, apresentando sintomas como estrabismo, desordens de movimentos dos olhos, demência, tremor, entre outros (ALFONSO et al., 2007). Já a mutação L444P apresenta um efeito maior sobre o fenótipo dos pacientes e está associada à forma neuronopática quando presente nos dois alelos do *GBA1* (DANDANA et al., 2016; GRABOWSKI, 2012), assim como a variante D409H (c.1342G>C; p.Asp448His) está associada a problemas cardiovasculares graves, sendo fatal em pacientes homozigotos (BOHLEGA et al., 2000).

Eventos de recombinação podem ocorrer entre *GBA1* e *GBAP*, resultando em alelos recombinantes, sendo o Rec*Nci*I o mais comum entre os alelos complexos. Esse alelo apresenta três mutações de ponto, sendo que em duas delas ocorre a substituição de aminoácidos (L444P e A456P) e a outra é uma variante silenciosa, na qual a mudança da base não altera o aminoácido (V460V), todas localizadas no éxon 10 do gene. Estudos sugerem que os pacientes heterozigotos para as alterações L444P e Rec*Nci*I apresentam um pior prognóstico, sendo o alelo L444P fortemente associado com a forma neuronopática da DG. O alelo recombinante em homozigose representa uma forma letal da doença, geralmente nos primeiros dias de vida, sugerindo que a presença de alelos complexos está associada à maior gravidade da DG (EL-MORSY et al., 2011; TAYEBI et al., 2003). Além disso, a variante L444P combinada com o alelo complexo Rec*Nci*I está associada ao tipo II da DG, enquanto que o genótipo L444P/L444P está associado ao tipo III da DG (HRUSKA et al., 2008).

Existem diversos fatores que estão agindo sobre o fenótipo da DG, atuando como modificadores, entre eles: alelos complexos, genes contíguos (inclusive o pseudogene), substratos alternativos, proteínas transportadoras, entre diversos modificadores genéticos e fatores ambientais (Figura 6) (SIDRANSKY, 2004).

Figura 6. Fatores que contribuem para a variabilidade fenotípica observada em pacientes com DG (adaptado de SIDRANSKY, 2004).

2.2.6 Modificadores do fenótipo

A DG apresenta uma ampla heterogeneidade fenotípica, apesar de algumas mutações já terem sido associadas a determinados fenótipos. A presença de uma mutação específica no *GBA1* não é suficiente para explicar as divergências fenotípicas (GOKER-ALPAN et al., 2005). Dessa forma, diversos fatores como modificadores genéticos parecem estar influenciando as diferentes apresentações clínicas dos pacientes (SIDRANSKY, 2004). De forma geral, genes modificadores podem alterar o fenótipo clínico por meio de quatro mecanismos básicos: alterando penetrância, expressividade, dominância e/ou pleiotropia (DAVIDSON et al., 2018). Os modificadores do fenótipo da DG podem estar envolvidos em diferentes processos, sejam em etapas metabólicas, em genes que codificam proteínas ativadoras, em erros durante o transporte, processamento ou degradação da GCase. Entender os mecanismos que envolvem a fisiopatogênese da doença é extremamente importante, bem como possíveis modificadores do fenótipo da DG, pois permitirão o desenvolvimento de novas estratégias terapêuticas e possibilitarão o estabelecimento de novas correlações

genótipo-fenótipo. Entre os principais genes modificadores do fenótipo da DG estão: *SCARB2*, *GBA2* e *PSAP*.

Estudos funcionais e clínicos sugerem que a proteína receptora LIMP-2 (*Lysosomal Integral Membrane Protein type 2*; proteína lisossomal integral de membrana do tipo 2), a qual transporta a GCase e é codificada pelo gene *SCARB2*, pode modificar a expressividade da DG (DAVIDSON et al., 2018). A proteína LIMP-2 é expressa constitutivamente e é responsável pela ligação e pelo direcionamento da GCase aos lisossomos (RECZEK et al., 2007). Essa ligação entre a enzima e a proteína é pH-dependente, sendo favorecida em pH neutro do retículo endoplasmático (RE) e interrompida ao chegar em pH ácido dentro dos lisossomos (ZACHOS et al., 2012), local em que a GCase é liberada do seu receptor LIMP-2, tornando-se disponível para degradação do substrato GC (RECZEK et al., 2007; ZACHOS et al., 2012). A deficiência da GCase também pode ser consequência de distúrbios que ocorrem durante o transporte e o direcionamento da enzima ao lisossomo. Dessa forma, um mau dobramento da enzima durante seu transporte para RE pode levar a sua degradação prematura via proteossomo. Até recentemente, era descrito que a LIMP-2 direcionava a GCase para o lisossomo apenas por uma rota independente de manose-6-fosfato. Porém, atualmente está estabelecido que essa proteína transporta a GCase para o lisossomo tanto por uma via independente como também como parte de um complexo com manose-6-fosfato (DAVIDSON et al., 2018).

Mutações no gene *SCARB2* estão associadas à síndrome de insuficiência renal com ação mioclônica, a qual apresenta herança autossômica recessiva e é caracterizada por dano renal, epilepsia mioclônica progressiva e ataxia (BLANZ et al., 2010). Alterações nesse gene também causam deficiência da proteína LIMP-2. Como consequência, a proteína fica retida dentro do RE e interfere na atividade da GCase, pois impede o transporte da enzima para os lisossomos (BLANZ et al., 2010). Dessa forma, a análise molecular do *SCARB2*, bem como a identificação de mutações e a análise de expressão gênica, sugerem que a proteína LIMP-2 tem importância fundamental na biogênese e na manutenção de endossomos tardios e lisossomos, inclusive na fusão entre lisossomos e autofagossomos (GLEICH et al., 2013). Mutações no *SCARB2* já foram identificadas em pacientes com DG com sintomas graves, associadas com a redução da quantidade da proteína LIMP-2 disponível e também com a redução da atividade da GCase (VELAYATI et al., 2011). Portanto, a LIMP-2 é essencial para o correto direcionamento da GCase aos lisossomos em pacientes com DG, sendo o *SCARB2* um forte candidato a modificador do fenótipo da DG. Um prejuízo na função de

LIMP-2 acarreta uma redução da atividade da GCase, ocasionando o acúmulo de GC, a liberação da GCase para o meio extracelular e/ou a degradação da enzima via proteossomo (VELAYATI et al., 2011).

O gene *GBA2* codifica uma glicocerebrosidade não-lisossomal associada à membrana do RE e ao complexo de Golgi, a qual é responsável pela hidrólise de O-glicosídeos e GC (KÖRSCHEN et al., 2013). Em pacientes com DG, a deficiência da atividade da GCase pode causar um aumento na expressão do *GBA2* como mecanismo compensatório (DAVIDSON et al., 2018). Quando há uma deficiência enzimática de GCase 1 e 2 concomitantemente, geralmente ocorre um maior acúmulo de GC dentro e fora dos lisossomos, em órgãos que são afetados na DG, tais como fígado, baço, medula óssea, sugerindo um papel do *GBA2* como fator modificador da DG (YILDIZ et al., 2013).

Da mesma forma que a LIMP-2, a Sap-C é essencial para a atividade da GCase devido ao seu papel de ativadora da enzima (GRABOWSKI, 2013; VACCARO et al., 2010), embora o mecanismo pelo qual essa ativação ocorra ainda não esteja bem estabelecido. Um possível mecanismo sugere que a Sap-C induz alterações na membrana, intermediando a exposição de GC e facilitando a interação entre a GCase e o substrato GC (TAMARGO et al., 2012). Além de ser responsável pela ativação da GCase, a Sap-C atua protegendo a enzima de degradação proteolítica (SUN; QI; GRABOWSKI, 2003).

Muito raramente, a DG pode ser causada por mutações patogênicas no gene *PSAP*, levando à deficiência da Sap-C, sem apresentar deficiência da GCase. Poucos casos de deficiência de Sap-C foram descritos na literatura e apesar de não terem sido identificados pacientes com deficiência de GCase e Sap-C concomitantemente, Sap-C parece ser um forte candidato a modificador do fenótipo da DG, visto que variantes do gene *PSAP* podem influenciar as manifestações clínicas bem como a intensidade dos sintomas apresentados pelos pacientes com DG (DAVIDSON et al., 2018; TAMARGO et al., 2012).

Visto que existem diversos fatores que estão influenciando as diferentes manifestações clínicas da DG, a possibilidade de ampliar o conhecimento desses modificadores do fenótipo da DG através da descoberta de novos genes envolvidos nas diferentes apresentações clínicas da doença é extremamente importante para compreender os mecanismos envolvidos na DG.

2.2.7 Diagnóstico

O diagnóstico da DG geralmente tem início com o aparecimento dos sintomas do indivíduo, levando à suspeita clínica da doença. O método padrão-ouro de diagnóstico é a medida da atividade da GCase em leucócitos e/ou fibroblastos, podendo ser também realizado a partir de sangue impregnado em papel filtro (SOZMEN; SEZER, 2017). Existem diversos protocolos utilizados para a análise da atividade enzimática, variável entre diferentes laboratórios e que pode ser realizada por diferentes métodos, como ensaio fluorimétrico e/ou espectrometria de massa em tandem (WOLF et al., 2018). A maioria deles utiliza um substrato sintético (4-metilumbeliferil-β-D-glicopiranosídeo) associado a detergentes para solubilizar e estabilizar a enzima (GRABOWSKI, 2013). Geralmente, pacientes com DG apresentam uma atividade residual da enzima cerca de 10-15% do valor de um indivíduo saudável (STIRNEMANN et al., 2017).

Mesmo sendo o teste padrão-ouro para diagnóstico, a dosagem da atividade enzimática pode não diferenciar indivíduos heterozigotos daqueles que não apresentam mutações no *GBA1*. Dessa forma, a análise genética pode ser indicada para identificação de heterozigotos, sendo também, uma ferramenta extremamente importante para o diagnóstico dos pacientes com DG, podendo auxiliar na classificação da forma clínica da DG ou na distinção entre as formas neuronopáticas e não-neuronopáticas da doença (GRABOWSKI, 2013). A investigação genética pode ser realizada a partir da busca das mutações mais frequentes no gene tais como N370S e L444P, como também do sequenciamento completo do *GBA1*, utilizando a metodologia de Sanger ou *Next-generation sequencing* (NGS).

2.2.8 Tratamento

Existem dois tratamentos principais disponíveis para pacientes com DG: a terapia de reposição enzimática (TRE) e a terapia de redução de substrato (TRS) (STIRNEMANN et al., 2017). De acordo com os dados do Ministério da Saúde, existem cerca de 700 pacientes com DG realizando tratamento no Brasil, sendo que aproximadamente 96% deles utilizam a TRE e 4% estão em uso da TRS (http://portalms.saude.gov.br).

A TRE consiste na administração intravenosa de uma forma recombinante da enzima GCase, sendo que a dose varia de acordo com as manifestações clínicas de cada paciente. Geralmente a infusão ocorre quinzenalmente, podendo variar em casos específicos e requer a supervisão de um profissional da saúde treinado. O objetivo dessa terapia é repor a GCase

que está deficiente em pacientes com DG através da administração exógena da enzima recombinante, permitindo que ocorra a redução do acúmulo do substrato GC e melhora dos sintomas apresentados pelos pacientes. É importante a presença de um farmacêutico para o monitoramento da administração da dose adequada a cada paciente. A TRE teve seu início com a utilização da enzima GCase purificada de placenta humana, denominada Alglucerase, que foi posteriormente substituída pela forma recombinante da enzima (Imiglucerase). As formas utilizadas de TRE que estão aprovadas pelo *Food and Drug Administration* (FDA) e pelo Ministério da Saúde (MS), e comercialmente disponíveis para o tratamento de pacientes com DG são: imiglucerase, alfavelaglicerase e alfataliglicerase. As diferenças entre essas três formas estão na forma como são produzidas e na sua composição. O tratamento com TRE apresenta algumas desvantagens, dentre as quais destacam-se: elevado custo (estima-se um gasto de até 300 mil dólares/ano por paciente) (HOLLAK et al., 2011), incapacidade da enzima atravessar a barreira hemato-encefálica (mais efetiva para pacientes com DG tipo I) (CHARROW; SCOTT, 2015; HOLLAK et al., 2009), administração intravenosa quinzenalmente, eficácia e distribuição da enzima não uniformes em diferentes tecidos e aumento da produção de anticorpos contra a enzima recombinante (WEINREB; LEE, 2013). A TRE tem sido altamente efetiva para reversão de manifestações viscerais, hematológicas e ósseas (MISTRY et al., 2017), apresentando uma redução de 50 – 60% do volume do baço em pacientes com DG no período de dois a cinco anos em tratamento; além de uma redução de 30 – 40% do tamanho do fígado em até cinco anos de tratamento; e aumento do número de plaquetas em torno de duas vezes dentro de cinco anos de TRE (ELSTEIN, 2013).

A TRS atua diretamente sobre o substrato acumulado e consiste de pequenos compostos inibidores da síntese do substrato GC, os quais podem se difundir rapidamente em vários tecidos, inclusive para os ossos e SNC (WAGNER et al., 2018). Em vez de repor a enzima que está deficiente, essa abordagem atua inibindo o acúmulo de GC no interior dos lisossomos, por meio da inibição da enzima glicosilceramida sintase, a qual é responsável pela síntese de GC. Os inibidores aprovados pelo FDA e MS que estão comercialmente disponíveis para o tratamento de pacientes com DG são: miglustate e eliglustate. A administração do medicamento é via oral e ocorre diariamente. Essa abordagem tem como vantagem a utilização de uma molécula pequena administrada via oral que não é capaz de provocar resposta imunológica e permite cruzar a barreira hemato-encefálica, podendo favorecer pacientes com as formas neuronopáticas da doença. Porém, ainda não se sabe se a TRS tem impacto na prevenção ou se pode reverter as manifestações neurológicas

apresentadas pelos pacientes (CERAVOLO et al., 2017). A TRS tem se mostrado efetiva para doença visceral, similarmente à TRE, porém as respostas hematológicas são mais lentas e menos efetivas comparadas à TRE (MISTRY et al., 2017). Os efeitos colaterais da TRS são mais expressivos que os da TRE e podem incluir diarréia, tremor, parestesia, cefaléia, artralgia, perda de peso, entre outros (VAN ROSSUM; HOLSOPPLE, 2016).

Outros tipos de tratamento para DG que estão emergindo como alternativas são: uso de chaperonas, terapia gênica, transplante de células-tronco, entre outros (TRAPERO; LLEBARIA, 2013). As chaperonas têm sido bastante exploradas não só para DG, mas também para outras doenças. Por terem um tamanho pequeno, essas moléculas se distribuem uniformemente entre os tecidos e têm potencial de atravessar a barreira hemato-encefálica, o que poderia prevenir o acúmulo do substrato GC no SNC de pacientes com a forma neuronopática da doença (Figura 7) (SÁNCHEZ-FERNÁNDEZ; GARCÍA FERNÁNDEZ; ORTIZ MELLET, 2016; TRAPERO; LLEBARIA, 2013). Uma estratégia terapêutica seria o uso de TRE ou TRS combinado com chaperonas farmacológicas podendo ser uma forma de tratamento mais efetivo do que o uso dessas moléculas de forma isolada. Além disso, o uso de terapia gênica vem crescendo consideravelmente nos últimos anos para diferentes doenças genéticas, inclusive para algumas DLs (EMERY, 2004; GIUGLIANI et al., 2016; GONZALEZ; BALDO, 2017; WILLIAMS, 2014).

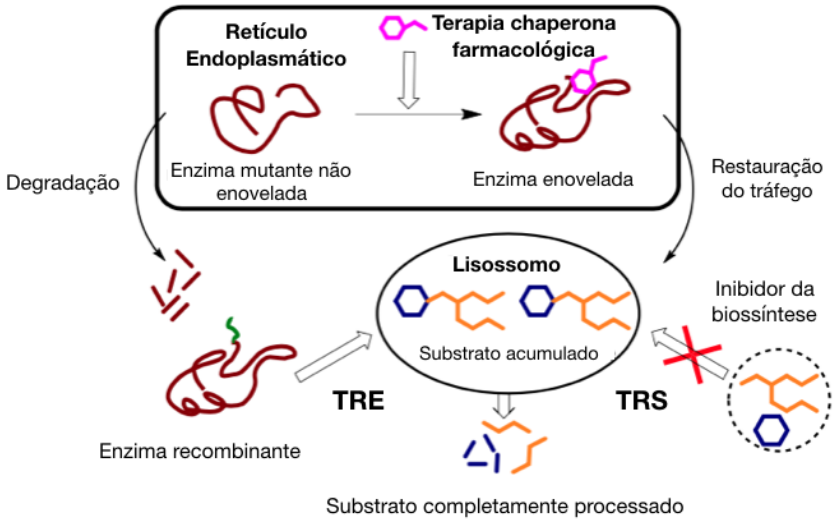

Figura 7. Representação esquemática de estratégias terapêuticas disponíveis para DG (Adaptado de SÁNCHEZ-FERNÁNDEZ; GARCÍA FERNÁNDEZ; ORTIZ MELLET, 2016).

2.2.9 Biomarcadores

Os biomarcadores são substâncias que podem ser mensuradas em amostras biológicas e são extremamente importantes para monitorar a resposta ao tratamento em pacientes com DG. Diferentes moléculas têm sido utilizadas como indicadores do estado da DG, auxiliando no diagnóstico, no prognóstico e no monitoramento da resposta ao tratamento. Essas substâncias são liberadas pelas CG ou por células com acúmulo de GC e seus níveis estão alterados em pacientes com DG quando comparados a indivíduos saudáveis. O acúmulo de GC é responsável pela ativação dos macrófagos, os quais liberam citocinas e diversas moléculas no plasma dos pacientes. Entre as moléculas liberadas pelos macrófagos ativados estão a enzima quitotriosidase (QT), a quimiocina *chemokine* (*C-C motif*) *ligand* 18/*Pulmonary and Activation-Regulated Chemokine* (CCL18/PARC) e a GlcSph, sendo estas, entre outras, consideradas biomarcadores da DG.

A QT é uma enzima pertencente à família das quitinases sendo sintetizada e secretada principalmente por macrófagos ativados (ELMONEM; VAN DEN HEUVEL; LEVTCHENKO, 2016). Os níveis plasmáticos de QT não são diretamente proporcionais à gravidade das manifestações da DG, mas tendem a diminuir após o início do tratamento (BODAMER; HUNG, 2010). Pacientes com DG geralmente apresentam níveis de atividade da QT aumentados, podendo chegar em até 1000 vezes os dos valores de referência (GRABOWSKI, 2013; HARMANCI; BAYRAKTAR, 2008). QT não é um biomarcador específico para DG, visto que este pode estar elevado em diferentes condições. Porém, esse biomarcador pode ser usado para monitorar a eficácia do tratamento, podendo ter um valor prognóstico (STIRNEMANN et al., 2017). Além disso, tanto pacientes com DG quanto indivíduos saudáveis podem apresentar deficiência da enzima QT devido à duplicação bialélica de 24 pares de bases (dup24; rs3831317) no éxon 10 do gene *CHIT1*, o qual codifica essa enzima (GRABOWSKI, 2013).

A CCL18/PARC é sintetizada e liberada pelas CG, resultando em níveis aumentados em até 50 vezes no plasma de pacientes com DG comparados aos indivíduos saudáveis e tem a função de mediar a quimiotaxia dos leucócitos. Essa proteína está diretamente associada com a quantidade de substrato acumulado nos macrófagos e com a resposta ao tratamento, sendo que altos níveis de CCL18/PARC estão associados com um pior prognóstico da doença (STIRNEMANN et al., 2017). Estudos mostram que altos níveis de CCL18/PARC aumentam o risco de desenvolvimento de complicações ósseas, principalmente osteonecrose

(PAVLOVA et al., 2011; PAVLOVA; DEEGAN; COX, 2012), porém esta proteína pode não ser um fator determinante para o diagnóstico da DG uma vez que está alterada em diversas doenças inflamatórias (STIRNEMANN et al., 2017). O uso dessa proteína como biomarcador pode ser uma alternativa para pacientes que apresentam a dup24 no *CHIT1*, e que, portanto, não apresentam níveis elevados de QT.

Outro biomarcador que atualmente tem sido considerado como o mais promissor para DG, por ser mais específico e sensível que QT e CCL18/PARC, é a GlcSph (STIRNEMANN et al., 2017). Esse biomarcador parece ter grande valor para o monitoramento dos pacientes com DG, mas ainda não está acessível à grande parte dos laboratórios (STIRNEMANN et al., 2017). Estudos têm mostrado que os níveis de GlcSph estão elevados no plasma especificamente em pacientes com DG. Pacientes não tratados apresentam níveis plasmáticos de GlcSph até 500 vezes maior quando comparado ao valor de referência. Enquanto a concentração dessa proteína no cérebro de pacientes com DG tipo I apresenta níveis normais, em pacientes com DG tipos II e III os valores de GlcSph podem chegar a mais de 1000 vezes o valor normal (NILSSON; SVENNERHOLM, 1982), destacando seu envolvimento no comprometimento do SNC (ORVISKY et al., 2002). Portanto, o acúmulo de GlcSph pode causar disfunção neuronal, levando principalmente aos sintomas neurológicos relacionados à DG (STIRNEMANN et al., 2017).

Os elevados níveis de GlcSph podem estar relacionados com o genótipo do paciente, o que poderia refletir no progresso e na melhora dos sintomas após o início do tratamento em pacientes com DG. Pacientes homozigotos para N370S apresentam níveis menores de GlcSph que pacientes heterozigotos compostos para N370S/L444P e homozigotos para L444P (DEKKER et al., 2011; ROLFS et al., 2013). Pacientes em tratamento com TRE apresentam níveis reduzidos de GlcSph após os primeiros seis meses (ROLFS et al., 2013), sendo observada uma redução ainda maior em pacientes em tratamento com TRS, mais especificamente com eliglustate (SMID et al., 2016). Estudos têm mostrado que a GlcSph apresenta efeitos tóxicos em diferentes tipos celulares, incluindo oligodendrócitos, células neuronais, células *natural killers* (NK) e osteoblastos, o que contribui para as características clínicas apresentadas pelos pacientes com DG (MISTRY et al., 2010; NAIR et al., 2015; SUEYOSHI; MAEHARA; ITO, 2001; TOHYAMA; MATSUDA; SUZUKI, 2001).

Em virtude da ativação dos macrófagos pelo acúmulo de GC em pacientes com DG, algumas funções do organismo podem ser afetadas, como por exemplo, o metabolismo do ferro. Para avaliar se há um mau funcionamento nesse processo, um importante biomarcador

a ser utilizado é a ferritina, a qual é responsável pelo direcionamento do ferro aos tecidos (LI et al., 2009; ORINO, 2016). Pacientes com DG tipo I geralmente apresentam níveis de ferritina aumentados bem como de hepcidina, a qual é o hormônio regulador da homeostase do ferro, enquanto que os níveis de saturação de transferrina e ferro sérico são mantidos normais. Pacientes com DG geralmente apresentam níveis significativamente reduzidos de ferritina após o início do tratamento (STIRNEMANN et al., 2011). Esse biomarcador é mais utilizado para avaliar a resposta ao tratamento do que como um indicador de gravidade da doença, visto que existem poucos estudos associando esse biomarcador a complicações ósseas e idade de início do tratamento (KOPPE et al., 2016; MEKINIAN et al., 2012; STEIN et al., 2010).

Há diversos biomarcadores descritos para DG, porém, nenhum deles é satisfatoriamente eficiente, específico e confiável para ser utilizado sozinho a fim de auxiliar no prognóstico e na avaliação da taxa de resposta ao tratamento. Portanto, existe uma necessidade de buscar novos biomarcadores que atendam essas características e que sejam específicos para DG. O uso combinado de diferentes biomarcadores em associação aos achados laboratoriais de pacientes com DG poderiam auxiliar no acompanhamento desses pacientes e na eficácia do tratamento.

2.2.10 Centro de Referência para doença de Gaucher no Rio Grande do Sul (CRDG-RS)

O CRDG-RS foi criado em 2003 visando a realização do diagnóstico, o acompanhamento e o tratamento de pacientes com DG de diferentes regiões do Estado do Rio Grande do Sul, e segue as orientações previstas no protocolo do Ministério da Saúde (MS). Esse Centro está localizado no Hospital de Clínicas de Porto Alegre (HCPA), onde há uma equipe multidisciplinar responsável pelo acompanhamento desses pacientes, por meio de consultas, avaliações a partir de exames, além do tratamento de TRE aos pacientes que são encaminhados para o Centro. Atualmente, o CRDG-RS acompanha um total de 45 pacientes, sendo 42 com DG tipo I e 3 com DG tipo III.

2.3 Vitamina B_{12}

A vitamina B_{12} (B_{12}, cobalamina, Cbl) é hidrossolúvel, sintetizada exclusivamente por um pequeno grupo de bactérias sendo estocada primariamente no fígado. A estrutura da B_{12} é representada por um íon cobalto (Co) localizado no centro da estrutura em anel tetrapirrólico, o qual é composto por um grupo nucleotídico, que consiste em uma base 5,6-dimetilbenzimidazol (DMB) e uma ribose fosforilada esterificada com 1-amino,2-propanol

(Figura 8). Essa vitamina está presente em alimentos de origem animal, tais como leite, ovos, carne, entre outros. Os seres humanos necessitam de uma ingestão diária de 2,4 µg dessa vitamina (HANNIBAL et al., 2018; STRAND et al., 2013). A Cbl tem um papel extremamente importante em diversos processos, como eritropoiese (RETIEF; GOTTLIEB; HERBERT, 1966), síntese de DNA (HERRMANN; OBEID, 2012), prevenção de problemas cardíacos (HERRMANN; GEISEL, 2002), manutenção da função neurológica (STRAND et al., 2013), além de estar associada ao risco de desenvolvimento de câncer (CHOI et al., 2004).

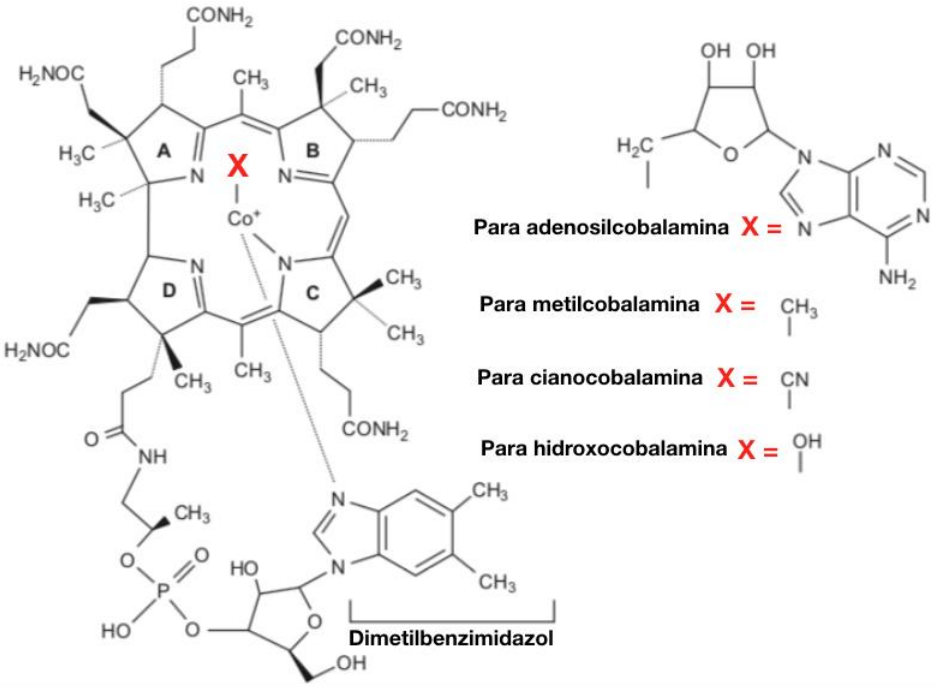

Figura 8. Estrutura da vitamina B_{12}. A estrutura de cianocobalamina é quando no lugar do X está o grupo – CN. As principais formas biológicas de cobalamina têm o grupo ciano substituído pelos grupos adenosil, metil ou hidroxil, os quais são encontrados nas formas adenosilcobalamina, metilcobalamina e hidroxocobalamina, respectivamente (adaptado de SMITH; WARREN; REFSUM, 2018).

2.3.1 Metabolismo da vitamina B_{12}

O metabolismo da B_{12} é complexo, requer vários processos e uma falha em alguma das etapas envolvidas pode levar à deficiência de B_{12}. Em humanos, a absorção de Cbl pela dieta envolve diferentes proteínas, tais como haptocorrina (HC), fator intrínseco (FI) e transcobalamina (TC). A via metabólica da Cbl começa quando a vitamina entra no estômago e é ligada à proteína de origem animal (P). No estômago, pepsina e ácido clorídrico (HCl)

agem rompendo a ligação da proteína (P) com a Cbl, liberando Cbl na forma livre. A maior parte da Cbl livre é então ligada à proteína-R, sendo esta secretada pelas células parietais e salivares. O FI também é secretado no estômago, porém sua ligação à Cbl é fraca na presença de proteína-R. Subsequentemente, o complexo Cbl-R é transportado até o duodeno, onde enzimas pancreáticas degradam esse complexo, liberando Cbl na forma livre. A Cbl é então ligada ao FI. O complexo Cbl-FI permanece intacto até a porção terminal do íleo, onde ocorre a absorção de Cbl por meio de receptores de células da mucosa, chamados de cubilina. Por fim, a Cbl é ligada a proteínas transportadoras, chamadas de TCI, TCII e TCIII. A TCII (embora represente apenas uma pequena fração (aproximadamente 10%) das TCs) é a mais importante, pois é capaz de transportar Cbl para todas as células do corpo. A Cbl ligada à TC é chamada de holo-TC, a qual corresponde à fração de vitamina B_{12} metabolicamente ativa. A Cbl é subsequentemente transportada sistemicamente via portal. Dentro de cada célula, o complexo Cbl-TCII é desfeito por endocitose, a Cbl é liberada e convertida enzimaticamente em duas formas de coenzimas: metilcobalamina (MeCbl) e adenosilcobalamina (AdoCbl) (Figura 9). (ANDRÈS et al., 2004).

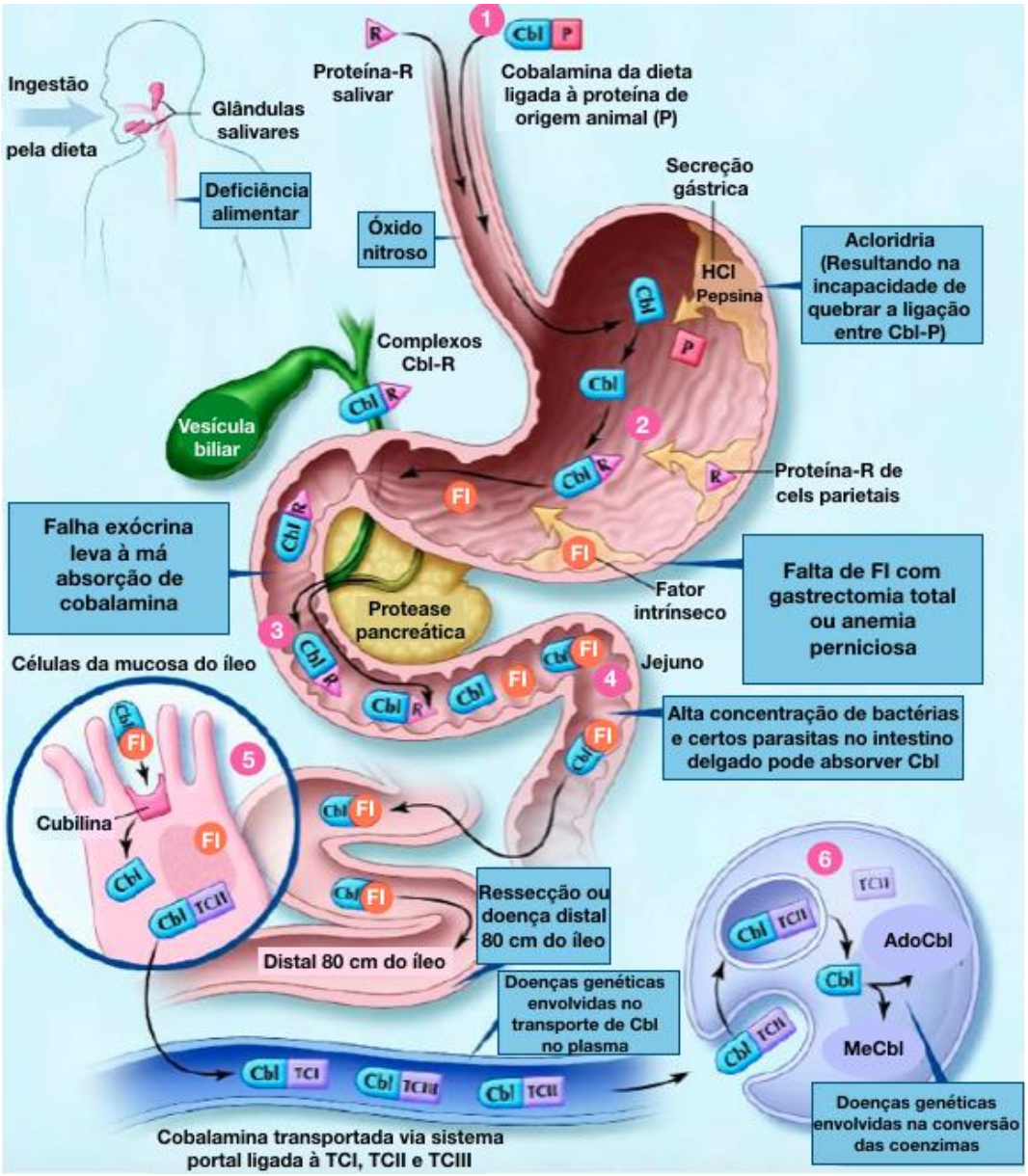

Figura 9. Metabolismo da vitamina B_{12}. Representação das etapas envolvidas desde a absorção de Cbl através da dieta até sua conversão em duas formas de coenzimas. A figura indica alguns erros que podem ocorrer durante o processo, bem como as doenças associadas (adaptado de ANDRÈS et al., 2004).

A B_{12} é absorvida pelas células via endocitose mediada por receptor, e este micronutriente é então liberado da proteína transportadora TC no lisossomo (QUADROS; SEQUEIRA, 2013). Cbl livre sai do lisossomo com o auxílio de duas proteínas, chamadas de transportadores cblF (LMBRD1) e cblJ (ABCD4) (COELHO et al., 2012; GAILUS et al., 2010; RUTSCH et al., 2011). Mutações nos genes *cblF* e *cblJ* (COELHO et al., 2012; GAILUS et al., 2010; RUTSCH et al., 2011), bem como distúrbios não relacionados à via

lisossomal e endocítica (STOCKLER et al., 2014; ZHAO et al., 2014), levam à deficiência funcional de vitamina B_{12} e ao início de deterioração neurológica.

A Cbl atua como cofator das enzimas metionina sintase (MS) e metilmalonil-CoA mutase (MCM), as quais estão envolvidas na síntese de metionina (Met) a partir de homocisteína (Hcy) e na conversão de metilmalonil-CoA a succinil-CoA, respectivamente (Figura 10). Dessa forma, quando há deficiência de Cbl, as concentrações séricas de homocisteína (Hcy) e ácido metilmalônico (MMA) estão aumentadas (HERRMANN; OBEID, 2012). Tal deficiência pode ocorrer por diferentes causas, seja nutricional devido a uma dieta pobre em Cbl ou funcional em decorrência de falhas na absorção, no processamento intracelular ou no transporte dessa vitamina (HANNIBAL; DIBELLO; JACOBSEN, 2013).

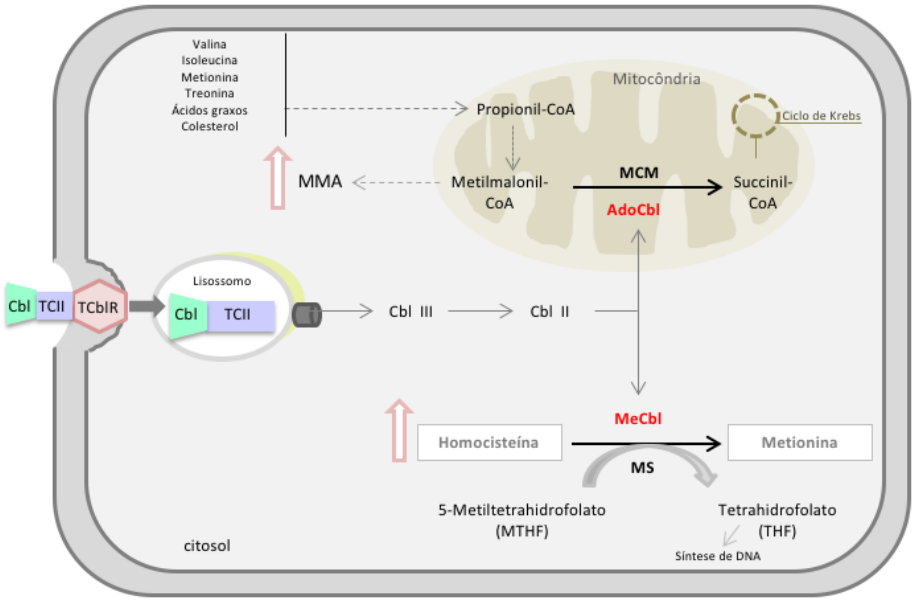

Figura 10. Representação esquemática das reações que necessitam de Cbl como cofator. No citosol, a Cbl atua na forma de MeCbl como cofator da enzima MS na conversão de Hcy a Met. Na mitocôndria, a Cbl atua na forma de AdoCbl como cofator da enzima MCM na conversão de metilmalonil-CoA a succinil-CoA (Autora, 2019).

2.3.2 *Status* de B_{12}

Os sintomas de deficiência de B_{12} variam desde anemia megaloblástica, problemas neurológicos, sintomas cardiovasculares e até mesmo acidúria metilmalônica, entre outras (SMITH; WARREN; REFSUM, 2018). Insuficiência de B_{12} em humanos é causada não

somente pela quantidade de ingestão desse micronutriente ou pela má absorção, mas também por doenças genéticas relacionadas a problemas na absorção e no transporte dessa vitamina, como alguns EIM causados por mutações em genes que codificam proteínas específicas envolvidas nesses processos (WATKINS; ROSENBLATT, 2013). São conhecidos diferentes grupos de genes, chamados de *cblA-G* e *mut*, os quais estão associados ao metabolismo e ao transporte da B_{12}. Uma alteração em cada um desses genes será responsável por uma determinada condição associada a um dado fenótipo (Figura 11) (HANNIBAL et al., 2016; SMITH; WARREN; REFSUM, 2018).

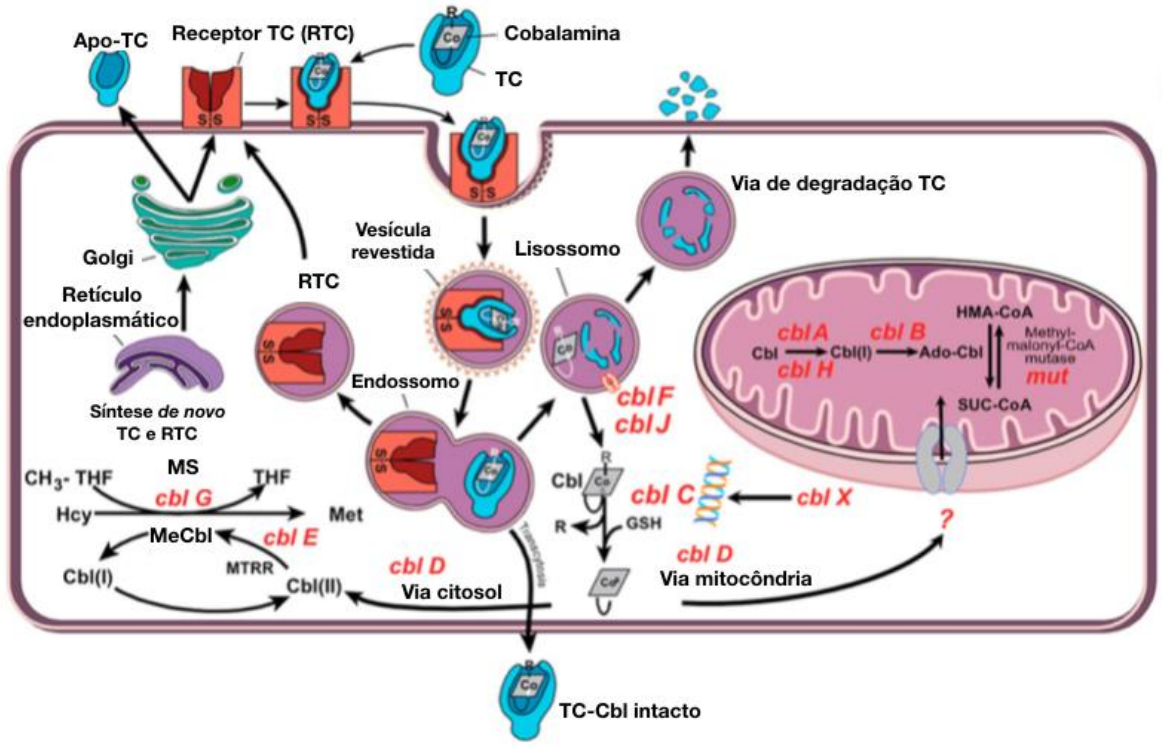

Figura 11. Processamento celular e tráfego da vitamina B_{12} proveniente da dieta. A Cbl entra na célula via endocitose mediada pelo receptor TC (RTC). No lisossomo, a Cbl é liberada, a apo-TC é degradada e o RTC é retornado à superfície celular para ser reutilizado. Cbl sai do lisossomo via transportadores cblF/cblJ. No citosol, Cbl sofre processamento pela enzima cblC. Após seu processamento, a Cbl é direcionada através da cblD para o citosol, onde irá atuar como cofator da MS ou para a mitocôndria, onde irá atuar como cofator da MCM. Deficiências nutricionais e funcionais da B_{12} levam à inativação das enzimas MS e MCM e aumento nos níveis dos metabólitos tHcy e MMA (adaptado de HANNIBAL et al., 2016).

A deficiência de B_{12} pode causar danos neurológicos irreversíveis, os quais necessitam de uma detecção precoce para o manejo dos pacientes. Sendo assim, a análise dos

níveis dos metabólitos tHcy e MMA é extremamente importante para avaliar o *status* desse micronutriente (HERRMANN; OBEID, 2013). Muitas vezes, a deficiência de B_{12} apresenta sintomas não específicos, como fadiga, mal-estar, vertigem e comprometimento cognitivo, os quais podem ser atribuídos à idade avançada. Portanto, uma detecção precoce da deficiência de B_{12} deve ser realizada visando evitar o agravamento dos sintomas apresentados (Figura 12) (PALACIOS et al., 2013). Recentemente, uma nova ferramenta para diagnóstico de insuficiência de B_{12} foi proposta, a qual utiliza dados combinados de quatro biomarcadores: B_{12}, holo-TC, tHcy e MMA como indicadores do *status* de B_{12} (FEDOSOV et al., 2015). Tal ferramenta encontra-se restrita à pesquisa, pois necessita de validação, principalmente quanto aos aspectos cognitivos (KVESTAD et al., 2017; SMITH; WARREN; REFSUM, 2018). O diagnóstico de insuficiência de B_{12} ainda é um desafio, visto que não há um teste padrão-ouro estabelecido para definir uma deficiência desse micronutriente e a avaliação clínica é um fator extremamente importante nesse processo (SMITH; WARREN; REFSUM, 2018).

Figura 12. Representação de algumas características clínicas apresentadas por indivíduos com deficiência de B_{12} (adaptado de HUNT; HARRINGTON; ROBINSON, 2014).

Existe uma tendência no aumento da prevalência de deficiência de B_{12} conforme o aumento da idade, mas isso não está bem elucidado (BAILEY et al., 2011). Muitas vezes, a deficiência desse micronutriente em idosos é acompanhada de alterações metabólicas devido à maior propensão de desenvolver disfunção gástrica e outras complicações que interferem na

absorção da vitamina B_{12} (HANNIBAL et al., 2016). De uma forma geral, a prevalência de deficiência de B_{12} é alta em diferentes grupos na população, variando de 30 – 60%. Em países como a Índia, onde grande parte da população é vegetariana, mais de 70% das mulheres grávidas apresentam níveis insuficientes de B_{12}, o que representa grandes implicações de saúde tanto para a mãe quanto para o bebê (SMITH; WARREN; REFSUM, 2018; YAJNIK et al., 2008). Cabe destacar que vegetarianos e veganos geralmente apresentam insuficiência de B_{12}, variando de 11 até 90% dos casos (RIZZO et al., 2016).

2.3.3 Biomarcadores do *status* de B_{12}

Estudos apontam que os metabólitos tHcy e MMA são considerados indicadores mais sensíveis dos níveis de vitamina B_{12} do que a dosagem dessa vitamina no soro (VOGIATZOGLOU et al., 2009). Um resultado normal de Cbl no soro pode não refletir o estado funcional adequado de B_{12} (DAVID SMITH; REFSUM, 2012). Apenas cerca de 20% da vitamina B_{12} total no soro está na forma ativa de holo-TC (NEXO; HOFFMANN-LÜCKE, 2011). Portanto, a dosagem de Cbl total no soro não é sensível para verificar a deficiência dessa vitamina. Para um diagnóstico mais preciso e para poder avaliar funcionalmente a B_{12} é necessário dosar os níveis de holo-TC (DEVALIA et al., 2014) e de seus biomarcadores funcionais MMA e tHcy no plasma (Tabela 1) (APARICIO-UGARRIZA et al., 2015; YETLEY et al., 2011). Uma deficiência de B_{12} não diagnosticada pode ser uma falha grave na prevenção de sintomas expressivos que os pacientes podem apresentar, principalmente em relação ao desenvolvimento neurológico (SPENCE, 2016).

Tabela 1. Biomarcadores do *status* de B_{12} com suas vantagens e limitações.

Biomarcador	Utilidade	Vantagens	Desvantagens
Vitamina B_{12}	Fornece informação sobre o *status* de B_{12} e estoque no fígado. É um indicador razoável do *status* de B_{12} em um grupo populacional. Correlacionado com a ingestão de B_{12}. Geralmente seu valor sobre o *status* de B_{12} discorda com outros biomarcadores.	Ensaio amplamente disponível e de baixo custo. Não sofre influência por ingestão recente de B_{12}. Não necessita de jejum antes da coleta da amostra. Não é influenciado pela idade do indivíduo.	Leva meses para aumentar após a ingestão pela dieta ou suplementação de baixa dose, mas responde a intervenções de alta dose. Pode resultar em falso positivo.
Holo-TC	Fornece informação da B_{12} disponível para as células. É correlacionada com a ingestão de B_{12}.	Maior sensibilidade à ingestão recente, respondendo em algumas horas. É um indicador mais sensível de deficiência do que a B_{12} sérica, mas a prevalência populacional de deficiência é similar. Não sofre influência por infecção.	Concentrações podem estar aumentadas pela ingestão recente se os estoques estiverem baixos. Os ensaios são limitados comparados à B_{12}. Não é um indicador funcional do *status* de B_{12}. Custo mais elevado que a B_{12} sérica e menos disponível nos laboratórios.
MMA	Reflete o nível de B_{12} para a função metabólica (atividade da MCM). Concentrações aumentam quando $B_{12} < 300$ pmol/L.	O biomarcador mais sensível para o *status* de B_{12}. Reflete estoque no fígado. Responde mais rapidamente a intervenções que a B_{12}, mas não à ingestão recente. Não sofre influência pelos níveis de folato nem pelo *status* de vitamina B_6.	Análise requer um equipamento de alto custo. Os níveis aumentam conforme a idade, não sendo totalmente decorrente de disfunção hepática. Aumenta em indivíduos com disfunção renal e necessita da mensuração de creatinina especialmente em idosos.
tHcy	Reflete o nível de B_{12} para a função metabólica (atividade da MS). Concentrações aumentam quando $B_{12} < 300$ pmol/L.	tHcy responde rapidamente à melhora no *status* de indivíduos com deficiência de B_{12}.	Não é específico para avaliar o *status* de B_{12}, visto que os níveis estão elevados em outras condições como deficiência de folato e vitamina B_6, além de insuficiência renal e hipotireoidismo.

B12: Vitamina B12; holo-TC: holo-transcobalamina; MMA: ácido metilmalônico; tHcy: homocisteína total (adaptado de ALLEN et al., 2018).

O teste preferencialmente utilizado para determinar o *status* de B_{12} é a mensuração dos níveis totais dessa vitamina no soro, sendo um ensaio disponível na maioria dos laboratórios (APARICIO-UGARRIZA et al., 2015; HUNT; HARRINGTON; ROBINSON, 2014). Os valores de referência variam de acordo com cada laboratório, mas as definições mais utilizadas pelos laboratórios são: normais (>250 pmol/L), baixos níveis (150-249 pmol/L) e deficiência aguda (<149 pmol/L) (CLARKE et al., 2003; MIRKAZEMI et al., 2012; SELHUB et al., 2008).

Estudos mostram que os níveis séricos de B_{12} nem sempre representam o *status* celular dessa vitamina (CARMEL, 2000; DEVALIA et al., 2014; LYSNE et al., 2016; SOLOMON, 2004). Pacientes com EIM da vitamina B_{12} podem apresentar níveis séricos normais de B_{12} e ao mesmo tempo uma deficiência dessa vitamina a nível celular (GREEN, 2017; HANNIBAL et al., 2016; SOLOMON, 2015). Solomon e colaboradores (2015) identificaram uma deficiência funcional de B_{12} devido ao estresse oxidativo em idosos que apresentavam valores séricos normais de B_{12}.

Apesar dos níveis séricos isolados de B_{12} não refletirem o *status* dessa vitamina, quando elevados auxiliam na avaliação de doenças tais como câncer (ARENDT et al., 2016) e síndrome linfoproliferativa autoimune (conhecida como ALPS) (BOWEN et al., 2012). Níveis elevados de vitamina B_{12} estão associados com inflamação e a maioria desses casos envolve fisiopatologicamente uma desordem de TCs que pode ser tanto quantitativa como qualitativa (ANDRÈS et al., 2013).

Em idosos, a holo-TC tem sido o biomarcador mais indicado para avaliar o *status* de B_{12} comparado aos metabólitos tHcy e MMA (VALENTE et al., 2011). Os valores de referência desse biomarcador em indivíduos saudáveis é de 20-125 pmol/L (VALENTE et al., 2011). Ainda não estão estabelecidos os mecanismos que controlam a homeostase de holo-TC tanto em indivíduos saudáveis quanto em pacientes com doenças que afetam o metabolismo da vitamina B_{12}. Estudos adicionais são necessários para elucidar esses mecanismos e para avaliar o valor diagnóstico desse biomarcador.

A Hcy é um metabólito do metabolismo de 1 carbono que é remetilado pela MeCbl dependente da enzima MS ou pela betaína-homocisteína metiltransferase (BHMT) como parte do ciclo da metionina, e degradado pela cistationina β-sintase (CBS) na via de transulfuração. A conversão de Hcy a Met pela enzima MS depende da disponibilidade de vitamina B_{12} e folato como cofatores. Portanto, deficiências nutricionais em algum desses

micronutrientes levam ao acúmulo de tHcy no soro e na urina. Além disso, em casos de EIM que afetam o processamento e o transporte de vitamina B_{12} ou de folato levam ao aumento desse metabólito, ocasionando uma condição chamada de hiperhomocisteinemia (HANNIBAL et al., 2016).

Os valores de referência dos níveis plasmáticos normais de tHcy em humanos é de 5-15 µmol/L (UELAND et al., 1993), sendo que em alguns casos os valores acima de 13 µmol/L podem ser considerados elevados em adultos (JACQUES et al., 1999). Além disso, estudos apontam que os intervalos de referência podem variar conforme o gênero e a idade do indivíduo (JACOBSEN et al., 1994; RASMUSSEN et al., 1996; VAN BEYNUM et al., 2005). Os níveis de tHcy são mais elevados no soro comparados aos níveis desse metabólito no plasma devido à liberação de Hcy ligada aos componentes celulares (JACOBSEN et al., 1994). Apesar de ser um biomarcador de deficiência de B_{12}, a tHcy não é um indicador específico de deficiência de B_{12}, visto que suas concentrações também são elevadas em outras condições como deficiência de folato, deficiência de B_6 e em pacientes com doença renal e hipotireoidismo (DEVALIA et al., 2014; HUNT; HARRINGTON; ROBINSON, 2014). Portanto, esse biomarcador não é indicado para determinar o *status* de B_{12}, se utilizado isolado (HANNIBAL et al., 2016).

Níveis de MMA aumentam quando ocorre a inativação da enzima MCM, a qual é dependente de AdoCbl na mitocôndria. Deficiências nutricional e funcional de B_{12} resultam na inativação da MCM levando ao acúmulo do seu substrato metilmalonil-CoA, o qual entra na circulação como MMA livre. A reação catalisada pela MCM não é afetada por outras vitaminas do metabolismo de um carbono, sendo esse biomarcador mais específico para determinar o *status* de B_{12} (CLARKE et al., 2003). Valores séricos de MMA entre 260 – 350 nmol/L indicam níveis elevados desse metabólito (CLARKE et al., 2003) Porém, cabe ressaltar que existem algumas condições patológicas, como insuficiência renal, que levam ao aumento dos níveis de MMA (IQBAL et al., 2013). Estudos já mostraram casos de indivíduos com níveis elevados de vitamina B_{12} que também apresentavam concentrações elevadas de MMA, o que poderia refletir a ocorrência de insuficiência renal nesses indivíduos em vez de deficiência de B_{12} (CLARKE et al., 2003).

Dessa forma, recomenda-se o uso combinado de diferentes biomarcadores para avaliar o *status* de B_{12} (HANNIBAL et al., 2016; IQBAL et al., 2013).

2.3.4 Vitamina B_{12} e doença de Gaucher

Algumas manifestações clínicas da DG são observadas em indivíduos com deficiência de vitamina B_{12}, principalmente em relação ao envolvimento neurológico, sugerindo que exista um mecanismo de patogênese comum a essas doenças. Uma hipótese é que a disfunção lisossomal na DG leva à deficiência funcional de vitamina B_{12} por uma falha em algum dos processos, tais como: absorção, degradação intralisossomal de TC ou no transporte de B_{12} para o citoplasma, comprometendo as reações bioquímicas em que a B_{12} atua como cofator (MS e MCM). Ainda não está estabelecido se o acúmulo de glicoesfingolipídeos afeta o tráfego de vitamina B_{12} dentro do lisossomo e se prejudica o seu transporte para o citoplasma (HANNIBAL et al., 2017).

Estudos que avaliaram precocemente o *status* de vitamina B_{12} em pacientes com DG reportaram altos níveis de TC circulante que não foram correlacionados com níveis séricos de B_{12}, sugerindo que o aumento dos níveis de TC na DG resultam de um processo inflamatório. Porém, cabe destacar que outros biomarcadores do *status* de B_{12}, como MMA e tHcy, não foram mensurados (GILBERT; WEINREB, 1976). Embora a holo-TC represente a fração bioativa de B_{12}, há uma limitação quanto ao uso isolado desse biomarcador na verificação do *status* de B_{12}. Isso porque apenas uma pequena fração desse micronutriente está ligada à holo-TC, permitindo que ocorram variações a nível sérico de holo-TC. Outros estudos identificaram baixos níveis de holo-TC em pacientes com diferentes doenças que não apresentavam deficiência de vitamina B_{12} (HANNIBAL et al., 2017).

Gielchinsky e colaboradores (2001) avaliaram uma coorte de 85 pacientes com DG, os quais eram judeus Ashkenazi e não realizavam tratamento para a doença, e 122 indivíduos saudáveis. Esse estudo mostrou uma alta incidência de baixos valores séricos de vitamina B_{12} e de elevados valores de tHcy e MMA; porém, esses achados não foram estatisticamente significativos em relação aos controles, visto que a maioria dos indivíduos saudáveis de origem Ashkenazi apresentam baixos níveis de vitamina B_{12}. Esse *status* de vitamina B_{12} nessa população específica pode ser devido ao fato de ser frequente a doação de sangue, o que poderia prejudicar o estoque desse micronutriente no fígado, além das diferenças étnicas entre esses indivíduos (ELSTEIN et al., 1998; GIELCHINSKY et al., 2001; HANNIBAL et al., 2017; VEINOT et al., 1999).

Elstein e colaboradores (1998) realizaram um estudo baseado em um questionário e identificaram uma alta incidência de complicações neurológicas em pacientes com as formas neuropáticas da DG, sendo que estes pacientes apresentaram concomitantemente deficiência

de vitamina B_{12} e gamopatias. Porém, os níveis dos metabólitos tHcy e MMA não foram analisados nesse estudo, o que dificulta a avaliação do *status* de vitamina B_{12} nesses pacientes.

Um estudo longitudinal e observacional de uma coorte envolvendo oito centros de diferentes países da Europa avaliou o *status* de vitamina B_{12} em pacientes adultos com DG tipo I na presença ou na ausência de polineuropatia. Os resultados mostraram uma elevação estatisticamente significativa nos níveis séricos de tHcy e MMA em pacientes com polineuropatia comparados aos que não tinham comprometimento neurológico, com valores variando de baixos a normais de vitamina B_{12} em ambos os grupos. Contudo, cabe ressaltar que os pacientes de ambos os grupos apresentaram níveis dos metabólitos tHcy e MMA dentro dos valores de referência, o que pode ter sido por estarem recebendo tratamento com TRE há mais de dois anos (BIEGSTRAATEN et al., 2010).

3. MARCO CONCEITUAL

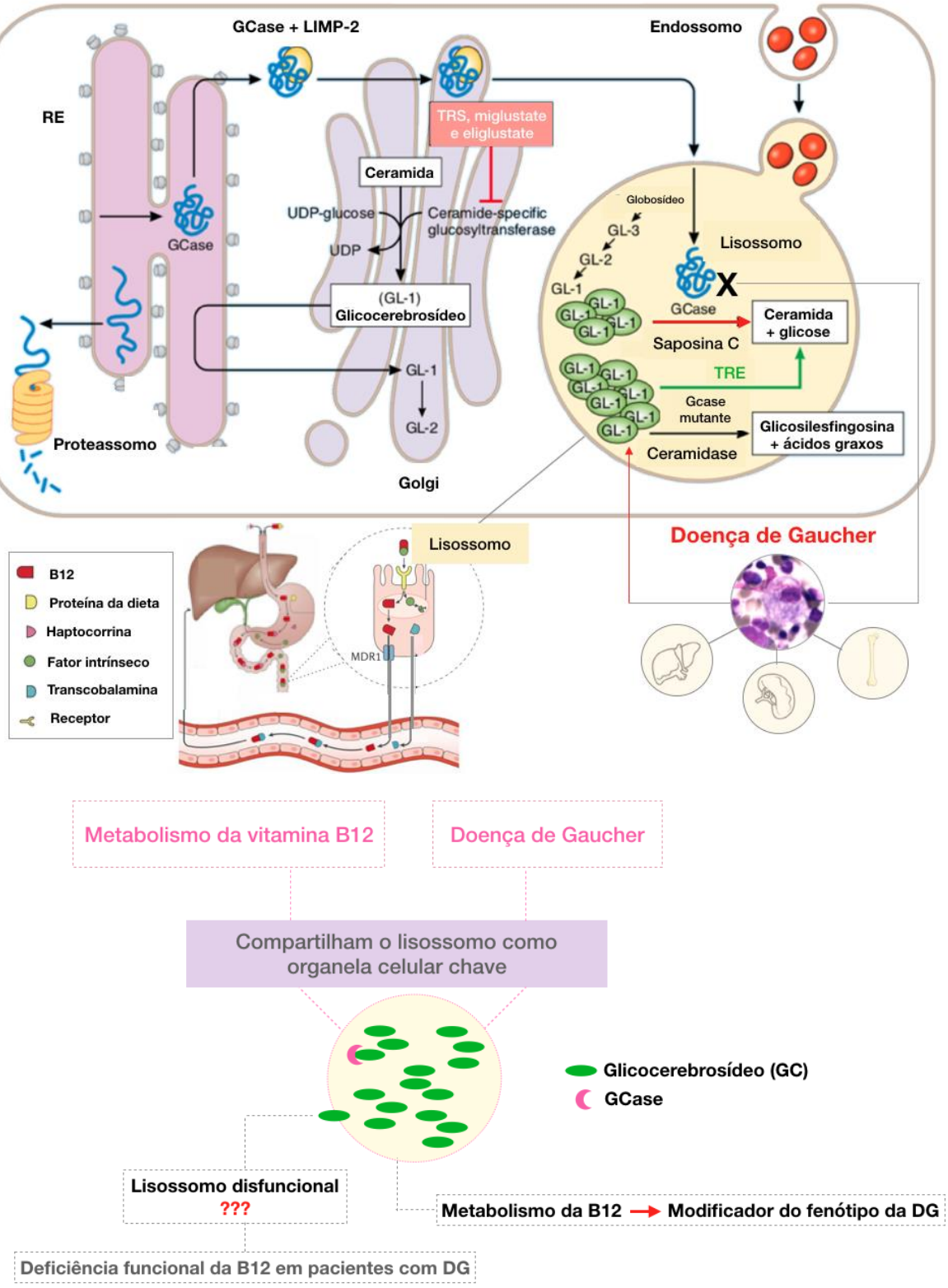

Figura 13. Marco conceitual da DG envolvendo o metabolismo da vitamina B_{12}.

4. JUSTIFICATIVA

A DG apresenta alta heterogeneidade fenotípica, apesar de ter sua causa definida. Embora cerca de 500 mutações no gene *GBA1* já tenham sido descritas como causadoras da doença, não há uma associação bem estabelecida entre determinada mutação e as diferentes manifestações clínicas apresentadas pelos pacientes. Dessa forma, sabe-se que existem diversos fatores, como modificadores genéticos, que contribuem para a variabilidade fenotípica observada em pacientes com DG. Portanto, conhecer os mecanismos fisiopatogênicos envolvidos na DG é extremamente importante para que seja possível melhorar o manejo desses pacientes. Apesar de o tratamento ser uma excelente estratégia terapêutica, há diferenças interpessoais na resposta clínico-laboratorial aos medicamentos utilizados.

Tendo em vista que a DG é causada por uma deficiência enzimática que ocorre devido a mutações patogênicas no gene que codifica essa enzima, o diagnóstico genético da DG é recomendado e deve ser realizado. Muitos laboratórios realizam a investigação das mutações mais comuns e acabam não identificando o genótipo completo dos pacientes. É importante que seja realizada a análise genética de todo o gene *GBA1* para identificação das mutações bialélicas. Em alguns casos, há pacientes que não apresentam o genótipo definido mesmo tendo realizado a análise completa do gene por sequenciamento. Cabe ressaltar que cada metodologia apresenta suas limitações e os pesquisadores devem ter isso em mente. Nesses casos, sabendo a clínica do paciente, é essencial realizar a análise molecular utilizando diferentes metodologias. A técnica de *Multiplex ligation-dependent probe amplification* (MLPA) permite detectar a presença de deleções e duplicações em determinadas regiões do genoma. O emprego dessa técnica para investigar alterações no gene *GBA1* em pacientes com DG é extremamente útil, visto que há casos em que não são identificadas as alterações causadoras da doença. Essa metodologia é inovadora e foi realizada pela primeira vez em pacientes com DG, o que permitirá que outras pessoas tenham conhecimento e acesso a este método, favorecendo o uso dessa técnica para investigar a presença de deleções e duplicações no gene *GBA1* em pacientes com DG que não apresentam genótipo estabelecido. Além disso, a identificação dessas alterações genéticas no *GBA1* pode contribuir para o melhor entendimento da DG.

Por se tratar de uma doença rara, existem poucos estudos de frequências alélicas de mutações no Brasil. Sendo assim, faz-se necessário a realização de um estudo mais abrangente, que possa representar os pacientes com DG de todas as regiões do país. A

iniciativa de caracterizar pacientes com DG de diferentes Centros de Referência para a doença partiu do CRDG-RS, o qual dispõe de laboratório de investigação genética, e que auxilia na caracterização genética de pacientes de outras regiões. O presente estudo proporcionou a parceria entre os Centros com objetivos em comum, que é fazer o melhor para os pacientes. Portanto, a caracterização de pacientes com DG de diferentes regiões do país é de suma relevância para estabelecer se existe um perfil alélico específico em determinadas regiões por meio da comparação das frequências alélicas das mutações identificadas nesses pacientes. Saber se existe ou não uma variabilidade de frequências alélicas no *GBA1* em todo o território brasileiro, poderá contribuir para um melhor entendimento da doença e auxiliar no manejo dos pacientes com DG.

Na DG, a deficiência da enzima GCase prejudica a conversão de GC em ceramida e glicose, resultando no acúmulo do substrato nos lisossomos. Esse acúmulo de glicoesfingolipídeo pode estar afetando o funcionamento adequado dessa organela. O presente estudo é o primeiro a investigar a relação entre uma doença lisossômica estabelecida e o metabolismo da vitamina B_{12}. O metabolismo desse micronutriente pode estar envolvido na modificação do fenótipo dos pacientes com DG, sendo relevante investigar o papel da vitamina B_{12} na progressão da doença tanto na parte clínica quanto molecular, uma vez que tanto o metabolismo da B_{12} quanto os distúrbios moleculares observados na DG envolvem o lisossomo como um compartimento celular chave. Além disso, foi relatado que níveis de vitamina B_{12} estão diminuídos na DG, níveis de holo-TC estão elevados e que pacientes com outras condições neurodegenerativas, como doença de Alzheimer, apresentam anormalidades envolvendo as vias lisossomal, endocítica e celular da vitamina B_{12}. Portanto, a investigação do metabolismo de B_{12} em pacientes com DG é inovador e importante para avaliar se existe uma deficiência funcional desse micronutriente nesses pacientes, a qual pode ser causada pelo mau funcionamento dos lisossomos devido ao acúmulo de GC.

5. OBJETIVOS

5.1 Objetivo geral

Caracterizar o genótipo e o metabolismo da vitamina B_{12} em pacientes com DG.

5.2 Objetivos específicos

5.2.1 Identificar, por meio de MLPA, mutações no *GBA1* em pacientes com DG que não possuíam o genótipo estabelecido;

5.2.2 Avaliar a existência de um perfil alélico específico dos pacientes com DG da região Sul e de outras regiões do Brasil;

5.2.3 Avaliar o metabolismo da vitamina B_{12} em células de pacientes com DG;

5.2.4 Analisar biomarcadores do *status* da vitamina B_{12} em pacientes com DG.

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7. CAPÍTULOS

Os resultados da tese serão apresentados nos capítulos a seguir.

7.1 CAPÍTULO 1 – Artigo 1

Título do artigo: "Use of a multiplex ligation-dependent probe amplification method for the detection of deletions/duplications in the *GBA1* gene in Gaucher disease patients"

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Use of a multiplex ligation-dependent probe amplification method for the detection of deletions/duplications in the *GBA1* gene in Gaucher disease patients

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Abstract

Gaucher disease (GD) is caused by the deficient activity of β-glucocerebrosidase due to pathogenic mutations in the *GBA1*. This gene has a pseudogene (*GBAP*) with 96% of sequence homology. Recombination (Rec) events in the *GBA1* seem to be facilitated by an increased degree of homology and proximity to the *GBAP*. The objectives of this study were to validate the P338-X1 GBA kit (MRC-Holland) for Multiplex Ligation-dependent Probe Amplification (MLPA) and to detect larger deletions/duplications present in *GBA1* in GD patients from Brazil. Thirty-three unrelated Brazilian GD patients, previously genotyped by the Sanger method (both pathogenic alleles identified = 29 patients, only one allele identified = 3 patients, no pathogenic alleles identified = 1 patient), were evaluated by the MLPA assay. MLPA was compatible with the previous results obtained by Sanger sequencing and identified an additional allele (a heterozygous deletion in intron 7 in one patient with only one mutation identified by Sanger). Our data suggest that, although larger deletions/duplications do not appear to be frequent in GD, the P338-X1 GBA kit for MLPA appears to be a good method for *GBA1* analysis. Additional investigations should be performed in order to characterize the remaining four uncharacterized alleles of our sample.

Keywords

Gaucher disease, *GBA1*, Molecular diagnosis, MLPA

1. Introduction

Gaucher disease (GD) is one of the most common lysosomal storage disorders. It is inherited as an autosomal recessive disorder and is caused by deficient activity of the β-glucocerebrosidase enzyme due to pathogenic mutations in the *GBA1* gene. The function of this enzyme is to catalyze the hydrolysis of the glycolipid glucocerebroside to ceramide and glucose [1]. Enzyme deficiency leads to the accumulation of non-degraded substrate in tissues, especially in the cells of the reticuloendothelial system, resulting in the formation of Gaucher cells, which will harm the regular function of certain organs [2]. GD frequency is estimated to be around 1 in 40,000–60,000 individuals in the general population and may affect 1 in 850 in the Ashkenazi Jewish population [3].

The disorder is divided into three types, based on the absence (type I) or presence and severity (types II and III) of primary involvement of the central nervous system (CNS) [1]. Type I, or nonneuronopathic GD, is by far the most common type, representing more than 90% of cases, and it includes patients with great variability in the progression and severity of the disease. Type II, or acute neuronopathic GD, is the rarest and the most severe form of the disease; these patients generally die before 2 years of age. Type III, or chronic neuronopathic GD, is the intermediate form of the disease because it commits the functions of CNS more slowly and gradually than type II; these patients survive until 20–30 years of age [1], [4].

The clinical features associated with GD include hepatosplenomegaly, anemia, thrombocytopenia, bone and lungs problems. The laboratory diagnosis of GD is based on the measurement of β-glucocerebrosidase in leukocytes and/or fibroblasts. Analysis of the *GBA1* gene is complementary to the biochemical diagnosis [5]. Often, the genotype-phenotype correlation is very difficult to establish because there is a wide phenotypic variability among patients with GD. Defining the patient's genotype is extremely important as it can help in the definition of the prognosis, the severity, and rate of progression of the clinical manifestations of the disease. The presence of at least one N370S allele prevents the development of neurological manifestations and confers type I disease, whereas the presence of the L444P allele in homozygosity is highly associated with CNS involvement [6].

The *GBA1* gene is located on chromosome 1q21 and spans 7.6 kb of genomic DNA divided into 11 exons. A highly homologous 5.7 kb pseudogene (*GBAP*) is located 16 kb downstream, with the same organization of exons [7]. The *GBAP* is transcribed, but it does not produce a functional protein [8]. *GBA1* and *GBAP* share more than 96% exonic sequence homology, enhancing the likelihood of homologous recombination. Recombination events between *GBA1* and *GBAP* have been identified, resulting from gene conversion, fusion or duplication and are responsible for several different identified mutant alleles [9]. To date, over 400 different pathogenic *GBA1* mutations have been reported. These include missense mutations, nonsense mutations, small insertions or deletions that lead to either frameshifts or in-frame alterations, splice junction mutations and complex alleles carrying two or more mutations in cis (http://www.hgmd.cf.ac.uk, September 2016; [3]).

Our aims in the present study were to validate the P338-X1 GBA kit (MRC-Holland) for MLPA and to detect large deletions and/or duplications in *GBA1* in GD patients from Southern Brazil.

2. Materials and methods

The study was approved by the institutional review board of Hospital de Clínicas de Porto Alegre, Brazil.

2.1. Patients

DNA samples from 33 unrelated patients with GD (type I = 27; type II = 4; type III = 2) were analyzed. The diagnosis of GD was established by the demonstration of deficient β-glucocerebrosidase activity in leukocytes and/or fibroblasts. *GBA1* gene was previously analyzed by Sanger sequencing in all patients (both mutations identified = 29 patients, only one mutation = 3, no identified mutation = 1; no pathogenic alleles identified = 5/66; point mutation alleles = 47/66; Rec alleles = 14/66) (Table 1). The location of the mutations found is as follow: R163* (exon 6); E236K and H311R (exon 8); N370S and G377S (exon 9); L444P, L444R, A456P and Rec*Nci*I (exon 10).

Table 1. Genotypes and phenotypes of Gaucher patients included in the present study.

Genotype by Sanger	MLPA results	Number of patients (33)	GD Type
N370S/N370S	Normal	1	I
G377S/G377S	Normal	1	I
N370S/R163*	Normal	1	I
N370S/L444P + A456P	Low signal exon 10	1	I
N370S/L444P	Low signal exon 10	7	I
N370S/Rec*Nci*I	Low signal exon 10	11	I
N370S/L444R	Low signal exon 10	2	I
N370S/?	Normal	2	I
L444P/Rec*Nci*I	Low signal exon 10	2	II
Rec*Nci*I/?	Low signal intron 7 and exon 10	1	II
L444P + E326K/H311R	Low signal exon 10	1	II
L444P/L444P	Low signal exon 10	2	III
No mutations found	Normal	1	I

Location of mutations –R163* (exon 6); E236K and H311R (exon 8); N370S and G377S (exon 9); L444P, L444R, A456P and Rec*Nci*I (exon 10).

2.2. Multiplex ligation-dependent probe amplification assay

The MLPA kit (P338-X1 GBA kit, MRC Holland) for the *GBA1* gene contains one probe for each of the following regions of *GBA1*: 5'UTR, exons 3, 4, 6, 8, 9, 10, and intron 7; it is used to determine gene or exon deletion/duplication (Fig. 1). The probes do not cover the entire length of the corresponding regions. The assay conditions and reactions were performed according to the manufacturer's recommendations [MRC Holland, Amsterdam, The Netherlands (www.mlpa.com)]. The amplified products were analyzed using the ABI 3500 equipment (Thermo Fisher Scientific). Data results were analyzed using Coffalyser software for MLPA [MRC Holland, Amsterdam, The Netherlands (https://coffalyser.wordpress.com/)]. Subjects with wild-type genotype were included as controls in all reactions.

Fig. 1. Schematic representation of probes used in the present study. The MLPA kit contains one probe for each of the following regions of *GBA1*: 5'UTR, exons 3, 4, 6, 8, 9, 10, and intron 7. Each probe is represented by a red star in the corresponding region.

3. Results

Table 1 shows the results of the Sanger sequencing and MLPA. No evidence for deletion/duplication was found.

Of 66 alleles, 13 (19.7%) were found to have L444P, and 2 (3.03%), L444R, confirming our sequencing results. However, we were unable to distinguish if the reduced signal was due to the presence of Rec*Nci*I (exon 10), L444P + A456P (exon 10) or L444P + E326K (exon 10). Only one patient presented a heterozygous deletion in intron 7; according to the sequencing, this patient is also heterozygous for Rec*Nci*I (Fig. 2d). These results were compatible with the previous analysis by Sanger sequencing. After MLPA analysis, 4 uncharacterized alleles from 4 GD patients still remain.

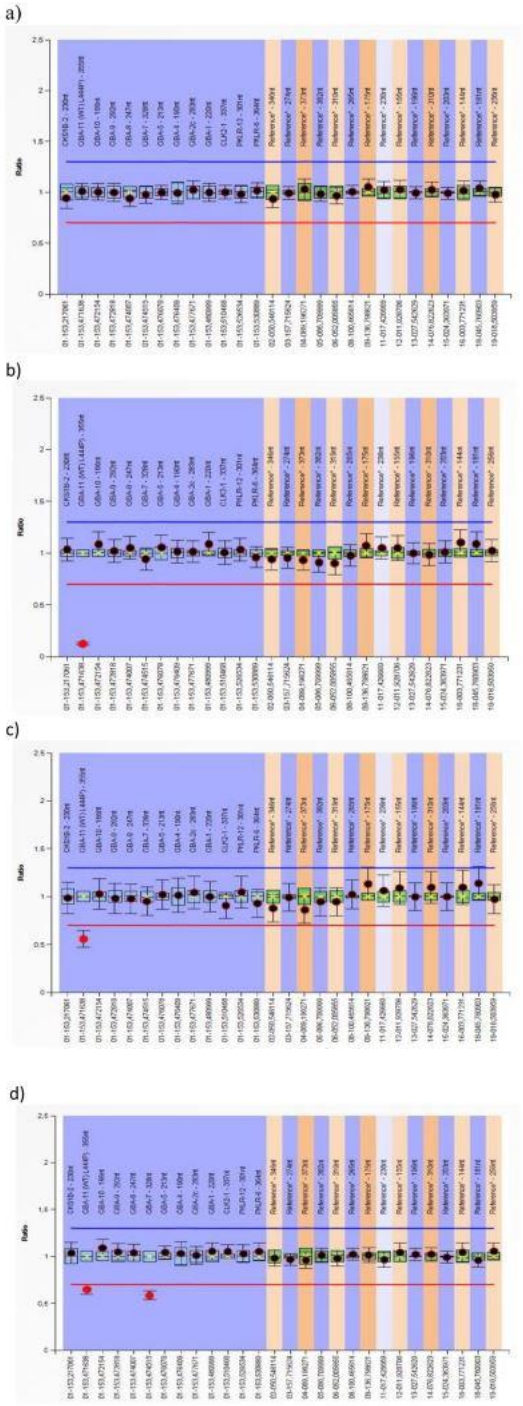

Fig. 2. The representative amplicon coverage plot of four GD patients. The horizontal axis shows the chromosome number and the genomic position. The vertical axis shows the final ratio. (a) The patient had no alteration in any of the MLPA probes. This patient had only one allele identified by Sanger sequencing, heterozygous for N370S mutation. (b) The patient had a very low signal of probe located on exon 10. This patient is homozygous for L444P mutation. For this reason, there was almost no signal for the corresponding probe. (c) The patient had a reduced signal for exon 10 probe. This patient has L444R mutation in one allele. (d) The patient had a reduced signal of exon 10 probe, which is in agreement with Sanger sequencing. This patients is heterozygous for RecNciI (L444P, A456P, V460V *in cis*). Also, there was a reduced signal in the intron 7 probe. The deletion is denoted by the red spots below the deletion cut-off line (red) in the ratio chart.

4. Discussion

This is the first analysis of the *GBA1* gene by the MLPA technique in Brazilian patients with GD. Genotyping of GD patients is usually made by the Sanger sequencing. The identification of both mutations in the *GBA1* can provide useful information regarding the prognosis of the patients, and it is essential for carrier diagnosis. However, large deletions and duplications that span entire exons are not detected by sequencing techniques, and additional investigation, such as MLPA, should be performed.

Restrictive factors of the MLPA kit are as follows: a) probes reach only part of the gene's coding region; and, b) not all coding regions are covered due to *GBAP* homology and do not cover the full extension of each exon, which may not detect some variation of the gene structure in the uncovered regions. This method is used for relative quantification of 40 different DNA sequences in a single reaction performance, and it only requires a thermocycler, a capillary electrophoresis equipment, and a concentration of 20 ng of human DNA. Among the different applications of this technique are: detection of deletions and duplications in exons of a variety of human genes; trisomy detection, such as Down syndrome; and characterization of chromosomal aberrations in cell lines and tumor samples [10]. This technique can also be used for prenatal diagnosis [11], and it allows molecular diagnosis of gene copy-number alterations in human genetic diseases [12]. Moreover, this technique can be used as screening for deletions/duplications as well as to exclude important missense mutation in *GBA1* (such as L444P) given that the specific probe covers the region in which the mutation is located. When the MLPA result suggests a single exon deletion, the result should be confirmed by an additional technique, such as Sanger sequencing of the target region. Although Sanger sequencing is used for various diseases, it is likely that the MLPA technique will be implemented as a basic technique for molecular analysis of genetic diseases and will be used in molecular diagnostic laboratories, both to confirm and to detect copy number variations (CNVs) on rare genetic diseases [12].

The L444P mutation is the second most common pathogenic variant in *GBA1*, and it may occur alone or in cis with other mutations. This mutation derives from the pseudogene sequence [13]. Many protocols of genetic analysis for GD patients include only the investigation of the most frequent mutations; this prevents the differentiation between L444P alleles and those resulting from recombination events. Among the most prevalent complex alleles is the Rec*Nci*I, which includes 3 distinct mutations located at exon 10 (L444P, A456P

and V460V) of *GBA1* [14].

The exon 10 probe generates a normal signal at the 444 position, but it generates reduced signal when L444P or L444R is present. Results herein showed that it is not possible to differentiate patients who have the Rec*Nci*I allele from those with L444P or L444R.

This technique is extremely necessary for the analysis of genes that comprise a large number of exons, such as the DMD gene, which contains 79 exons and is associated with Duchenne Muscular Dystrophy (DMD). The deletion and/or duplication analysis of this gene by another method, such as Southern blot, would be more laborious when compared to the MLPA technique, which is performed in 48 h [15].

MLPA can also be applied not only in patients with GD but also in patients with other lysosome storage disorders (LS, such as Fabry, MPS II, and Niemann-Pick type C disease). Presently, there are MLPA probes also available for Krabbe, Tay-Sachs and Pompe disease [16].

5. Conclusions

In the present study, all variants at the 444 amino acid position were detected by a reduced signal in the exon 10 MLPA probe set. However, alterations among L444P, L444R or Rec*Nci*l could not be distinguished based solely on the MLPA results. After MLPA analysis, 4 uncharacterized alleles from 4 GD patients still remain. Giving that the probes do not cover all regions of the gene, an additional approach is necessary to evaluate uncovered regions of *GBA1* (exons 1, 2, 5, 11, promoter and 3'UTR). Also, the GD patient that had a variant in *GBA1* intron 7 will be further studied by Sanger sequencing and mRNA analysis to evaluate the splicing alteration.

The MLPA technique can contribute for a better understanding of the pathophysiology of GD and should be implemented in molecular diagnosis laboratories, increasing the detection of non-identified variants. The use of this approach in the diagnosis of GD is innovative and will serve as reference to future studies regarding the real prevalence of large deletions/duplications not only in GD but also in other LSD.

Conflict of interest

No disclosure to declare.

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7.2 CAPÍTULO 2 – Artigo 2

Título do artigo: "*GBA1* allele frequencies in Gaucher patients from different regions of Brazil"

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Situação: Em elaboração

GBA1 allele frequencies in Gaucher patients from different regions of Brazil

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Abstract

Background: Gaucher disease (GD) is an autosomal recessive lysosomal disorder caused by pathogenic variants in *GBA1* which result in the deficient activity of glucocerebrosidase (GCase). The most frequent mutations described in Brazil are p.Asn409Ser (N370S) and p.Leu483Pro (L444P). The Brazilian territory is extensive, and its population is a result of interethnic ancestral crosses between Amerindians, Europeans, Jews, and Africans. These crosses were different depending on Brazilian region. **Aim:** To compare the *GBA1* variants frequencies in GD patients from South Brazil (SB) to patients from different regions of Brazil. **Methodology:** Seventy-two unrelated GD patients (type I= 63 , type II= 4 , type III= 5; male= 31) were included in this study. Forty patients were from SB, and 32 were from other regions of Brazil (Others). *GBA1* gene was analyzed by Sanger sequencing and/or next-generation sequencing by Ion Torrent PGM platform. **Results:** In total, 31 pathogenic variants were identified, being six common to SB and Others. Twelve variants were found only in patients from SB and 13 mutations were identified exclusively in patients from other regions of the country. Three variants (N370S, L444P and Rec*Nci*I) corresponded to 76.3% (61/80) of total alleles in SB and only 51.6% (33/64) in Others. Two novel variants were described: p.Gln109Argfs*9 and c.690G>A. **Conclusions:** The most frequent allele in Brazil was N370S. This study suggests that SB has a different profile of *GBA1* alleles compared to other regions of Brazil.

Keywords: Gaucher disease, *GBA1*, allele frequency, mutations, Brazilian patients.

Introduction

Gaucher's disease (GD) is an inborn error of metabolism caused by the deficient activity of β-glucosidase enzyme (GCase; E.C. 3.2.1.45). This deficiency leads to an accumulation of glucocerebroside inside the lysosomes, especially in the reticuloendothelial system. The excessive storage of this substrate is found in the liver, spleen, bone, and bone marrow of GD patients[1].

GD is an autosomal recessive disorder, resulting from pathogenic mutations on GCase gene, *GBA1*. This gene is located on chromosome 1q21 and comprises 11 exons. More than 500 different mutations have already been described according to the Human Gene Mutation Database[2]. The most frequent mutations in *GBA1* are N370S [c.1226A>G; p.Asn409Ser] and L444P [c.1448T>C; p.Leu483Pro], corresponding to 63.6% and 13.0% worldwide, respectively. The frequencies of these mutations differ among populations, for instance, N370S represents 83.2% of Ashkenazi-Jewish with GD[3].

Brazil is a country composed by several interethnic ancestral crosses among Amerindians, Europeans and Africans. These crosses are derived from immigration waves that occurred since XV century, that were different depending on Brazilian regions[4] (Figure 1). For instance, Northeast individuals have a strong African ancestry due to slavery period, North had a large influence of Amerindians and the South was mostly settled by European immigrants[4]. Individuals from South Brazil (SB) present an almost exclusively European ancestry[5,6].

Previous studies described the *GBA1* allele frequencies in Brazilian GD patients (Table 1). Rozenberg et al.[7] analyzed 40 unrelated type I GD patients and detected 34 different mutations. The most frequent alleles were N370S (35%), W184R, R120W and V398I (5%) followed by L444P, IVS2+1G>A, I489T and R120Q (3.75%).

In other study[8], the same author described the high frequency of mutation G377S in Brazilian GD type III patients. This study analyzed 262 unrelated GD patients to establish the frequency of the most common mutations. The frequency of the three most common GD-causing mutation identified among Brazilian patients were N370S (47%), L444P (27.1%) and G377S (2.2%).

Sobreira et al.[3] described phenotypic and genotypic heterogeneity in GD type I in patients from Brazil and the rest-of-the-world. This study identified the N370S and L444P alleles as the most frequent in Brazilian patients, corresponding to 48.2% and 27.2%, respectively. The second most common allele, L444P, was more frequent in Brazilian cohort compared to the rest-of-world cohort.

Siebert et al.[9] identified the G377S (11.1%) allele as the third most frequent mutation among 48 Brazilian GD patients, after N370S (44.1%) and L444P (28.5%). This study suggested that this mutation needed to be included in preliminary screens of Brazilian GD patients.

However, little is known about the frequencies of other GD-causing alleles in Brazil. Therefore, the objective of this work was to compare the *GBA1* variants' frequencies in GD unrelated patients from South Brazil to patients from different regions of Brazil (Others).

Materials and methods

It was an observational study with convenient sampling assessment, which was approved by the local Ethics Committee. Patients from Rio Grande do Sul (n=36) came from the local Gaucher Reference Center. Other (n=38) were included after contacting geneticists and hematologists from all over Brazil.

Subjects

A total of 72 unrelated patients with biochemical diagnosis of GD previously performed by the measurement of β-glucosidase (GCase) activity in leukocytes (type I= 63 , type II= 4 , type III= 5; male= 31) were included in this study. Forty patients (55.6%) were from SB, and 32 (44.4%) were from four other regions in Brazil (Southeast = 13/32, Northeast = 12/32, Center-West = 6/32 and North = 1/32) (Others).

Genotype analysis

Genomic DNA was extracted from peripheral blood collected into EDTA-containing tubes using Easy-DNA™ kit (Thermo Scientific™) according to the manufacturer' instructions. All patients had their *GBA1* gene analyzed by Sanger sequencing using the ABI 3500 Genetic Analyzer (Thermo Scientific™) following Stone et al.[10] experiment designs and/or next-generation sequencing using the Ion Torrent PGM platform (Thermo Scientific™) comprising all exons and exon/intron junctions using a customized AmpliSeq Panel (Thermo Scientific™). The results were analyzed using Chromas (Technelysium), Ion Reporter™ (Thermo Scientific™) and Enlis Genome (Enlis, LCC) softwares. The genome and reference sequence of the *GBA1* gene were GRCh37 and NM_001005742.2, respectively.

***In silico* functionality prediction analysis**

Mutation Taster[11] and Human Splicing Finder[12] were used to predict functional effects of sequence variations.

Statistical analysis

Comparison of *GBA1* allele frequencies between SB and the other regions (named as Others) was performed by χ^2-test.

Results

The genotype of all patients was determined (Supplementary Table 1). Thirty-one different pathogenic mutations were found, being 6 found both in SB and in Others (Figure 1 and 2). Of the 25 remain variants, 12 were found only in patients from SB, and 13 mutations were identified exclusively in patients from other regions of the country. SB had 18/80 (22.5%) different alleles found, and Others, 19/64 (29.7%). The genotype N370S/Rec*Nci*I was the most frequent in Brazil (total= 23.6%; SB= 11/40, 27.5%; Others= 6/32, 18.8%).

N370S was the most frequent allele considering all sample (60/144; 41.7%) and both SB (33/80; 41.3%) or others (27/64; 42.2%). The general frequency for Rec*Nci*I was 13.9% (SB= 14/80, 17.5%; others= 6/64, 9.4%) and for L444P, 13.2% (SB= 14/80, 17.5%; others= 5/64, 7.8%). There was no difference in relation to the frequency of N370S, L444P and Rec*Nci*I alleles between SB and Others (p=0.909; p=0.087; p=0.161, respectively). In SB, the 3 most frequent alleles (N370S, L444P and Rec*Nci*I) corresponded to 76.3% (61/80) of total alleles and in others to 51.6% (33/64).

Two novel mutations were found in Others. One is a deletion located on exon 4, c.326delA (p.Gln109Argfs*9). This deletion determines a frameshift alteration and induces a new acceptor splice site (score 0.47, confidence score of the newly created splice site is >0.3). This mutation was found in a type I GD patient, 29 years old, from Northeast. The patient's genotype was p.Gln109Argfs*9/p.Arg535His (pt #54). This mutation was classified as pathogenic by American College of Genetics and Genomics (ACMG)[13] guidelines.

The other mutation is c.690G>A leading to a synonymous mutation, p.Val230= (V191V). This patient from Southeast was a type I GD with genotype p.Val230=/p.Asn409Ser (pt #48). *In silico* analysis showed that this mutation could gain a

donor splice site, score 0.86 (confidence score of the newly created splice site is >0.3). This mutation was classified as a variant of uncertain significance by ACMG.

Discussion

This study highlights the differences in allele frequencies between SB and the rest of Brazil. Given the results presented herein, SB seems to have a different profile of *GBA1* pathogenic alleles compared to other regions. Three alleles (N370S, Rec*Nci*I and L444P) were overrepresented in SB, with frequency around 76%, however, those represent only about 51% in Others. This could be explained by the immigration process in Brazil. Northeast individuals have a strong African ancestry, North had a large influence of Amerindians and the South was mostly settled by European immigrants. Population genetic studies showed that SB has the lowest admixture rate in Brazil (~82% of population are from European ancestry)[14]. Furthermore, the frequency of alleles which cause, in homozygosity, types II and III GD (L444P and Rec*Nci*I) appears to be higher in SB when compared to the rest of the country.

Whole *GBA1* sequencing is reported to be the best strategy for accurate genotyping. Genotyping based on PCR screening for a few common mutations will miss not only rare alleles but also recombinant alleles that comprise more than one point mutation[15]. Even though the best way of GD genetic diagnosis is by complete *GBA1* sequencing, in SB, it is reasonable to expect the alleles N370S, Rec*Nci*I and L444P alleles are present in the majority of cases. Thus, this could lead to a focused strategy depending on the region/country it is analyzed.

This present study evaluated the complete *GBA1* gene by sequencing. This methodology was performed within 2 weeks and provided a genotype of GD patients. The Rec*Nci*I allele was investigated using primers specific for *GBA1* gene. Many protocols of

genetic analysis for GD patients include only the investigation of the most frequent mutations; this prevents the differentiation between L444P alleles and those resulting from recombination events. Therefore, the determination of allele frequencies in GD patients' needs to consider the whole *GBA1* analysis, whereas GD patients can present complex alleles and recombination events. The Rec*Nci*I frequency in Brazilian GD patients was relatively high and this may be due to the analysis performed that allows the detection of recombinant alleles, which can contribute to a better understanding of genotype-phenotype correlation in GD.

Some studies suggest that L444P and the recombinant alleles usually result in poor prognosis. Furthermore, the L444P allele is strongly associated with neuronopathic GD, a combination of L444P and Rec*Nci*I leads to type II, suggesting that complex alleles increase the severity of the GD, and homozygous for L444P is found in GD type III[15]. Although the N370S, L444P and Rec*Nci*I alleles are responsible for more than 75% of the variants identified in Brazilian patients, the technique used for analysis is fundamental to obtain a complete genotype. Therefore, it is essential to make sure that the analysis covers the different types of mutations.

Rozenberg et al.[7], evaluated 40 unrelated type I GD patients by a combination of restriction fragment length polymorphism (RFLP), denaturing high performance liquid chromatography (dHPLC) and DNA sequencing methods. The initial test used was RFLP to identify the most frequent GD causing mutations (N370S, G377S, and L444P), and the others methods were used to analyze one or both of the alleles remained unidentified. This study detected 34 different mutations and the most frequent alleles was N370S (35%). This strategy used for analysis of *GBA1* gene presents as a limitation a longer time to define the genotype of the patients, which may delay the initiation of treatment in patients with a more severe form of the disease.

Rozenberg et al[8] analyzed 262 unrelated GD (type I=247, type II=3, type III=12) patients to establish the frequency of the most common mutations. Among 247 GD type I patients, N370S mutation was detected in 47% of all the alleles. Three GD type II patients presented the L444P allele in homozygous with additional mutations (E326K or recombinant alleles), which probably lead to more severe phenotypes. The molecular analysis was performed by RFLP to identify the N370S, L444P, G377S, 84insG, IVS2+1G>A, 55del, V460V, D409H, and E326K mutations. All patients were screened for N370S, L444P, and G377S, which is the third most frequent mutation in Portugal. The initial strategy was to investigate these three mutations and in the absence of these mutations in one of the alleles, a group of patients was screened for 84insG and IVS2+1G>A. For the neuronopathic form of the GD, patients were also tested for 55del, D409H and E326K.

Sobreira et al.[3] described the phenotypic and genotypic heterogeneity in GD type I in patients from Brazil and the rest-of-the-world. A total of 221 Brazilian cohort was compared to both the rest-of-the-world (n=1477) cohort, which comprised non-Brazilian patient and the rest-of-the-world non-Ashkenazi (n=692) cohort, consisting of non-Ashkenazi ancestry. The genotype differed in those three cohorts, especially in Brazilian patients compared to the other cohorts. The most common genotype in the Brazilian cohort was N370S/L444P (47%); this genotype occurred in only 16% of the rest-of-the-world cohort and 26% of the rest-of-the-world non-Ashkenazi cohort. The genotype N370S/N370S was the most frequent in the rest-of-the-world cohort representing 40% and corresponded only 7% in the Brazilian cohort. This study highlights the genetic and phenotypic heterogeneity among geographic populations of type I GD patients, being that the more severe form of the disease appears in Brazilian patients compared to the rest-of-world. This study was evaluated based on reported genotype by the International collaborative Gaucher group (ICGG) and the methodology used was not reported.

These allelic frequencies observed in both Rozenberg et al.[7,8] and Sobreira et al.[3] studies may be overlapping, since both studies may have analyzed common patients. It should also be noted that the regions of each sample analyzed in these studies are not described. Thus, it is not known if the patients are from a specific region of the country or if the patients represent all regions of Brazil.

Siebert et al.[9] designed a strategy for analysis of the entire *GBA1* gene in 48 unrelated non-Jewish GD patients from Brazil. The two most common mutations, N370S and L444P, were screened by genotyping based on real time- PCR using TaqMan probes. For 84insG and IVS2+1G>A were screened by Amplification Refractory Mutation System-PCR (ARMS-PCR) and PCR-RFLP, respectively. This study analyzed one patient that was included in the present study (pt #19).

Chaves et al.[16] evaluated the founder effect for the G377S mutation among GD patients in a population from Northeastern Brazil. All GD patients were homozygous for G377S. The identification of a single mutation in homozygotes from different generations, suggested that the high prevalence of GD in this population may be due to a combination of consanguinity and founder effect of this mutation identified. This study performed a screening for four most frequent mutations (N370S, L444P, G377S and 55del) using the DNA sequencing method.

Conclusions

The most frequent allele in Brazil was N370S. This study suggests that SB has a different profile of *GBA1* alleles compared to other regions of Brazil.

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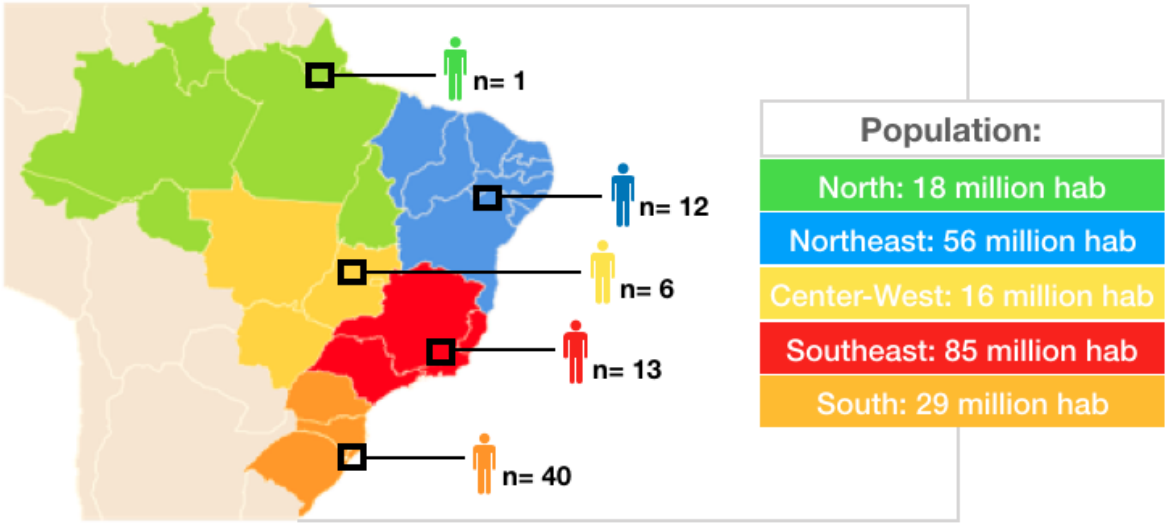

Figure 1. Brazilian regions. In this study, Others comprises four regions: Southeast, Northeast, North and Center-West. N= patients included in this study; hab= habitants.

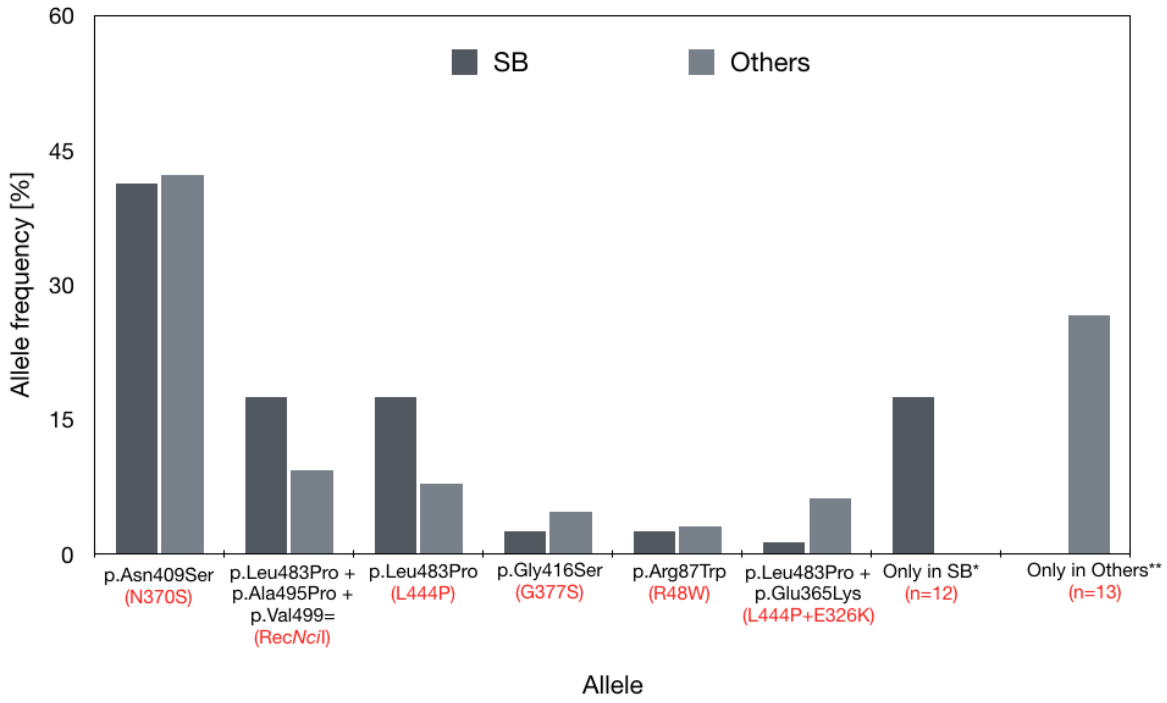

Figure 2. Allele frequencies of South Brazil (SB) vs. other regions (Others). Mutation nomenclature (HGVS), followed by the usual nomenclature *in parenthesis*. *Mutations: p.Asn435Thr, p.Leu483Arg, p.Arg159Trp, p.Arg202*, p.Gly241Arg, intron 6 deletion, p.Glu388Lys, p.Ser405Gly, p.Ser405Asn, c.1328+1G>A, p.Leu483Pro + p.Ala495Pro, p.Leu500Pro + c.1505+1G>T **Mutations: p.Trp417Cys, p.Arg502Cys, p.Arg535His, p.Gln109Argfs*9, p.Arg159Gln, p.Trp223Arg, p.Val230=, p.Pro284Thr, p.His350Arg, p.Arg392Trp, p.Val437Ile, c.1263_1317del, p.Asp448His + p.Leu483Pro + p.Ala495Pro + p.Val499=.

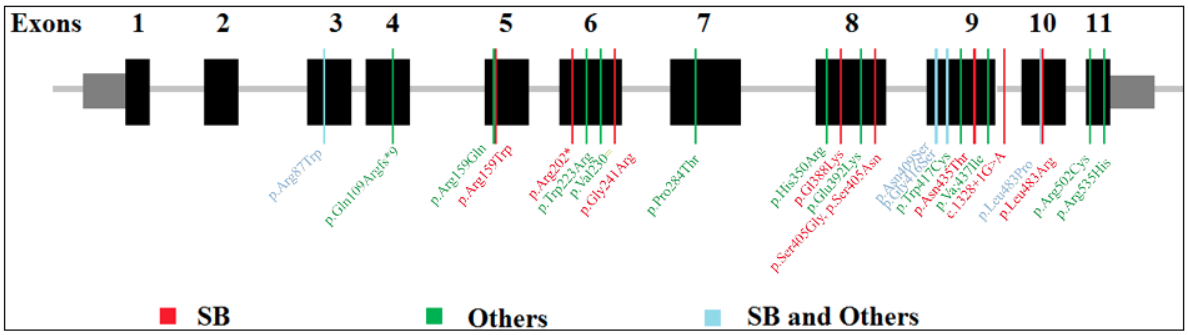

Figure 3. Location of *GBA1* missense mutations found in GD patients from SB, Others and both SB and Others,

Table 1. Studies evaluating allelic frequencies in Brazilian GD patients.

Reference	Total patients (I, II, III)	Region of Brazil	Methodology	Most frequent allele	Observations
Rozenberg et al. 2006	40 (all GD type I)	N/A	RFLP, dHPLC and DNA sequencing	N370S (35%)	*Whole *GBA1* analyzed
Rozenberg et al. 2006	262 (247, 3, 12)	N/A	RFLP	N370S (47%)	*Screening for 9 variants
Sobreira et al. 2007	221 (all GD type I)	N/A	ICGG Gaucher Registry evaluation	N370S (48.2%)	*ICGG Gaucher Registry
Siebert et al. 2013	48 (N/A)	N/A	TaqMan PCR, ARMS-PCR and DNA sequencing	N370S (44.1%)	**Whole *GBA1* analyzed
Chaves et al. 2015	5 (all GD type I)	NE	DNA sequencing	G377S (100%)	Screening for 4 variants
This study (2019)	72 (63,4,5)	5 (N, NE, CW, SE, S)	Sanger and Next-generation sequencing	N370S (41.7%)	**Whole *GBA1* analyzed

Abbreviations: I, GD type I; II, GD type II; III, GD type III; N/A, not available; ICGG, International Collaborative Gaucher Group; RFLP, restriction fragment length polymorphism; dHPLC, denaturing high performance liquid chromatography; ARMS-PCR, Amplification Refractory Mutation System-PCR; *Patient overlap (Rozenberg and Sobreira studies); **These two studies presented a patient in common.

Supplemental material

Table S1. Characterization of Gaucher disease patients from different regions of Brazil (n= 72)

N	Gender	GD type	*GBA1* genotype	cDNA [allele 1; allele 2]	protein [allele 1; allele 2]	Origin
1	M	II	L444P/Rec*Nci*I	c.[1448T>C;1448T>C+1483G>C+1497G>C]	p.[Leu483Pro;Leu483Pro+Ala495Pro+Val499=]	South
2	M	I	N370S/IVS9+1G>A	c.[1226A>G;1328+1G>A]	p.[Asn409Ser;***]	South
3	F	I	N370S/L444R	c.[1226A>G;1448T>G]	p.[Asn409Ser;Leu483Arg]	South
4	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
5	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
6	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
7	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
8	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
9	M	III	L444P/L444P	c.[1448T>C;1448T>C]	p.[Leu483Pro;Leu483Pro]	South
10	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
11	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
12	M	I	N370S/L444P+A456P	c.[1226A>G;1448T>C+1483G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
13	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
14	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
15	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
16	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
17	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
18	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
19	F	I	N370S/L461P+IVS10+1G>T	c.[1226A>G;1499T>C+1505+1G>T]	p.[Asn409Ser;Leu500Pro+***]	South
20	F	I	N370S/R163*	c.[1226A>G;604C>T]	p.[Asn409Ser;Arg202*]	South
21	M	I	N370S/G202R	c.[1226A>G;721G>A]	p.[Asn409Ser;Gly241Arg]	South
22	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
23	M	I	N370S/R120W	c.[1226A>G;475C>T]	p.[Asn409Ser;Arg159Trp]	South
24	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser/;Leu483Pro]	South

25	F	I	E349K/S366N	c.[1162G>A;1214G>A]	p.[Glu388Lys;Ser405Asn]	South
26	M	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	South
27	F	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	South
28	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
29	M	I	N370S/L444R	c.[1226A>G;1448T>G]	p.[Asn409Ser;Leu483Arg]	South
30	M	III	L444P/L444P	c.[1448T>C;1448T>C]	p.[Leu483Pro;Leu483Pro]	South
31	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
32	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	South
33	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	South
34	M	II	L444P/Rec*Nci*I	c.[1448T>C;1448T>C+1483G>C+1497G>C]	p.[Leu483Pro;Leu483Pro+Ala495Pro+Val499=]	South
35	M	I	G377S/G377S	c.[1246G>A;1246G>A]	p.[Gly416Ser;Gly416Ser]	South
36	F	I	R48W/R48W	c.[259C>T;259C>T]	p.[Arg87Trp;Arg87Trp]	South
37	F	I	N370S/S366G	c.[1226A>G;1213A>G]	p.[Asn409Ser;Ser405Gly]	South
38	M	*	N396T/Rec*Nci*I	c.[1304A>C;1448T>C+1483G>C+1497G>C]	p.[Asn435Thr;Leu483Pro+Ala495Pro+Val499=]	South
39	F	II	Rec*Nci*I/intron 6 deletion	c.[1226A>G;**]	p.[Asn409Ser;**]	South
40	F	I	R463C/ R463C	c.[1504C>T;1504C>T]	p.[Arg502Cys;Arg502Cys]	South
41	M	III	P245T/del55	c.[850C>A;1263-317]	p.[Pro284Thr;Leu422Profs]	Southeast
42	F	I	L444P+E326K/R496H	c.[1448T>C+1093G>A;1604G>A]	p.[Leu483Pro+Glu365Lys;Arg535His]	Southeast
43	F	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	Southeast
44	M	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	Southeast
45	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Southeast
46	M	II	L444P+E326K/H311R	c.[1448T>C+1093G>A;1049A>G]	p.[Leu483Pro+Glu365Lys;His350Arg]	Southeast
47	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Southeast
48	M	I	N370S/**V191V**	c.[1226A>G;**690G>A**]	p.[Asn409Ser;**Val230=]**	Southeast
49	F	I	N370S/R120Q	c.[1226A>G;476G>A]	p.[Asn409Ser;Arg159Gln]	Southeast
50	M	I	N370S/W184R	c.[1226A>G;667T>C]	p.[Asn409Ser;Trp223Arg]	Southeast
51	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Southeast
52	F	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	Southeast
53	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Southeast

54	F	I	R496H/**p.Gln109Argfs*9**	c.[1604G>A;**326delA**]	p.[Arg535His**;Gln109Argfs*9]**	Northeast
55	F	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Northeast
56	M	I	N370S/W378C	c.[1226A>G;1251G>C]	p.[Asn409Ser;Trp417Cys]	Northeast
57	F	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	Northeast
58	M	I	N370S/L444P+E326K	c.[1226A>G;1448T>C+1093G>A]	p.[Asn409Ser;Leu483Pro+Glu365Lys]	Northeast
59	M	I	N370S/L444P+E326K	c.[1226A>G;1448T>C+1093G>A]	p.[Asn409Ser;Leu483Pro+Glu365Lys]	Northeast
60	M	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	Northeast
61	F	I	R48W/R48W	c.[259C>T;259C>T]	p.[Arg87Trp;Arg87Trp]	Northeast
62	F	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	Northeast
63	M	I	N370S/W378C	c.[1226A>G;1251G>C]	p.[Asn409Ser;Trp417Cys]	Northeast
64	M	I	N396T/L444P+E326K	c.[1304A>C;1448T>C+1093G>A]	p.[Asn435Thr;Leu483Pro+Glu365Lys]	Northeast
65	F	III	G377S/W378C	c.[1246G>A;1251G>C]	p.[Gly416Ser;Trp417Cys]	Northeast
66	M	I	N370S/L444P	c.[1226A>G;1448T>C]	p.[Asn409Ser;Leu483Pro]	Center-West
67	F	I	N370S/N370S	c.[1226A>G;1226A>G]	p.[Asn409Ser;Asn409Ser]	Center-West
68	F	III	L444P/L444P	c.[1448T>C;1448T>C]	p.[Leu483Pro;Leu483Pro]	Center-West
69	F	I	R353W/RecTL	c.[1174C>T;1342G>C+1448T>C+1483G>C+1497G>C]	p.[Arg392Trp;Asp448His+Leu483Pro+Ala495Pro+Val499=]	Center-West
70	M	I	N370S/Rec*Nci*I	c.[1226A>G;1448T>C+1483G>C+1497G>C]	p.[Asn409Ser;Leu483Pro+Ala495Pro+Val499=]	Center-West
71	F	I	G377S/G377S	c.[1246G>A;1246G>A]	p.[Gly416Ser;Gly416Ser]	Center-West
72	F	I	N370S/V398I	c.[1226A>G;1309G>A]	p.[Asn409Ser;Val437Ile]	North

Abbreviations: N, patients; M, male; F, female; GD, Gaucher disease; *unknown GD type; **MLPA method identified this deletion; ***mutation in exon/intron junctions; Novel mutations are set in bold.

7.3 CAPÍTULO 3 – Artigo 3

Título do artigo: "Assessment of cellular cobalamin metabolism in Gaucher disease"

Autores: Suelen P. Basgalupp, Marina Siebert, Charles Ferreira, Sidney Behringer, Ute Spiekerkoetter, Luciana Hannibal, Ida Vanessa D. Schwartz.

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Assessment of cellular cobalamin metabolism in Gaucher disease
Suelen Porto Basgalupp; Marina Siebert; Charles Ferreira; Sidney Behringer; Ute Spiekerkoetter; Luciana Hannibal; Ida Vanessa Doederlein Schwartz

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Assessment of cellular cobalamin metabolism in Gaucher disease

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Abstract

Background: Gaucher disease (GD), one of the most prevalent lysosomal diseases, is caused by biallelic pathogenic mutations in the *GBA1* gene that encodes β-glucosidase (GCase). Clinically, GD manifests with heterogeneous multiorgan involvement mainly affecting hematological, hepatic and neurological axes. At the cellular level, deficiency of *GBA1* disturbs lysosomal storage with buildup of glucocerebroside. The consequences of disturbed lysosomal metabolism on biochemical pathways that also require lysosomal processing are unknown. Abnormal systemic markers of cobalamin (Cbl, B_{12}) metabolism have been reported in patients with GD, suggesting impairments in lysosomal handling of Cbl or in its downstream processing and trafficking events.

Methods: Dermal fibroblasts isolated from healthy subjects (n=3), patients with GD types I (n=1), II (n=1), III (n=1) and a heterozygous asymptomatic carrier were examined for their GCase enzymatic activity and lysosomal compartment intactness. Healthy human and GD fibroblasts were cultured in growth medium with and without 500 nM hydroxocobalamin (HOCbl) supplementation. Cellular cobalamin status was examined via determination of metabolomic markers in cell lysate (intracellular) and conditioned culture medium (extracellular). The expression of transcobalamin (TC), hosted in the lysosome early in the processing of incoming cobalamins, was examined by Western blot.

Results: Cultured dermal fibroblasts from GD patients exhibited reduced GCase activity compared to healthy individuals and the heterozygous carrier, demonstrating a preserved disease phenotype in this cell type. The concentrations of total homocysteine (tHcy), methylmalonic acid (MMA), cysteine (Cys) and methionine (Met) in GD cells were comparable to control levels, except in one patient with GD III. The response of these metabolomic markers to supplementation with HOCbl yielded variable results. Intracellular

expression and retention of transcobalamin was comparable in healthy individuals and GD patients, suggesting a preserved cobalamin uptake-and processing axis.

Conclusions: Our results showed that cobalamin transport and cellular processing pathways are overall protected from lysosomal storage damage in GD cells. To our knowledge, this is the first study to investigate cellular metabolism of vitamin B_{12} in Gaucher disease. GD patients who present with concomitant cobalamin deficiency should be examined on a case-specific manner with respect to potential use of and response to cobalamin.

Keywords: Gaucher disease, vitamin B_{12}, cobalamin, β-glucosidase, methylmalonic acid, homocysteine, transcobalamin.

Background

Gaucher disease (GD) is an autosomal recessive inborn error of metabolism caused by deficient activity of glucocerebrosidase (GCase) enzyme due to pathogenic mutations in the *GBA1* gene (OMIM 606463), located on chromosome 1 (1q21). This enzyme catalyzes the conversion of the glycolipid glucocerebroside to ceramide and glucose, and its deficiency leads to the accumulation of the substrate in tissues, especially in the cells of reticuloendothelial system, resulting in dysfunction of different organs such as liver, spleen and bone marrow [1]. GD frequency is estimated to be around 1 in 40,000-60,000 individuals in general population, being more common in Ashkenazi Jews, affecting 1 in 800 people [2, 3]. This disorder is classified into three main types, based on the absence (type I) or presence and severity (types II and III) of involvement of the central nervous system (CNS) [4]. The diagnosis of GD is performed by measurement of the GCase activity in leukocytes and fibroblasts of individuals with clinical suspicion of the disease. Analysis of the *GBA1* gene is also performed to identify the genotype of the patients. The standard method for variant analysis in GD is full-gene sequencing of *GBA1*. Complementary techniques such as Multiplex Ligation-dependent Probe Amplification (MLPA) can be used to identify deletions or duplications of any region of this gene [5].

The mechanisms of GD pathology are likely multifactorial, with the contribution of genetically unrelated disease modifiers remaining largely unexplored. Abnormalities in systemic markers of cobalamin (Cbl) status have been noted in GD patients, raising the question of whether cellular handling of this micronutrient is sensitive to aberrant lysosomal storage [6]. Specifically, studies showed reduced plasma Cbl and elevated holo-transcobalamin (holo-TC) in GD patients [7]. The value of these systemic biomarkers in diagnosing vitamin B_{12} deficiency is limited as they do not measure the status of the two Cbl-dependent enzymes in humans [8, 9]. Cbl deficiency inactivates the two Cbl-dependent

enzymes methionine synthase and methylmalonyl-CoA mutase, which results in elevation of their substrates, homocysteine (Hcy) and methylmalonic acid (MMA), respectively. Thus, in the absence of folate deficiency (which also leads to elevated tHcy), tHcy and MMA are direct reporters of cellular Cbl status. Associations between impaired endocytosis and lysosomal metabolism and transient Cbl deficiency have been found in Alzheimer's disease [10] and in a patient with mutations in the rabenosyn-5 gene [11]. The cellular utilization of vitamin B_{12} requires a functional lysosomal metabolism [8]. Mutations in the lysosomal *cblF* and *cblJ* genes [12-14] responsible for Cbl shuttling from the lysosome into the cytosol, as well as unrelated disturbances of the lysosomal and endocytic pathways [10, 11], lead to functional vitamin B_{12} deficiency and the onset of neurological deterioration. It is currently unknown whether abnormal accumulation of glucocerebroside may affect Cbl transit in and out of the lysosome [6]. Herein, GCase activity, intracellular and extracellular functional markers of Cbl status tHcy and MMA, and expression of the cellular Cbl transporter TC were measured in cultured fibroblasts from healthy individuals and from GD patients. This is the first study to demonstrate an intact Cbl transport and processing axis in Gaucher disease cells. The variable response of cultured GD cells to metabolite reduction upon hydroxocobalamin (HOCbl) supplementation suggests that GD patients presenting with concomitant Cbl deficiency should be examined on a case-specific basis.

Methods

Cell culture

Fibroblasts derived from untreated patients with GD type I (GM00852), type II (GM00877), type III (GM20272) and one individual heterozygous carrier (GM00878) were obtained from the Coriell Institute for Medical Research (Table 1). Healthy fibroblasts were obtained commercially (NHDF), from the Lerner Research Institute, Cleveland Clinic, USA (HFF) [15] or from our clinic from individual without any metabolic diseases (Control-W).

Healthy human and GD fibroblasts were cultured in 25 cm^2 flasks with 5 mL of growth medium (DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C) until 80-90% confluency. This culture medium contains no vitamin B_{12}, except that present in the 10% FBS as holo-TC (60-70 pM). Trypsin-digestion for cell passages was performed at a ratio of 1:3. Culture medium was exchanged every 2 days, until the beginning of the experiment.

Cell cultures were synchronized such that healthy individuals and GD patients cell lines were grown simultaneously, beginning on day 1, under the exact same experimental conditions. The experiment was performed in vitamin B_{12}-free medium and in medium supplemented with 500 nM HOCbl. Each cell line and condition was grown in triplicate. A sample of culture medium with and without HOCbl supplementation was taken on day 1 of the experiment, and frozen at – 80°C for further analysis. A total of 42 flasks of cells were maintained in culture at 37°C for 5 days. Then, conditioned culture medium and cell pellets from each flask were collected and stored at – 80°C until further analysis.

β-Glucosidase enzymatic activity assay

Preparation of whole cell lysates. Cells were lysed in 400 mM citrate phosphate buffer containing a protease inhibitor cocktail (Sigma-Aldrich, product Nr. P8340-5ML), triton X-100 and sodium-taurodeoxycholate and centrifuged at 13,000 rpm for 15 minutes at room temperature (RT). The concentration of proteins in the samples was determined with the Bradford reagent (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as standard.

β-glucosidase activity in a 96-well microplate assay. A previously reported method for the assessment of β-glucosidase activity [16] was adapted to a 96-well plate assay format for whole cell extract. This assay uses the synthetic substrate 4-methylumbelliferyl-β-D-glucopyranoside (4MUβ-Glucopyranoside, Sigma-Aldrich). Briefly, cell lysates containing

0.1 µg of protein were transferred to a 96-well microplate, each sample by triplicate. The reaction buffer consisted of 400 mM citrate phosphate buffer (pH 5.2), 14.3 mM sodium taurodeoxycholate and 6 mM 4MUβ-Glucopyranoside (90 µL). Samples were incubated in the reaction buffer for 18 hours at 37°C. Reaction was stopped by the addition of 110 µL of glycine buffer (0.5 M, pH 10.4). Amount of fluorescent product formed was measured with an Infinite® 200 PRO plate reader (Tecan, Life Sciences) set up with fluorescence excitation at 355 nm and fluorescence emission at 460 nm. As a control of lysosomal integrity, we measured α-glucosidase enzymatic activity assay using the substrate 4-methylumbelliferyl-α-D-glucopyranoside (Sigma-Aldrich). This lysosomal protein is expected to be intact in GD cell lines. Assay conditions were exactly as described herein for β-glucosidase activity, except that 4-methylumbelliferyl-α-D-glucopyranoside was used as the substrate.

Expression of transcobalamin in whole cell lysates

Expression of TC in healthy human and GD fibroblasts was examined by Western blot, with whole cell extracts prepared under near-native conditions as described above for the β-glucosidase activity measurement. From that extract, 15–20 µL (30 µg of total protein) was loaded on an SDS-PAGE for Western blotting. The primary antibody (rabbit anti-human TC; 189871; Abcam, Cambridge, United Kingdom) was used at a dilution of 1:500 and the secondary antibody at 1:1000 (polyclonal goat anti-rabbit) as reported previously [17].

Analysis of intracellular and extracellular tHcy, Cys and Met by LC-ESI-MS/MS

Extraction of aminothiols from cultured cells. Cultured fibroblasts were isolated by trypsinization, washed with PBS, and frozen at − 80°C until further analysis. After thawing the cell pellets, 0.1 mL of 20 mM dithiothreitol (DTT) was added. Lysis was performed by freeze-thawing of cells by alternating between dry-ice and RT, three times. An aliquot of 10 µL of lysate was separated and stored at − 80°C for further measurement of concentration of proteins with the Bradford reagent (Bio-Rad, Hercules, CA, USA). Then, 0.1 mL 10%

trifluoroacetic acid (TFA) was added to precipitate proteins. The extracts were incubated at RT for 15 minutes, and then centrifuged at 13,000 rpm for 15 minutes at RT. An aliquot of 3.16 μL of aminothiol-containing supernatant (intracellular aminothiols) or culture medium (extracellular aminothiols) was transferred into a clean Eppendorf tube. A 20 μL aliquot of Internal Standard (Stable isotopically labelled Hcy, cysteine (Cys) and methionine (Met) was then added to each tube followed by 20 μL of 0.5 M DDT solution and 100 μL of methanol with 0.1% formic acid. The mixtures were vortexed for 10 seconds at a medium speed and incubated for 20 minutes at RT. After that, the samples were centrifuged at 10,000 g for 5 minutes. An aliquot of 60 μL of supernatant was transferred into high performance liquid chromatography (HPLC) vials and 10 μL of each sample was injected into the HPLC equipment. Two commercial controls having known amounts of Hcy, Cys and Met were used as a quality control ("Special Assays in Serum" and "control amino acids", MCA Laboratory, Queen Beatrix Hospital in 7101 BN Winterswijk, The Netherlands). The concentration of aminothiols was determined with respect to a calibration curve and the addition of stable isotopically labelled internal standards. Briefly, calibrators were prepared by preparing a master mix containing 100 μL of 0.5 M DDT, 100 μL of Hcy (998.6 μM), 150 μL of Met (998.5 μM), 300 μL of Cys (1007 μM), 150 μL of methionine sulfoxide (1000 μM) and 200 μL of H_2O (Calibrator 1). A serial dilution was performed by pipetting 200 μL of Calibrator 1 into an Eppendorf tube containing 200 μL H_2O (Calibrator 2), up to calibrator 7. The calibrators were vortexed. Aliquots of 3.16 μL of each calibrator were pipetted into HPLC vials and 20 μL of internal standard solution (D_4-Homocysteine (20 μM), D_4-Methionine (60 μM), $^{13}C_3$-Cysteine (102.3 μM) in H_2O) was added to each vial followed by 20 μL of 0.5 M DDT solution and 100 μL of methanol with 0.1% formic acid. After vortexing, 10 μL of the samples were injected into the liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) machine (QTrap 6500+, Sciex). Values of intracellular and

extracellular tHcy-, Cys- and Met- concentration were normalized to total protein concentration.

Analysis of intracellular and extracellular methylmalonic acid by LC-ESI-MS/MS

MMA was determined based on a previously published method with modifications [18]. Cultured fibroblasts were lysed using the same protocol described for aminothiols. For sample preparation, an aliquot of 100 μL of cell lysate (intracellular MMA) and conditioned culture medium (extracellular MMA) of each healthy human and Gaucher fibroblasts was pipetted into a clean microcentrifuge filter (Amicon, 30 kDa MW cut-off, Merck Millipore) tube and 100 μL of 0.8 μM D_3-MMA (CDN isotopes) Internal Standard solution was added to each tube. Samples were vortexed for 10 seconds and centrifuged at 14,000 g for 30 minutes at 10°C. After centrifugation, 100 μL of the filtrate was transferred into HPLC vials and acidified with 10 μL of 4% formic acid. Then, 10 μL of the sample was injected into the LC-ESI-MS/MS (QTrap 6500+, Sciex). Two commercial controls (Special Assays in Serum, MCA Laboratory, Queen Beatrix Hospital in 7101 BN Winterswijk, The Netherlands) that have known concentrations of MMA were used for analysis. Quantification of MMA was performed by use of a calibration curve. Briefly, 100 μL of MMA standards (0.1, 0.25, 0.5, 0.75, and 1.0 μM) were pipetted into Eppendorf tubes and 100 μL of 0.8 μM D_3-MMA internal standard solution was added to each tube. After vortexing, a 100 μL aliquot was transferred into HPLC vials and acidified with 10 μL of 4% formic acid. Then, 10 μL of each sample was injected into the LC-ESI-MS/MS system. Stock solutions of MMA (Sigma Aldrich) and D_3-MMA were prepared in deionized H_2O and kept at − 20°C. Values of intracellular and extracellular MMA were normalized to total protein concentration of the cell lysates.

Statistical analysis

Regarding the data processing, database double entry, review and analysis were performed using the SPSS, version 18.0. [SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.].

Quantitative data was expressed by median and 95% Confidence Interval [95%CI]. To compare medians between groups (healthy *versus* GD) or Cbl supplementation (with *versus* without the presence of HOCbl supplementation), the Mann-Whitney test for independent samples was used. For assessing possible interactions between both factors, the Kruskal-Wallis test for independent samples, with Dunn *post hoc* test, was applied. The level of significance was set at 5% for all analysis.

Results

The genotypes and phenotypes of healthy individuals, GD patients and asymptomatic heterozygous carrier individual are displayed in Table 1.

α- and β-glucosidase activities of fibroblasts derived from healthy individuals, Gaucher disease patients and an asymptomatic heterozygous carrier

β-glucosidase activity was measured in whole cell extracts of healthy individuals and GD fibroblasts. β-glucosidase activities in the GD (types I, II and III) cell lines were consistently lower than those in control and heterozygous cells (Fig. 1a). The enzymatic assay confirmed that GD cells homozygous or compound heterozygous for *GBA1* mutations had no detectable β-glucosidase activity (Fig. 1a). Additionally, the results demonstrate the deficiency of GCase in GD fibroblasts and that heterozygous for *GBA1* had β-glucosidase activity almost as high as healthy cells, suggesting a compensatory mechanism provided by the wild type *GBA1* allele. The β-glucosidase activity was not markedly affected by the presence of HOCbl supplementation in the culture medium (black bars).

To evaluate lysosomal integrity, the lysosomal α-glucosidase activity was measured and revealed that healthy individuals and GD fibroblasts exhibit an intact α-glucosidase

activity (Fig. 1b). These results exclude the occurrence of pleiotropic effects induced by mutations in the *GBA1* gene as well as unwanted damage of lysosomal components during the sample preparation protocols.

Expression of transcobalamin, the cellular transporter of cobalamin

Because Cbl reaches all cells in the body bound to transporter protein TC, and once inside the cell, release of Cbl for downstream use is preceded by lysosomal degradation of TC, it was examined whether abnormal lysosomal storage brought about by mutations in *GBA1* affect the TC in cells. Western blot analysis of whole cell lysates indicated normal content of TC in healthy individuals and GD fibroblasts (Fig. 2). Thus, previously reported abnormalities in plasma holo-TC in GD patients [7] do not seem to arise from abnormal biosynthesis/turnover of this protein in GD cells.

Intracellular tHcy, Cys, Met and MMA in Gaucher disease and response to supplementation with hydroxocobalamin

Aiming to access cellular Cbl status in GD (n=3) and healthy individuals (n=3), it was examined the intracellular concentration of metabolite markers of Cbl status, tHcy and MMA, as well as markers of the methionine cycle, Met, and of the trans-sulfuration pathway, Cys.

A group effect was observed in Met, since GD participants showed increased median compared to control group (Mann-Whitney test, $p=0.015$; Table 2). Additionally, no group, Cbl supplementation or interaction effect was observed in all other assessed variables intracellularly (Mann-Whitney test or Kruskal-Wallis test with Dunn *post hoc,* $p>0.05$ for all, Table 2).

Extracellular tHcy, Cys, Met and MMA in Gaucher disease and response to supplementation with hydroxocobalamin

Cells maintain healthy intracellular concentrations of tHcy and MMA partly through the export of these metabolites into the extracellular milieu (to circulation in whole organisms, and to conditioned culture medium in cultured cells). The levels of tHcy, Cys, Met and MMA were comparable between healthy (n=3) and GD (n=3) conditioned medium (Table 3). The concentration of tHcy was significantly decreased after supplementation with HOCbl in healthy individuals and GD fibroblasts (Mann-Whitney test, $p=0.041$), with no effect of this supplementation in Cys, Met and MMA (Mann-Whitney test, $p>0.05$). Furthermore, no group effect or interaction was observed among all other assessed variables extracellularly (Mann-Whitney test or Kruskal-Wallis test with Dunn *post hoc*, $p>0.05$ for all, Table 3).

Discussion

Our results suggest that there is no functional deficiency of vitamin B_{12} in GD patients. Biomarker levels of B_{12} status showed values within normal limits and the data did not present significantly differences between the different types of GD, that is, between the neuropathic and non-neuropathic forms of the disease. A distinct correlation between serum markers of Cbl (from published work) [7, 19, 20] and cellular status of the micronutrient is not observed, and this merits further investigation.

Cultured human fibroblasts are an invaluable resource in the diagnosis of metabolic diseases. Dermal fibroblasts from Gaucher patients exhibit a severe deficiency of GCase activity [21], which facilitates the investigation of metabolic abnormalities *in vitro*. The results confirmed the deficiency activity of GCase in GD fibroblasts types I, II and III, and showed that a heterozygous carrier for GD had enzymatic activity similar to healthy human cells. This *in vitro* result is consistent with the asymptomatic phenotype of patients heterozygous for *GBA1*, and suggests that one functional allele is sufficient to compensate GCase activity. The presence of a normal α-glucosidase activity confirmed that under our cell culture and sample preparation conditions, the lysosomal compartment is overall

preserved in the presence of mutations in the *GBA1* locus. The investigation of Cbl metabolism in GD was partly motivated by previous reports indicating a high incidence of low serum vitamin B_{12} in untreated GD patients [22], increased circulating levels of transcobalamin II (TCII) in GD patients [7], and slightly increased Hcy and MMA in polyneuronopathic GD type I patients compared to non-neuronopathic patients [19]. In the latter study, it is worth mentioning that the tHcy and MMA elevation documented in polyneuropathic GD type I patients versus those without signs of polyneuropathy was mild, within the reference range considered normal. The assessment of Cbl status should include preferentially a combination of functional markers (tHcy, MMA) and systemic markers (plasma B_{12} and holo-TC) [20, 23-25]. This is the first time that Cbl status is assessed in cultured cells isolated from patients with GD. Intracellular tHcy was slightly increased in GD cells, but the difference with respect to control did not hold statistical significance. We found comparable levels of intracellular and extracellular Cys and Met in control and GD cells, suggesting a functional methionine cycle and steady state of Cys concentration. Intracellular MMA was elevated in GD type III cells and did not respond to supplementation with hydroxocobalamin, but this was not observed in GD types I and II or in the heterozygous asymptomatic carrier. Extracellular tHcy, Met and Cys were comparable in healthy individuals and GD participants, with the exception of GD type III exhibiting elevated Met and Cys compared to control, none of which responded to HOCbl supplementation.

Extracellular MMA was only elevated in GD type III and did not respond to HOCbl supplementation, whereas in the other GD cells levels of this metabolite were comparable to healthy human fibroblasts. Regardless of exogenous HOCbl supplementation all GD cells expressed TC comparable to the levels observed in healthy cells. Altogether, these results indicate that GD cells preserve normal Cbl metabolism. Because was examined only one case of GD type III, we are unable to conclude whether the metabolite elevations identified in this

participant (tHcy, Met, Cys and MMA) are relevant to other GD type III patients, or if this is case-specific. Further, it possible that the metabolite elevations identified in this GD type III participant are not pathogenic. These aspects warrant further investigation in a larger cohort of GD patients.

Conclusion

Our results showed that cobalamin transport and processing pathways are overall preserved in GD cells. GD patients who present with concomitant Cbl deficiency should be examined on a case-specific manner with respect to potential use of and response to Cbl.

Abbreviations

GD: Gaucher disease; GCase: β-glucosidase enzyme; Cbl: Cobalamin; B_{12}: Vitamin B_{12}; TC: Transcobalamin; tHcy: Total homocysteine; MMA: Methylmalonic acid; Cys: Cysteine; Met: Methionine; CNS: Central nervous system; MLPA: Multiplex Ligation-dependent Probe Amplification; Holo-TC: Holo-transcobalamin; FBS: Fetal bovine serum; HOCbl: Hydroxocobalamin; RT: Room temperature; BSA: Bovine serum albumin; 4MUβ-Glucopyranoside: 4-methylumbelliferyl-β-D-glucopyranoside; DDT: Dithiothreitol; TFA: Trifluoroacetic acid; HPLC: High performance liquid chromatography; LC-ESI-MS/MS: Liquid chromatography electrospray ionization tandem mass spectrometry.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SPB designed and executed experiments, analyzed data and wrote the manuscript; MS revised the manuscript; CF contributed to statistical analyses; SB performed and analyzed the biochemical experiments (performed LC-ESI-MS/MS measurements); US designed experiments and revised the manuscript; LH designed the experiments, analyzed data, supervised the writing and revised the manuscript; and IVDS designed the experiments, supervised the writing and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

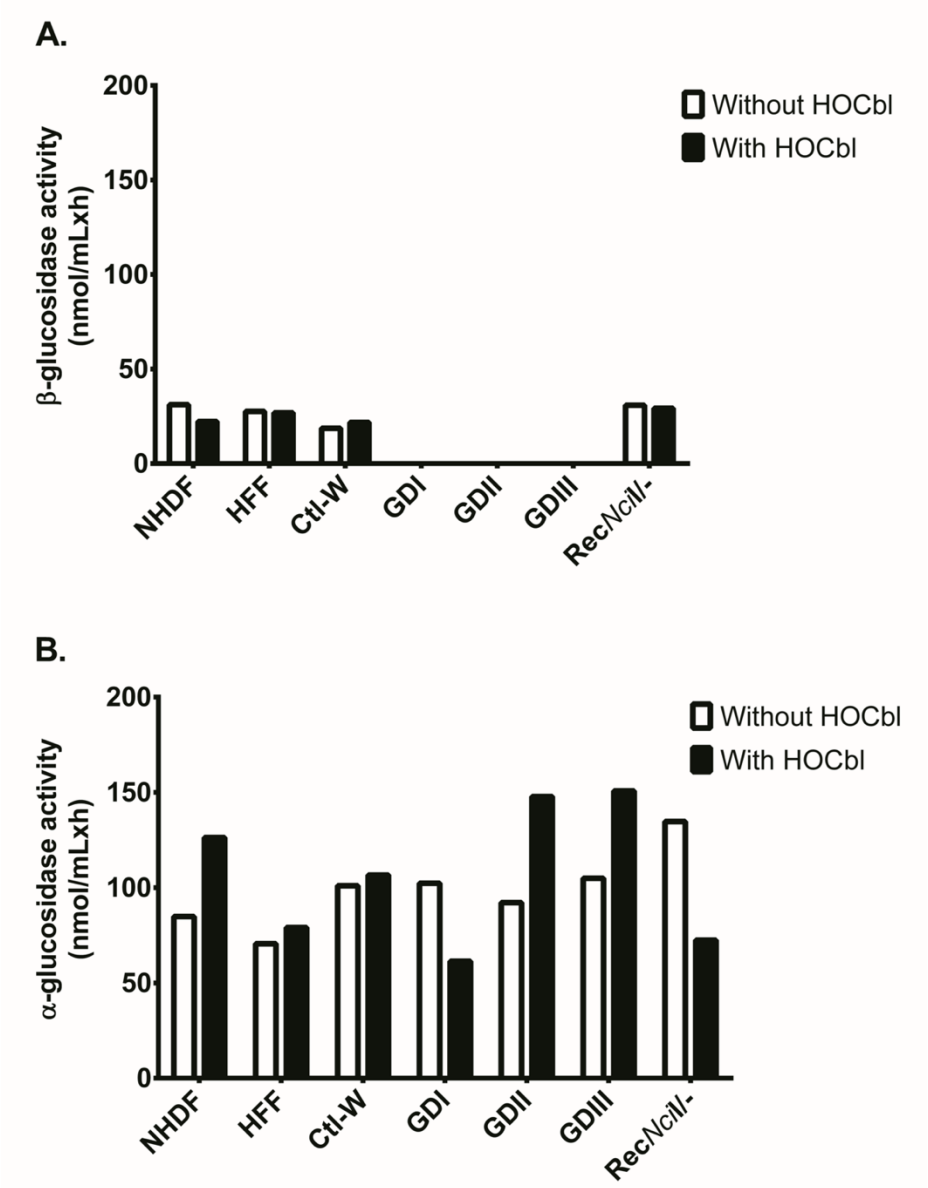

Fig. 1 α- and β-glucosidase activity in healthy individuals, Gaucher disease patients and an asymptomatic heterozygous carrier. (**a**) Gaucher patients (n=3) had no detectable activity of β-glucosidase (nmol/mLxh), whereas the asymptomatic carrier (n=1) exhibited β-glucosidase activity comparable to those of healthy individuals (n=3); (**b**) All examined subjects presented comparable α-glucosidase activity (nmol/mLxh), suggesting preserved activity of lysosomal components not associated with the *GBA1* mutation. Legend: without HOCbl – culture medium without hydroxocobalamin; with HOCbl – culture medium with hydroxocobalamin, GD – Gaucher disease, Rec*Nci*I/- – asymptomatic heterozygous carrier.

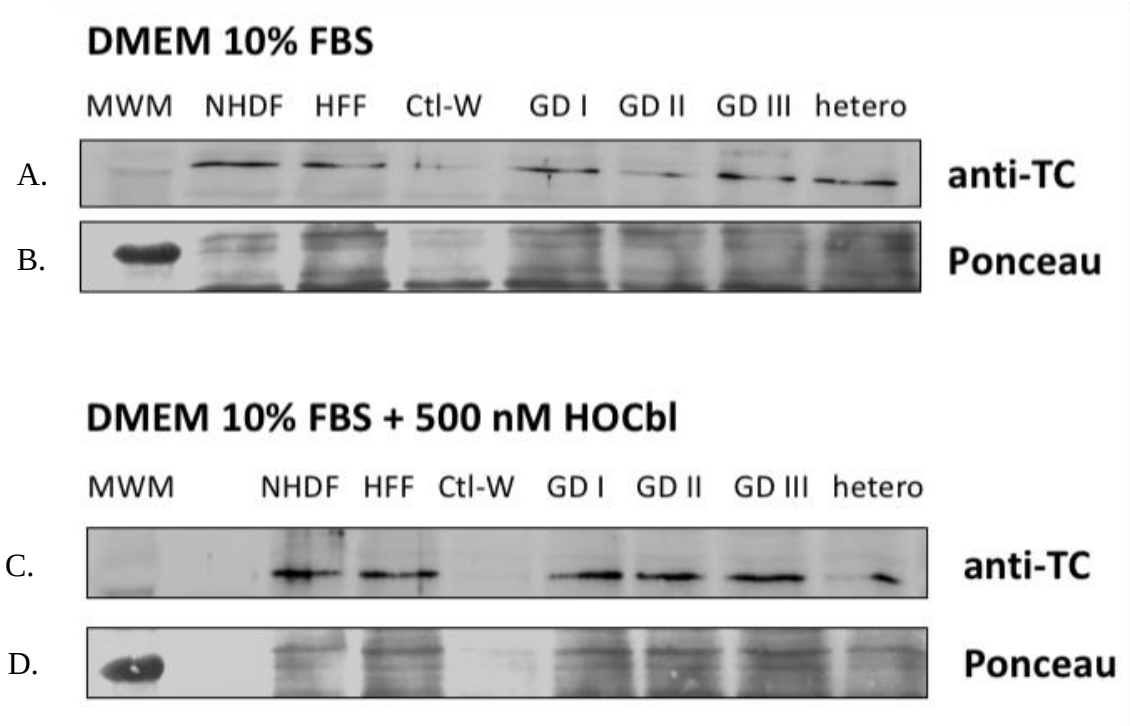

Fig. 2 Expression of transcobalamin in healthy individuals, Gaucher disease patients and an asymptomatic heterozygous carrier. Whole cell lysates (30 µg) of healthy human and GD fibroblasts were examined for intracellular content of transcobalamin, with and without HOCbl supplementation. Under our experimental conditions, no differences were identified between healthy and GD cells, suggesting normal expression of transport protein transcobalamin. A. and C. panels: Western blot results obtained by probing whole cell lysates with anti-TC (rabbit anti-human, dilution 1:500) and secondary goat anti-rabbit IgG-HRP (1:1000) antibody. B. and D. panels: Ponceau staining of nitrocellulose membrane after semi-dry blot transfer as protein loading control.

Table 1 Genotype and phenotype of healthy and Gaucher disease fibroblasts utilized in this study.

Sample	Gender	GD type	Genotype	Remarks
GM00852	Male	I	N370S/84GG	Hepatosplenomegaly; Slowed horizontal saccades.
GM00877	Male	II	L444P/Rec*Nci*I	Hepatosplenomegaly; Strabismus; Trismus.
GM20272	Male	III	L444P/L444P	Hepatosplenomegaly; Slowed horizontal saccades.
GM00878	Female	Heterozygous	Carrier for Rec*Nci*I	Clinically unaffected mother of GM00877.
NHDF	Male	Normal human	No *GBA1* mutation	Healthy dermal fibroblast
HFF	Male	Normal human	No *GBA1* mutation	Healthy dermal fibroblast
Control-W	Female	Normal human	No *GBA1* mutation	Healthy dermal fibroblast

Table 2 Intracellular levels of tHcy, Cys, Met and MMA in Gaucher disease and healthy human fibroblasts in the absence and in the presence of HOCbl.

Variable	Healthy human fibroblasts		GD fibroblasts		**p-value		
	Without HOCbl (n=3)	With HOCbl (n=3)	Without HOCbl (n=3)*	With HOCbl (n=3)*	Group	Cobalamin supplementation	Interaction
tHcy (nmol/mg)	0.21[0.04–0.45]	0.17[0.12–0.22]	0.26[-0.12–0.77]	0.25[0.10–0.40]	0.180	0.180	0.154
range	0.19–0.34	0.15–0.19	0.19–0.53	0.19–0.31			
Cys (nmol/mg)	34.2[10.21–53.57]	36.26[6.80–64.97]	31.67[-10.99–87.88]	27.93[-22.75–102.04]	1.000	0.818	0.954
range	22.24–39.23	23.99–47.40	22.82–60.85	22.53–68.48			
Met (nmol/mg)	27.72[15.90–40.19]	32.49[20.65–40.67]	39.73[22.03–55.31]	38.59[23.84–59.61]	**0.015**	0.589	0.103
range	23.33–33.09	26.04–33.45	31.50–44.77	36.62–49.96			
MMA (nmol/mg)	0.01[0.01–0.02]	0.01[0.01–0.03]	0.02[-0.01–0.05]	0.02[-0.02–0.06]	0.699	0.818	0.965
range	0.01–0.02	0.01–0.02	0.01–0.03	0.01–0.04			

Data expressed as Median [95% Confidence Interval], range. n=3 for each group. Legend: without HOCbl – culture medium without hydroxocobalamin; with HOCbl – culture medium with hydroxocobalamin; GD – Gaucher disease. p – statistical significance. **Mann-Whitney test (factors: group – healthy human and GD patients, cobalamin supplementation) or Kruskal-Wallis test with Dunn *post hoc* (factors: group – healthy human and GD; cobalamin supplementation; interaction) for independent samples. Significance set at 5% for all analysis. *GD types I (n=1); II (n=1) and III (n=1).

Table 3 Extracellular levels of tHcy, Cys, Met and MMA in Gaucher disease and healthy human fibroblasts in the absence and in the presence of HOCbl.

Variable	Healthy human fibroblasts		GD fibroblasts		**p-value		
	Without HOCbl (n=3)	With HOCbl (n=3)	Without HOCbl (n=3)*	With HOCbl (n=3)*	Group	Cobalamin supplementation	Interaction
tHcy (nmol/mg)	3.74[3.01–4.45]	2.15[0.22–3.44]	3.73[1.87–5.27]	1.93[-0.88–5.92]	0.699	**0.041**	0.187
range	3.44–4.02	1.08–2.25	2.82–4.16	1.54–4.08			
Cys (nmol/mg)	142.88[5.70–280.12]	159.23[26.33–271.79]	284.72[-115.81–648.73]	266.75[-192.00–807.51]	0.132	1.000	0.459
range	87.69–198.16	95.36–192.59	104.26–410.40	130.24–526.28			
Met (nmol/mg)	116.05[31.86–212.08]	126.66[36.86–214.55]	156.44[-53.85–368.93]	148.51[-74.35–424.63]	0.589	0.699	0.887
range	89.02–160.84	89.47–160.98	73.00–243.18	90.70–286.20			
MMA (nmol/mg)	0.14[0.01–0.26]	0.01[-0.09–0.46]	0.23[0.01–0.37]	0.23[-0.01–0.49]	0.310	0.394	0.536
range	0.08–0.18	0.06–0.27	0.10–0.23	0.14–0.34			

Data expressed as Median [95% Confidence Interval]. Legend: without HOCbl – culture medium without hydroxocobalamin; with HOCbl – culture medium with hydroxocobalamin; GD – Gaucher disease. p – statistical significance. **Mann-Whitney test (factors: group – healthy human and GD patients, cobalamin supplementation) or Kruskal-Wallis test with Dunn *post hoc* (factors: group – healthy human and GD; cobalamin supplementation; interaction) for independent samples. Significance set at 5% for all analysis. *GD types I (n=1); II (n=1) and III (n=1).

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7.4 CAPÍTULO 4 – Artigo 4

Título do artigo: "Evaluation of B_{12} vitamin status in Gaucher disease type 1 patients"

Autores: Suelen Porto Basgalupp, Luciana Rosès Rizzon, Marina Siebert, Filippo Pinto e Vairo, Sidney Behringer, Ute Spiekerkoetter, Luciana Hannibal, Ida Vanessa D. Schwartz.

Situação: Em elaboração

Evaluation of B_{12} vitamin status in Gaucher disease type 1 patients

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Abstract

Gaucher disease (GD), one of the most prevalent lysosomal storage disorders, is caused by pathogenic mutations in the *GBA1* gene that encodes the enzyme glucocerebrosidase (GCase). GD manifests with considerable phenotypic variation and it is characterized mainly by hematological, visceral, skeletal and neurological manifestations. Abnormal systemic markers of cobalamin (Cbl, B_{12}) metabolism have been reported in GD patients, suggesting impairments in lysosomal handling of Cbl or its downstream processing and trafficking events. **Aim**: To investigate the B_{12} status in GD patients. **Methodology:** Total B_{12}, holo-transcobalamin (holo-TC), total homocysteine (tHcy) and methylmalonic acid (MMA), were prospectively measured in 52 plasma samples (pre-treatment: n=16; post-treatment: n=36) from 10 GD type I patients (male=5; averages of plasma measured per patient were 1.6 and 4.5 in pre-treatment and post-treatment samples, respectively) followed by a Reference Center for GD from Southern Brazil. **Results:** No patient presented low levels of total-B_{12}. In pre-treatment samples, one patient (pt #10) showed high levels of B_{12} and holo-TC with elevated MMA levels and tHcy level within the superior limit of normality. Two patients (pts #3 and #9) had low holo-TC levels, normal values for MMA. Pt #3 presented tHcy level within the reference range, whereas the pt #9 showed low tHcy levels. For tHcy measurement, one patient had high level (pt #1) and two patients presented low tHcy levels (pts #8 and #9). Only one patient (pt #10) had MMA value outside of reference range. Comparing pre and post-treatment samples, one patient (pt #3) showed decreased levels of holo-TC under treatment, whereas this patient had a low holo-TC levels before treatment and presented levels of B_{12} relatively low compared to other patients. The patient #8 had a decreased level of holo-TC and the value was lower than reference range. Three subjects (pts #3, #5 and #8) presented decreased holo-TC levels under GD treatment. **Conclusions:** The results showed that Brazilian GD patients present normal levels of B_{12}, in contrast with

previous studies that demonstrated a high prevalence of low B_{12} in untreated patients with GD from the Ashkenazi Jewish population. Furthermore, no patient presented biomarker levels related to functional deficiency of B_{12}. Therefore, glucocerebroside accumulation in lysosomes seems does not affect the processing and trafficking of B_{12} and it does not cause functional impairment of B_{12} in patients with GD type I.

Keywords: Gaucher disease, vitamin B_{12}, cobalamin, methylmalonic acid, homocysteine, transcobalamin.

Introduction

Gaucher disease (GD) is a rare autosomal recessive disorder caused by deficient activity of glucocerebrosidase (GCase) enzyme due to biallelic pathogenic mutations in the *GBA1* gene (OMIM 606463), located on chromosome 1 (1q21). This enzyme hydrolyzes glucosylceramide (GlcCer) into ceramide and glucose and its deficiency leads to the accumulation of the GlcCer substrate in macrophages, inducing their transformation into Gaucher cells, which mainly infiltrate bone marrow, liver and spleen (STIRNEMANN et al., 2017). The disease's frequency is around 1/40,000 to 1/60,000 individuals in the general population, but it can reach 1 in 800 people in the Ashkenazi Jewish population (GRABOWSKI, 2013; STIRNEMANN et al., 2017). There are three types of GD, based on the absence (type I) or presence and severity (types II and III) of involvement of central nervous system (CNS), being type I the most common, reaching around 90% of cases (SIDRANSKY, 2012). The diagnosis of GD is performed by measuring of the GCase activity in leukocytes and/or fibroblasts. Genetic analysis is also realized to identify the biallelic pathogenic mutations in the *GBA1* gene. Two primary forms of treatment for GD are enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) (BENNETT; MOHAN, 2013).

GD is a monogenic disease, but it presents a broad phenotypic variability (HRUSKA et al., 2008; SIDRANSKY, 2004). Despite having been identified around 500 mutations in the *GBA1* gene (http://www.hgmd.cf.ac.uk, December 2018; GRABOWSKI, 2013) and, although some genotypes are often associated with mild or severe symptoms, the genotype-phenotype correlation is still not well explained (GOKER-ALPAN et al., 2005; KOPRIVICA et al., 2000). Thus, unraveling the factors contributing to the heterogeneity in this single gene disorder may have a direct impact on studies of the pathophysiology and therapeutic options available for GD, and also very relevant to other diseases. Therefore, genetically unrelated

disease modifiers can be actively contributing to modify the phenotype presented by GD patients (EL-MORSY et al., 2011; HRUSKA et al., 2008; HUANG; ZHANG; CHEN, 2015; SIDRANSKY, 2004).

Some clinical manifestations of GD are observed in individuals with vitamin B_{12} deficiency, mainly concerning neurological involvement, suggesting that there is a mechanism of pathogenesis common to these diseases. Although genotype may play a role in determining the degree of neurological involvement, the mechanisms by which a defined genotype leads to a particular phenotype still remain unknown. One hypothesis is that lysosomal dysfunction in GD leads to the functional deficiency of vitamin B_{12} due to a failure in some of the processes, such as absorption, intralysosomal degradation of transcobalamin, or transport of B_{12} to the cytoplasm, compromising the biochemical reactions in which B_{12} acts as a cofactor (HANNIBAL et al., 2017).

Abnormalities in systemic markers of B_{12} status have been described in GD patients, raising the question of whether cellular handling of this micronutrient is sensitive to impaired lysosomal storage (HANNIBAL et al., 2017). Furthermore, it is also reported that plasma B_{12} levels are decreased in GD, holo-TC levels are elevated, and that patients with other neurodegenerative conditions, such as Alzheimer's disease, exhibit abnormalities involving lysosomal, endocytic and cellular B_{12} pathways (ZHAO et al., 2014).

B_{12} deficiency inactivates the two B_{12}-dependent enzymes methionine synthase (MS) and methylmalonyl-CoA mutase (MCM), which results in elevation of their substrates, Hcy and MMA, respectively (SMITH; WARREN; REFSUM, 2018). Thus, tHcy and MMA are direct biomarkers of cellular B_{12} status. It is currently unknown whether the abnormal accumulation of GlcCer may affect B_{12} transit in the lysosome (HANNIBAL et al., 2017). Herein, we analyzed functional (tHcy and MMA) and direct (total B_{12} and holo-TC) markers of B_{12} status in plasma samples from GD patients.

Methods

Study design

A retrospective study investigated the prevalence of low B_{12} and high tHcy levels in a cohort of Brazilian patients with GD. The results showed that 14% (6/42) of GD patients had a reduction of B_{12} levels in at least one measured analyzed. Herein, we evaluated 10 GD patients which were selected to investigate the B_{12} deficiency using the biomarkers of B_{12} status (Figure 1).

Subjects

Fifty-two plasma samples (pre-treatment: n=16; post-treatment: n=36) from 10 patients with GD type I (male= 5) followed by a Reference Center for GD from Southern Brazil were included in this study (Table 1). No patient had Ashkenazi Jewish ascendency, and all patients deny vegetarian and vegan habits. The diagnosis of GD was established by measurement of GCase activity in leukocytes and by Sanger sequencing of the *GBA1* gene. Plasma samples were obtained from peripheral venous blood samples collected in tubes with heparin, centrifuged for 5 min at 3000 rpm. Clinical variables such as age, treatment history, genotype, and symptoms were obtained by a review of medical records.

Ethics Statement

This study was approved by the ethics committee of Hospital de Clínicas de Porto Alegre (HCPA/no 17-0094), and all patients and/or their legal guardians provided written informed consent.

Analysis of plasma tHcy by LC-ESI-MS/MS

tHcy plasma concentration was measured by high-performance liquid chromatography (HPLC). Briefly, 3.16 μL of plasma sample was pipetted into Eppendorf tube, followed by 20

μL of the internal standard solution, 20 μL of 0.5 M DDT solution and 100 μL of methanol with 0.1% formic acid. After vortexing and incubation for 20 min at room temperature, the samples were centrifuged at 10,000 g for 5 min. Then, the supernatant was transferred to HPLC vials, and 10 μL of the sample was injected into the equipment. Two commercial controls with known amounts of aminothiols were used as quality control (MCA Laboratory, Queen Beatrix Hospital in 7101 BN Winterswijk, The Netherlands). To determine the concentration of aminothiols, calibrators were prepared by preparing a master mix containing 100 μL of 0.5 M DDT, 100 μL of Hcy (998.6 μM), 150 μL of Met (998.5 μM), 300 μL of Cys (1007 μM), 150 μL of Met sulfoxide (1000 μM) and 200 μL of H_2O (Calibrator 1). Then, a serial dilution was performed by pipetting 200 μL of Calibrator 1 into an Eppendorf containing 200 μL H_2O (Calibrator 2), up to calibrator 7. After vortexing, 3.16 μL of each calibrator was pipetted into HPLC vials, and 20 μL of internal standard solution was added to each vial followed by 20 μL of 0.5 M DDT solution and 100 μL of methanol with 0.1% formic acid. Lastly, after vortexing again, 10 μL of the sample was injected into the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) machine (QTrap 6500+, Sciex).

Analysis of plasma MMA levels by LC-ESI-MS/MS

MMA was determined based on a previously published method with some modifications (BLOM; VAN ROOIJ; HOGEVEEN, 2007). Briefly, 100 μL of plasma sample was pipetted into a clean microcentrifuge filter (Amicon, 30 kDa MW cut-off, Merck Millipore) tube and 100 μL of 0.8 μM D_3-MMA (CDN isotopes) Internal Standard solution was added to each tube. After vortexing and centrifugation at 14,000 g for 30 min at 10°C, 100 μL of the filtrate was transferred into HPLC vials and acidified with 10 μL of 4% formic acid. Then, 10 μL of the sample was injected into the LC-ESI-MS/MS system (QTrap 6500+, Sciex). Two

commercial controls with known amounts of MMA were used as quality control (MCA Laboratory, Queen Beatrix Hospital in 7101 BN Winterswijk, The Netherlands). It was performed a calibration curve to determine the concentration of MMA by preparing a mix containing 100 μL of MMA standard (0.1, 0.25, 0.5, 0.75, and 1.0 μM) and 100 μL of 0.8 μM D_3-MMA internal standard solution. After vortexing, 100 μL of each calibrator was transferred into HPLC vials and acidified with 10 μL of 4% formic acid. Then, 10 μL of each sample was injected into the LC-ESI-MS/MS system. Stock solutions of MMA (Sigma Aldrich) and D_3-MMA were prepared in deionized H_2O and kept at – 20°C.

Determination of total plasma vitamin B_{12} and holo-TC

Total plasma vitamin B_{12} was determined using the standardized chemiluminescent immunoassay (CLIA). The reference range for normal B_{12} concentration using this methodology is 197-771 pg/mL. Plasma holo-TC was measured using a standardized immunoassay (EIT). The reference range for normal holo-TC level was > 50 pmol/L. Both measurements were performed by MVZ Clotten Labor Dr. Hass, Dr. Raif & Kollegen GbR, Freiburg, Germany.

Results

Biomarkers of vitamin B_{12} status in pre-treatment samples from GD patients

Of the 10 patients with GD type I, five were male. The mean age at diagnosis of GD was 40.6 years, ranged from 1 to 62 years. All GD patients included in this study had at least one sample collected pre-treatment sample. The characterization of plasma samples is represented in Table 2. Before treatment, patients had a mean tHcy level of 8.6 μmol/L (95% CI = 7.3-10.2), a mean MMA level of 0.09 μmol/L (95% CI = 0.08-0.12), a mean B_{12} level of 772.2 pg/mL (95% CI = 491.3-1213.7) and a mean holo-TC level of 61.6 pmol/L (95% CI = 48-79).

No GD patient presented low levels of total-B_{12}. The total B_{12} and holo-TC measurements were not available from all patients. One patient (pt #10) showed high levels of B_{12}, holo-TC, MMA and tHcy within the superior limit of normality. Two patients (pts #3 and #9) had low holo-TC with normal values for MMA biomarker and the pt #3 presented tHcy within the reference range, whereas the pt #9 showed low tHcy levels. For tHcy measurement, one patient had it high (pt #1) and two patients presented it low (pts #8 and #9). For patients #1 and #5 B_{12} and holo-TC values were not available, therefore, it was not possible to evaluate functional deficiency of B_{12} in these two GD patients. Only one patient (pt #10) had MMA outside reference range.

Biomarkers of vitamin B_{12} status in the post-treatment follow-up of GD patients

Comparing pre and post-treatment samples, one patient (pt #3) showed decreased levels of holo-TC under treatment, whereas this patient had a low holo-TC levels before treatment and presented levels of B_{12} relatively low compared to other patients. This patient had B_{12} measured only in pre-treatment sample. The patient #8 had a decreased level of holo-TC and the value was lower than reference range.

Regarding B_{12} measurement, one patient (#4) showed a decreased in B_{12} levels under ERT. Two patients (#1 and #5) had B_{12} and holo-TC measured only in post-treatment samples. The patient #5 had a low holo-TC level under treatment with B_{12} of 351 pg/mL and presented values of tHcy and MMA biomarkers within the reference range. One patient (#2) presented increased levels of B_{12} at follow-up under ERT. Two related patients (#7 and #8) presented B_{12} measurement in pre and post-treatment samples and both showed increased levels of B_{12} under therapy. The patient #8 had a low holo-TC level after treatment, it was outside the reference range, and showed an increase of tHcy level after treatment. The holo-

TC levels showed an decreased in 3 patients (#3, #6 and #8) and increased levels in two patients (#4 and #7).

Discussion

This study is the first to evaluate the relationship between direct (B_{12} and holo-TC) and functional (tHcy and MMA) biomarkers of B_{12} status in patients with GD. Previous studies reported high prevalence of low B_{12} levels in GD (GIELCHINSKY et al., 2001), high levels of holo-TC (GILBERT et al., 1976), and abnormalities involving the lysosomal, endocytic and cellular B_{12} pathways in patients with Alzheimer's disease (ZHAO et al., 2014). Whereas it is unknown whether the abnormal accumulation of GlcCer may affect B_{12} transit in and out of the lysosome, this study investigated if the lysosome, which is the central cellular compartment in both GD and B_{12} metabolism, is dysfunctional when occurring the accumulation of the substrate, leading to functional deficiency of B_{12}.

No patient presented low levels of total B_{12}. Vitamin B_{12} deficiency is caused by either inadequate intake, inadequate bioavailability or malabsorption. Disruption of B_{12} transport in the blood, or impaired cellular uptake or metabolism causes an intracellular deficiency and may cause unspecific irreversible neurological symptoms which necessitate the early detection and management. There is no single test to diagnose vitamin B_{12} deficiency. The distribution of serum concentrations of total vitamin B_{12} overlaps between deficient and non-deficient individuals. Diagnostic biomarkers for B_{12} status include decreased levels of circulating total B_{12} and holo-TC, and abnormally increased levels of tHcy and MMA. However, the exact cut-offs to classify clinical and subclinical deficiency remain debated (GREEN, 2017).

MMA and tHcy accumulate when concentrations of B_{12} are inadequate; tHcy also accumulates with inadequate amounts of folate. These functional biomarkers are useful for

identifying subclinical B_{12} status and reflect early changes in B_{12} status. Some factors may affect the functional biomarkers. Impaired renal functions, genetic variation, disease conditions, pregnancy may alters MMA and tHcy concentrations (YETLEY et al., 2011). One advantage of using MMA as a functional biomarker of B_{12} deficiency is that this metabolite is specific for B_{12} metabolism pathway, whereas tHcy acts in routes other than vitamin B_{12}, such as trans-sulfuration and folate (HANNIBAL et al., 2016; VASHI, 2016) . It is recognized that tHcy levels may be affected by different factors, such as age (BRATTSTRÖM et al., 1994), smoking status (BAZZANO et al., 2003), body mass index (BMI) (ZHU et al., 2006), diabetes mellitus (HULTBERG et al., 1991; MUNSHI et al., 1996), kidney function (DENNIS; ROBINSON, 1996) and serum concentrations of vitamin B_{12} and folate (UBBINK et al., 1993).

Several studies have showed that holo-TC is a more sensitive marker than total B_{12} for investigating B_{12} status (NEXO; HOFFMANN-LÜCKE, 2011; PALACIOS et al., 2013; WOO et al., 2010; YETLEY et al., 2011). About 50% of patients with subclinical B_{12} deficiency have values of this micronutrient within the reference range. When B_{12} concentrations present normal values, the functional biomarkers, tHcy and MMA will be elevated in asymptomatic subjects (VASHI et al., 2016). Therefore, it is essential to measure additional biomarkers to investigate and establish the B_{12} deficiency. The National Health and Nutrition Examination Survey (NHANES) suggests the measurement at least one biomarker of circulating concentrations of B12 (B12 or holo-TC) and one biomarker of functional B_{12} status (tHcy or MMA) (YETLEY et al., 2011). Many laboratories use a diagnostic strategy that involves more than one biomarker, most often using B_{12} levels as the initial test and MMA or tHcy levels as the second line test. The holo-TC measurement is expansive and it not always available worldwide. Recently, this approach has been further improved by the development of an equation that includes two to four biomarkers (GREEN, 2017).

In the present study, one patient had high B_{12} and holo-TC levels with elevated levels of MMA. This patient presented severe hepatic impairment due to cirrhosis. Several liver diseases like acute hepatitis, cirrhosis, hepatocellular carcinoma and metastatic liver disease can also be accompanied by an increase in circulating B_{12}. This phenomenon is predominantly caused by B_{12} release during hepatic cytolysis and/or decreased B_{12} clearance by the affected liver. Altogether it can be concluded that an observed elevation of B_{12} in blood merits the a full diagnostic work up to assess the presence of disease. The paradox of high plasma B_{12} and elevated MMA has actually been described in patients with cirrhosis and chronic myelocytic leukemia (CML) (ERMENS; VLASVELD; LINDEMANS, 2003).

Gilbert et al. (1976) suggested that the elevated circulating TCII levels may be a complementary test for the confirmation of GD. These researchers noted that TCII levels are significantly higher in splenectomized GD patients suggesting that TCII measurement can be established in following the clinical course of Gaucher´s disease, whereas splenectomized patients present more severity of the disease. Cases of Gaucher disease, systemic lupus, rheumatoid arthritis and Still's disease with high serum B_{12} have also been reported. High serum B_{12} in dysimmune and inflammatory diseases may be linked to an increase in TCB II during the acute phase of inflammation (ANDRÈS et al., 2013).

Elevated levels of TCII have been identified in patients with acute myelogenous leukemia, in CML and refractory anemia (ZITTOUN et al., 1975). Some studies confirmed this TCII elevation in acute leukemia and lymphoma in your active stage (RACHMILEWITZ et al., 1972). These researchers suggested that TCII levels may be a marker of acute cell proliferation and that the reticuloendothelial system can play a part in TCII metabolism (GILBERT; WEINREB, 1976).

The results showed decreased levels of holo-TC in two GD patients. One of them had B_{12} level of 296 pg/mL, which is considered low level according some laboratories. The tHcy and MMA levels were within the reference value. This patient may receive cobalamin supplementation to prevent further reduction of B_{12} levels. This finding highlights the importance of monitoring these patients regarding the potential use of cobalamin as supplementation.

Conclusion

The results suggested that Brazilian GD patients present normal levels of B_{12}, in contrast with previous studies that demonstrated a high prevalence of low B_{12} in untreated patients with GD from the Ashkenazi Jewish population. Furthermore, no patient presented biomarker levels related with functional deficiency of B_{12}. Therefore, glucocerebroside accumulation in lysosomes seems does not affect the processing and trafficking of B_{12}, and it does not cause functional impairment of B_{12} in patients with GD type I. This study highlights the importance of monitoring the GD patients regarding the early B_{12} deficiency diagnosis and the potential use of cobalamin as supplementation.

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Competing interests

The authors declare that they have no competing interests.

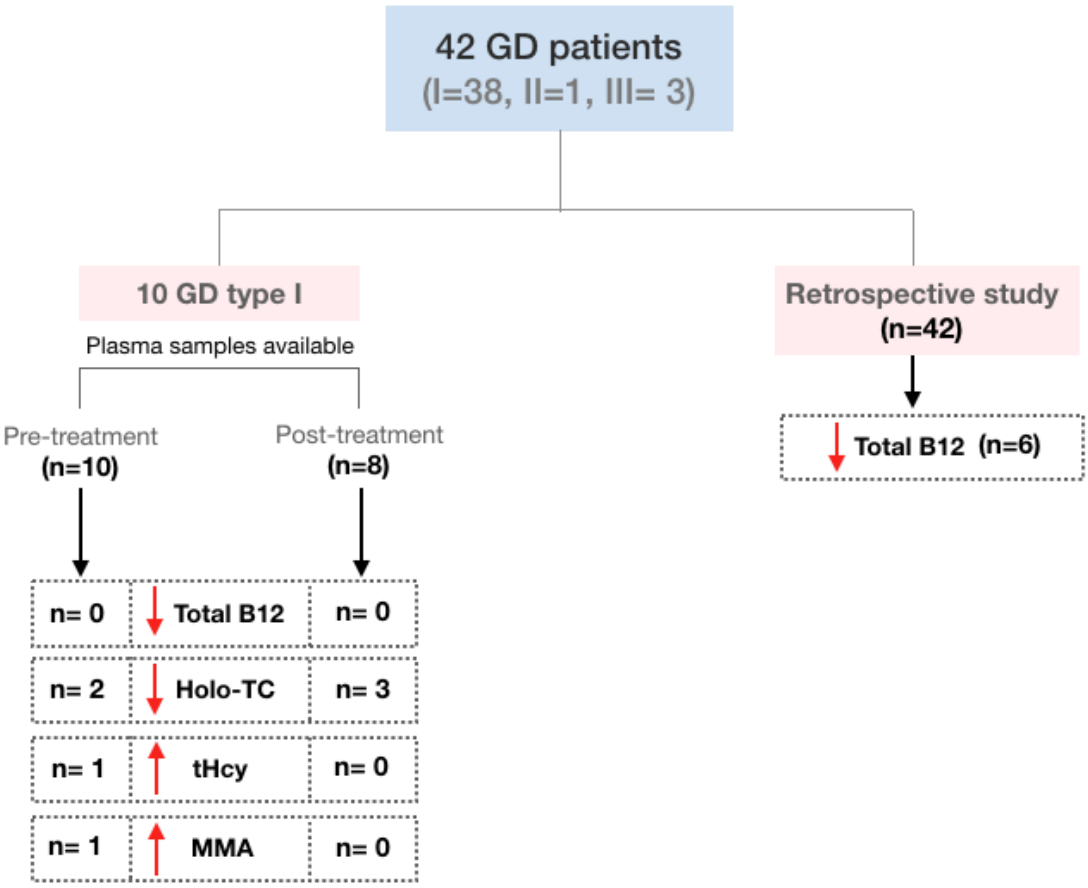

Figure 1. Schematic representation of study design. The retrospective study was performed by review of medical records and it presented the total B_{12} measurement. The present study analyzed the direct (total B_{12} and holo-TC) and functional (tHcy and MMA) biomarkers of B_{12} status in 10 GD type I patients.

Table 1. Characterization of 10 GD type I patients included in this study.

Patient	Gender	Age (y)*	Genotype	Treatment	Dose	B_{12} [pg/mL]		Holo-TC [pmol/L]		tHcy [µmol/L]		MMA [µmol/L]	
						0	1	0	1	0	1	0	1
1**	M	49/52	N370S/Rec*Nci*I	Imiglucerase	15 UI/Kg	N/A	641	N/A	116	**19.8**	11.8	0.06	0.11
2**	F	57/57	N370S/Rec*Nci*I	Imiglucerase	15 UI/Kg	642	720	N/A	60.1	8.5	8.4	0.09	0.10
3**	M	44/47	N370S/Rec*Nci*I	Imiglucerase	20 UI/Kg	296	N/A	**39.9**	**38.7**	11.1	11.7	0.19	0.17
4	M	43/44	N370S/L444P	Imiglucerase	15 UI/Kg	399	299	86.3	**128**	7	8	0.06	0.14
5	F	35/36	N370S/Rec*Nci*I	Imiglucerase	15 UI/Kg	N/A	351	N/A	**32**	10.9	10.9	0.03	0.07
6	M	29/32	N370S/L444P	Taliglucerase	20 UI/Kg	N/A	578	119	91.9	7.4	8.0	0.21	0.07
7**	F	46/47	E349K/S366N	Miglustat	3 pills/day	436	470.4	61.7	102.8	8.1	7.0	0.09	0.15
8**	F	42/43	E349K/S366N	Imiglucerase*	30 UI/Kg	407	453	55.1	**44.5**	**4.5**	5.8	0.08	0.04
9	F	4	N370S/G202R	N/A	N/A	616.4	N/A	**47.9**	N/A	**4.4**	N/A	0.06	N/A
10	M	62	N370S/Rec*Nci*I	N/A	N/A	**2,000**	N/A	**128**	N/A	13.3	N/A	**0.30**	N/A

y, years; * Age of patient at first plasma sample collection (before and after treatment); 0, pre-treatment sample; 1, post-treatment sample; ** Related patients (1, 2 and 3; 7 and 8); UI/Kg, units per kilogram per infusion; N/A, not applicable; *Patient 9 was treated with imiglucerase, taliglucerase and miglustat; B_{12}, vitamin B_{12}; Holo-TC, holo-transcobalamin; tHcy, total homocysteine; MMA, methylmalonic acid. *Reference values of: tHcy: 5–15 µmol/L (Skovby, 2003); MMA: 0-0.26 µmol/L (Clarke, 2011); Holo-TC: > 50 pmol/L and B_{12}: 197-771 pg/mL (MVZ Clotten Labor Dr. Hass, Dr. Raif & Kollegen GbR, Freiburg, Germany); Bold type indicates values outside of reference range.

Table 2. Characterization of the 52 samples from GD type I patients included in the present study.

Variables	Plasma samples (n=52)	
	Pre-treatment	Post-treatment
Patients (n)	10	8
Plasma samples	16	36
Male:Female	5:5	4:4
ERT (n)		
Imiglucerase	N/A	6
Velaglucerase	N/A	0
Taliglucerase	N/A	2
SRT (n)		
Miglustat	N/A	2
Use of vitamin B_{12} (n)	0	0
B_{12} [pg/mL]	772.2	528.3
Holo-TC [pmol/L]	61.6	70.4
tHcy [µmol/L]	8.6	8.5
MMA [µmol/L]	0.09	0.11

GD, Gaucher disease; n, number of patients; F, female; M, male; ERT, enzyme replacement therapy; SRT, substrate reduction therapy; N/A, not applicable; tHcy, total homocysteine; MMA, methylmalonic acid; B_{12}, vitamin B_{12}; Holo-TC, holo-transcobalamin; *Reference values of: tHcy: 5–15 µmol/L (Skovby, 2003); MMA: 0-0.26 µmol/L (Clarke, 2011); Holo-TC: > 50 pmol/L and B_{12}: 197-771 pg/mL (MVZ Clotten Labor Dr. Hass, Dr. Raif & Kollegen GbR, Freiburg, Germany).

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7.5 CAPÍTULO 5 – Artigo 5

Título do artigo: "Is holo-TC elevated in Gaucher disease type II? A case report in a Brazilian patient"

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Situação: Em elaboração

Is holo-TC elevated in Gaucher disease type II? A case report in a Brazilian patient

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Abstract

Gaucher disease (GD), one of the most common lysosomal disorders, is caused by deficient activity of the β-glucocerebrosidase enzyme. Based on the absence or presence and extent of neurological complications it has been divided into three types. The primary clinical manifestations are hepatosplenomegaly, thrombocytopenia, anemia and bone involvement. Herein, we report a case of GD type II who was admitted to the hospital presenting with jaundice and hepatosplenomegaly at 60 days of life. This patient showed high levels of vitamin B_{12} and holo-transcobalamin (holo-TC). This study suggests that the vitamin B_{12} metabolism may be impaired in neuronopathic forms of GD.

Introduction

Gaucher disease (GD) is an autosomal recessive disorder caused by deficient activity of lysosomal enzyme β-glucocerebrosidase (GCase) due to biallelic pathogenic mutations in the *GBA1* gene. Enzyme deficiency leads to the accumulation of non-degraded glucocerebroside substrate in tissues resulting in the formation of Gaucher cells, which will harm the regular function of specific organs (SIDRANSKY, 2004). This disorder is divided into three types, based on the absence (type I) or presence and severity (types II and III) of involvement of the central nervous system (CNS).

GD type I corresponds to more than 90% of GD cases in the world. It is characterized by high variability in the progression and severity of the disease, and the most common clinical manifestations are hepatosplenomegaly, anemia, thrombocytopenia, bone and lung problems (ZIMRAN et al., 2018). GD type II is the rarest type and is characterized by early onset of systemic clinical signs and involvement of the CNS, resulting in death within the first two years of life due to the rapid progression of the disease (SIDRANSKY, 2004; STIRNEMANN et al., 2017). In this type of DG, compromise of the brain, liver, spleen, and

lungs occurs, with pulmonary failure (MIGNOT et al., 2006). Among the primary clinical features of type II are developmental delay, ocular problems such as strabismus, vertical gaze paralysis, opisthotonus, spasticity, fetal hydrops, congenital ichthyosis (GOKER-ALPAN et al., 2003). DG type III corresponds to the intermediate form of GD, since it compromises CNS functions more slowly and gradually compared to DG type II (SIDRANSKY, 2012). This form includes patients with systemic involvement and neurological impairment that may manifest over time, usually accompanied by epilepsy, ataxia, vertical gaze paralysis, and dementia. Also, patients with GD type III may present with valvular heart disease (HRUSKA et al., 2008).

Patients with GD are usually treated with enzyme replacement therapy (ERT) or substrate reduction therapy (SRT), but the treatment is not always indicated for patients with GD type II due to the severity of the disease and limited prognosis (BEUTLER, 2006). Vitamin B_{12} (cobalamin, Cbl, B_{12}) levels can be decreased in untreated GD type I, although there are no reports on B_{12} levels in GD types II and III (GIELCHINSKY et al., 2001).

Abnormalities in biomarkers of B_{12} status have been described in GD patients, raising the question of whether cellular handling of this micronutrient is sensitive to impaired lysosomal storage (HANNIBAL et al., 2017). Previous studies reported that plasma B_{12} levels are decreased in GD, holo-transcobalamin (holo-TC) levels are elevated, and that patients with other neurodegenerative diseases exhibit abnormalities involving the lysosomal, endocytic and cellular B_{12} pathways (ZHAO et al., 2014). B_{12} deficiency can be caused by nutritional deficiency or defects that disrupt B_{12} absorption, processing and trafficking pathways and it may lead to the accumulation of total homocysteine (tHcy) and methylmalonic acid (MMA) (HANNIBAL et al., 2017). In the past, high B_{12} levels were identified in a GD type II patient followed by our group. However, the investigation of B_{12}

profile was incomplete. We report herein on a new case of GD type II presenting an increase of B_{12}.

Case report

A 2-month-old male first born to a non-consanguineous couple, with no history of perinatal abnormalities, presented jaundice at 10 days of age. He was admitted to the Hospital de Clínicas de Porto Alegre (HCPA) for clinical investigation. The maternal and paternal ages were 26 and 27, respectively. He was vaginally delivered at 39 weeks and 5 days of gestation without any complication. Maternal prenatal were low risk, without intercurrences, ultrasound and other tests were normal. The mother denied previous or gestational diseases, use of medications, alcohol, and cigarette during pregnancy. Apgar score was 6 at the first minute and 8 at fifth minute. Body weight was 2460 g, length was 47 cm, and head circumference was 32 cm. The patient was discharged from the hospital at 2 days of age.

On admission, cholestasis and ascites were confirmed, septicemia and thrombocytopenia were suggesting. Diagnosis of pulmonary stenosis was performed. The ophthalmologic evaluation was unremarkable. At 53 days of age, the newborn presented a new episode of sepsis. Newborn screening blood spot test showed increased galactose 13.6 mg/dL (RV up to 10 mg/dL). At 60 days of age, the physical examination was performed at HCPA and showed jaundice, opisthotonos to manipulation and he did not follow stimuli with the eye, body weight, length and head circumference were 3340 g, 48.5 cm, and 34.5 cm, respectively. The abdomen was distended, and he presented ichthyosis skin. Liver and spleen were palpable in the iliac fossa.

The previous tests (initial laboratory examination) showed platelets of 41 $10^3/\mu L$ (RV=210-650 $10^3/\mu L$), total bilirubin of 20.5 mg/dL (RV=up to 1 mg/dL), direct bilirubin of 15.67 mg/dL (RV=up to 0.3 mg/dL), aspartate transaminase (AST) of 483 U/L (RV=15-40

U/L) and alanine transaminase (ALT) of 234 U/L (RV=10-40 U/L). The karyotyping was performed, and the result was 46, XY. The abdominal ultrasound showed hepatosplenomegaly. The chitotriosidase and β-glucosidase activity in leukocytes were performed at 36 days of age, and the results were chitotriosidase 4987 nmol/h/mL of protein (RV=8.8-132 nmol/h/mL) and GCase activity 0.65 nmol/h/mg of protein (RV=10-45 nmol/h/mg of protein). The examination for infectious etiologies was negative, including cytomegalovirus and rubella.

A new echocardiogram was performed in HCPA and showed normal results. An abdominal ultrasound displayed a sequelae portal vein thrombosis with portal hypertension. The levels of ferritin, total homocysteine and vitamin B_{12} were 4175 ng/mL (RV=30-400 ng/mL), 8.3 μmol/L (RV=5-15 μmol/L), and 1459 pg/mL (RV=211-946 pg/mL), respectively. Analysis of *GBA1* gene was performed and showed the L444P+E326K/Rec*Nci*I genotype. The clinical and laboratory tests confirmed the diagnosis of GD type II.

The patient showed abnormal liver function enzymes, prolonged prothrombin time activity (48.3%; RV=more than 70%), and high levels of vitamin B_{12}. The level of methylmalonic acid (MMA) was normal (0.18 μmol/L; RV=0.16-0.60 μmol/L). A second blood sample was analyzed, and high levels of B_{12} and of holo-transcobalamin (holo-TC; the bioactive form of vitamin B_{12} in circulation; >128 pmol/L; RV=35-50 pmol/L) were confirmed. The patient had never received vitamin supplementation, although he was fed with an infant formula at 27 days of age following the Nutrologist recommendation. A gene panel, including 16 genes involved in iron metabolism, identified 3 heterozygous non-pathogenic variants in genes associated to hemochromatosis type I, neuroferritinopathy and iron-refractory iron deficiency anemia (p.Ser65Cys in *HFE*, p.Gln4His in *FTL*, and p.Val736Ala in *TMPRSS6*). After discharge, he stayed at home for 10 days and died at 76 days of life.

Discussion and conclusions

GD type II, or acute neuronopathic form, is the most severe and progressive form of the disease, it presents with neurological involvement within first months of life, followed by death within the first years of life (STONE et al., 2000). Although newborns often appear healthy at birth, they frequently develop neurological impairment with a rapid decline in quality of health within the first years (WEISS et al., 2015). It comprises about 1% of all GD cases, and it is encountered in all ethnicities. Perinatal-lethal GD (PLGD) forms are exceptionally rare. While GD type II is generally associated with specific mutations in the *GBA1* gene, there is also significant genotypic heterogeneity and genotype-phenotype correlation is not entirely understood (EBLAN; GOKER-ALPAN; SIDRANSKY, 2005).

Bhutada et al. (2018) have reported a case of PLGD, which is a rarer variant of GD type II, presenting prenatally with thrombocytopenia, transfusion refractory severe anemia, and non-immune fetal hydrops. In this case report, the genetic analysis revealed that the fetus was homozygous for the Rec*Nci*I mutation, which comprises 3 variants [L444P (p.Leu483Pro; c.1448T>C) + A456P (p.Ala495Pro; c.1483G>C) + V460V (p.Val499=; c.1495G>C)], in the *GBA1* gene. Evaluation of *GBA1* in the parents' sample was performed, and the results showed that both were heterozygous for this pathogenic variant. GD patients homozygous for the Rec*Nci*I alteration usually have a severe phenotype (BHUTADA et al., 2018). This pathogenic variant occurs due to recombination events between *GBA1* and its highly homologous pseudogene located 16kb downstream, known as *GBAP* (TAYEBI et al., 2003). This recombinant events referred to as recombinant alleles, are relatively common and are often found in patients with GD type II (HRUSKA et al., 2008). For this reason, the genetic analysis can be challenging as several mutations originate from the pseudogene sequence, and it is essential to differentiate between *GBA1* and *GBAP* sequences. Then, Rec*Nci*I variant can be easily missed by testing on next-generation sequencing (NGS)

platforms for the GD.

In opposite to B_{12} deficiency, pathophysiology and clinical consequences of high B_{12} serum levels are not often reported. However, it is currently considered that an increase in plasma levels of B_{12} may be an indicator of the functional deficit with clinical consequences similar to those of B_{12} deficiency. High serum vitamin B_{12} can yield clinical signs indicating a functional and qualitative B_{12} deficiency. The majority causes of elevated B_{12} levels is related to quantitative anomalies of transcobalamins. Therefore, a better knowledge of these proteins, sites of production, distribution and physiological functions are vital to understand the pathophysiological mechanisms and etiological implications of high serum B_{12}. Some studies show that the primary etiologies of this condition are neoplasms, myeloproliferative blood disorders, liver diseases, kidney failure, autoimmune disease and bronchopulmonary dysplasia (ANDRÈS et al., 2013; CARMEL, 1975; DENEUVILLE et al., 2009).

Chiche et al. (2008) found a statistically significant association between high levels of B_{12} and the presence of a malignant blood disease, suggesting an investigation for possible blood disorder in cases of elevated B_{12} (CHICHE et al., 2008). Similarly, Deneuville et al. (2009) demonstrated a significant association between high levels of B_{12} and liver diseases (DENEUVILLE et al., 2009). Examples of other diseases, such as GD, systemic lupus, rheumatoid arthritis and Still's disease with elevated B_{12} levels have also been reported (CHICHE et al., 2008; KALYONCU et al., 2010; MOLAD et al., 1990). Furthermore, high levels of B_{12} in inflammatory disease may be linked to an increase in holo-TC during the acute phase of inflammation (SOLOMON, 2007). Holo-TC, the active fraction of serum vitamin B_{12}, is an essential protein in the delivery of B_{12} to cells and tissues and it is responsible for the transport around 20% of circulating cobalamins (HANNIBAL et al., 2016). Severe disorders are observed in congenital deficiencies in holo-TC, including developmental neuropsychiatric disorders, hematological disorders, and megaloblastic

anemia (ANDRÈS et al., 2013).

Hemophagocytic syndrome, which presents some clinical manifestations common to GD, is characterized by fever, lymphadenopathy, hepatosplenomegaly, cytopenia, high levels of ferritin, triglycerides, liver enzymes, and hemophagocytosis by activated macrophages (KALYONCU et al., 2010). Holo-TC is synthesized by different cells such as hepatocytes, fibroblasts, macrophage, and myeloblasts. Fehr et al. (1985) have shown that holo-TC is a marker for macrophage proliferation in malign and reactive macrophage proliferation (FEHR; DE VECCHI, 1985). In GD, the accumulation of glucocerebroside may be the trigger that initiates a systemic inflammatory reaction, characterized by macrophage activation (KACHER; FUTERMAN, 2006) and it can explain the results of B_{12} and holo-TC levels in our case report.

High level of ferritin is one of the hallmarks of GD, being considered a consequence of inflammation associated with the storage of glucocerebroside in macrophages. Although the high levels of B_{12} and holo-TC could be partially explained by the ingestion of infant formula, which contains relatively high amounts of vitamin B_{12} and may potentially mask a "mild" B_{12} deficiency, this case suggests B_{12} metabolism may be impaired in GD, especially in the neuropathic forms. Alternatively, increased B_{12} could be a marker of macrophage activation. The use of B_{12} and holo-TC as biomarkers of GD should be explored.

Conflict of interest

None of the authors have any conflicts of interest associated with this paper.

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8. CONSIDERAÇÕES FINAIS

As conclusões da tese serão apresentadas abaixo, de acordo com os objetivos específicos.

1. Identificar, por meio de MLPA, mutações no GBA1 em pacientes com DG que não possuíam o genótipo estabelecido;

A partir da realização da técnica de MLPA foi possível identificar uma deleção em uma paciente com DG que apresentava mutação identificada em apenas um alelo. Os resultados foram concordantes com as análises prévias realizadas por sequenciamento de Sanger. O método MLPA é uma excelente ferramenta diagnóstica que permite detectar deleções e inserções de determinadas regiões genômicas, podendo ser utilizado para diagnóstico genético de diversas doenças. Os resultados sugerem que, embora esse tipo de alteração não pareça ser tão frequente em pacientes com DG, essa técnica pode auxiliar no diagnóstico genético de DG, especialmente em casos de pacientes que apresentam genótipo incompleto. A técnica utilizada pode contribuir para melhor compreender alguns mecanismos envolvidos na DG e poderá ser implementada em laboratórios de diagnóstico genético, o que poderá aumentar a detecção de variantes que não foram identificadas por outras metodologias.

2. Avaliar a existência de um perfil alélico específico em pacientes com DG da região Sul e de outras regiões do Brasil;

A partir da análise das frequências alélicas de pacientes com DG de diferentes regiões do Brasil, o estudo sugere que existe um perfil alélico específico no *GBA1* diferente entre os pacientes da região Sul comparado aos pacientes de outras regiões do país. Os alelos N370S, Rec*Nci*I e L444P foram mais representativos na região Sul, totalizando mais de 75% dos alelos identificados, enquanto que os mesmos alelos representaram cerca de 50% do total nas demais regiões do Brasil. Essa diferença de perfil alélico entre as diferentes regiões pode ser decorrente do processo de imigração, visto que o Brasil é composto por uma população heterogênea, tais como Europeus, Africanos e Ameríndios. Portanto, os três alelos identificados que representam a maior fração do Sul do Brasil podem ser advindos de origem ancestral, visto que a maior parte dessa população é de origem Européia. Uma análise de

haplótipos em uma amostra de pacientes que represente todas as regiões do país poderá fornecer informações sobre a origem dos diferentes alelos, o que poderá definir as diferenças de perfis alélicos entre os pacientes brasileiros com DG.

3. Avaliar o metabolismo da vitamina B_{12} em células de pacientes com DG;

Os resultados obtidos sugerem que não existe uma deficiência funcional da vitamina B_{12} em pacientes com DG. Os níveis dos biomarcadores do *status* de B_{12} foram analisados e os dados mostraram valores dentro da normalidade, os quais não foram significativamente diferentes nas formas neuronopática e não-neuronopática da DG. Porém, embora o transporte e o processamento de B_{12} pareçam estar preservados nas células de pacientes com DG, é importante que os pacientes com DG que apresentam deficiência de B_{12} sejam monitorados e avaliados quanto ao uso de cobalamina. Novos estudos com uma coorte de pacientes com DG são extremamente necessários para confirmar esses achados, visto que o estudo foi realizado em linhagens celulares comerciais e o número de fibroblastos utilizados nesse estudo era reduzido.

4. Analisar biomarcadores do status de vitamina B_{12} em pacientes com DG;

A partir da análise de biomarcadores do *status* de vitamina B_{12} em amostras de plasma de pacientes com DG, o estudo mostrou que pacientes brasileiros com DG tipo I apresentam níveis normais de vitamina B_{12}, o que difere de um estudo prévio que demonstrou uma alta prevalência de níveis reduzidos de vitamina B_{12} em pacientes com DG da população judeus Ashkenazi. Além disso, nenhum paciente apresentou níveis de biomarcadores funcionais (tHcy e MMA) sugestivos de deficiência de B_{12}. Portanto, o acúmulo de glicocerebrosídeo nos lisossomos parece não afetar o processamento e o tráfego da vitamina B_{12}, não ocasionando uma deficiência funcional de B_{12} em pacientes com DG tipo I. O presente estudo ressalta a importância do monitoramento de pacientes com DG quanto à deficiência de B_{12} diagnosticada em estágio inicial e o uso potencial de cobalamina como suplementação.

9. PERSPECTIVAS FUTURAS

A DG é uma doença rara e, muitas vezes, acaba sendo sub-diagnosticada devido ao número restrito de médicos e profissionais da saúde que atuam na área da genética, mais especificamente em EIM. Visto que a DG apresenta uma heterogeneidade fenotípica, faz-se necessário investigar possíveis fatores modificadores do fenótipo da doença, os quais poderão identificar futuros alvos terapêuticos para a DG.

Apesar de a TRE ser uma excelente estratégia terapêutica para os pacientes com DG, muitos deles apresentam diferenças na resposta clínico-laboratorial aos medicamentos. Dessa forma, as intervenções médicas na DG devem ser aprimoradas por meio de um diagnóstico mais preciso, o que levará a uma melhor resposta ao tratamento. Além disso, o desenvolvimento de novas estratégias terapêuticas que possam auxiliar as terapias já existentes, representa uma maneira de aprimorar e otimizar o manejo desses pacientes.

Visando adequado diagnóstico dos pacientes com DG, para os casos em que não foram identificadas as mutações causadoras da doença, diferentes técnicas devem ser realizadas para definir o genótipo desses pacientes. Como perspectiva do trabalho realizado, a implementação da técnica de MLPA como rotina no laboratório de diagnóstico genético, poderá ser realizada proporcionando um aumento da taxa de detecção de variantes não identificadas por outras metodologias. Além disso, a técnica de MLPA poderá ser utilizada não só para o diagnóstico de DG, mas também para a investigação de outras doenças, inclusive aquelas em que as mutações mais comuns são do tipo deleção ou inserção.

A partir da análise do perfil das frequências alélicas de mutações no gene *GBA1* em pacientes com DG de diferentes regiões do Brasil, o próximo passo do estudo será avaliar a origem alélica nessas populações por meio da análise de haplótipos nesses pacientes provenientes de diferentes centros de referência para a DG.

O presente estudo avaliou o metabolismo celular da vitamina B_{12} em fibroblastos de pacientes com DG, os quais foram adquiridos comercialmente, sendo analisados somente um paciente de cada tipo de DG, bem como o mesmo número de indivíduos saudáveis. Para avaliar um efeito maior, a caracterização do metabolismo celular da B_{12} poderá ser realizada em fibroblastos de uma coorte de pacientes com DG, os quais serão mais representativos, além da vantagem de ter dados disponíveis sobre as manifestações clínicas desses pacientes. A análise dos biomarcadores de B_{12} ajudará a interpretar o *status* de B_{12} na coorte de pacientes com DG com maior acurácia e confiabilidade.

Uma outra abordagem a ser realizada é a investigação do metabolismo do ferro como fator modificador da DG. A hiperferritinemia tem sido, cada vez mais, associada à DG e ainda é discutido sobre a etiologia da ferritina na DG. Sabe-se que a ferritina é originada a partir da ativação de macrófagos. Porém, não se sabe se na DG a ferritina seria resultado de um processo inflamatório influenciado por citocinas, se seria motivada por sobrecarga de ferro nos macrófagos ou se seria de uma etiologia compartilhada entre esses dois fatores. Um estudo recente demonstrou que a hiperferritinemia observada em pacientes com DG tipo I está relacionada a um defeito no metabolismo do ferro e ao sequestro de ferro nas células Gaucher. Dessa forma, a análise de mutações em genes envolvidos em diferentes vias do metabolismo do ferro é uma estratégia promissora que poderá fornecer informações relevantes dos mecanismos envolvidos na DG.

10. ANEXOS

10.1 ANEXO I

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

COMISSÃO CIENTÍFICA

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

Projeto: 160101
Data da Versão do Projeto: 29/02/2016

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
FILIPPO PINTO VAIRO
SUELEN PORTO BASGALUPP
MARINA SIEBERT

Título: ANÁLISE MOLECULAR DO GENE GBA1 EM PACIENTES BRASILEIROS COM DOENÇA DE GAUCHER

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

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

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

Porto Alegre, 09 de junho de 2016.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

10.2 ANEXO II

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nº do projeto GPPG ou CAAE_____

Título do Projeto: **ANÁLISE MOLECULAR DO GENE *GBA1* EM PACIENTES BRASILEIROS COM DOENÇA DE GAUCHER**

Você está sendo convidado a participar de uma pesquisa cujo objetivo é identificar a presença de variantes na estrutura do gene *GBA1*, o qual codifica a enzima glicocerebrosidase que está deficiente em pacientes com doença de Gaucher (DG) devido a mutações nesse gene. Alguns estudos indicam que podem ocorrer diferenças nos sintomas clínicos apresentados pelos pacientes com a mesma forma da doença. Existem diferentes fatores envolvidos na manifestação de diferentes características clínicas da doença e um deles pode ser a presença de diferentes variações no gene envolvido na DG. Os achados do presente estudo serão correlacionados com as manifestações clínicas dos pacientes com DG, o que poderá contribuir para um melhor entendimento de como a doença está progredindo, por que os pacientes apresentam sintomas diferentes e o motivo pelo qual existem diferentes respostas ao tratamento entre os pacientes. Esta pesquisa está sendo realizada pelo laboratório B.R.A.I.N (*Basic Research and Advanced Investigations in Neurosciences*) do Hospital de Clínicas de Porto Alegre (HCPA).

Se você aceitar participar da pesquisa, os procedimentos envolvidos em sua participação são os seguintes: Coletar 5 mL de sangue para análise molecular do gene *GBA1*; consultar prontuários para buscar dados clínicos e laboratoriais. Se você permitir, o material coletado que restar após a realização da análise molecular deste estudo será armazenado e utilizado em estudos futuros, desde que você consinta novamente. Em relação ao armazenamento do material biológico, você declara que:

( ) Autorizo o armazenamento do material biológico que restar após a realização da análise molecular do gene *GBA1*;

( ) Não autorizo o armazenamento do material biológico que restar após a realização da análise molecular do gene *GBA1*.

Os possíveis riscos ou desconfortos decorrentes da participação na pesquisa são semelhantes aos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). O desconforto e os riscos associados à coleta de sangue serão minimizados pela sua realização por profissional treinado.

Os possíveis benefícios decorrentes da participação na pesquisa são indiretos, ou seja, a participação na pesquisa não lhe trará benefícios diretos, porém, contribuirá para um melhor entendimento da doença, o que futuramente poderá levar ao desenvolvimento de uma terapia mais

efetiva. Não existe nenhum prazo para que você receba o resultado originado desta pesquisa, mas este lhe será informado assim que estiver disponível. Você pode optar por não saber o resultado do teste.

Sua participação na pesquisa é totalmente voluntária, ou seja, não é obrigatória. Caso você decida não participar, ou ainda, desistir de participar e retirar seu consentimento, não haverá nenhum prejuízo ao atendimento que você recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra alguma intercorrência ou dano, resultante de sua participação na pesquisa, você receberá todo o atendimento necessário, sem nenhum custo pessoal.

Os dados coletados durante a pesquisa serão sempre tratados confidencialmente. Os resultados serão apresentados de forma conjunta, sem a identificação dos participantes, ou seja, o seu nome não aparecerá na publicação dos resultados.

Caso você tenha dúvidas, poderá entrar em contato com a pesquisadora responsável Dra. Ida Vanessa D. Schwartz, pelo telefone (51) 3359 8011 ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 3359 7640, ou no 2º andar do HCPA, sala 2227, de segunda à sexta, das 8h às 17h.

Esse Termo é assinado em duas vias, sendo uma para o participante e outra para os pesquisadores.

_____
Nome do participante da pesquisa

_____
Assinatura

_____
Nome do pesquisador que aplicou o Termo

_____
Assinatura

Local e Data: _____

10.3 ANEXO III

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

COMISSÃO CIENTÍFICA

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

Projeto: 170094
Data da Versão do Projeto: 15/02/2017

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
TATIELE NALIN
SUELEN PORTO BASGALUPP
MARINA SIEBERT

Título: Enzimas lisossomais e receptores envolvidos no metabolismo de glicoesfingolipídeos e vitamina B12: um estudo bioquímico baseado na doença de Gaucher

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

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

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

Porto Alegre, 25 de janeiro de 2018.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

10.4 ANEXO IV

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nº do projeto GPPG ou CAAE_____

Título do Projeto: **Enzimas lisossomais e receptores envolvidos no metabolismo de glicoesfingolipídeos e vitamina B_{12}: um estudo bioquímico baseado na doença de Gaucher**

Você está sendo convidado a participar de uma pesquisa cujo objetivo é caracterizar o metabolismo da vitamina B_{12} em pacientes com diferentes tipos da doença de Gaucher (DG). O presente estudo proporciona uma excelente oportunidade para investigar o papel da vitamina B_{12}, a qual pode estar influenciando a ocorrência dos diferentes sintomas clínicos apresentados pelos pacientes. Você está sendo convidado a participar porque já possui o diagnóstico de DG. Existem diversos fatores envolvidos na manifestação de diferentes características clínicas da doença e um deles pode ser uma anormalidade no metabolismo dessa vitamina. Os achados do presente estudo serão relacionados com as manifestações clínicas dos pacientes com DG, o que poderá contribuir para um melhor entendimento de como a doença está progredindo, porque os pacientes apresentam sintomas diferentes e o motivo pelo qual existem diferentes respostas ao tratamento entre os pacientes. Se você aceitar participar da pesquisa, os procedimentos envolvidos em sua participação são os seguintes: Coletar 10 mL de sangue para análise de metabólitos envolvidos no metabolismo da vitamina B_{12}; coletar uma biópsia de pele para análise do metabolismo da vitamina B_{12} e autorizar a consulta do seu prontuário para buscar dados clínicos e laboratoriais relacionados à doença. Se você permitir, o material coletado que restar após a realização da análise bioquímica deste estudo será armazenado e utilizado em estudos futuros, sendo que entraremos em contato com você novamente para que você autorize a utilização. Em relação às coletas, você declara que autorizou a coleta de:

( ) 10 mL de sangue para análise de metabólitos envolvidos no metabolismo da vitamina $B_{12;}$

( ) Biópsia de pele para análise do metabolismo da vitamina B_{12} em fibroblastos.

Em relação ao armazenamento do material biológico, você declara que:

( ) Autorizo o armazenamento do material biológico que restar após a realização da análise bioquímica relacionada ao metabolismo da vitamina B_{12}, sabendo que serei recontactado;

( ) Não autorizo o armazenamento do material biológico que restar após a realização da análise bioquímica relacionada ao metabolismo da vitamina B_{12}, devendo o mesmo ser devidamente descartado.

Os possíveis riscos ou desconfortos decorrentes da participação na pesquisa são semelhantes aos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). O desconforto e os riscos associados à coleta de sangue serão minimizados pela sua realização por profissional treinado.

A participação na pesquisa não lhe trará benefícios diretos, porém, contribuirá para um melhor entendimento da doença, o que futuramente poderá levar ao desenvolvimento de uma terapia mais efetiva para outros pacientes. Não existe nenhum prazo para que você receba o resultado originado desta pesquisa, e o mesmo não altera o prognóstico e tratamento da sua doença, no entanto, você poderá ter acesso ao resultado, caso você desejar, assim que estiver disponível. Sua participação na pesquisa é totalmente voluntária, ou seja, não é obrigatória. Caso você decida não participar, ou ainda, desistir de participar e retirar seu consentimento, não haverá nenhum prejuízo ao atendimento que você recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra alguma intercorrência ou dano, resultante de sua participação na pesquisa, você receberá todo o atendimento necessário, sem nenhum custo pessoal.

Os dados coletados durante a pesquisa serão sempre tratados confidencialmente. Os resultados serão apresentados de forma conjunta, sem a identificação dos participantes, ou seja, o seu nome não aparecerá na publicação dos resultados.

Caso você tenha dúvidas, poderá entrar em contato com a pesquisadora responsável Dra. Ida Vanessa D. Schwartz, pelo telefone (51) 3359 8011 ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 3359 7640, ou no 2º andar do HCPA, sala 2227, de segunda à sexta, das 8h às 17h.

Esse Termo é assinado em duas vias, sendo uma para o participante e outra para os pesquisadores.

_____

Nome do participante da pesquisa

_____

Assinatura

Data: _____

_____

Nome do pesquisador que aplicou o Termo

_____

Assinatura

Data: _____

10.5 ANEXO V

Ficha para coleta de dados dos exames clínicos, laboratoriais e de imagem

Nome do paciente: _____

Sexo: ( ) Feminino ( ) Masculino

Data de nascimento: ___/___/____

Cidade: _____ Estado: _____ Centro de vínculo: _____

Esplenectomizado: ( ) Sim ( ) Não

Idade do início dos sintomas: _____

Data do diagnóstico: ___/___/____ Idade ao diagnóstico: _____

Dosagem de β-glicosidase: _____

Genótipo: _____/_____

Escore de gravidade SSI: _____

Tipo de DG: _____

Exames laboratoriais:

EXAME	**ANTES DO TRATAMENTO**	**APÓS TRATAMENTO (6, 12 e 18 MESES)**	**VALOR DE REFERÊNCIA**
Ferritina			
Saturação de transferrina			
Ferro sérico			
Quitotriosidase			
Hemoglobina			
Plaquetas			
Aspartato aminotransferase (AST)			
Alanina aminotransferase (ALT)			
Tempo de protrombina (TP)			
Lactato desidrogenase (LDH)			
Quantificação de Fe em tecido hepático por RM			
Bone marrow burden por RM			

Apresentação clínica antes do tratamento:

Hepatomegalia: ( ) Sim ( ) Não
Esplenomegalia: ( ) Sim ( ) Não
Anemia: ( ) Sim ( ) Não
Plaquetopenia: ( ) Sim ( ) Não
Alterações ósseas: ( ) Sim ( ) Não
Comprometimento neurológico: ( ) Sim ( ) Não
Enfermidades não relacionadas a DG: ( ) Sim ( ) Não

Observações: _____

Apresentação clínica após 6 meses de início do tratamento:

Hepatomegalia: ( ) Sim ( ) Não
Esplenomegalia: ( ) Sim ( ) Não
Anemia: ( ) Sim ( ) Não
Plaquetopenia: ( ) Sim ( ) Não
Alterações ósseas: ( ) Sim ( ) Não
Comprometimento neurológico: ( ) Sim ( ) Não
Enfermidades não relacionadas a DG: ( ) Sim ( ) Não

Observações: _____

Apresentação clínica após 12 meses de início do tratamento:

Hepatomegalia: ( ) Sim ( ) Não
Esplenomegalia: ( ) Sim ( ) Não
Anemia: ( ) Sim ( ) Não
Plaquetopenia: ( ) Sim ( ) Não
Alterações ósseas: ( ) Sim ( ) Não
Comprometimento neurológico: ( ) Sim ( ) Não
Enfermidades não relacionadas a DG: ( ) Sim ( ) Não

Observações: _____

Apresentação clínica após 18 meses de início do tratamento:

Hepatomegalia: ( ) Sim ( ) Não
Esplenomegalia: ( ) Sim ( ) Não
Anemia: ( ) Sim ( ) Não
Plaquetopenia: ( ) Sim ( ) Não
Alterações ósseas: ( ) Sim ( ) Não
Comprometimento neurológico: ( ) Sim ( ) Não
Enfermidades não relacionadas a DG: ( ) Sim ( ) Não

Observações: _____

10.6 ANEXO VI

Review Article

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SAGE

Hampered Vitamin B12 Metabolism in Gaucher Disease?

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Abstract

Untreated vitamin B_{12} deficiency manifests clinically with hematological abnormalities and combined degeneration of the spinal cord and polyneuropathy and biochemically with elevated homocysteine (Hcy) and methylmalonic acid (MMA). Vitamin B_{12} metabolism involves various cellular compartments including the lysosome, and a disruption in the lysosomal and endocytic pathways induces functional deficiency of this micronutrient. Gaucher disease (GD) is characterized by dysfunctional lysosomal metabolism brought about by mutations in the enzyme beta-glucocerebrosidase (Online Mendelian Inheritance in Man (OMIM): 606463; Enzyme Commission (EC) 3.2.1.45, gene: *GBA1*). In this study, we collected and examined available literature on the associations between GD, the second most prevalent lysosomal storage disorder in humans, and hampered vitamin B_{12} metabolism. Results from independent cohorts of patients show elevated circulating holotranscobalamin without changes in vitamin B_{12} levels in serum. Gaucher disease patients under enzyme replacement therapy present normal levels of Hcy and MMA. Although within the normal range, a significant increase in Hcy and MMA with normal serum vitamin B_{12} was documented in treated GD patients with polyneuropathy versus treated GD patients without polyneuropathy. Thus, a functional deficiency of vitamin B_{12} caused by disrupted lysosomal metabolism in GD is a plausible mechanism, contributing to the neurological form of the disorder but this awaits confirmation. Observational studies suggest that an assessment of vitamin B_{12} status prior to the initiation of enzyme replacement therapy may shed light on the role of vitamin B_{12} in the pathogenesis and progression of GD.

Keywords

Gaucher disease, vitamin B12, lysosomal disease, homocysteine, methylmalonic acid, transcobalamin, lysosomal trafficking, polyneuropathy, cobalamin, enzyme replacement therapy

Introduction

Lysosomal storage disorders (LDs) are a broad group of more than 50 rare, life-threatening diseases characterized by abnormal degradation of glycans, carbohydrates, lipids and proteins, and lysosomal transporter and trafficking.[1]

The concept of LDs or lysosomal disorders was developed in the early 1960s, after the discovery that Pompe disease was caused by a deficiency in the lysosomal enzyme α-glucosidase.[2] Lysosomal storage disorder may be caused not only by defective enzymes but also by enzyme activator proteins (eg, Prosaponin [PSAP] deficiency), membrane proteins (eg, Danon disease), transporters (eg, cystinosis), or enzyme signaling (eg, mucolipidosis type II). Lysosomal storage disorders are characterized by an abnormal storage of a variety of molecules, including triglycerides, sterols, sphingolipids, sulfatides, sphingomyelin, gangliosides, and lipofuscins.[3] The buildup of substrates within lysosomes results in impaired function of the affected organs (eg, liver, spleen, bone, and nervous system), causing a wide and diverse range of clinical features. In addition, the release of lysosomal acid hydrolases into the

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cytoplasm will cause cellular damage, which may worsen disease progression. Also, dysregulation of apoptosis may cause disease manifestations in some LDs. Indeed, increased apoptosis has been noted in a number of the sphingolipidoses and in neuronal ceroid lipofuscinoses.[4] Since different mechanisms related to apoptosis, cholesterol metabolism dysregulation, inflammation, and alteration in signal transduction are also related to the pathogenesis of these conditions, we prefer to use the term "lysosomal disorder" than "lysosomal storage disorder."[5] To date, more than 60 different proteins were identified as causing LDs.[6] Individually, LDs are rare, inherited disorders with an estimated frequency from 1 in 25 000 to 1 in 250 000 live newborns, but the overall incidence of all LDs is estimated to be 1 in 7000 live newborns, which makes them a relevant public health issue. The frequency may be underestimated because nowadays more individuals with mild disease and/or adult-onset forms of the diseases are being identified.[7]

Gaucher disease (GD) is an autosomal recessive, multiorgan disorder caused by mutations in the lysosomal enzyme β-glucocerebrosidase (GCase, OMIM: 606463; EC 3.2.1.45, gene: *GBA1*), which catalyzes the conversion of the glycolipid glucosylceramide to ceramide and glucose.[8] The *GBA1* gene is located on chromosome 1q21, comprises 7.6 kb of genomic DNA, and it is divided into 11 exons.[9] The *GBA1* messenger RNA (mRNA) has approximately 2 kb and produces a mature protein of 497 amino acids with 56 kDa.[10,11] The expression levels of mRNA produced from *GBA1* varies considerably between different cell types and has no direct correlation with GCase enzyme activity.[10] *GBA1* has a pseudogene (*GBAP*) of approximately 5 kb, which is highly homologous (96%) with the functional gene. *GBAP* has an identical genomic organization and is located 16 kb downstream of *GBA1*.[9,10] The high degree of homology between the gene and the pseudogene must be taken into account in the investigation of mutations in patients with GD, since some of the mutations found in patients are also present in the *GBAP* sequence.[10-12]

A dysfunctional or absent β-GCase protein leads to the buildup of glucosylceramide in the lysosome, in particular within macrophages of the reticuloendothelial system, and to a defective production of ceramide, the hydrophobic membrane anchor for all sphingolipids in the cell.[13]

This review covers clinical and molecular aspects of GD, the second most prevalent inborn error of LD, with an emphasis on lysosomal metabolism and the potential occurrence of abnormalities in vitamin B_{12} status.

Gaucher Disease

Gaucher disease has been classified into 3 major types based on the absence (type 1) or presence (types 2 and 3) of neurological impairments.[14] The prevalence of GD in the general population has been estimated to be about 1:60 000[15]; however, as seen in other autosomal recessive disorders, GD exhibits ethnical preference.

At the cellular level, GD is characterized by the presence of macrophages with an altered morphology due to abnormal lipid storage, also known as "Gaucher cells." Gaucher cells are typically found in affected organs including spleen, liver, and bone marrow.[16] In addition to exhibiting an abnormal morphology, macrophages from GD have a distinct pattern of expression of pro-inflammatory effectors.[17,18]

At the subcellular level, GD features abnormalities in lysosomal pathways, which is accompanied by mitochondrial dysfunction and accumulation of α-synuclein in the mitochondrion.[19] Lysosomal lipid storage in GD reduces the efficiency of lysosomes to fuse with autophagosomes, thereby impairing cellular clearance of unnecessary substrates, protein aggregates, and dysfunctional mitochondria.[16] This triggers an inflammatory response that ultimately leads to cellular death.[16] Interestingly, mitochondrial dysfunction and increased deposition of α-synuclein are 2 features of GD that are shared with other peripheral neuropathies such as Parkinson and Alzheimer diseases.[20]

The molecular mechanism underlying GD pathogenesis remains elusive. A few metabolic hallmarks have been identified in GD, including increased chitotriosidase (EC 3.2.1.14), angiotensin-converting enzyme (EC 3.4.15.1), C-C Motif Chemokine Ligand 18 (CCL18) (P55774), tartrate-resistant acid phosphatase (EC 3.1.3.2), and serum ferritin (P02792 and P02794), and some of these continue to be utilized as diagnostic and prognostic tools in clinical practice.[21] An interesting aspect of GD is the presumptive abnormality in vitamin B_{12} status.[22-26] The earliest observations linking GD with vitamin B_{12} metabolism were documented about 4 decades ago by Gilbert and Weinreb[26] and by Rachimilewitz and Rachimilewitz.[27] These studies demonstrated elevated levels of circulating holotranscobalamin (holo-TC) in GD patients, which was not associated with decreased serum levels of vitamin B_{12} or any other vitamin B_{12} binders.[26] The levels of holo-TC were directly proportional to the severity of GD.[26] Very few follow-up studies were conducted thereafter, and to this date, the associations between GD and vitamin B_{12} metabolism are open for investigation, as also will be shown below.

Clinical Manifestations

Gaucher disease is classically divided into 3 clinical types based upon the severity and onset of neurological involvement; however, overlap is often seen among the phenotypes. Gaucher disease type 1 (OMIM 230800), the nonneuronopathic type, is the most common form of the disorder in the western hemisphere and the most prevalent type overall (90%-95% of the patients), with an incidence of 1 in 70 000 live newborns worldwide.[28] In Ashkenazi Jews, the incidence is 1 in 400 live newborns.[29] Type 1 is characterized by multiorgan involvement, especially hepatic, splenic, bone, hematologic, and pulmonary systems. The life expectancy depends on the time of diagnosis, severity of visceral involvement, and treatment. Patients with GD type 1 are treated with specific therapies such as enzyme replacement therapy (ERT) or substrate reduction therapy. Most treated patients usually have a

normal life expectancy. The absence of early-onset primary central nervous system (CNS) is essential for the diagnosis of GD type 1.[30] During the last 2 decades, population studies have shown an association between GD and Parkinson disease. Carriers of *GBA1* mutations are also at risk of developing parkinsonism.[31] Also, GD type 1 patients are at an increased risk of developing cholelitiasis[32] and hematological malignancies as multiple myeloma.[33]

Gaucher disease type 2 (OMIM 230900), the acute neuronopathic type, is the less frequent phenotype, with an incidence of 1 in 100 000 live newborns and is characterized by cholestasis, hepatosplenomegaly, and early-onset and rapidly progressive CNS manifestations with bulbar involvement. Hydrops fetalis and collodion baby may be present in the most severe forms. The life expectancy varies from hours to a few months.[34] There is no specific treatment for patients with GD type 2.

Gaucher disease type 3 (OMIM 231000), the subacute or chronic neuronopathic type, is particularly prevalent in Asian and Arab countries.[14] Gaucher disease type 3 is characterized by an intermediate phenotype with visceral manifestations as GD type 1 and CNS manifestations are less severe than GD type 2. Patients may have severe bone involvement, with kyphoscoliosis, ataxia, myoclonic epilepsy, strabismus, horizontal gaze palsy, and dementia. Some patients may present corneal clouding and cardiac valvular calcifications. Enzyme replacement therapy is indicated to treat the visceral signs and symptoms of GD, but it fails to alleviate CNS manifestations. The life expectancy is 20 to 30 years.[35]

Diagnosis

Clinical manifestations, such as hepatosplenomegaly, bone lesions, hematologic changes, and/or CNS involvement, are important signs that would suggest the presence of GD.[23,36] However, the diagnosis of GD should not be based exclusively on the clinical evaluation of the patient. There are other LDs that may present with symptoms similar to GD, which may complicate the establishment of a precise diagnosis.

The standard diagnostic method for GD is the evaluation of β-GCase (acid β-glucosidase) activity in dried blood spots, peripheral blood leukocytes, cultured skin fibroblasts, or other nucleated cells. Molecular analysis of *GBA1*, which encodes GCase, and the identification of 2 disease-causing mutations may assist the patient's clinical classification into a determined subtype or at least make it possible to distinguish between neuronopathic and nonneuronopathic forms.[37] Genetic testing enables the confirmation and a better characterization of the patient's condition and is considered an essential tool for GD diagnosis.[38] Up to date, more than 400 different disease-causing mutations have been described in the *GBA1* gene (www.hgmd.cf.ac.uk[37,39]). *GBA1* mutations may alter GCase stability and/or impair its catalytic function.[37,38]

The identification of disease-causing mutations in *GBA1* may be challenging due to the *GBAP*. The most accurate method for mutation analysis in GD is full-gene sequencing of *GBA1*.[39] In order to describe a recombinant allele, a combination of direct sequencing along with an additional method, such as Southern blot or qPCR, is strongly recommended.[40,41] The occurrence of deletion and/or duplication of any region of *GBA1* can be specifically addressed by multiplex ligation-dependent probe amplification.[40,41]

Vitamin B_{12} Metabolism

Vitamin B_{12} is an essential micronutrient synthesized only by a select group of bacteria and archaea. Humans completely rely on a dietary intake of minimally 2 to 3 µg of vitamin B_{12} per day,[42] which is indispensable to support the activities of cytosolic methionine synthase (MS) and mitochondrial methylmalonyl-CoA mutase (MCM). Dietary vitamin B_{12} is absorbed in the lower portions of the ileum after sequential relay by the dedicated transporters haptocorrin, intrinsic factor, and transcobalamin.[43,44] Vitamin B_{12} bound to transcobalamin, that is, holo-TC is distributed via systemic circulation to all cells in the body. Cells take up holo-TC via receptor-mediated endocytosis, aided by the transcobalamin receptor (CD320),[45,46] which shuttles vitamin B_{12} into lysosomes. The protein binder TC undergoes degradation in the lysosome, liberating vitamin B_{12} that is subsequently exported out of this compartment using the transporters LMBR1 Domain Containing 1 (LMBRD1)[47,48] and ATP Binding Cassette Subfamily D Member 4 (ABCD4).[49,50] Once in the cytosol, newly internalized vitamin B_{12} undergoes processing and trafficking by proteins, CblC[51-57] and CblD,[58-63] respectively, to finally reach acceptor proteins, MS in the cytosol and MCM in the mitochondrion.

Insufficient intake and certain inborn errors of metabolism impairing the cellular transport, trafficking, and utilization of vitamin B_{12} manifest as functional cobalamin deficiency, with either isolated or combined homocystinuria and methylmalonic aciduria. Untreated vitamin B_{12} deficiency causes hematological abnormalities, subacute combined degeneration of the spinal cord, and polyneuropathy.[64] However, its precise role in the progression of neurological diseases (measured as the onset of dementia in only 1 large study[65]) has been debated.[65] It is possible that sufficiency of vitamin B_{12} is important for preventing nerve deterioration. Vitamin B_{12} administration only partially reverses clinically established nerve degeneration and neuropathies.

Lysosomal Disorders of Vitamin B_{12}

The lysosome is an essential compartment in cellular vitamin B_{12} metabolism by connecting uptake and downstream utilization of the micronutrient. Two genetic disorders affecting lysosomal proteins LMBRD1 (cblF)[47,48] and ABCD4 (cblJ)[49,50,66] have been described, leading to trapping of vitamin B_{12} inside the lysosome and the concomitant onset of functional cobalamin deficiency by inactivation of the B_{12} acceptors MS and MCM. LMBRD1 and ABCD4 mediate the export of vitamin B_{12} from the lysosome.[47-50,66]

Apart from these canonical defects of lysosomal vitamin B_{12} transporters, 2 other reports documented abnormal vitamin B_{12}

Table 1. Demographics, Treatment, Vitamin B_{12} Status, and Clinical Manifestations of Adult GD Type 1 in a Cohort of European Patients.[a,b,c]

	Polyneuropathy (n = 17)	No Polyneuropathy (n = 86)	*P* Value
Demographics			
Age in years, median (range)	61 (41-75)	39 (18-67)	<.001
Gender, male/female, n (%)	11 (64.7)/6 (35.3)	38 (44.2)/48 (55.8)	NS
Enzyme replacement therapy			
Receiving ERT, n (%)	15 (88.2)	74 (86.0)	NS
Duration in years, median (range)	3.0 (0.2-10.9)	2.1 (0.0-13.6)	NS
Dosage in IU/kg/m, median (range)	30.0 (11.1-112.2)	58.2 (12.3-156.3)	.013
Vitamin B_{12} status			
Vitamin B_{12} (pmol/L), median (range)	208 (88-593)	237 (86-886)	NS
Homocysteine (µmol/L), median (range)	11.4 (7.5-30.4)	9.7 (4.6-26.5)	.013
Methylmalonic acid (µmol/L), median (range)	0.18 (0.09-0.98)	0.12 (0.03-0.54)	.001
Systemic GD type 1 manifestations, n (%)			
Splenomegaly	16 (94.1)	77 (89.5)	NS
Hepatomegaly	16 (94.1)	64 (74.4)	NS
Thrombocytopenia	10 (58.8)	66 (76.7)	NS
Bleeding tendencies	9 (52.9)	38 (44.2)	NS
Anemia	9 (52.9)	34 (39.5)	NS
Bone/joint pain	9 (52.9)	24 (27.9)	NS
Bone crisis	6 (35.3)	25 (29.1)	NS

Abbreviations: ERT, enzyme replacement therapy; GD, Gaucher disease; NS, not statistically significant.
[a]N = 103.
[b]Table Modified from Biegstraaten et al.[82]
[c]Normal ranges: methylmalonic acid, <30 µmol/L; homocysteine, pre-menopausal females, 6 to 15 µmol/L, males, and postmenopausal females, 8to 18 µmol/L.

metabolism caused by genetic mutations that impair the endocytic and lysosomal pathways independent of vitamin B_{12} metabolism. These include the occurrence of abnormal lysosome acidification in a patient with Alzheimer disease[67] and impaired endocytosis in a patient with mutations in the rabenosyn-5 gene.[67,68] In both cases, cellular vitamin B_{12} deficiency was documented.[68]

Vitamin B_{12} Status in GD

The overlap of clinical manifestations of GD and vitamin B_{12} deficiency concerning neurological impairments suggests shared mechanisms of pathogenesis. One hypothesis is that lysosomal dysfunction in GD leads to functional deficiency of vitamin B_{12} by disrupting the uptake, intralysosomal degradation of transcobalamin, or the export of free vitamin B_{12} from the organelle into the cytoplasm, thereby compromising the downstream reactions of MS and MCM. Further, it is unknown whether the abnormal accumulation of glycosphingolipids, in particular *N*-acyl-sphingosyl-1-*O*-β-D-glucoside, may affect vitamin B_{12} transit in and out of the lysosome.

The earliest assessment of vitamin B_{12} status in GD reported abnormally high levels of circulating TC,[26,29] which did not correlate with serum levels of vitamin B_{12} or any other B_{12}-transport protein levels. It was suggested that increased TC levels in GD resulted from a general status of inflammation. Unfortunately, no other biomarkers of vitamin B_{12} status were measured.[69] Indeed, although holo-TC represents the bioactive fraction of vitamin B_{12} that is available for cellular uptake,[70-73] this has limitations as a stand-alone marker of vitamin B_{12} status in that the majority of circulating vitamin B_{12} is bound to haptocorrin (80%), and thus, fluctuations in the serum level of holo-TC (representing 6%-20% of total serum vitamin B_{12}[70-73]) may not be accurate marker of vitamin B_{12} sufficiency (reviewed by Djaldetti et al[74]). For instance, low levels of holo-TC have been determined in patients with several disorders not featuring vitamin B_{12} deficiency.[75-78] At present time, it is unknown whether and how holo-TC levels vary in various disease states, and thus, the diagnostic and prognostic value of holo-TC as a first-line test awaits further investigation.

A study performed within an Ashkenazi Jewish cohort of 85 untreated GD patients and 122 neighbor controls showed a high incidence of low-serum vitamin B_{12}, elevated plasma homocysteine (Hcy), and methylmalonic acid (MMA); however, these findings were not statistically significant with respect to controls, due to an overall low vitamin B_{12} status among healthy Ashkenazi individuals.[23] The generally low vitamin B_{12} status in this Ashkenazi Jewish population has been suggested to arise from frequent blood donation that would exhaust blood and liver storages of vitamin B_{12} as well as to ethnical differences.[79,80] A questionnaire-based study identified a high incidence of neurological complaints in patients with nonneuronopathic forms of GD, with concomitant vitamin B_{12} deficiency and gammopathies.[81] Unfortunately, no laboratory measurements of marker metabolites Hcy and MMA were performed in these studies, which makes it difficult to ascertain the role of vitamin B_{12} status in this cohort of GD patients.[81]

A 2-year prospective, longitudinal, observational cohort study involving 8 centers across 7 countries in Europe examined vitamin B_{12} status in adult patients with GD type 1 with (n = 17) and without (n = 86) polyneuropathy.[82] The study found statistically significant elevation of serum Hcy and

MMA in patients with polyneuropathy compared to those without neuropathic impairment, with low–normal values of serum vitamin B_{12} in both groups.[82] However, both groups of patients displayed metabolite levels still within the normal range (MMA, <0.4 µmol/L; Hcy, premenopausal females, 6-15 µmol/L, males, and postmenopausal females, 8-18 µmol/L).[82] Importantly, both groups of patients had received ERT for at last 2 to 3 years. Table 1 summarizes the data that represent the largest examination of vitamin B_{12} status in GD to date. Patients with polyneuropathy had received a lower dose of ERT compared to patients without polyneuropathy.[82] In the absence of vitamin B_{12} biomarker (Hcy and MMA) values before ERT, it is difficult to state whether the reported values represent a partially corrected vitamin B_{12} metabolism. In sum, a disturbed vitamin B_{12} metabolism in GD is plausible, but the available data beg for additional investigation.

Outlook

Lysosomal storage disorders and GD in particular are complex diseases affecting various facets of metabolism. The occurrence of vitamin B_{12} deficiency as a general manifestation of GD awaits further confirmation, but available studies point to disturbances in vitamin B_{12} metabolism that may originate from abnormal lysosomal metabolism or a general status of inflammation as it has been reported in 2 other human disorders,[73,74] not intrinsically related to vitamin B_{12} metabolism. It has been suggested that aging leads to increased impairments in lysosomal metabolism, and that this could explain the concomitant disturbances of vitamin B_{12} pathways often found in association with diseases featuring peripheral neuropathies, such as Alzheimer and Parkinson disorders.[83,84] This further supports the need for vitamin B_{12} supplementation in the aging population, beyond the known limitations in gastric absorption of the micronutrient, which also becomes less efficient with aging. Our revision of the available literature points to the importance of assessing vitamin B_{12} status prior to the initiation of ERT in GD patients, in order to establish involvement of this micronutrient on the onset of symptoms and possibly in its associated peripheral neuropathies.

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Declaration of Conflicting Interests

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10.7 ANEXO VII

Tabela. Itens essenciais que devem ser descritos em estudos observacionais, segundo a declaração Strengthening the Reporting of Observational Studies in Epidemiology (STROBE). 2007.

Item	Nº	Recomendação
Título e Resumo	1	Indique o desenho do estudo no título ou no resumo, com termo comumente utilizado
		Disponibilize no resumo um sumário informativo e equilibrado do que foi feito e do que foi encontrado
Introdução		
Contexto/Justificativa	2	Detalhe o referencial teórico e as razões para executar a pesquisa.
Objetivos	3	Descreva os objetivos específicos, incluindo quaisquer hipóteses pré-existentes.
Métodos		
Desenho do estudo	4	Apresente, no início do artigo, os elementos-chave relativos ao desenho do estudo.
Contexto (*setting*)	5	Descreva o contexto, locais e datas relevantes, incluindo os períodos de recrutamento, exposição, acompanhamento (follow-up) e coleta de dados.
Participantes	6	Estudos de Coorte: Apresente os critérios de elegibilidade, fontes e métodos de seleção dos participantes. Descreva os métodos de acompanhamento. Estudos de Caso-Controle: Apresente os critérios de elegibilidade, as fontes e o critério-diagnóstico para identificação dos casos e os métodos de seleção dos controles. Descreva a justificativa para a eleição dos casos e controles Estudo Seccional: Apresente os critérios de elegibilidade, as fontes e os métodos de seleção dos participantes. Estudos de Coorte: Para os estudos pareados, apresente os critérios de pareamento e o número de expostos e não expostos. Estudos de Caso-Controle: Para os estudos pareados, apresente os critérios de pareamento e o número de controles para cada caso.
Variáveis	7	Defina claramente todos os desfechos, exposições, preditores, confundidores em potencial e modificadores de efeito. Quando necessário, apresente os critérios diagnósticos.
Fontes de dados/ Mensuração	8ª	Para cada variável de interesse, forneça a fonte dos dados e os detalhes dos métodos utilizados na avaliação (mensuração). Quando existir mais de um grupo, descreva a comparabilidade dos métodos de avaliação.
Viés	9	Especifique todas as medidas adotadas para evitar potenciais fontes de vies.
Tamanho do estudo	10	Explique como se determinou o tamanho amostral.
Variáveis quantitativas	11	Explique como foram tratadas as variáveis quantitativas na análise. Se aplicável, descreva as categorizações que foram adotadas e porque.
Métodos estatísticos	12	Descreva todos os métodos estatísticos, incluindo aqueles usados para controle de confundimento. Descreva todos os métodos utilizados para examinar subgrupos e interações. Explique como foram tratados os dados faltantes ("missing data") Estudos de Coorte: Se aplicável, explique como as perdas de acompanhamento foram tratadas. Estudos de Caso-Controle: Se aplicável, explique como o pareamento dos casos e controles foi tratado. Estudos Seccionais: Se aplicável, descreva os métodos utilizados para considerar a estratégia de amostragem. Descreva qualquer análise de sensibilidade.
Resultados		
Participantes	13ª	Descreva o número de participantes em cada etapa do estudo (ex: número de participantes potencialmente elegíveis, examinados de acordo com critérios de elegibilidade, elegíveis de fato, incluídos no estudo, que terminaram o acompanhamento e efetivamente analisados) Descreva as razões para as perdas em cada etapa. Avalie a pertinência de apresentar um diagrama de fluxo
Dados descritivos	14ª	Descreva as características dos participantes (ex: demográficas, clínicas e sociais) e as informações sobre exposições e confundidores em potencial. Indique o número de participantes com dados faltantes para cada variável de interesse. Estudos de Coorte: Apresente o período de acompanhamento (ex: média e tempo total)

Continua

Tabela continuação

Item	Nº	Recomendação
Desfecho	15[a]	Estudos de Coorte: Descreva o número de eventos-desfecho ou as medidas-resumo ao longo do tempo Estudos de Caso-Controle: Descreva o número de indivíduos em cada categoria de exposição ou apresente medidas-resumo de exposição. Estudos Seccionais: Descreva o número de eventos-desfecho ou apresente as medidas-resumo.
Resultados principais	16	Descreva as estimativas não ajustadas e, se aplicável, as estimativas ajustadas por variáveis confundidoras, assim como sua precisão (ex: intervalos de confiança). Deixe claro quais foram os confundidores utilizados no ajuste e porque foram incluídos. Quando variáveis contínuas forem categorizadas, informe os pontos de corte utilizados. Se pertinente, considere transformar as estimativas de risco relativo em termos de risco absoluto, para um período de tempo relevante.
Outras análises	17	Descreva outras análises que tenham sido realizadas. Ex: análises de subgrupos, interação, sensibilidade.
Discussão		
Resultados principais	18	Resuma os principais achados relacionando-os aos objetivos do estudo.
Limitações	19	Apresente as limitações do estudo, levando em consideração fontes potenciais de viés ou imprecisão. Discuta a magnitude e direção de viéses em potencial.
Interpretação	20	Apresente uma interpretação cautelosa dos resultados, considerando os objetivos, as limitações, a multiplicidade das análises, os resultados de estudos semelhantes e outras evidências relevantes.
Generalização	21	Discuta a generalização (validade externa) dos resultados.
Outras Informações		
Financiamento	22	Especifique a fonte de financiamento do estudo e o papel dos financiadores. Se aplicável, apresente tais informações para o estudo original no qual o artigo é baseado.

[a] Descreva essas informações separadamente para casos e controles em Estudos de Caso-Controle e para grupos de expostos e não expostos, em Estudos de Coorte ou Estudos Seccionais.

Nota: Documentos mais detalhados discutem de forma mais aprofundada cada item do *checklist*, além de apresentarem o referencial teórico no qual essa lista se baseia e exemplos de descrições adequadas de cada item (Vandenbroucke et al.[24,25] A *checklist* do STROBE é mais adequadamente utilizada um conjunto com esses artigos (disponíveis gratuitamente no site das revistas PLoS Medicine [www.plosmedicine.org], Annals of Internal Medicine [www.annals.org] e Epidemiology [www.epidem.com]). No website da iniciativa STROBE (www.strobe-statement.org) estão disponíveis versões separadas de checklist para Estudos de Coorte, Caso-Controle ou Seccionais. Reproduzida de von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. Declaração STROBE: Diretrizes para a comunicação de estudos observacionais[material suplementar na internet]. Malta M, Cardoso LO, tradutores.In: Malta M, Cardoso LO, Bastos FI, Magnanini MMF, Silva CMFP. Iniciativa STROBE: subsídios para a comunicação de estudos observacionais. *Rev Saude Publica*. 2010;44(3):559-65.