UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

O SISTEMA IMUNE NA DOENÇA DE GAUCHER

FILIPPO PINTO E VAIRO

Porto Alegre

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

Orientadora: Prof. Dra. Ida Vanessa Doederlein Schwartz

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APRESENTAÇÃO

Conforme formato requerido pelo Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, esta tese está dividida em: *Introdução*; *Objetivos*; *Capítulos* (substituirão as seções de Material e Métodos e Resultados descrevendo os resultados obtidos no período sob forma de artigos científicos publicados, artigo no prelo e artigo submetido); *Discussão e Perspectivas*; *Conclusões*. Este trabalho foi desenvolvido no Serviço de Genética Médica e no Serviço de Imunologia do Hospital de Clínicas de Porto Alegre. O estudo foi financiado pelo Fundo de Incentivo à Pesquisa e Eventos (FIPE) do Hospital de Clínicas de Porto Alegre e Programa de Apoio a Núcleos de Excelência (PRONEX) do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e da Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) e *unrestricted grants* da empresa Shire. O aluno recebeu bolsa de estudos concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) via Edital Especial de Seleção de Candidatos para Bolsas Especiais de Doutorado para Pesquisa Médica (Edital Nº 14/2008). Todos os experimentos apresentados neste trabalho estão incluídos em projeto de pesquisa aprovado em seus aspectos éticos e metodológicos pelo Comitê de Ética e Pesquisa do Grupo de Pesquisa e Pós-Graduação (GPPG) do Hospital de Clínicas de Porto Alegre sob o número 09-398.

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SUMÁRIO

ABREVIATURAS E SIGLAS

ANVISA: Agência Nacional de Vigilância Sanitária

APC: Célula apresentadora de antígeno

BDNF: *Brain-derived neurotrophic factor*

CCL: Quimiocina CC (cisteína-cisteína) ligante

CD: *Cluster of differentiation*

CXCL: Quimiocina CXC (cisteína-aminoácido-cisteína) ligante

DG: Doença de Gaucher

EMA: *European Medicines Agency*

EUA: Estados Unidos da América

FDA: *U.S. Food and Drug Administration*

GBA: Glicocerebrosidase, beta-glicosidase ácida

GCSF: *Granulocyte colony-stimulation*

GMCSF: *Granulocyte-macrophage colony-stimulation factor*

HCPA: Hospital de Clínicas de Porto Alegre

HGF: *Hepatocyte growth fator*

HLA: *Human leukocyte antigen*

IL: Interleucina

Ig: Imunoglobulina

KIR: *Killer immunoglobulin like receptor*

LAMP: Proteína de membrana associada ao lisossomo

LRC: *Leukocyte Ig-like receptor complex*

MCSF: *Macrophage colony-stimulation factor*

MGUS: Gamopatia monoclonal de significância indeterminada

MHC: *Major Histocompatibility Complex*

MIP: Proteína inflamatória macrofágica

MM: Mieloma múltiplo

MMP: *Matrix metaolloproteinase*

NK: *Natural killer cells*

NO: Óxido nítrico

NOS: Óxido nítrico sintetase

OPN: Osteopontina

PCR: *Polimerase chain reaction*

SSP: *Sequence specific primers*

SUS: Sistema Único de Saúde

TCR: Receptor de célula T

Tfh: Linfócito T folicular

TGF-β: Fator de crescimento transformador beta

Th: Linfócito T helper (auxiliador)

TNF-α: Fator de necrose tumoral alfa

TRE: Terapia de reposição enzimática

Treg: Linfócitos T regulatórios

TRS: Terapia de redução de substrato

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RESUMO

Introdução: A Doença de Gaucher (DG) é causada pela atividade reduzida da enzima lisossomal glicocerebrosidase, o que leva ao acúmulo de glicocerebrosídeo em macrófagos. Embora o macrófago venha sendo a célula mais estudada, alguns achados sugerem que outras células do sistema imune possam ter papel importante na fisiopatologia da DG. Os pacientes com DG apresentam níveis elevados de imunoglobulinas e uma maior incidência de malignidades hematológicas, possivelmente devido ao desbalanço da regulação de citocinas inflamatórias. **Objetivo:** Caracterizar o envolvimento do sistema imune na DG ao analisar alterações clínicas e bioquímicas, variabilidade dos genes *HLA* e *KIR* e a expressão de citocinas em uma coorte de pacientes com DG do Estado do Rio Grande do Sul. **Metodologia:** Para o estudo relacionado aos genes *HLA* e *KIR,* 31 pacientes com DG tipo I foram analisados e comparados a 250 controles saudáveis. Para os estudos relacionados à variação de citocinas, foram obtidas amostras de 14 pacientes com DG tipo I fora de tratamento e após 6 meses de tratamento regular. **Resultados/Discussão:** O alelo HLA B37 foi mais frequente em pacientes com DG do que nos controles (p=0,01). A idade de início dos sintomas foi associada à combinação das variantes KIR2DL2 e KIR2DS2 com seu ligante HLA-C1 (p=0,038). Pacientes que apresentam a variante HLA-C2 parecem apresentar maior susceptibilidade a desenvolver bandas mono ou policlonais na eletroforese de proteínas (p=0,007, OR=21,3). Foi encontrada associação entre os alelos DR11 (p=0.008) e DR13 (p=0.011) e gravidade da doença. O BDNF está diminuído em pacientes com DG em relação a controles saudáveis e aumenta significativamente após a TRE, enquanto a osteopontina parece ser mais sensível que a quitotriosidase para avaliação de resposta ao tratamento. Ao avaliar a variação das citocinas, encontramos uma diminuição significativa de TNF-α, MIP-1α, MIP-1β e MDC e um aumento significativo de GRO, PAI-1 e leptina após tratamento. **Conclusão:** Nossos dados sugerem uma possível associação entre variantes dos genes *KIR* e *HLA* e a expressão fenotípica de pacientes com DG. Além disso, demonstramos a variabilidade de diferentes

citocinas após o tratamento, podendo algumas delas ser utilizadas para monitorização de resposta ao tratamento dos pacientes.

ABSTRACT

Background: Gaucher disease (GD) is caused by the reduced activity of the lysosomal enzyme glucocerebrosidase, which leads to the accumulation of glucocerebroside in the macrophages and a chronic stimulation of the immune system. Although the macrophage has been better studied, there are some findings regarding the role of other immunological cells in the pathophysiology of GD. GD patients have elevated levels of immunoglobulins and an increased incidence of hematological malignancies, possibly due to the imbalance of regulatory cytokines. **Objectives:** To characterize the involvement of the immune system in GD by analyzing clinical and biochemical features, variability of *HLA* and *KIR* genes and the cytokines expression in a cohort of patients from Rio Grande do Sul, Brazil. **Methodology:** Regarding the *HLA* and *KIR* genes study, DNA samples from 31 patients with GD type I were analyzed and compared to 250 healthy controls. For studies related to the variation of cytokines, samples from 14 patients with Gaucher type I off treatment and after 6 months of regular treatment were compared. **Results/Discussion:** The HLA B37 allele was more frequent in patients with GD than in controls ($p = 0.01$). The age of onset was associated with KIR2DL2 and KIR2DS2 combination with its ligand HLA-C1 ($p = 0.038$). Patients with the HLA-C2 appear to exhibit increased susceptibility to develop monoclonal or polyclonal bands on protein electrophoresis ($p = 0.007$, OR = 21.3). An association between the DR11 ($p=0.008$) and DR13 ($p=0.011$) alleles and disease severity was found. BDNF is decreased in patients with GD compared to healthy controls and increases after ERT while osteopontin seems to be more sensitive than chitotriosidase for assessing response to treatment. When evaluating the variation of cytokines, we found a significant decrease of TNF-α, MIP-1α, MIP-1β and MDC and significant increased GRO, PAI-1 and leptin after treatment. **Conclusion:** Our data suggest a possible association between variants of *KIR* and *HLA* genes and phenotypic expression presented by GD patients. Furthermore, we demonstrate the variability of different cytokines after treatment and some of them can be used for monitoring the response to treatment of patients.

1) INTRODUÇÃO

1.1) Doença de Gaucher

Até o momento, foram descritas mais de 50 doenças lisossômicas com uma incidência estimada de 1 em cada 5000 nascidos vivos (Meikle *et al.*, 2006), embora estudos recentes baseados em triagem neonatal demonstrem uma incidência de 1 em cada 2315 nascidos vivos (Mechtler *et al.*, 2012).

A Doença de Gaucher (DG) é uma das mais frequentes doenças lisossômicas, com uma incidência estimada de 1 em cada 57.000 nascidos vivos no mundo (Meikle *et al.,* 1999), porém em judeus Ashkenazi a incidência chega a 1 em cada 400 nascidos vivos (Sobreira *et al.*, 2007). É causada por mutações em ambos os alelos do gene *GBA1*, que codifica a enzima lisossomal glicocerebrosidase (GBA) ou beta-glucosidase ácida (EC 3.2.1.45), responsável pela hidrólise de glicocerebrosídeo em glicose e ceramida (Beutler *et al.,* 2006) (Figura 1). Como consequência, há acúmulo de glicocerebrosídeo nos macrófagos, principalmente no baço, fígado, medula óssea e pulmão, caracterizando a DG como uma doença multissistêmica, com heterogeneidade fenotípica. Embora a DG tenha sido descrita há mais de 125 anos, uma terapia específica e efetiva só foi descoberta há menos de 25 anos.

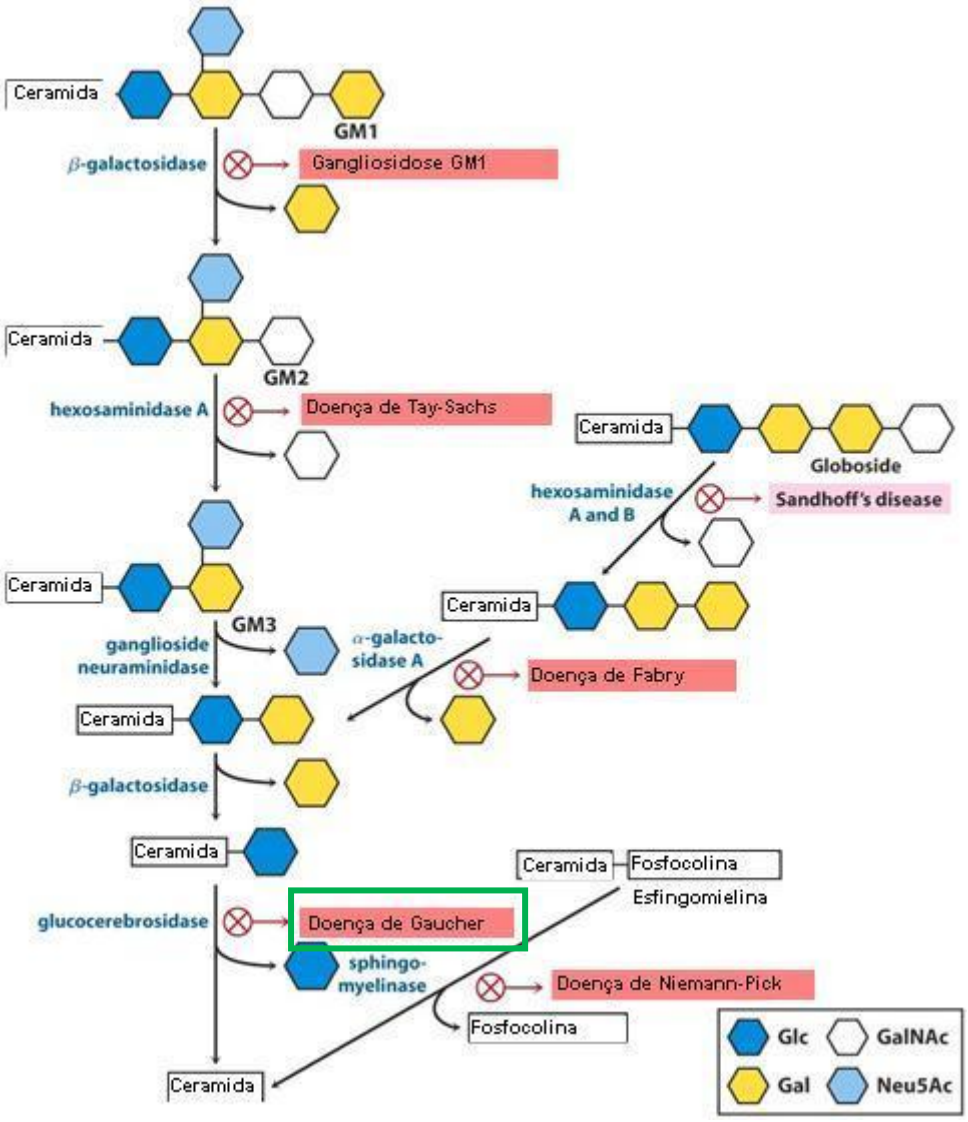

Figura 1: Rota da degradação dos glicoesfingolipídios. Adaptado de Lehninger, 2005

1.1.2) História

Em 1882, o Dr Phillipe Gaucher, em sua tese de doutorado, descreveu uma mulher de 32 anos com o baço aumentado de volume devido a células ingurgitadas e, naquele momento, foi suspeitada ser uma doença maligna. Quando em 1885 outro caso similar foi relatado, foi nomeada doença de Gaucher (Gaucher, 1882). Em 1904, Brill sugeriu que a doença poderia ser herdada e demonstrou que o fígado, os linfonodos e os ossos também estavam

envolvidos (Brill *et al.,*1904). Os primeiros pacientes com envolvimento neurológico foram descritos nos anos 1920 (Brady *et al.,* 1997) e em 1934, Aghion demonstrou que órgãos do sistema reticulo endotelial eram infiltrados por células de Gaucher que eram preenchidas por glicocerebrosídeo, um glicoesfingolipídio (Aghion, 1934). A base bioquímica para o acúmulo lipídico na DG foi determinada nos anos 1960, quando Brady demonstrou que a via de biossíntese do glicocerebrosídeo era normal em pacientes com DG, porém o primeiro passo da via de degradação, pela glicocerebrosidase, estava comprometido (Brady *et al.,* 1966). Após isso, a glicocerebrosidase foi identificada como um enzima lisossomal, sendo a DG caracterizada como doença de depósito lisossômico (Brady *et al.,* 1972). Utilizando um substrato natural e marcado com material radioativo, Kampine e Brady demonstraram que a deficiência de glicocerebrosidase poderia ser detectada em leucócitos (Kampine *et al.,* 1967), técnica modificada por Beutler, com a utilização de um substrato artificial fluorogênico (Beutler *et al.,* 1970), sendo utilizada até hoje para diagnóstico dos pacientes. Em 1974, Brady provou o conceito de terapia de substituição enzimática, após testar a infusão de glicocerebrosidase purificada de placentas humanas em 3 pacientes, com diminuição do glicocerebrosideo hepático e plasmático (Brady *et al.,* 1974), culminando em um ensaio clínico com 12 pacientes em 1991, base para a aprovação da primeira terapia de reposição com enzima recombinante (Barton *et al.,* 1991). Em 1989, o gene *GBA1*, que codifica a enzima glicocerebrosidase foi descrito, localizado no cromossomo 1 (Horowitz *et al.,* 1989).

1.1.3) Genética

A DG é herdada de forma autossômica recessiva. O gene *GBA1* está localizado na região 1q21.31 e compreende uma região de 7,6 kb (Cormand *et al.,* 1997). É composto por 11 éxons e possui um pseudogene com 5 kb localizado 16 kb *downstream* que contribui para algumas mutações recombinantes (Horowitz *et al.,* 1989). Já foram descritas 421 mutações nesse gene e dessas, 62% são substituições de nucleotídeos únicos, enquanto

inserções, deleções e outros alelos complexos são responsáveis pelos outros 38% (Human Gene Mutation Database, novembro 2014).

Existem três mutações mais frequentes nos pacientes com DG. A mutação c.1226A>G (N370S), que compreende 53% dos alelos mutados (Charrow *et al.,* 2000) é uma mutação de sentido trocado que resulta em atividade enzimática residual. É encontrada mais comumente em europeus não-judeus e em judeus Ashkenazi (Beutler *et al.,* 1993). A mutação c.1448T>C (L444P), compreende 18% dos alelos mutados (19), é mais comum na Suécia e no norte da Europa e está geralmente associada a um fenótipo neuropático quando presente em homozigose (Dahl *et al.,* 1990). A mutação nula c.84dupG (84GG) representa 7% dos alelos mutados (Charrow *et al.,* 2000) e até o momento não foram descritos pacientes homozigotos, possivelmente pela letalidade pré-natal. Um único alelo p.N370S, independente da segunda mutação, parece ser suficiente para a proteção de um fenótipo neuropático. Em judeus Ashkenazi, as três mutações constituem 94% dos alelos mutantes, sendo a N370S responsável por 70%.

1.1.4) Fisiopatogênese

A DG é causada pela deficiência da enzima lisossomal GBA, uma glicoproteína enzimática necessária para o metabolismo da glicosilceramida. Esse glicoesfingolipídio é a base para a fabricação de globosídeos e gangliosídeos que são importantes componentes das membranas celulares, com diversos receptores e *rafts* lipídicos (Messner *et al.,* 2010). A saposina C é um cofator e ativador essencial da GBA e, em conjunto com uma segunda proteína transportadora é necessária para a transferência da GBA do retículo endoplasmático para o lisossomo (Tamargo *et al.,* 2012). Raros casos de DG, a maioria com apresentação neuropática, foram atribuídos à deficiência de saposina C (Tylki-Szymanska *et al.,* 2007). A deficiência de GBA leva ao acúmulo intracelular de glicosilceramida e outros glicoesfingolipídios, como o gangliosídeo GM3, uma molécula sinalizadora importante e a glicosilesfingosina, um lipídio potencialmente tóxico (Dekker *et al.*, 2011).

Embora níveis elevados de glicosilceramida ocorram em diversos tipos celulares, o acúmulo em macrófagos é mais proeminente devido à sua função fagocítica (Schaefer *et al.,* 1981). Como os macrófagos estão presentes em maior número na medula óssea, fígado, baço e pulmões, esses órgãos chegam a acumular de 20 a 100 vezes mais glicosilceramida que o normal em pacientes com DG (Svennerholm *et al.,* 1982).

As principais manifestações clínicas da DG resultam do acúmulo progressivo de macrófagos ingurgitados com glicolipídios (células de Gaucher), nos diferentes órgãos e tecidos já citados. No entanto, histologicamente, as células de Gaucher são responsáveis por apenas uma fração pequena do aumento do volume do baço e do fígado, sendo a maior parte relacionada a componentes celulares hiperplásicos inflamatórios (Allen *et al.,* 1997). As células de Gaucher possuem de 20-100µm de diâmetro, apresentam o núcleo excêntrico e o citoplasma com estrias. A detecção de marcadores de superfície de macrófagos e a intensa atividade fagocítica confirmam a ontogenia de fagócitos mononucleares (Boven *et al.,* 2004). Todas as células derivadas de macrófagos incluindo não somente as células de Kupffer e macrófagos do baço e da medula óssea, mas também osteoclastos, micróglia, macrófagos alveolares, linfonodos estão envolvidos na patogênese da DG (Pastores *et al.,* 1997).

As células de Gaucher e outras células do sistema imune secretam enzimas lisossomais e catepsinas. Além disso, mediadores inflamatórios como IL-6, IL-8, IL-10 e proteínas inflamatórias macrofágicas (MIP) 1α e 1β são detectáveis nos órgãos afetados e no sangue periférico (Hollak *et al.,* 1997). O acúmulo de glicocerebrosídeo nos macrófagos leva a uma mudança de fenótipo conhecido como ativação alternativa devido à expressão de moléculas de superfície e citocinas distintas das encontradas na ativação dependente de interferon gama, a qual é chamada de ativação clássica. Essa ativação alternativa está envolvida na inflamação crônica, processo de cicatrização e fibrose (Boven *et al.,* 2004).

Em pacientes com formas neuropáticas de DG, a glicosilesfingosina, a forma deacetilada da glicosilceramida, está aumentada em células neuronais e

participa de forma importante no dano neurológico apresentado por desregular a homeostase do cálcio (Pelled *et al.,* 2005).

1.1.5) Manifestações clínicas e variabilidade fenotípica

A DG envolve órgão viscerais, medula óssea e ossos em todos os pacientes afetados. A gravidade da doença pode ir desde pacientes assintomáticos até a forma letal perinatal, com suas manifestações clínicas em idades variáveis. Classicamente a DG é dividida em três formas principais, definidas pelas características clínicas, curso da doença e prevalência étnica. No entanto, há uma gama de achados que se sobrepõem entre as formas clássicas, levando a avaliar a DG como um espectro contínuo e não como três subtipos distintos (Beutler and Grabowski, 2001; Sidransky, 2004).

A DG tipo I (MIM #230800) é a forma mais prevalente e ocorre com maior frequência na população de judeus Ashkenazi, embora a maioria dos pacientes com DG tipo I não sejam judeus. As DG tipo II e III são menos comuns e ocorrem em todas as etnias. O tipo I se distingue do tipo II (MIM #230900) e do tipo III (MIM #231000) pelo não envolvimento do sistema nervoso central, embora alguns estudos documentem características neurológicas em pacientes do tipo I diferentes das vistas em pacientes com tipo II ou III (Biegstraaten *et al.,* 2008). Pacientes com DG que apresentam envolvimento neurológico (DG neuropática) são designados como tipo II ou tipo III de acordo com a natureza aguda ou crônica, respectivamente (Tabela 1).

Características de todos os tipos de DG ao diagnóstico (Charrow *et al.,* 2000;Kaplan *et al.,* 2006)

- Esplenomegalia (85% dos pacientes)

- Hepatomegalia (63% dos pacientes)

- Anemia (34% dos pacientes)

- Trombocitopenia (68% dos pacientes)

- Sangramento

- Osteopenia e fraturas patológicas (osteopenia – 55%; fraturas – 7%; crises ósseas – 7%)

- Dor óssea (33% dos pacientes)

- Retardo de crescimento (36% dos pacientes)

O tipo II constitui 1% dos pacientes e o tipo III, 7%. As manifestações mais comuns das formas neuropáticas são: atraso do desenvolvimento, estrabismo, paralisia do olhar vertical (tipo II e III), hidropsia fetal não imune, ictiose congênita (tipo II) e demência progressiva, ataxia e mioclonias (tipo III).

DG tipo I

A DG tipo I (não neuropática) é a forma mais frequente, entre 90 e 95% dos casos. Sua incidência varia de 1 em cada 20.000 a 1 em cada 200.000 nascidos vivos na população mundial (Altarescu *et al.,* 2000), alcançando 1 em cada 400 nascidos vivos entre os judeus Ashkenazi (Zimran *et al.,* 1991). De acordo com a Associação Brasileira de Pacientes com Doença de Gaucher há em torno de 700 pacientes diagnosticados no Brasil.

Essa forma de DG afeta crianças e adultos de qualquer idade e as manifestações clínicas típicas incluem hepatoesplenomegalia, anemia, trombocitopenia e doença óssea. As citopenias ocorrem devido ao sequestro esplênico e infiltração da medula óssea. O acúmulo de células de Gaucher na medula óssea leva a dores crônicas, osteopenia, lesões líticas, fraturas e osteonecrose. A progressão da doença varia e a sobrevida pode ser normal, dependendo da gravidade das complicações (Barranger *et al.,* 2001). As manifestações clínicas que se apresentam durante a primeira ou segunda década de vida, geralmente, são mais agressivas e progridem com gravidade maior do que as que aparecem em estágios mais avançados de vida.

Espenomegalia é a manifestação visceral mais comum. O baço pode estar aumentado até 75 vezes, mas a média é 15,2 vezes o tamanho normal (Kaplan *et al.,* 2006). Hepatomegalia não é incomum, com tamanho do fígado entre 2 a 3 vezes do normal, em média. Embora fibrose hepática possa ocorrer como progressão natural da doença, com a instituição do tratamento específico, cirrose, hipertensão portal e falência hepática, raramente ocorrem (Charrow *et al.,* 2000). Os pacientes submetidos à esplenectomia devido a graves citopenias ou devido ao desconforto abdominal, tendem a apresentar doença óssea e doença hepática mais proeminentes (Deegan *et al.,* 2011; Lachmann *et al.,* 2000). Sabidamente a esplenectomia melhora as citopenias, porém, sem um tratamento específico, a medula óssea apresenta infiltração progressiva causando anemia e trombocitopenia.

A doença óssea é responsável por grande parte da morbidade apresentada pelos pacientes com DG. Dores ósseas e nas articulações, às vezes associadas à crise de dor (devido a infartos agudos) podem ser debilitantes (Landgren *et al.,* 2007). Necrose avascular pode levar a alterações irreversíveis de articulações do quadril, joelhos e ombros. Lesões osteolíticas, fraturas patológicas e compressão medular por fratura ou massas extraósseas (Gaucheromas) também contribuem para a morbidade.

As crianças afetadas tendem a apresentar atraso da velocidade de crescimento e a puberdade pode ser atrasada em até 60% dos pacientes (Kaplan *et al.,* 2006).

Doença pulmonar intersticial com grave hipoxemia devido à proliferação de macrófagos alveolares ingurgitados pode ocorrer. A hipertensão pulmonar é independente da doença infiltrativa e é devida à oclusão dos capilares pulmonares e a lesões induzidas por citocinas inflamatórias, representando uma complicação com risco de morte (Ross *et al.,* 1997). A síndrome hepatopulmonar devido ao *shunt* anormal dos pulmões também leva à hipoxemia e cirrose hepática (Kim *et al.*, 1999; Bouguila *et al.,* 2012). Esses sintomas geralmente ocorrem em pacientes que foram submetidos à esplenectomia.

Tradicionalmente a DG tipo I é considerada não-neuropática, porém sequelas neurológicas, principalmente em pacientes de meia idade e idosos tem sido cada vez mais reportadas, especialmente polineuropatia (Biegstraaten *et al.,* 2010). Por razões ainda não elucidadas, parkinsonismo é encontrado de forma mais frequente em portadores de mutações em *GBA1*, além de ser encontrado mais do que o esperado em pacientes com DG (Rosenbloom *et al.,* 2011). Esses pacientes apresentam sintomas parkinsonianos em idade mais jovem do que pacientes com doenças de Parkinson típica, além de apresentarem manifestações atípicas como demência progressiva resistente às medicações comumente utilizadas para o tratamento de doenças de Parkinson.

Os pacientes com DG possuem ainda risco aumentado para diferentes tipos de malignidades como linfomas, leucemias e mieloma múltiplo (Landgren *et al., 2007;* Cheung *et al., 2007*), possivelmente precipitados pelo *status* pró-inflamatório e anormalidades do sistema imune.

DG tipo II

A forma neuropática aguda ocorre em menos de 1 em cada 100.000 nascidos vivos e geralmente afeta crianças entre 4 e 5 meses de vida, com comprometimento do cérebro, baço, fígado e pulmões. A clínica neurológica é grave com envolvimento bulbar (estridor, estrabismo, dificuldades para engolir) e piramidal (opistótono, espasticidade e trismo). A evolução é rápida e os pacientes falecem até o final do segundo ano de vida, principalmente devido à falência pulmonar (Mignot *et al.,* 2006).

DG tipo III

A incidência da forma neuropática subaguda é em torno de 1 em cada 100.000 nascidos vivos. Distribui-se por todas as populações, mas predomina em regiões do nordeste da Suécia (Dahl *et al.,* 1990). Os pacientes com DG tipo III podem apresentar manifestações sistêmicas semelhantes a pacientes com o tipo I e o comprometimento neurológico pode se manifestar em qualquer idade, geralmente com epilepsia, ataxia, paralisia do olhar vertical ou demência

(Davies *et al.,* 2007). Alguns pacientes podem apresentar opacificação de córnea, doença cardíaca valvular e calcificação progressiva. A expectativa de vida é de 20 a 30 anos (Tylki-Szymanska *et al.,* 1999).

Tabela 1: Comparação dos tipos de Doença de Gaucher (Balwani *et al.,* 2010; Gupta *et al.,* 2011; Cox *et al.,* 1997)

	Tipo I	**Tipo II**	**Tipo III**
Início dos sintomas	Infância até adulto	Primeiro ano de vida	Infância
Hematológico	Anemia, trombocitopenia	Mínima trombocitopenia	Anemia
Ossos	Osteopenia, osteoesclerose	Mínimo comprometimento	Osteopenia, osteoesclerose
Neurológico	Não	Epilepsia, hipertonia, atraso neuropsicomotor grave, apnéia	Mioclonias, demência progressiva, ataxia
Outros sistemas	Fibrose hepática, hipertensão pulmonar, gamopatias	Ictiose congênita	Ocular, cardíaco e vascular
Progressão	Lenta	Rápida	Variável
Expectativa de vida	Diminuída, mas pode ser normal	Morrem antes dos 2 anos de vida	20-30 anos
Mutação mais associada	c.1226A>G (N370S)	Várias	c.1448T>C (L444P)
Prevalência étnica	100 vezes mais comum em judeus Ashkenazi	Não	Suecos

Diversos genes modificadores, genes contíguos, proteínas transportadoras e fatores ambientais podem influenciar o fenótipo dos pacientes com DG.

Dentre os fatores genéticos, está a expressão do gene *PSAP* (localizado no cromossomo 10q21), que codifica o cofator saposina C, uma proteína ativadora da glicocerebrosidase. Indivíduos homozigotos para mutações no gene *PSAP* podem também desenvolver a DG (Pampols *et al.,* 1999).

A eficiência do mecanismo de transporte da glicocerebrosidase para os lisossomos é influenciada por mutações nos genes que codificam proteínas de membrana associadas aos lisossomos (LAMP-1 e LAMP-2), e que participam do transporte intracelular da glicocerebrosidase a partir do retículo

endoplasmático, podendo influenciar o fenótipo de pacientes com DG (Whitfield *et al.,* 2002).

Um fator possivelmente relacionado às manifestações ósseas é a presença de mutações no gene que codifica a interleucina-6 (IL-6), secretada por macrófagos, que estimula a reabsorção óssea (Altarescu *et al.,* 2003).

Apenas os mecanismos acima não são suficientes para explicar a variabilidade clínica apresentada pelos pacientes com DG, já que há descrição de fenótipos diferentes em gêmeos monozigóticos, corroborando para a existência de genes modificadores e indicando uma possível importância da influência ambiental (Biegstraaten *et al.,* 2011; Lachmann *et al.,* 2004).

1.1.6) Achados laboratoriais

Trombocitopenia e anemia são características marcantes em pacientes com DG. Outra alteração hematológica mais rara observada é a leucopenia, porém não suficiente para causar aumento do número ou gravidade de infecções. Os pacientes podem apresentar elevação aguda de enzimas hepáticas, o que pode ser atribuído à colecistite já que colelitíase é um achado mais frequente em pacientes com DG do que na população geral (Taddei *et al.,* 2010). Alguns biomarcadores estão elevados em pacientes com DG como a enzima conversora de angiotensina, fosfatase ácida resistente ao tartarato, CCL18/PARC, ferritina e quitotriosidase (Hollak *et al.,* 1994; Boot *et al.,* 2004; Stein *et al.,* 2010). A magnitude da elevação dos biomarcadores não se correlaciona com a gravidade clínica apresentada pelos pacientes, porém é útil para monitorar a resposta ao tratamento. Estudos sobre novos biomarcadores estão emergindo e a glicosilesfingosina parece ser uma molécula mais específica do que as anteriores para diagnóstico e monitorização da DG (Rolfs *et al.,* 2013). As células de Gaucher estão presentes em biópsias de medula óssea, fígado e baço, porém não são patognomônicas, pois já foram relatadas em outras doenças como leucemia mielóide crônica, anemia diseritropoética, talassemia, anemia falciforme, mieloma múltiplo e infecções por micobactérias,

por exemplo (Bain *et al.,* 2010; Busarla *et al.,* 2013; Sharma *et al.,* 2007; Sharma *et al.,* 2014).

1.1.7) Achados de imagem

A partir de um registro internacional de DG (Charrow *et al.,* 2000) os seguintes achados foram reportados:

- A deformidade em frasco de Erlenmeyer do fêmur distal, causada pelo remodelamento ósseo, foi encontrada em 46% dos pacientes.

- Lesões ósseas irreversíveis foram encontradas em 17% dos pacientes homozigotos para a mutação N370S e 26% dos pacientes heterozigotos compostos com essa mutação.

- Infiltração da medula óssea avaliada por ressonância nuclear magnética foi encontrada em 40% dos pacientes, embora essa observação seja provalmente subreportada pela dificuldade de realização do exame e da existência de examinadores experientes. Em outras populações com DG, quando o estudo é realizado sob condições controladas, a prevalência de detecção de infiltração na medula óssea dos pacientes atinge os 100%.

- Osteopenia avaliada por densitometria óssea foi encontrada em 42% dos pacientes.

1.1.8) Diagnóstico

Bioquímica

O diagnóstico bioquímico definitivo de DG requer confirmação da deficiência da atividade da enzima glicocerebrosidase em leucócitos ou fibroblastos. Indivíduos com suspeita clínica e níveis enzimáticos não característicos requerem confirmação com análise em fibroblastos ou análise molecular do gene *GBA.* Os valores de referência variam de acordo com o

laboratório e a técnica utilizada. A atividade residual enzimática não se correlaciona com a gravidade da doença, além disso, a atividade enzimática de indivíduos heterozigotos pode se sobrepor aos valores de indivíduos saudáveis ou afetados (Michelin *et al.,* 2004).

Pacientes com DG apresentam níveis elevados da enzima quitotriosidase plasmática (sintetizada por macrófagos ativados). Embora possa auxiliar no diagnóstico, é importante lembrar que em torno de 6% da população apresentam deficiência dessa enzima (Hollak *et al.,* 1994), o que não leva a repercussões clínicas.

Genético

A análise de mutações comuns do gene *GBA* é um método eficaz para confirmar o diagnóstico de DG e a primeira escolha para identificar portadores entre os familiares de pacientes com genótipo conhecido. No entanto, ao não se encontrar ambas as mutações, há possibilidade de realização de sequenciamento completo do gene, método já disponível clinicamente.

A análise de DNA auxilia na classificação dos pacientes e na predição de achados clínicos. Por exemplo, até 75% dos indivíduos homozigotos para a mutação c.1226A>G (N370S) apresentam sintomas de DG (Strasberg *et al.,* 1994), porém sem envolvimento neurológico (Sidransky *et al.,* 1994). A mutação D409H está associada a envolvimento cardíaco e da córnea (Chabas *et al.,* 1996).

De acordo com um estudo de 221 pacientes, o genótipo mais comum em pacientes brasileiros é o N370S/L444P (Sobreira *et al.,* 2007).

1.1.9) Tratamento

Durante muitos anos, a DG foi manejada com tratamento sintomático e com medidas paliativas, tais como a esplenectomia, utilizada para atenuar o atraso no crescimento, as citopenias e a compressão abdominal. O transplante

de medula óssea foi utilizado com sucesso, porém após o desenvolvimento de enzimas recombinantes na década de 90 a terapia de reposição enzimática (TRE) é o tratamento de escolha. Essa terapia tem permitido a melhora da qualidade de vida para os pacientes através da reversão de muitos sinais e sintomas (Mistry *et al.,* 2007; Hollak *et al.,* 2009). Entretanto, a quantidade de enzima necessária para manter a qualidade de vida e a reversão dos sintomas ainda é controversa. Dados de um registro internacional de DG mostram que baixos níveis de hemoglobina atingem níveis normais após 12 meses de tratamento, enquanto os níveis de plaquetas aumentam significativamente nos primeiros dois anos de tratamento e, em pacientes que não tem seus níveis normalizados, o número de plaquetas continua a aumentar mais lentamente nos anos subsequentes. A respeito da doença visceral, a hepatomegalia geralmente é resolvida após dois anos enquanto a esplenomegalia diminui em 60%, embora pacientes que iniciam TRE com esplenomegalia importante podem nunca ter o baço menor do que 5 vezes o volume normal (Weinreb *et al.,* 2002). Mais de 50% dos pacientes com doença óssea apresentam significativa melhora e 90% dos pacientes que apresentavam crises ósseas, não apresentaram recorrência (Poll *et al.,* 2002).

A TRE utilizada nos pacientes com DG é um tratamento de alto custo (de 100 a 300 mil dólares/ano por paciente) (Hollak *et al.,* 2011; de Souza *et al.,* 2010). O tratamento deve ser administrado mediante infusões do medicamento a cada 14 dias, sob a supervisão de um profissional da saúde treinado (médico, enfermeiro, etc.). No manejo da medicação, principalmente em locais que atendem a diversos pacientes, é importante a presença de um farmacêutico para o monitoramento da adequação e fracionamento das doses. A resposta à TRE é distinta e de acordo com os tipos da DG, sendo que os pacientes do tipo II e III respondem pior que os pacientes do tipo I, visto que ores a glicocerebrosidase recombinante não ultrapassa a barreira hemato-encefálica (Hollak *et al.,* 2009).

Segundo o Ministério da Saúde, encontram-se atualmente em tratamento com TRE, no Brasil, em torno de 670 pacientes. A venda mundial da enzima recombinante, que em 2005 foi ofertada ao restrito grupo de aproximadamente 4.000 pacientes no mundo inteiro, chega a um bilhão de

dólares por ano (Pastores *et al.,* 2005). O Brasil é o terceiro país do mundo em número de pacientes em tratamento, portanto é fácil perceber a importância econômica desse tratamento no país, onde a terapia é subsidiada pelo sistema público de saúde, demostrando que a DG pode ser de baixa incidência, mas de significativo impacto econômico.

Até 2009, existia somente a imiglucerase (produzida pelo laboratório *Genzyme Corporation,* Cambridge, MA, EUA) como enzima recombinante para TRE. Atualmente, novas opções surgiram no mercado brasileiro e internacional como a alfavelaglicerase (*Shire HGT,* Dublin, Irlanda) e a taliglucerase alfa (*Protalix,* Carmiel, Israel). Apesar da histórica utilização de imiglucerase, estudos demonstram que as três enzimas recombinantes são similares tanto estruturalmente quanto na manutenção de parâmetros clínicos adequados pelos pacientes (Elstein *et al.,* 2011). Cerca de 1% dos pacientes tem reações adversas à TRE com imiglucerase e produzem anticorpos contra a enzima sintética, fazendo com que a manutenção das infusões de tal enzima nesses pacientes seja feita com parcimônia. Os eventos adversos incluem febre, calafrios, *rash* e sintomas gástricos. O mecanismo de reação é imunomediado, sendo reações relacionadas à IgE, raras e potencialmente fatais. Em torno de 13-15% dos pacientes desenvolvem anticorpos IgG contra imiglucerase, 6% contra taliglucerase alfa e 1% contra alfavelaglicerase (Pastores *et al.,* 2014; Zimran *et al.,* 2013; van Dussen *et al.,* 2013), embora essas diferenças não pareçam ser clinicamente significantes.

Há um tratamento alternativo aprovado pela EMA, FDA e ANVISA: a terapia de redução de substrato (TRS) com miglustate (Zavesca®, *Actelion Pharmaceuticals*, Freiburg, Alemanha) indicada para pacientes adultos com DG que apresentam contraindicação ao uso de imiglucerase. Essa terapia é feita com a utilização de inibidores da síntese de substrato que atuam na via dos esfingolipídios (Pastores *et al.,* 2005; Platt *et al.,* 1997; Cox *et al.,* 2000). Em vez de substituir a enzima deficiente, a terapia de redução de substrato visa diminuir a síntese do glicocerebrosídeo que se acumula nos tecidos dos pacientes com DG. A vantagem do miglustate é que, por ser uma molécula pequena, de administração oral, não provoca resposta imunológica e pode cruzar a barreira hemato-encefálica. Além de atuar como inibidor da

glicosiltransferase, envolvida na síntese do glicocerebrosídeo, esse mesmo composto pode funcionar como uma chaperona, aumentando a atividade enzimática da β-glicosidase quando usado em baixas concentrações (Alfonso *et al.,* 2005). No entanto, os efeitos colaterais dessa terapia são mais significativos que os da TRE, incluindo diarreia, tremor e parestesia, entre outros (McCormack *et al.,* 2003). Em agosto de 2014, foi aprovada pelo FDA uma nova opção para TRS, o eliglustate (Cerdelga®, *Genzyme Corporation,* Cambridge, MA, EUA), uma molécula análoga à glicosilceramida, que se apresentou eficaz e com menos efeitos adversos que o miglustate (Poole *et al.,* 2014), porém não ultrapassa a barreira hemato-encefálica.

Outros tratamentos para as doenças lisossômicas estão em desenvolvimento e representam uma perspectiva futura de tratamento para a DG, incluindo o uso de chaperonas (Motabar *et al.,* 2010), a terapia gênica (Jin *et al.,* 2011; Giraldo *et al.,* 2011) e o transplante de células-tronco (Ringden *et al.,* 2006). Ademais, o alto custo da TRE pode restringir seu potencial de uso, o que já ocorre em alguns países (Connock *et al.,* 2006; Mrsic *et al.,* 2007; Correa *et al.,* 2009). Eventualmente esses novos tratamentos poderão ser utilizados de forma complementar à TRE (Elstein *et al.,* 2011).

1.2) Doença de Gaucher no Centro de Referência do Rio Grande do Sul

O Centro de Referência Estadual para DG foi implementado em 2003, no Hospital de Clínicas de Porto Alegre (HCPA), e segue todas as orientações previstas no protocolo do Ministério da Saúde. Todos os processos administrativos, solicitando ao Rio Grande do Sul o fornecimento de enzima recombinante, são avaliados pelo médico do centro; caso tenha o seu processo deferido, o usuário é encaminhado para atendimento neste local. Dos 41 pacientes diagnosticados com DG no estado (dois falecidos, um com DG tipo I e um com DG tipo II), uma paciente está em uso de miglustate, duas pacientes em uso de eliglustate (em protocolo de pesquisa clínica), 2 pacientes fora de tratamento e 34 pacientes estão em tratamento com TRE (31 pacientes com DG tipo I e 3 pacientes com DG tipo III). Oito pacientes atualmente realizam as

infusões no Centro e os demais realizam as infusões em outros locais. Entretanto, todos os pacientes, inclusive os que não realizam as infusões no Centro estão sendo acompanhados e monitorizados no HCPA, por meio de avaliações trimestrais, conforme previsto no protocolo.

Até 2010, a medicação escolhida pelo Ministério da Saúde para fornecimento a todos os pacientes brasileiros era a imiglucerase. Devido à contaminação na fabricação da enzima, houve interrupção no fornecimento a partir da metade de 2010 até o início de 2011. Em caráter emergencial, a enzima taliglucerase alfa foi aprovada pela ANVISA por um período de quatro meses para suprir a falta da imiglucerase, sendo a medicação de escolha, protocolada pelo Ministério da Saúde, até o final de 2011, quando foi publicada uma nova portaria regularizando a utilização das três enzimas recombinantes (imiglucerase, taliglucerase alfa e alfavelaglicerase), além da terapia de redução de substrato com miglustate. No momento, há pacientes em tratamento com as três enzimas recombinantes e com miglustate.

1.3) O sistema imune na Doença de Gaucher

A DG é a doença lisossômica com maior envolvimento da imunidade, tanto que uma das hipóteses existentes para explicar a expressão fenotípica desta doença é a existência de um estímulo crônico do sistema imune (Schoenfeld *et al.,* 1982). O consequente acúmulo de glicosilceramida e glicosilesfingosina nos lisossomos é o cerne da patogênese da DG, com o envolvimento de macrófagos de órgãos viscerais, cérebro e ossos (Mizukami *et al.,* 2002), porém há cada vez mais estudos sobre o envolvimento de monócitos, células dendríticas, linfócitos T, linfócitos B, células NK e suas inter-relações (Figura 2).

Os pacientes com DG apresentam níveis elevados de IgA, IgG e IgM, auto anticorpos (Jurecka *et al.,* 2011; Shoenfeld *et al.,* 1995) e uma maior incidência de gamopatia policlonal, gamopatia monoclonal de significância

indeterminada (MGUS) e mieloma múltiplo (MM) (de Fost *et al.,* 2008; Hughes *et al.,* 2009). Esta resposta imune poderia gerar uma resposta inflamatória levando a dano celular, mediado por citocinas. Algumas das citocinas encontradas elevadas em pacientes com DG incluem IL-1, IL-6, IL-10, TNF-α, M-CSF, dentre outras (Hollak *et al.,* 1997; Barak *et al.,* 1999). Como citocinas exercem um papel regulatório importante em células do sistema imune, alguns autores atribuem o seu desbalanço a um estado pró-inflamatório e ao desenvolvimento de malignidades hematológicas (Brody *et al.,* 2006; Costello *et al.,* 2006; Hawkesford *et al.,* 2011; Rosenbloom *et al.,* 2005).

Abaixo encontra-se um resumo dos tipos celulares e moléculas do sistema imune envolvidas na fisiopatogênese da DG.

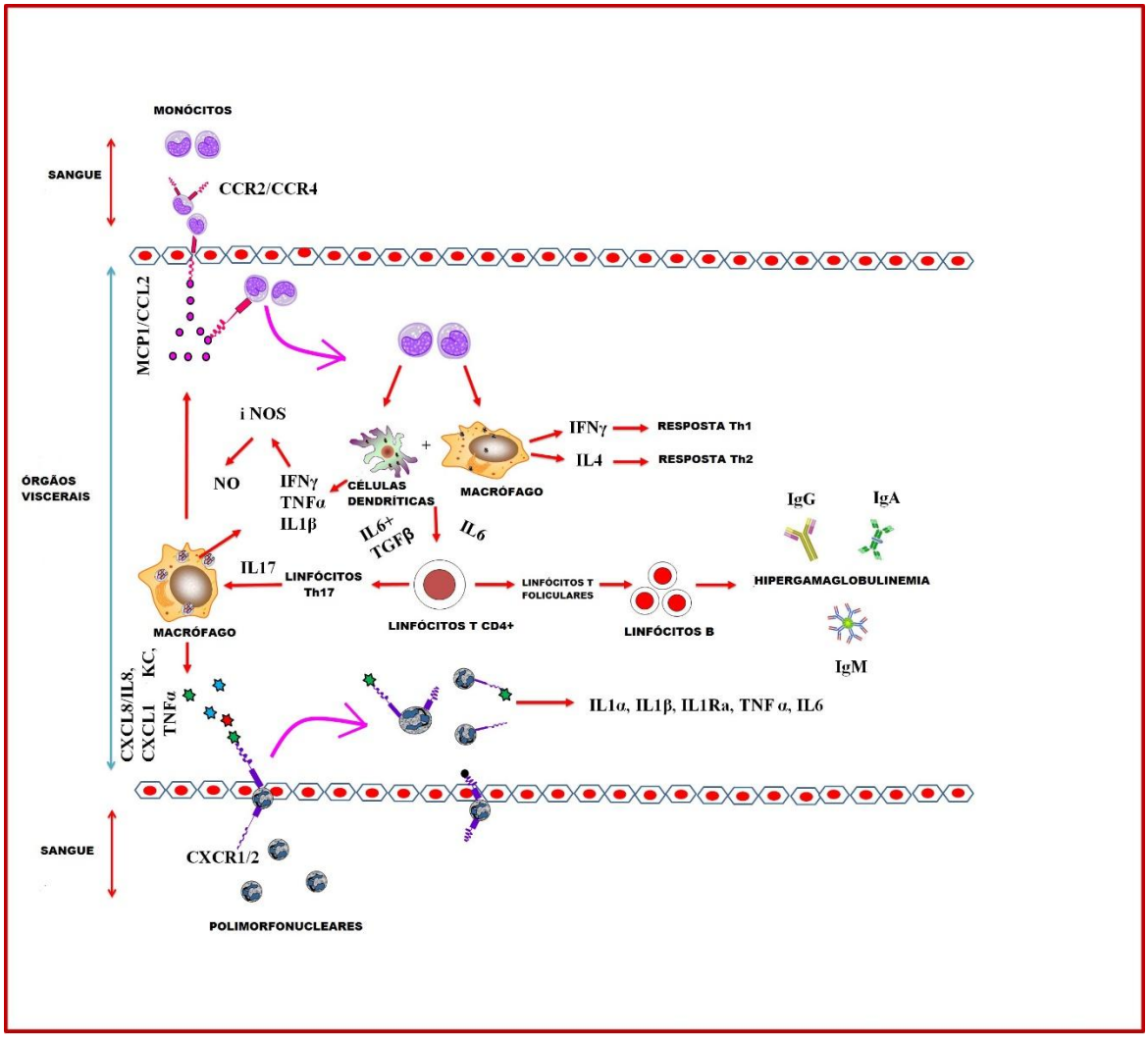

Figura 2: Modelo esquemático da propagação inflamatória na Doença de Gaucher. A ativação dos macrófagos devido ao excesso de glicosilceramida (GC) dá início à liberação de quimiocinas CC, por exemplo MCP1 ou CCL2, que causam o recrutamento de monócitos do sangue até os órgãos periféricos. Essas células maturam-se em macrófagos e células dendríticas que são ativadas e liberam interferon gama (INF-γ), interleucina 4 (IL-4), IL-6, e fator de crescimento transformador beta (TGF-β). As citocinas INF-γ e IL-4 estimulam respostas mediadas por linfócitos T helper 1 (Th1) e T helper 2 (Th2), enquanto a IL-6 facilita o desenvolvimento de linfócitos T foliculares (Tfh). A IL-4 ainda estimula respostas mediadas por linfócitos B. Essas respostas levam à formação e ativação do centro germinal que estimula a diferenciação de linfócitos B e produção de imunoglobulinas (IgG, IgA e IgM) e hipergamaglobulinemia. A IL-6 em conjunto com TGF-β estimula o desenvolvimento de linfócitos Th17, que produzem IL-17 e subsequentemente há produção de CXCL8/IL-8. Macrófagos ingurgitados com GC secretam CXCL1, IL-1β, IFN-γ e TNF-α que são críticos para o recrutamento dos polimorfonucleares (neutrófilos) e liberação de seus produtos (TNF-α, IL-6, IL-1α, IL-1β e IL-1Ra) nos órgãos viscerais. Também, TNF-α e IL-1β induzem a formação de óxido nítrico sintase induzida (iNOS) após a produção de óxido nítrico (NO) o que estimula a inflamação na Doença de Gaucher. Adaptada de Pandey *et al*., 2013.

Monócitos

São células mononucleares presentes no sangue, medula óssea e baço. Após ativação, eles migram do sangue para os tecidos, fabricam citocinas inflamatórias e se diferenciam em macrófagos ou células dendríticas. Os monócitos são definidos pela expressão de CD11b, CD11c e CD14, e ausência de marcadores de linfócitos B, T e células NK (Muller *et al.,* 2001). Em pacientes com DG, os monócitos apresentam atividade microbicida diminuída devido à supressão de geração de superóxido (Liel *et al.,* 1994). Em contraste, com o aumento da expressão de moléculas MHC classe II e moléculas CD1d, os monócitos aumentam a ativação de linfócitos T CD4+ (Balreira *et al.,* 2005). Além disso, INF-γ, IL-4 e MCSF, que estão aumentados em pacientes com DG, são críticos para a diferenciação dos monócitos em células inflamatórias ativadas.

Macrófagos

O ganhador do prêmio Nobel, Metchnikoff, classificou os fagócitos em macrófagos (“comedores grandes”), micrófagos (um tipo celular fagocítico menor) e os leucócitos polimorfonucleares, agora conhecidos como granulócitos. Ele estabeleceu que todos os tipos celulares fagocíticos apresentavam papel importante na defesa do hospedeiro contra infecções (Kaufmann *et al.,* 2008) e determinou sua relação com o baço, linfonodos, medula óssea e tecido conjuntivo, levando ao termo sistema macrofágico (Gordon *et al.,* 2008). Algumas citocinas como o INF-γ, GM-CSF, TNF-α e produtos microbianos (lipopolissacarídeos) estimulam a ativação do macrófago e induzem a extensa cascata pró-inflamatória necessária para a defesa contra patógenos intracelulares. Na DG, os macrófagos são funcionalmente e numericamente importantes para o desenvolvimento dos sinais e sintomas preponderantes da doença e, por estarem ingurgitados com glicosilceramida, são chamadas de células de Gaucher. Estudos de expressão gênica em modelos animais de DG determinaram um significativo aumento da via do INF-γ (CCL2, CCL3, CCL9, NOS2, TNF, e IL-6) e da via da IL-4 (CD163 e MMP12) (Xu *et al.,* 2011).

Polimorfonucleares

Também chamados de granulócitos ou neutrófilos são os leucócitos mais abundantes do sangue periférico, medula óssea e locais de inflamação aguda, que influenciam a resposta imune adaptativa através da condução dos patógenos para os linfonodos e modulação das respostas Th1 e Th2 (Tacchini-Cottier *et al.,* 2000). Além de sua ação como fagócito, os polimorfonucleares expressam uma grande variedade de citocinas e quimiocinas em resposta a estímulos fisiológicos e apresentam um papel central nas reações imunes e inflamatórias, por serem as primeiras células recrutadas para o local de inflamação aguda (Jacobs *et al.,* 2010). Em DG, alguns estudos demonstraram um aumento da expressão de quimiocinas CXC (CXCL1 e CXCL8/IL-8), IL-1α, TNF-α e MIP-1α que são importantes para a ativação e recrutamento dos polimorfonucleares (Johnson *et al.,* 2011; Smart *et al.,* 1994). Esses estudos são importantes, visto que uma deficiência na migração dessas células levaria

a um aumento na susceptibilidade à infecções piogênicas, o que já foi sugerido em pacientes com DG, por avaliações *in vitro* (Aker *et al.,* 1993) e corroborado pela experiência clínica em algumas coortes de pacientes não tratados com TRE (Zimran *et al.,* 1994).

Células dendríticas

As células dendríticas foram assim chamadas devido à sua morfologia e, junto com os macrófagos são células apresentadoras de antígeno (APC) que estão presentes em tecidos linfóides e não linfóides (Steinman *et al.,* 1991). São derivadas da medula óssea e migram como precursores pelo sangue para tornarem-se residentes em tecidos específicos como as células de Langerhans na epiderme, por exemplo. Após a invasão do patógeno, são recrutadas para o local da inflamação, capturam o invasor, migram para os linfonodos, apresentam o antígeno e iniciam a imunidade celular tecido-específica (Mellman *et al.,* 2001). Em pacientes com DG não tratados, as células dendríticas estão diminuídas no sangue periférico, porém com atividade normal em comparação a controles saudáveis (Micheva *et al.,* 2006).

Linfócitos B

Os linfócitos B são células críticas para a resposta humoral e tem função similar à APC para gerar resposta imune T celular (Mauri *et al.,* 2012), além de expressar altos níveis de moléculas MHC classe II (Janeway *et al.,* 1994). A ativação do linfócito B influencia a diferenciação de células T *naïve* para um fenótipo Th2 (Croft *et al.,* 1997), demonstrando um papel importante na manutenção da homeostase imunológica. Os linfócitos B são classificados em dois subgrupos: linfócitos B1, presentes em cavidades pleural e peritoneal, baço e placas de Peyers no intestino, enquanto os linfócitos B2 estão presentes no baço e linfonodos (Kroese *et al.,* 1992). As células B2 possuem um papel central na patogênese de doenças autoimunes como a artrite reumatoide, lúpus, esclerose múltipla, tireoidite e diabetes autoimune, enquanto as células B1 podem desenvolver leucemia linfocítica crônica (Chiorazzi *et al.,*

2005). Pacientes com DG apresentam risco de desenvolver malignidades associadas às células B e plasmócitos, além de apresentarem hiperimunoglobulinemia G e M que melhoram após esplenectomia ou terapia de reposição enzimática (Wine *et al.,* 2007; Arikan-Ayyildiz *et al.,* 2011).

Linfócitos T

As células T são classificadas em dois grupos principais: linfócitos T helper CD4+ e T citotóxico CD8+. As células T helper são subdivididas em Th1, Th2, Th17, T regulatória (CD4+CD25+) e T folicular (Tfh), baseadas na produção de citocinas e ativação por fatores de transcrição específicos. As células Th1 produzem IFN-γ, as células Th2 produzem IL-4 e IL-13, as células Th17 produzem IL-17, sendo importantes para a eliminação de patógenos intracelulares (Abbas *et al.,*1996). As células Treg são críticas para a tolerância imunológica, em que o TGF-β tem um papel fundamental (Lee *et al.,* 2009). As células Tfh auxiliam as células B para produzirem anticorpos e desenvolver centros germinativos. As células T são importantes para eliminar células infectadas por patógenos virais (Russell *et al.,* 2002). Os pacientes com DG podem desenvolver linfomas de células T e deficiência de células CD4+ e CD8+ foi encontrada no sangue periférico dos pacientes (Lacerda *et al.,* 1999). A expressão de IL-6 é crítica para respostas mediadas por Tfh, além de ser importante para a maturação de centros germinativos de células B (Crotty *et al.,* 2011). A associação de aumento de IL-6 e linfomas de células T e B e hipergamaglobulinemia em pacientes com DG sugerem a necessidade de maiores estudos sobre as células Tfh, já que a conexão de Tfh e os subtipos B1 e B2 pode explicar o mecanismo fisiopatológico dessas alterações na DG (King *et al.,* 2008).

Células Natural-Killer (NK)

As células NK são linfócitos responsáveis pela interface entre a imunidade inata e adaptativa. Contribuem diretamente para a defesa imune através de funções efetoras como citotoxicidade e secreção de citocinas e

indiretamente, regulando as células APC e as respostas adaptativas dos linfócitos T (Long *et al.,* 2013). Expressam pelo menos um receptor inibitório cuja interação com moléculas de HLA classe I (HLA A, B e C) exerce um importante controle para evitar a resposta a células normais do organismo (Moretta *et al.,* 2004). Essas células reconhecem vários antígenos lipídicos e glicolipídicos através do sistema de apresentação CD1 que é expresso pelas APC (Porcelli *et al.,* 1999). Existem cinco classes de CD1 baseadas na similaridade de sequências. CD1a, -b e -c constituem o grupo I, enquanto o CD1d forma o grupo II. O CD1e representa um intermediário entre os dois grupos e age como chaperona para facilitar o transporte lipídico de CD1b para CD1d (de la Salle *et al.,* 2005).

Um aumento da expressão de CD1d e moléculas MHC classe II foi encontrado em macrófagos de pacientes com DG. O aumento de CD1d foi relacionado à alteração do tráfico de lipídios intracelulares enquanto o aumento de MHC classe II e moléculas HLA-DR foi atribuído ao status pró-inflamatório dos pacientes (Balreira *et al.,* 2005; Florena *et al.,* 2006), além disso, o número de células NK está diminuído na DG (Burstein *et al.,* 1987).

Receptores KIR (Killer cell immunoglobulin-like receptor)

Os receptores KIR são representantes da família das imunoglobulinas e estão presentes na superfície das células NK (Moretta *et al.,* 1990) e em alguns linfócitos T (NKT) (Van Kaer *et al.,* 2007). Até o momento, foram descritos 15 genes *KIR (KIR*2DL1, *KIR*2DL2, *KIR*2DL3, *KIR*2DL4, *KIR*2DL5A, *KIR*2DL5B, *KIR*2DS1, *KIR*2DS2, *KIR*2DS3, *KIR*2DS4, *KIR*2DS5, *KIR*3DL1, *KIR*3DL2, *KIR*3DL3 e *KIR*3DS1) e 2 pseudogenes (*KIR*2DP1 e *KIR*3DP1) (Marsh *et al.,* 2003) localizados na região 19q13.4 (Suto *et al.,* 1996) e, tipicamente, cada um deles possui 9 éxons (Martin *et al.,* 2000) que codificam sequências-líder (éxons 1 e 2), domínios extracelulares (D0, D1, D2; que correspondem aos éxons 3, 4 e 5, respectivamente), a cauda (éxon 6, entre o domínio extracelular e a membrana), a porção transmembrana (éxons 7) e a cauda intracitoplasmática (éxons 8 e 9) (Wilson *et al.,* 2000).

Os receptores KIR são resultado de um sistema polimórfico e estão divididos em grupos ativadores e inibitórios. Os receptores com sinal intracelular inibitório evitam a lise da célula alvo e os ativatórios auxiliam em sua execução (Fan *et al.,* 1997; Biassoni *et al.,* 1996). Possuem uma cauda intracitoplasmática comprida, por isso receberam em sua denominação a letra "L" (do inglês *long*). Já os receptores ativadores possuem cauda curta e receberam a letra "S" (do inglês *short*) (Figura 3).

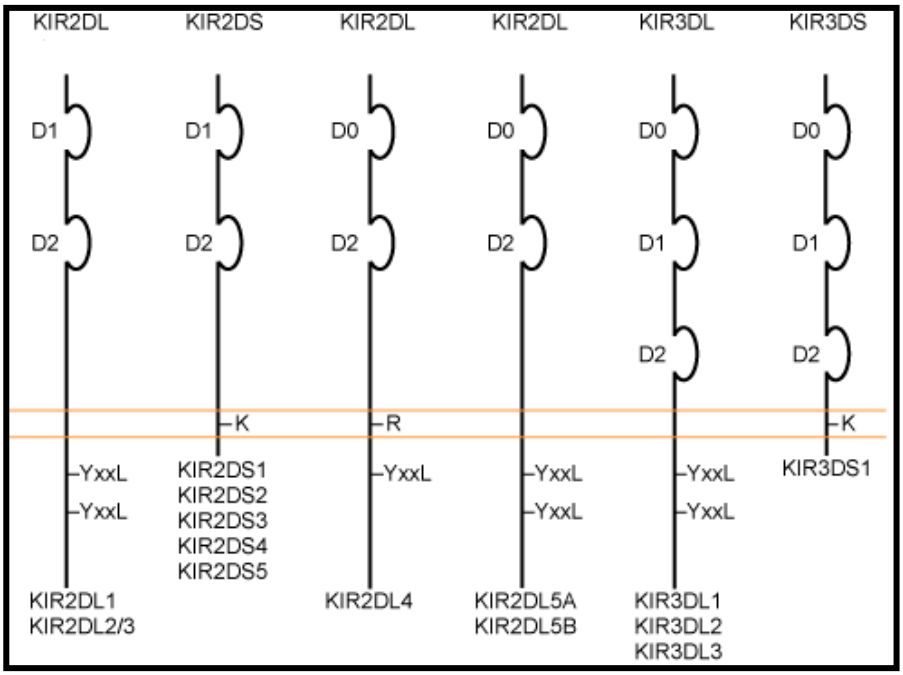

Figura 3: Receptores KIR na membrana celular. Adaptado de Jobim and Jobim, 2008.

Os domínios extracelulares são responsáveis pelo reconhecimento da célula alvo. Alguns possuem dois domínios (denominados 2D, divididos em D1 e D2), com especificidade para HLA-C e outros possuem três domínios (denominados 3D, divididos em D0, D1 e D2) (Wagtmann *et al.,* 1995), com especificidade para HLA-A ou HLA-B. Por exemplo, KIR2DL1 se liga a HLA-Cw2, HLA-Cw4, HLA-Cw5 e HLA-Cw6 (chamados de grupo C2), enquanto KIR2DL3 se liga a HLA-Cw1, HLA-Cw3, HLA-Cw7 e HLA-Cw8 (chamados grupo C1) (Campbell *et al.,* 2011; Ugolotti *et al.,* 2011) (Tabela 2).

Tabela 2: Receptores KIR e seus ligantes HLA (Thielens *et al.,* 2012)

Receptor	Ligante
KIR2DL1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DL2	HLA-C1: C*01, C*03, C*07, C*08 Alguns HLA-C2: C*0501, C*0202, C*0401 Alguns HLA-B: B*4601, B*7301
KIR2DL3	HLA-C1: C*01, C*03, C*07, C*08 Alguns HLA-C2: C*0501, C*0202 Alguns HLA-B: B*4601, B*7301
KIR3DL1	Alguns HLA-A e HLA-B que expressam o epítopo Bw4 HLA B*08, B*27, B*57, B*58 HLA-A: A*24, A*23, A*32
KIR3DL2	Alguns HLA-A: A*03, A*11
KIR3DL3	Desconhecido
KIR2DL5A e B	Desconhecido
KIR2DL4	HLA-G
KIR2DS1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DS2	HLA-C1: C*01, C*03, C*07, C*08 Alguns HLA-C2: C*0501, C*0202, C*0401 Alguns HLA-B: B*4601, B*7301
KIR2DS3	Desconhecido
KIR2DS4	HLA-C: C*0501, C*1601, C*0202 Alguns HLA-A: A*1102
KIR2DS5	Desconhecido
KIR3DS1	Desconhecido

Os genes *KIR* estão na região do complexo de receptores leucocitários (LRC) e entre eles há cerca de 2 kb de intervalo (Parham *et al.,* 2005), formando haplótipos, classificados como A e B (Wende *et al.,* 1999). O haplótipo A possui 9 genes *KIR*, sendo apenas um ativador (KIR2DS4), cinco inibitórios e três estruturais. Já o haplótipo B possui alta diversidade de genes,

tanto ativadores como inibitórios (Figura 4). Quatro genes estão presentes na maioria dos haplótipos (3DL3, 3DP1, 2DL4 e 3DL2), o que sugere conservação e estabilidade em relação à recombinação gênica (Martin *et al.,* 2003). A frequência desses haplótipos varia significantemente em diferentes populações (Middleton *et al.,* 2008).

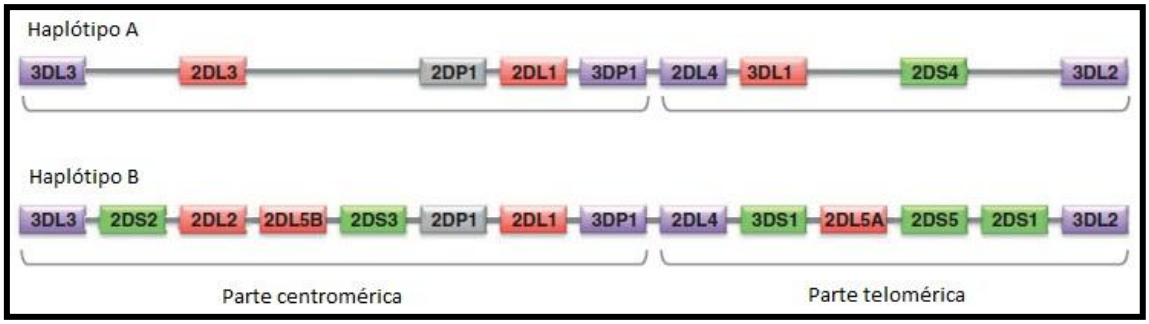

Figura 4: Representação esquemática dos haplótipos A e B no locus *KIR***.** Exemplos de haplótipos A e B. Pseudogenes estão indicados em cinza, receptores ativadores em verde e receptores inibidores em vermelho. Genes conservados, que podem codificar receptores ativadores ou inibitórios ou serem pseudogenes estão indicados em roxo. Cada fragmento centromérico pode se combinar com fragmentos teloméricos, o que aumenta a diversidade dos haplótipos KIR. Modificado de Thielens *et al*, 2012.

Cada vez mais os genes *KIR* estão sendo relacionados a variabilidade de resposta a patologias virais (HIV, CMV, hepatites) (Tiemessen *et al.,* 2011; Stern *et al.,* 2008; Marangon *et al.,* 2011) e a doenças autoimunes como esclerose sistêmica, artrite reumatoide e psoríase, por exemplo (Martin *et al.,* 2003; Salim *et al.,* 2010; Jobim *et al.,* 2008). Devido à sua diversidade haplotípica, há interesse em avaliar a associação com outras doenças. Por exemplo, foi encontrada associação de ligantes HLA e receptores KIR no desenvolvimento de pré-eclâmpsia (Hiby *et al.,* 2004), neoplasias hematológicas, como linfomas e leucemias (Leung *et al.,* 2007; Karabon *et al.,* 2011), além de tumores sólidos (Romagne *et al.,* 2009).

Até o momento, não foram localizados, na literatura, estudos que estabeleçam relação direta ou indireta entre tipagem dos genes *HLA* e *KIR* no desenvolvimento, variabilidade fenotípica ou prognóstico da DG.

Citocinas inflamatórias e óxido nítrico

Cada um dos linfócitos CD4+ é caracterizado pela produção de citocinas específicas. Além das citocinas efetoras, como IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17), IL-35 e TGF-β (Treg), IL-6 e IL-21 (Tfh), todas as células T produzem IL-10, uma citocinas com propriedades imunoregulatórias (Jankovic *et al.,* 2010). As células Th1 produzem IFN-γ, IL-2 e TNF-α para eliminar patógenos intracelulares e evocar a imunidade celular, enquanto as células Th2 produzem IL-4, IL-5 e IL-13 para eliminar patógenos extracelulares e evocar respostas humorais (Bottomly *et al.,* 1988). Ao contrário da diferenciação de Th1 e Th2, a qual depende das citocinas efetoras, a diferenciação Th17 não requer IL-17, porém necessita de IL-6 e TGF-β. As células Treg, ao produzir IL-10 e TGF-β, levam à tolerância imunológica e inibição da síntese de IFN-γ, além de impedirem a diferenciação de células T helper *naïve* em efetoras (Amarnath *et al.,* 2011). Muitos estudos demonstram o aumento de diferentes citocinas em pacientes e modelos animais de DG (Tabela 3). O acúmulo progressivo de glicosilceramida aumenta a ativação macrofágica e a expressão de genes relacionados à imunidade como INFγ, TNF, IL-1ra, IL-4, IL-6, CCL2, CCL3, CCL6, CCL9, CXCL1, CXCL12, CCL17 e CCL22. Além disso, induzem a produção de quimiocinas (MCP1/CCL2, CXCL8/IL8, e KC/CXCL1), o que aumenta o recrutamento de polimorfonucleares e a transformação de monócitos em macrófagos ativados que iniciam a reação inflamatória nos tecidos de pacientes com DG.

A produção de óxido nítrico (NO) por diferentes tipos celulares é um importante efetor para a produção de várias citocinas e quimiocinas (Bogdan *et al.,* 2000). NO é produzido a partir de L-arginina por óxido nítrico sintases (NOS), codificadas por três genes: *NOS1*, *NOS2* e *NOS3.* A NOS2, também chamada de iNOS (NOS induzida) é expressa por células imunológicas após a ativação por IFN-γ e TNF-α (Portillo *et al.,* 2012). O aumento dos níveis dessas citocinas em pacientes com DG pode estar relacionado a um aumento da expressão de *NOS2.* Essa hipótese é embasada pela associação de NO e espécies reativas de oxigênio detectadas em cérebros de modelos animais de DG (Hong *et al.,* 2006).

Tabela 3: Citocinas e quimiocinas na Doença de Gaucher. Adaptada de Pandey *et al.*, 2013.

Citocina	Nome
IFN-γ	Interferon gama
TNFα	Fator de necrose tumoral alfa
IL-1α	Interleucina 1 alfa
IL-1β	Interleucina 1 beta
IL-1Ra	Antagonista do receptor de IL-1
sIL-2R	Receptor de IL-2 solúvel
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-10	Interleucina 10
IL-18	Interleucina 18
TGF-β1	Fator de crescimento transformador beta 1
HGF	Fator de crescimento de hepatócitos
M-CSF	Fator estimulador de colônia de macrófagos
G-CSF	Fator estimulador de colônia de granulócitos
MCP-1/CCL2	Proteína quimiotática de monócitos 1 / Quimiocina CC ligante 2
MIP-1α/CCL3	Proteína inflamatória de macrófago 1 alfa / Quimiocina CC ligante 3
MIP-1β/CCL4	Proteína inflamatória de macrófago 1 beta / Quimiocina CC ligante 4
CCL6	Quimiocina CC ligante 6
CCL9	Quimiocina CC ligante 9
CCL17	Quimiocina CC ligante 17
PARC/CCL18	Quimiocina pulmonar e regulada por ativação / Quimiocina CC ligante 18
CCL22	Quimiocina CC ligante 22
CXCL1	Quimiocina CXC ligante 1
IL-8/CXCL8	Interleucina 8 / Quimiocina CXC ligante 8
CXCL12	Quimiocina CXC ligante 12

Os mecanismos detalhados sobre a fisiopatogênese da DG ainda precisam ser elucidados. Sem dúvida, a base para o início e propagação do envolvimento de células do sistema imune na DG deve ser estudada para prover novas abordagens terapêuticas para essa e outras doenças lisossomais.

1.4) Biomarcadores na Doença de Gaucher

Como a terapia de reposição enzimática é custosa e pode necessitar de algum tempo até que benefícios clínicos sejam evidentes, há necessidade de identificação de biomarcadores que possam indicar uma resposta precoce ao tratamento.

Além do aumento de citocinas inflamatórias, os pacientes com DG apresentam elevação de certas proteínas séricas como a ferritina, a fosfatase ácida resistente a tartarato, a enzima conversora de angiotensina, as hexosaminidases e a lisozima, que eram utilizadas como biomarcadores, com algumas restrições (Aerts *et al.,* 1997). Nenhuma delas é específica para DG e seus valores podem se sobrepor aos observados em indivíduos saudáveis. A detecção do aumento da atividade da enzima quitotriosidase em plasma de pacientes com DG melhorou o acompanhamento e manejo da doença (Hollak *et al.,* 1994). No entanto, a quitotriosidase não é um biomarcador perfeito, pois pode estar elevada em outras doenças, como talassemia, doença de Niemann-Pick tipo A, B e C, além de infecções e ainda, em torno de 6% da população apresenta deficiência dessa enzima. Por essas razões, há grande interesse na busca de biomarcadores sensíveis e específicos para a DG, o que levou à determinação do PARC/CCL18 (Boot *et al.,* 2004) e da glicosilesfingosina que parece ser uma proteína mais específica (Dekker *et al.,* 2011).

A osteopontina (OPN) é uma proteína codificada pelo gene *SPP1* e já foi identificada em células tumorais (Senger *et al.,* 1989) e células ósseas (Oldberg *et al.,* 1986), além de ser produzida por diferentes células do sistema imune como linfócitos T e macrófagos (Weber *et al.,* 1996). Em modelos *in vivo*, monócitos expressam OPN em níveis baixos e assim que se diferenciam em macrófagos, esses níveis se elevam (Atkins *et al.,* 1998). Muitas citocinas estimulam a expressão de OPN, particularmente, INF-γ, IL-10, IL-6, TNF-α e IL-1β (Konno *et al.,* 2006; Li *et al.,* 2003; Rollo *et al.,* 1996). A OPN afeta a expressão de IL-2, IL-10, CCL4 (O'Regan *et al.,* 2000; Zheng *et al.,* 2009), além de estar envolvida no processo de diferenciação, migração e fagocitose dos macrófagos (Nystrom *et al.,* 2007; Giachelli *et al.,* 1998; Pedraza *et al.,* 2008). Até o momento, não há estudos sobre o papel da osteopontina na fisiopatogênese da DG.

1.5) Neurotrofinas na Doença de Gaucher

Mutações no gene *GBA* estão associadas a um aumento do risco de desenvolvimento de doenças neurodegenerativas, como doença de Parkinson e demência com inclusão de corpos de Lewy (Sidransky *et al.,* 2009; Nalls *et al.,* 2013). O mecanismo molecular exato envolvido na interação entre *GBA* e a α-sinucleína, a principal proteína associada a essas doenças, permanece sem resolução.

O *Brain-derived neurotrophic factor*, ou BDNF, é membro da família das neurotrofinas, um grupo de fatores de crescimento neuronal, que inclui o fator de crescimento de nervos (NGF), a neurotrofina 3 (NT-3) e a neurotrofina 4 (NT-4) (Binder *et al.,* 2004). Essas proteínas são importantes para o desenvolvimento do sistema nervoso central e periférico, além de terem papel fundamental na sobrevivência neuronal e na plasticidade sináptica do cérebro adulto (Arancio *et al.,* 2007). A expressão de BDNF já foi descrita como diminuída em pacientes com doença de Parkinson, Alzheimer e demência com inclusão de corpos de Lewy (Murer *et al.,* 2001; Imamura *et al.,* 2005) e, recentemente, estudos demonstraram que alterações no suporte de fatores neurotróficos no cérebro, em particular o BDNF, podem contribuir para neurodegeneração (Balaratnasingam *et al.,* 2012). Além disso, pacientes com depressão apresentam níveis circulantes de BDNF diminuídos que se elevam após a instituição de tratamento medicamentoso (Lee *et al.,* 2010; Bocchio-Chiavetto *et al.,* 2010).

Já foram publicados dados sobre níveis diminuídos de BDNF em cérebros de camundongos com DG, resultando em morte neuronal massiva e neurodegeneração (Kim *et al.,* 2006), porém não há estudos sobre BDNF em pacientes com DG.

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2) OBJETIVOS

2.1) Objetivo geral

Caracterizar o envolvimento de marcadores do sistema imune na doença de Gaucher, por meio de avaliação clínica, bioquímica, genética, imunoensaios e determinar se esses achados estão relacionados à variabilidade do fenótipo apresentada pelos pacientes com doença de Gaucher acompanhados no Centro de Referência Estadual do Rio Grande do Sul.

2.2) Objetivos específicos

A partir de uma amostra de pacientes com doença de Gaucher acompanhados no Centro de Referência Estadual do Rio Grande do Sul

1- Avaliar a frequência de hipergamaglobulinemia apresentada pelos pacientes e compará-la a outras coortes já publicadas.

2- Descrever o perfil imunogênico das três enzimas recombinantes disponíveis para tratamento da doença de Gaucher em um paciente com DG tipo III.

3- Determinar a existência de variantes de genes *KIR* e de combinações de variantes *KIR*-*HLA* associadas à gravidade do fenótipo clínico dos pacientes.

4- Avaliar a relação da tipagem do MHC de classe I (*HLA*-A, *HLA*-B e *HLA*-C) e de classe II (*HLA*-DR) com características apresentadas pelos pacientes.

5- Determinar a concentração sérica de diferentes citocinas durante um período sem tratamento específico e após o reinício do tratamento em

pacientes com doença de Gaucher tipo I e compará-las com características clínicas e bioquímicas em busca de associações com a variabilidade fenotípica apresentada pelos pacientes.

3) CAPÍTULOS

3.1) CAPÍTULO 1

Hyperimmunoglobulinemia in Pediatric Gaucher Patients in Southern Brazil

Título do manuscrito: *Hyperimmunoglobulinemia in Pediatric Gaucher Patients in Southern Brazil*

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LETTER TO THE EDITOR
Hyperimmunoglobulinemia in Pediatric Gaucher Patients in Southern Brazil

To the Editor: We read with great interest the brief report published by Arikan-Ayyildiz et al. [1] about immunoglobulin abnormalities and enzyme replacement therapy (ERT) in children with Gaucher disease (GD). We would like to point out some issues based on the findings of the cohort of Gaucher patients followed in our clinics.

Since 2003, all patients (30 GD type I and 3 GD type III) are followed with clinical assessment and complementary exams every 3 months and after achievement of main goals, laboratory exams, including immunological profile (IgG, IgM, IgA, and IgE, as well as electrophoresis of proteins) are done on a yearly basis [2,3]. Evaluation of immunological data at the diagnosis was performed for 11 patients (9 GD type I and 2 GD type III) who were under the age of 18 years at the diagnosis. All but one pediatric patient (GD type III patient due to allergic reaction) have been receiving ERT with imiglucerase (30 U/kg/inf for GD type I and 60 U/kg/inf for GD type III) every 2 weeks. The mean age at diagnosis was 6.4 years (range: 2–16 years) and the mean age at the start of treatment was 7 years (range: 2–17 years). The mean time of ERT was 12.7 years (range: 8–16 years).

We had access to immunological profiles of eight pediatric patients since 2007 (7 GD type I patients and 1 GD type III patient) and four patients since 2008 (3 GD type I patients and 1 GD type III patient). Hyperimmunoglobulinemia was present in 10 (90.9%) pediatric patients at the first evaluation (IgA: 1/10 patients, IgE: 8/10 patients, IgM: 2/10 patients, IgG: 4/10 patients). The only GD type III patient, who was without ERT, presented IgG monoclonal gammopathy, but after a few months, even without treatment, the monoclonal pike disappeared. Four patients (36.4%) presented polyclonal gammopathy at the first evaluation. One GD type I patient (after 19 months of ERT) and one GD type III patient (after 15 months of ERT) resolved their gammopathy. Even in regular therapy with imiglucerase, nine (90%) patients still present with hyperimmunoglobulinemia (IgE: 6/9 patients, IgM: 2/9 patients, IgG: 6/9 patients).

Our data differs from previous studies, including the Brief Report by Ayyildiz et al. [4] that reported a frequency of hyperimmunoglobulinemia in pediatric GD patients ranging from 71% to 77% at diagnosis. Ninety percent of our patients presented hyperimmunoglobulinemia at the first evaluation (23–29% of difference from other studies) and 40% of the patients showed increased levels of IgG, similar to what has been reported [4]. After ERT, the number of patients with hyper IgE decreased 14%, hyper IgM increased 2% and hyper IgG increased 26%. None of our patients resolved their gammopathy.

Our data suggest that the cohort of patients in Southern Brazil presents more hyperimmunoglobulinemia than the previous reports and ERT was less effective in normalize immunoglobulin levels. We agreed that the initiation of early ERT might help to decrease the risk of malignancies, but other studies are needed.

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BRIEF REPORT
Immunoglobulin Abnormalities and Effects of Enzyme Replacement Therapy in Children with Gaucher Disease

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Hyperimmunoglobulinemia is documented in patients with Gaucher disease of all ages. We investigated the frequency of hyperimmunoglobulinemia in 12 pediatric patients with type I and III Gaucher disease and the effects of enzyme replacement therapy on these abnormalities. The incidence of hyperimmunoglobulinemia was 77%, 66%, and 60% at the diagnosis, before and after ERT, respectively. Immunoglobulin G abnormalities were the most commonly seen isotype abnormality. After enzyme replacement therapy normalization of IgA and IgM levels were recorded but decline in IgG levels was less likely to occur. This study indicated the higher frequency of hyperimmunoglobulinemia in pediatric Gaucher patients. Pediatr Blood Cancer 2011;56:664–666.

Key words: children; enzyme replacement therapy; Gaucher; Hyperimmunoglobulinemia

INTRODUCTION

Gaucher disease, the most prevalant lysosomal storage disorder, is caused by glucocerebrosidase deficiency. Enyzme replacement therapy (ERT) in Gaucher disease type I and III is a well accepted treatment which has been shown to diminish or stabilize many of the clinical manifestations and improve the quality of life.

Hyperimmunuglobulinemia is a documented clinical feature in Gaucher disease [1–3]. The frequency of hyperimmunuglobulinemia in series of adult Gaucher patients is around 50% and ERT has been shown to decrease the immunoglobulin (Ig) values of all three isotypes but not monoclonal gammopathies [4,5]. To date, there is only one study about hyperimmunoglobulinemia in pediatric Gaucher disease which shows that 71% of the patients have elevation of more than one Ig isotype [6]. In this study we determined the incidence of hyperimmunoglobulinemia in our patients with Gaucher disease and the effect of ERT on Ig levels.

METHODS

Patients with type I and type III Gaucher disease who were under the age of 18 years at diagnosis with any Ig levels available at pediatric age, were included in the study. All patients with type I Gaucher disease received enzyme replacement therapy (Cerezyme®) for 30–60 U/kg and type III for 120 U/kg every 14 days for at least six months. The Ig levels available before and after ERT were recorded. Immunoglobulin G, IgM, and IgA levels were adjusted for age using published age-spesific reference intervals. Values were considered as high if the patient's Ig exceed the upper normal limit. Ratio of Ig values to upper normal age specific values were also recorded. The data were analyzed using descriptive statistics and are presented as means. Approval for extraction of data from the files of patients was received from the Hospital Ethics Board.

Mutation analysis of the patients were done by "Vienna Lab Gaucher Strip A assay" and DNA sequence analysis. Severity score index (SSI) was calculated for each patient [7]. In symptom severity scoring, 0–10 points denotes mild disease, 11–20 points denotes moderate disease, and 21–30 points denotes severe involvement.

RESULTS

Twelve patients' data were reviewed. The mean age at diagnosis was 4.5 years (1–10 years). Mean age at the start of treatment was 8.8 years (1.8–22 years). Mean treatment period was 36.5 ± 26.8 months (range: 6–102 months). Patient characteristics are shown at Table I.

Nine patients' Ig levels were available at diagnosis. Overall, hyperimmunoglobulinemia was present in seven out of nine (77.7%) patients at diagnosis. In this group of patients, IgG elevation (mean ratio of 1.5) was present in six (66%), IgM elevation (mean ratio of 1.5) was found in four (44.4%), and IgA elevation (mean ratio of 2.1) in four also (44.4%). Two patients' Ig levels were within normal limits. Both of the groups with and without hyperimmunoglobulinemia were in mild Gaucher group except the patient with type III Gaucher disease who is classified in severe group and had hyperimmunoglobulinemia. Immunoglobulin isotypes elevated at the time of diagnosis in pediatric patients with Gaucher disease are shown in Table II.

Before initiation of ERT, 10 patients' Ig levels were available. As one of our patients was older than 18 years when ERT was started, we did not use her results (Patient 3). Out of nine patients,

Additional supporting information may be found in the online version of this article.

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TABLE I. Clinical Characteristics of the Patients Involved in the study

Patient	Sex	Phenotype	Genotype	Age at diagnosis (year)	Bone disease Before ERT	Spleen status Before ERT	Age at ERT (year)	ERT period (month)	ERT dose (initial) U/kg	Age at Post ERT review year	SSI (before ERT)	SSI (after ERT)
1.	F	I	S366T/S366T	8.4	Yes	Intact	13.5	48	60	17.5	9	1
2.	F	I	N370S/?	10.0	Yes	Intact	12.6	6	30	13	3	1
3.	F	I	N370S/?	8.6	?	Splenectomy	16.9	42	60	20.4	16	9
4.	F	I	N370S/?	4.1	Yes	Intact	4.5	48	60	8.5	7	1
5.	F	I	N370S/?	1.0	No	Intact	4.6	6	40	5.1	5	3
6.	M	I	N370S/D399N	3.4	Yes	Intact	5.8	102	60	14.3	9	1
7.	M	III	D409H/D409H	1.75	No	Intact	1.8	12	120	2.8	22	20
8.	F	I	R463C/?	7.0	Yes	Splenectomy	22	12	60	23	20	16
9.	F	I	L296V/L296V	3.0	?	Splenectomy	11.6	48	60	15.6	14	10
10.	F	I/III	L444P/L444P	2.0	Yes	Splenectomy	3	36	40	6	10	9
11.	F	I	N370S/?	4.0	No	Intact	8.1	30	60	10.6	16	6
12.	F	I/III	L444P/?	1.58	Yes	Intact	2	48	60	6	8	7

ERT, Enzyme replacement therapy; SSI, Severity score index.

six (66.6%) had hyperimmunoglobulinemia. Immunoglobulin levels were normal in three of the children while IgG abnormality (mean ratio of 1.4) was present in six (66.6%), IgM abnormality (mean ratio of 1.6) in two (22.2%), and IgA abnormality (mean ratio of 1.7) was present in three (33.3%) patients. In the group of patients of which Ig levels were normal before ERT, one patient was in the moderate severity group while others belonged to mild severity group. There were three patients in moderate severity group and one in severe group (patient with type III Gaucher disease) among the patients who had hyperimmunoglobulinemia before ERT.

After ERT all of the patients' Ig levels were assessed. Two patients were older than 18 at the assessment (Patient 3 and 8). Out of 12 patients, seven (58.3%) had hyperimmunoglobulinemia. By means of pediatric age group six out of 10 had hyperimmunoglobulinemia (60%). IgG abnormality (mean ratio of 1.1) was found in five (50%) children while IgM abnormality (ration of 1.1) was found in one (10%) and IgA abnormality (ratio of 1) was also detected in one (10%) patient.

Possible effects of ERT on hyperimmunoglobulinemia were observed in five pediatric patients whose data were available before and after ERT. Immunoglobulin changes in this group of patients could be compared as shown in Table II and Supplemental Table I and II. One of the two patients who showed post-ERT normalisation of hyperimmunoglobulinemia was in the mild and the other in the severe SSI group, while all the others without normalisation were in mild severity group.

TABLE II. Immunoglobulin Isotypes Elevated Before and After ERT in Pediatric Gaucher Patients

Immunoglobulin (Ig) isotypes	At the time of diagnosis Patients (*n*)	Before ERT (*n*)	After ERT (*n*)
IgG, IgA, IgM	2	1	—
IgA and IgM	1	—	—
IgA and IgG	1	2*	1
IgG and IgM	1	1	—
Only IgG	2	2	4
Only IgM	—	—	1
Total	7	6	6

*Data of one patient in this group was not taken into account after ERT because of the age which is older than 18.

In four patients who were splenectomised after diagnosis, we compared the effect of splenectomy on Ig levels. In two of the patients all Ig levels were normal after splenectomy while the levels before splenectomy were only available in one and it was normal. Two other patients with high Ig levels, ratio of IgA levels were elevated 11.5% and 7.5%, while ratio of IgG levels tend to decrease 1% and 17%, respectively, after splenectomy.

DISCUSSION

This is the second reported data of Ig levels in children with Gaucher disease. Although the subset is small, we recorded that Ig abnormalities occur in 77%, 66%, and 60% at diagnosis, before initiation of ERT and after ERT, respectively. Our data of frequency of hyperimmunoglobulinemia is similar to the first report in which the 71% of study population had abnormal Ig results [6]. Elevation of all isotypes of Ig was present in two of our patients (28.5%) at diagnosis and it was only in one before initiation of ERT. Immunoglobulin G abnormalities were the most commonly seen isotype abnormalities at diagnosis, before and after ERT.

A previous study concluded that age at diagnosis, frequency of splenectomy, bone involvement, timing of initiation of ERT support the link of disease severity to hyperimmunoglobulinemia in pediatric Gaucher patients [6]. However, no correlation was noted between the presence of polyclonal gammopathy and parameters of disease severity in another study [4]. Although statistical analysis were not possible because of the small numbers of cases in our study, we did not observe any correlation between hyperimmunoglobulinemia and disease severity.

In studies with adult Gaucher patients, it was shown that enzyme replacement therapy decreases elevated Ig levels [4,5,8]. In these studies, the common conclusion is the tendency of only IgG abnormalities to persist. It was reported that IgM

levels decreased only after an ERT period of 10 years but IgG and IgA hyperimmunoglobulinemia was unaffected [5]. In our study, it seems that mean ratios of all immunoglobulins to upper normal limit came near by 1.0 after ERT but it still means high normal. IgG was the leading isotype abnormality which tend to persist after ERT in our study (in three patients). Enzyme replacement therapy was also successful in children of which IgG levels were persistently high. One report of adult patients showed 11.7% decrease for IgG, 6.5% decrease for IgM, and 4.3% decrease for IgA after ERT [4]. Pediatric data have revealed 53.3% and 29% decreases of IgM and IgA levels, respectively, after ERT; however, IgG abnormalities persisted [6]. Change in IgG level after ERT was also the least in our study.

Splenectomy has been shown to decrease mostly IgM levels while IgA and IgG levels remain unchanged [9]. We also observed that in two of our patients who had splenectomy, IgA levels remained still high while IgG levels tend to decrease but they were still higher than normal. These two patients' IgG levels were also higher than normal after four years of enzyme replacement therapy but IgA levels decreased to normal ranges.

Decreased antigen clearance because of the overload of reticuloendothelial system, chronic antigenic stimulation by the abnormal lipid storage, defects in immunoregulation of B-cell function has been postulated to be the mechanisms of immune system dysfunction in Gaucher disease [2,8]. Plasma levels of some pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α were found to be increased in Gaucher disease and these cytokines have been shown to be involved in pathogenesis of Ig abnormalities [10,11,12]. Although it is known that Gaucher cells, the source of pro-inflammatory cytokines, decrease with enzyme replacement therapy, the mechanisms leading to persistence of abnormal IgG levels still need to be identified.

In conclusion, our study suggests that higher frequency of hyperimmunoglobulinemia occurs in pediatric patients in comparison to adults with Gaucher disease. Enzyme replacement therapy decreases Ig levels, particularly IgA and IgM. These findings can suggest that IgG gammopathy is a feature of Gaucher disease, but the question of why this feature is less effected by ERT remains unclear. As it is known that Gaucher disease is associated with monoclonal gammopathies and B cell neoplasms, initiation of early ERT might help to decrease the risk of these malignancies including multiple myeloma and lymphoma. This also needs further evaluation.

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3.2) CAPÍTULO 2

Enzyme Replacement Therapy in a Patient with Gaucher Disease Type III: A Paradigmatic Case Showing Severe Adverse Reactions Started a Long Time after the Beginning of Treatment

Título do manuscrito: *Enzyme Replacement Therapy in a Patient with Gaucher Disease Type III: A Paradigmatic Case Showing Severe Adverse Reactions Started a Long Time after the Beginning of Treatment*

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CASE REPORT

Enzyme Replacement Therapy in a Patient with Gaucher Disease Type III: A Paradigmatic Case Showing Severe Adverse Reactions Started a Long Time After the Beginning of Treatment

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Abstract *Introduction:* There are three recombinant enzymes available for the treatment of Gaucher disease (GD): imiglucerase, velaglucerase alfa, and taliglucerase alfa.

Case report: A male GD type III patient, 14 years old, genotype p.L444P/L444, diagnosed at 2 years old. He had been treated with imiglucerase for 9 years since the diagnosis. In 2008, however, he presented a severe adverse reaction to imiglucerase, characterized by cough, laryngeal stridor, and periorbital edema. The infusions were suspended for 3 months when imiglucerase was restarted with premedication and a slower infusion rate. After 5 months, he presented a new adverse reaction with vomiting, tachypnea, cough, and periorbital edema. Intradermal testing confirmed IgE-mediated reaction but serological tests were negative. After 2 years and 10 months with no specific treatment and a significant worsening of the clinical picture, taliglucerase alfa was prescribed, with premedication and a slower infusion rate. At the first infusion, he presented moderate adverse reaction and the infusions were suspended. After 2 months, velaglucerase alfa was initiated uneventfully. He maintains day-hospital infusions without premedication and shows improvement of clinical and laboratory parameters.

Conclusion: This is the first report of the use of velaglucerase alfa in patients with GD type III. The use of recombinant enzymes is safe for the majority of GD patients, but severe reactions may occur even many years after the beginning of the treatment. Premedication and slower infusion rate reduce the incidence of adverse reactions but may not solve the problem. This case report further demonstrates the different safety profile among all the recombinant enzymes available for the treatment of GD.

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Introduction

Gaucher disease (GD) is the most common lysosomal storage disorder, with an estimated worldwide incidence of

1 per 57,000 live births in the general population (Meikle et al. 1999) and up to 1 per 850 live births among Ashkenazi Jews (Mistry et al. 2011). Classically, GD is subdivided into three main forms (types I, II, and III), defined by clinical characteristics, disease course, and ethnic prevalence. Nevertheless, there is a wide range of findings that overlap across the classical forms, which has led to a new assessment of GD as a continuous spectrum of disorders rather than a disease with three distinct subtypes (Beutler and Grabowski 2001; Sidransky 2004).

The incidence of subacute neuronopathic (type III) GD is approximately 1 per 100,000 live births. Its distribution is ubiquitous, although the populations of some regions in Northeast Sweden are disproportionately affected (Dahl et al. 1990). Patients with GD type III may exhibit systemic manifestations similar to those of type I patients. Neurological involvement may arise at any age, and usually presents as epilepsy, ataxia, vertical gaze palsy, or dementia (Davies et al. 2007). Some patients may have corneal opacities and valvular heart disease with progressive calcification. The life expectancy is 20–30 years (Tylki-Szymanska and Czartoryska 1999).

For many years, GD was managed with supportive care and palliative measures alone, such as splenectomy to mitigate growth delays, cytopenias, and abdominal discomfort due to splenic enlargement. Since the 1990s, enzyme replacement therapy (ERT) has been the treatment of choice. ERT has improved quality of life among GD patients by reversing many signs and symptoms (Mistry et al. 2007; Hollak et al. 2009). However, the amount of enzyme required to maintain quality of life and reverse the course of symptoms is controversial. Until 2009, imiglucerase (Genzyme Corporation, Allston, MA), obtained from Chinese hamster ovary (CHO) cell lines, was the only ERT agent available. New alternatives have since entered the market, such as velaglucerase alfa (Shire HGT, Dublin, Ireland), which is obtained from human cells and received FDA and EMA approval in 2010, and taliglucerase alfa (Protalix, Carmiel, Israel), which is obtained from carrot cells and received FDA approval in 2012. Despite the longer history of imiglucerase, studies have shown that all three recombinant enzymes are similar in terms of efficacy (Elstein 2011). Approximately 1% of patients develop adverse reactions to imiglucerase ERT, which can be related or not to the production of IgG or IgE antibodies against the synthetic enzyme, forcing judicious use of maintenance infusions. The rate of infusion reactions appears to be higher with taliglucerase alfa and lower with velaglucerase alfa (Zimran et al. 2011; Morris 2012). Substrate reduction therapy with miglustat (Zavesca®, Actelion Pharmaceuticals, Freiburg, Germany) is also available and is mostly indicated for adult GD patients in whom ERT is contraindicated (Platt et al. 1997; Cox et al. 2000; Pastores et al. 2005). The results of therapy with eliglustat (Genzyme Corporation, Allston, MA), another substrate reduction agent, appear promising, but it is still at the clinical trial stage (Lukina et al. 2010).

The management of GD type III is hindered by the fact that recombinant enzymes cannot cross the blood–brain barrier efficiently and act on the CNS. In patients with neuronopathic GD, enzyme dosage is currently adjusted according to the severity of visceral manifestations, with the maximum dosage being 60 IU/kg/infusion every 2 weeks (Vellodi et al. 2009).

This report describes the case of a patient with GD type III who has received all three recombinant ERT forms available, the adverse effects to each formulation, and the clinical outcomes obtained.

Case Report

A 14-year-old male received a diagnosis of GD type III (β-glucocerebrosidase activity, 2 nmol/h/mg [reference range: 10–45]; genotype p.L444P/L444P) at age 2 due to hepatosplenomegaly, kyphoscoliosis, horizontal supranuclear gaze palsy, and cognitive and pulmonary involvement. During workup, the patient was found to be heterozygous for a 24-bp duplication in exon 10 of the *CHIT1* gene, causing partial chitotriosidase deficiency. Shortly after diagnosis, the patient was started on imiglucerase ERT (60 IU/kg/infusion every 2 weeks) at our hospital. Two years after the start of treatment, a central venous catheter was implanted so the patient could receive infusions at his hometown, located 360 km from our service; infusions were provided this way for a total of 4 years and after this through a peripheral access, although the central venous catheter was not removed. Nine years after the start of treatment, while receiving an infusion at a local health facility in his hometown, the patient developed a severe adverse reaction characterized by cough, laryngeal stridor, and periorbital edema within 5 min of the start of infusion. The infusion was ceased at once and the patient was given IV dexamethasone and oral dexchlorpheniramine, with complete resolution of symptoms. C3 and C4 levels were within normal limits, and the IgE level was 1629 UI/mL (reference range for age, <200 UI/mL) 4 days after the adverse event. We chose to discontinue ERT and wait for the results of the serum anti-imiglucerase antibody test, which was performed by the drug manufacturer and carried out on a blood sample collected 40 days after the reaction. The patient remained ERT-free for 3 months waiting for the results of testing, which were ultimately negative for anti-imiglucerase IgG and IgE antibodies (ELISA). Therefore, imiglucerase ERT was restarted at the same dosage (60 IU/kg/infusion every 15 days), now at our service, in a hospital

setting, with loratadine 10 mg PO as premedication and a slower rate of infusion (total infusion time 2 h 30 min). As the patient did not develop any adverse reactions to this scheme, infusions were restarted at his hometown after the third post-reaction infusion. Four months later, the patient developed another reaction, now presenting as vomiting, redness at the catheter site (we could not ascertain whether this was associated with a catheter-related infection), tachypnea, cough, and periorbital edema of 40 min duration. The infusion was ceased and the patient received hydrocortisone 400 mg IV, with complete resolution of symptoms. After this episode, ERT was again discontinued and the patient underwent skin testing for hypersensitivity. The test was performed in two stages, in an ICU setting, in accordance with a test protocol provided by the drug manufacturer. The first step, consisting of a similar standard prick test for common allergens, was negative. The second test included intradermal testing, whereby doses of increasingly concentrated imiglucerase were injected into the dermis. An IgE-mediated reaction was confirmed by the appearance of a >20-mm wheal-and-erythema response within 15 min of injection of imiglucerase 1:10 and 1:100. In view of the anaphylactoid nature of the reaction and the good clinical condition of the patient, we chose to discontinue imiglucerase treatment altogether. Furthermore, neither miglustat nor velaglucerase/taliglucerase alfa were available in the public health system in Brazil at the time (2008).

The patient continued to receive regular follow-up every 3 months for monitoring of clinical and laboratory parameters. At 34-month follow-up, as the patient's condition had deteriorated significantly (episodes of epistaxis, hepatosplenomegaly, hypoalbuminemia, and lower extremity edema) and taliglucerase alfa had recently become available in Brazil, and after discussing this option with the patient's family and securing their informed consent, as patients with allergic reactions to imiglucerase were excluded from clinical trials of taliglucerase alfa, we decided to attempt ERT with this novel medication. The patient was premedicated with loratadine 10 mg PO, ranitidine 150 mg PO, and hydrocortisone 400 mg IV and the infusion rate was titrated slowly (1 mL/15 min, 2 mL/15 min, 4 mL/15 min, 8 mL/15 min, 16 mL/15 min, and 32 mL thereafter). However, after infusion of 5.8 mL of taliglucerase alfa at a dosage of 60 IU/kg, the patient developed epigastric pain, vomiting, rash, and headache. Dexchlorpheniramine 2 mg PO, promethazine 25 mg IV, and metoclopramide 10 mg IV were administered and there was improvement of symptoms. The infusion was halted and the decision was made to discontinue taliglucerase alfa therapy. Two months after this reaction, velaglucerase alfa was provided for this patient as a compassionate use. After discussing this option with the patient's family and securing their informed consent, as no data were available on treatment of GD type III with this enzyme, the decision was made to attempt ERT once more. An anti-imiglucerase antibody test performed by Shire HGT in November 2011 (electrochemiluminescence immunoassay for anti-imiglucerase and anti-velaglucerase antibodies) was negative for IgG and IgE antibodies.

The patient was admitted to our hospital for stabilization of clinical parameters and a battery of tests to determine baseline laboratory values. After 2 weeks of hospitalization, velaglucerase alfa was administered at a dosage of 60 IU/kg, after premedication with hydrocortisone 400 mg IV and promethazine 25 mg IV and an infusion rate titrated to 200 mL over the course of 4 h. The infusion was completed uneventfully, and the patient was started on twice-monthly infusions on an outpatient basis. Premedication was gradually reduced over the course of five sessions, with no ill effects. After eight infusions at our hospital, the patient returned to his hometown, where he continues to receive periodic infusions. He no longer requires premedication and the infusion time has been shortened to 2 h. We chose to wait for further clinical improvement before removal of the central venous catheter.

The patient's neurological condition remains stable and his anemia, hyperproteinemia, and lower extremity edema have resolved completely. Thrombocytopenia has improved substantially and abdominal volume and chitotriosidase levels are reduced (Table 1). In addition to these improvements in objective parameters, application of the SF-36 and WHOQoL questionnaires (completed by proxy by the patient's mother) revealed improvement in quality of life (data not shown).

Discussion

Recombinant enzyme replacement therapy is safe for most GD patients, but 1.5% to 25% may develop adverse reactions, depending on the medication regimen (Starzyk et al. 2007; Zimran et al. 2011). Some reports have described premedication and manipulation of infusion rates for the management of imiglucerase-related adverse effects (Peroni et al. 2009), but these measures are not always effective. In view of a worldwide shortage of imiglucerase (Hollak et al. 2010), the Brazilian National Health Surveillance Agency (ANVISA), the regulatory counterpart of the U.S. FDA and the European EMA, granted emergency marketing authorization for taliglucerase alfa in 2010. In 2011, an updated version of the Brazilian Ministry of Health guidelines for GD disease was approved, which included all the three recombinant enzymes available on the market (imiglucerase, taliglucerase alfa, and velaglucerase alfa) and substrate reduction therapy (miglustat). Currently, there are Brazilian patients on all four forms of treatment. Although X-ray structures of all three enzymes are very similar, they show some differences in their sequence and glycan structure. Taliglucerase alfa has

Table 1 Follow-up of laboratory parameters and imaging findings

	Pre-treatment[b]	Before first imiglucerase reaction	34 months without treatment	Before first velaglucerase alfa infusion	After 6 velaglucerase alfa infusions	After 12 velaglucerase alfa infusions
Age (years)	2	11	13.3	14.3	14.6	14.9
Height (cm)[a]	73	122	132	132	132	132
Weight (kg)	9.0	23.6	29.7	29.7	30.1	31
Hemoglobin (g/dL)	8.3	13	8.6	8	10.7	12.6
Platelets (1,000/mm^3)	133	280	65	56	62	115
Chitotriosidase (nmol/mL/h)	8,627	1,808	15,117	19,878	15,814	13,074
Liver[c]	8.2 cm (longest axis)	889 cm^3	Normal	5,367 cm^3	5,369 cm^3	ND
Spleen[c] (longest axis, in cm)	12.1	9.5	17.5	27	17	ND
Albumin (g/dL)	ND	ND	3	2.9	ND	3.48
Bone changes[d]	Kyphoscoliosis	Kyphoscoliosis	Osteolytic and osteoblastic lesions, Erlenmeyer flask deformity, and kyphoscoliosis	ND	ND	ND
BMD (T score)	−5.7	ND	−4.6	ND	ND	ND
BMB score	ND	ND	ND	14	ND	ND
Spirometry	ND	FEV1/FVC: 73% – air flow preserved	FEV1/FVC: 34.5 % – severe restrictive ventilatory defect	FEV1/FVC: 31.3 % – severe restrictive ventilatory defect	ND	ND
Severity Score Index (SSI)[28]	24	28	31	33	32	29

[a] Difficult to measure due to bone changes

[b] Shortly before first imiglucerase infusion

[c] On ultrasound

[d] On X-rays

BMD Bone mineral density – DEXA (Z score was not available), *BMB score* bone marrow burden (MRI), *FEV_1* Forced expiratory volume in 1 s, *FVC* Forced vital capacity, *ND* Not done

two additional amino acids at the N-terminus, and it has additional seven amino acids at the C-terminus in relation to the "wild" human counterpart. Besides that, the amino acid composition of both imiglucerase and taliglucerase alfa differs from the human β-glucocerebrosidase at residue 495. Velaglucerase alfa has the same amino acid sequence as the human enzyme. Regarding the glycosylation process, taliglucerase alfa differs from the other two enzymes as it contains xylose and fucose derivatives, which are unique to plant-derived proteins (Brumshtein et al. 2010).

Despite no detectable serum anti-imiglucerase IgE or IgG antibodies, our patient had a positive intradermal test response and almost instant adverse response to imiglucerase (after 9 years of infusions without any intercurrence) and taliglucerase alfa (at the first infusion). This may be indicative of a hypersensitivity reaction to some element present during the manufacturing process of imiglucerase – an element possibly used in manufacturing of taliglucerase alfa as well. The patient does not seem to present an hyper-IgE syndrome since he did not present any clinical symptoms associated with hyper-IgE syndrome such as skin abscesses, recurrent pneumonia, pneumatoceles, early eczema, and late loss of primary dentition (Sowerwine et al. 2012).

Interestingly, our patient presented an anaphylactoid reaction after many years of imiglucerase ERT. This could have implications for some countries in which home therapy is widely available; for safety reasons, we suggest the patient should not be alone during home infusions.

Throughout the course of this case, we attempted to follow existing adverse reaction management protocols for patients with GD and other lysosomal storage disorders (Kim et al. 2008) and create our own, but the patient could not adapt to imiglucerase or taliglucerase alfa ERT despite these measures. Miglustat was not trialed because, despite marketing approval, there was no available stock at the time of the patient's reactions. Furthermore, the patient was extremely debilitated and underweight, and was thus not a candidate for substrate reduction therapy.

After the availability of other recombinant forms of β-glucocerebrosidase in several countries in 2010, the scenario for management of patients who tolerate imiglucerase poorly or have discontinued ERT for other reasons has improved, as the switch to substrate reduction therapy (Elstein et al. 2007) or another recombinant enzyme has proved safe and effective (Elstein et al. 2012; van Dussen et al. 2012).

This is the first report of velaglucerase alfa therapy in a patient with GD type III. We suggest, on the basis of our findings, although this enzyme has not received formal approval for use in patients with GD type III, it should be assessed for use in such patients who develop adverse reactions to imiglucerase or taliglucerase alfa. The Brazilian Ministry of Health guidelines for treatment of GD does not mention any contraindications to the use of velaglucerase alfa in patients with type III disease. In addition to describing the success of velaglucerase alfa therapy, this report demonstrates the differences in safety profile of the three enzymes available for ERT for Gaucher disease for this patient, which are most likely related to distinct manufacturing processes and can occur at any time after the beginning of therapy.

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Contributors

FV designed data collection, monitored data collection, analyzed the data, drafted and revised the paper. He is the guarantor. AD, CN, SM, MW, DD, KM, CBR, AQ, TV, TN, and SL analyzed the data, and revised the paper. IVDS designed data collection, monitored data collection, analyzed the data, drafted and revised the paper.

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3.3) CAPÍTULO 3

KIR genes and HLA class I ligands in Gaucher disease

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KIR genes and HLA class I ligands in Gaucher disease ☆

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ABSTRACT

Gaucher disease (GD) is caused by reduced activity of the lysosomal enzyme glucocerebrosidase, which leads to a buildup of glucocerebroside within the cells and chronic stimulation of the immune system. GD is associated with clinical variability even in the same family, which suggests the influence of modifier genes. Natural killer (NK) cells play an important role in the immune response, and their number is decreased in GD. Killer-cell immunoglobulin-like receptors (KIR) regulate the activity of NK cells through an interaction with specific human leukocyte antigen (HLA) class I molecules on target cells.

Objectives: To analyze the variability of *KIR* genes in a sample of GD patients from Southern Brazil, and look for associations between variants and clinical manifestations.

Methodology: Thirty-one GD patients (24 mild, 4 moderate, and 3 severe) were included in the study. Fifteen *KIR* genes, HLA-C and HLA-Bw4 were analyzed using SSP-PCR. Clinical, biochemical, and radiological data were collected by means of a chart review.

Results/Discussion: Age at symptom onset was associated with KIR2DL2 and KIR2DS2 in combination with the ligand HLA-C1 ($p = 0.038$). Patients who have the HLA-C2 variant appear to have more mono- and polyclonal bands on protein electrophoresis ($p = 0.007$, OR 21.3). There was no between-group significant difference in the frequencies of KIR/HLA variants.

Conclusion: Although exploratory our data suggest a possible association of KIR/HLA variants and the severity of GD. Further study of KIR/HLA variants is required, as they seem to be a phenotype-modifying factor in this disease.

1. Introduction

Gaucher disease (GD, OMIM 230800) is the most common lysosomal storage disorder, with an estimated worldwide incidence of 1 case per 57,000 live births (Meikle and Hopwood, 2003), although this rate can reach 1 in 400 among Ashkenazi Jews (Sobreira et al., 2007).

GD is caused by mutations in both alleles of the *GBA* gene, which codes for lysosomal glucocerebrosidase (EC 3.2.1.45), an enzyme responsible for catalyzing the hydrolysis of glucocerebroside into glucose and ceramide (Beutler, 2006). Consequently, there is intracellular accumulation of glucocerebroside in macrophages, particularly in the spleen, liver, bone marrow, and lungs. GD is therefore a multisystem disorder, and exhibits phenotypic heterogeneity. Although the genetic changes and biochemical pathways that underlie GD have been well characterized, the mechanisms whereby accumulation of glucocerebroside leads to clinical manifestations have yet to be fully determined (Hughes and Pastores, 2010; Jmoudiak and Futerman, 2005).

Gaucher cells (GCs, lipid-laden macrophage derivatives) have eccentric nuclei and cytoplasmic striations. Their expression of macrophage surface markers and marked phagocytic activity confirm the ontogeny of GCs as mononuclear phagocytes (Boven et al., 2004). In addition to lipid substrate buildup, the pathogenesis of GD can be explained by defects in enzyme conformation and endoplasmic reticulum stress (Ron and Horowitz, 2005), defects in calcium homeostasis (one of the mechanisms responsible for the neuropathology seen in

Abbreviations: ERT, enzyme replacement therapy; GCs, Gaucher cells; GD, Gaucher disease; HLA, human leukocyte antigen; KIR, killer-cell immunoglobulin-like receptors; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; NK, natural killer; REDOME, National Bone Marrow Donors Registry; SSI, severity score index; SSP, sequence-specific primers.

☆ Take-home message: Further study of KIR/HLA variants is required as they seem to be phenotype modifiers in GD.

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patients with acute neuronopathic GD) (Pelled et al., 2005), increased sensitivity to oxidative stress (Deganuto et al., 2007), and changes in autophagy mechanisms (Sun and Grabowski, 2010). It has also been speculated that changes in macrophage function may contribute to some components of GD, particularly hepatosplenomegaly and bone involvement (Mizukami et al., 2002).

One of the most widespread hypotheses to explain phenotypic variation in GD is the presence of a chronic immune stimulus (Shoenfeld et al., 1982). Patients with GD exhibit elevated levels of IgA, IgG, and IgM, autoantibodies (Jurecka et al., 2011; Shoenfeld et al., 1995), and increased incidence of polyclonal gammopathy, monoclonal gammopathy of undetermined significance (MGUS), and multiple myeloma (MM) (de Fost et al., 2008; Hughes, 2009). This immune response might generate an inflammatory response, leading to cytokine-mediated cell damage. Some of the cytokines found at elevated levels in GD patients include IL-1, IL-6, IL-10, TNF-α, and M-CSF (Barak et al., 1999; Hollak et al., 1997). As cytokines play an important role in the regulation of immune system cells, some authors ascribe their imbalance to a pro-inflammatory state and to the onset of hematological malignancies (Brody et al., 2006; Costello et al., 2006; Hawkesford et al., 2011; Rosenbloom et al., 2005). Another evidence of a pro-inflammatory response in GD patients is the presence of elevated levels of antigen-presenting molecules, including MHC class II antigens (HLA-DR) (Balreira et al., 2005; Florena et al., 1996).

Natural killer (NK) cells are lymphocytes that can be distinguished from T and B cells by their larger size and granular cytoplasm. NK cells are a component of the innate immune system and play a role in the defense against infectious pathogens and malignancy, exhibiting inflammatory cytokine-mediated cytotoxic and cytolytic effects (Leavy, 2012). NK cells express at least one inhibitory receptor that interacts with HLA class I (HLAs A, B, and C) to exert an important controlling effect and prevent immune response from targeting healthy cells (Moretta et al., 2004).

Killer cell immunoglobulin-like receptors (KIR) are members of the immunoglobulin family found on the surface of NK cells (Moretta et al., 1990) and some T lymphocytes (NKT) (Van Kaer, 2007). Thus far, 15 *KIR* genes (*KIR*2DL1, *KIR*2DL2, *KIR*2DL3, *KIR*2DL4, *KIR*2DL5A, *KIR*2DL5B, *KIR*2DS1, *KIR*2DS2, *KIR*2DS3, *KIR*2DS4, *KIR*2DS5, *KIR*3DL1, *KIR*3DL2, *KIR*3DL3 e *KIR*3DS1) and two pseudogenes (*KIR*2DP1 and *KIR*3DP1) have been described (Marsh et al., 2003), located on the 19q13.4 region (Suto et al., 1996). KIR receptors are the result of a polymorphic system and are divided into activating and inhibitory isoforms. Receptors that mediate an inhibitory signal possess a long cytoplasmic tail (and are thus denoted by the letter "L") and prevent target cell lysis, whereas activating KIRs possess a short tail (denoted by the letter "S") and aid cell lysis (Biassoni et al., 1996; Fan et al., 1997).

KIR genes have increasingly been associated with variability in response to viral diseases (HIV, CMV, viral hepatitides) (Marangon et al., 2011; Stern et al., 2008; Tiemessen et al., 2011) and with autoimmune diseases such as scleroderma, rheumatoid arthritis, and psoriasis (Jobim et al., 2008; Martin et al., 2003; Salim et al., 2010). Due to the haplotype diversity of *KIR* genes, there is interest in analyzing their potential association with other disease states. For instance, an association between HLA ligands and KIR receptors has been implicated in the development of preeclampsia (Hiby et al., 2004), hematologic neoplasms (Karabon et al., 2011; Leung et al., 2007), and solid tumors (Romagne et al., 2009). Therefore, as GD is associated with intra-familial variability (Amaral et al., 1994) and it is also associated with a reduction in NK cells (Burstein et al., 1987), with hematological malignancies and with an increase in the levels of natural autoantibodies (Shoenfeld et al., 1995), we may speculate that *KIR*/*HLA* variants are phenotype modifying factor of GD.

This is an exploratory study which aimed to ascertain whether correlations exist between *KIR* genes, their HLA ligands and clinical features in a cohort of GD patients from Southern Brazil.

2. Methods

2.1. Patients and controls

The study sample comprised 31 patients (from 28 unrelated families) with a biochemical and molecular diagnosis of GD followed at the State Reference Center for Gaucher disease of Rio Grande do Sul, Brazil, from 2003 to 2011. Of these 31 patients, 28 were receiving enzyme replacement therapy (ERT) with imiglucerase (n = 23) or taliglucerase alfa (n = 5) with a mean time of treatment of 97.21 months (range 4–215). All patients were European descendants, had been born in the South region of Brazil, and denied Ashkenazi Jewish ancestry. Patients (or the legal guardians of patients under the age of 18) provided written informed consent for participation in the study, and clinical, biochemical, and radiological data were collected by means of a chart review. The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

The following clinical parameters were assessed: age at onset of symptoms (when available) or age at diagnosis; the highest GD severity score index (SSI) (Zimran et al., 1992) presented for each patient during follow-up (use of this score was implemented at our service in 2007; therefore, patients diagnosed in previous years have no record of pretreatment SSI scores); presence of osteopenia or osteoporosis (as assessed by bone density scanning) or osteonecrosis (as diagnosed on plain radiographs) on at least one occasion; presence of hyperimmunoglobulinemia on at least one occasion; and presence of polyclonal or monoclonal bands on serum protein electrophoresis on at least one occasion.

The frequencies of the variants were compared with the data obtained from 250 healthy European descendants, bone marrow donors from Rio Grande do Sul, Brazil, drawn from the National Bone Marrow Donors Registry (REDOME) (Jobim et al., 2010).

2.2. KIR and HLA typing

Fifteen *KIR* genes (*2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, *3DS1*, *2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5*, *3DL1*, *3DL2*, *3DL3* e *2DP1*), HLA-C and HLA-Bw4 were analyzed using the PCR amplification with sequence-specific primers (SSP-PCR) technique as previously described (Bunce et al., 1995). The results of HLA-C typing were separated into two groups: HLA-C group 1 (C1), consisting of HLA-C 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04), and HLA-C group 2 (C2), consisting of HLA-C 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18 (Jobim et al., 2008). HLA-C group 1 (C1) molecules bind to KIR2DS2, KIR2DL2 and KIR2DL3, whereas group 2 (C2) molecules bind to KIR2DS1 and KIR2DL1. Bw4 molecules bind to KIR3DL1 and KIR3DL1 (Biassoni et al., 1995).

2.3. Statistical analysis

Pearson's chi-square test with continuity correction was used to compare *KIR* gene and HLA-C frequencies between the GD and control groups, and Bonferroni correction for multiple comparisons was performed. In a few cases where the expected difference between the two groups was small, Fisher's exact test was employed. There was no adjustment for population stratification. Odds ratios (OR), confidence intervals (95%CI), and p values ($p<0.050$) were calculated using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL).

3. Results

The clinical and demographic profile of the study sample is shown in Table 1.

There were no significant differences between the GD and control groups in frequency of *KIR* gene, HLA-C, and HLA-Bw4 variants (Table 2). Frequencies were similar to those reported in previous

Table 1
Clinical and demographic features of the study sample.

		Patients (n = 31)
Gender (female:male ratio)		13:18
Mean age ± SD (years)		29.48 ± 15.19
Mean age at onset of symptoms ± SD (years)[b]		37.5 ± 16.7
Disease severity (SSI)[a]	Mild (0–10)[c]	24
	Moderate (11–25)[d]	4
	Severe (>25)[e]	3
Hyperimmunoglobulinemia		24
Monoclonal/Polyclonal bands on protein electrophoresis		14
Osteopenia/Osteoporosis		18
Osteonecrosis		9

[a] According to Zimran et al. (1992). SD: standard deviation.
[b] When unavailable, age at diagnosis was used instead.
[c] Mean age ± SD (years): 31 ± 16.31.
[d] Mean age ± SD (years): 30.25 ± 8.01.
[e] Mean age ± SD (years): 16.33 ± 4.04.

studies of the Brazilian population (Middleton et al., 2008; Rudnick et al., 2008). The frequency of *KIR* gene variants in combination with their respective ligands was also determined and compared between the GD and control groups; again, there were no significant differences (Table 2).

Comparison of the frequencies of specific *KIR* gene variants in the GD group and analysis of the potential association of these variants with age at diagnosis or symptom onset, presence of hyperimmunoglobulinemia, presence of mono- or polyclonal bands on protein electrophoresis, and osteoporosis or osteonecrosis revealed a significant association ($p = 0.038$) between the absence of the KIR2DS2 and KIR2DL2/HLA-C1 combination and symptom onset after childhood (16 out of 19 did not have this combination, whereas 7 of the 12 patients in whom symptom onset occurred at age > 18 years did).

A significant association was also found between HLA-C typing and changes on serum protein electrophoresis. Patients who were homozygous for group HLA-C2 had an OR = 21.3 (95% CI: 2.18–208.7, $p = 0.007$) for the presence of monoclonal or polyclonal bands, whereas patients with at least one C1 allele (C1/C1 or C1/C2 genotype) had an OR = 0.047 (95% CI: 0.005–0.459, $p = 0.004$) for showing those findings (Table 3).

No other significant associations were found between other clinical and demographic parameters and *KIR* gene frequencies.

Table 2
KIR gene, ligand frequencies and their association in Gaucher disease (GD) patients (n = 31) and in controls (n = 250).

KIR gene, ligands and association	Controls		GD		p-Value[a]
	N	%	N	%	
2DL1	244	97.6	31	100.0	1.000
2DL2	136	54.4	15	48.4	0.549
2DL3	216	86.4	28	90.3	0.589
2DL4	250	100.0	31	100.0	1.000
2DL5	124	49.6	18	58.1	0.819
3DL1	244	97.6	31	100.0	1.000
3DL2	200	100.0	31	100	1.000
3DL3	200	100.0	31	100.0	1.000
2DS1	91	36.4	13	41.9	0.825
2DS2	134	53.6	14	45.2	0.407
2DS3	83	33.2	9	29.0	0.510
2DS4	238	95.2	31	100.0	0.612
2DP1	200	100.0	31	100.0	1.000
2DS5	85	34.0	16	51.6	0.084
3DS1	106	42.4	9	29.0	0.171
Bw4	179	71.6	22	71.0	1.000
C1	180	72.0	22	71.0	0.984
C2	179	71.6	24	77.4	0.403
2DL2/C1	102	40.8	10	32.3	0.432
2DS2/C1	100	40.0	10	32.3	0.340
2DL3/C1	153	61.2	20	64.5	0.778
2DL1/C2	176	70.4	24	77.4	0.499
2DS1/C2	63	25.2	10	32.3	0.565
3DL1/Bw4	174	69.6	22	71.0	1.000
3DS1/Bw4	77	30.8	4	12.9	0.075

C1 group: HLA-Cw 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04).
C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18.
[a] Bonferroni-adjusted alpha for significance $p < 0.002$.

4. Discussion

GD is the most prevalent lysosomal storage disorder, and that in which immune system involvement plays the greatest role (Castaneda et al., 2008). The main hypothesis is that a buildup of glucocerebroside within macrophage lysosomes leads to persistent inflammatory stimulation, but the exact mechanism is still unclear. Some of the features of the characteristic immune involvement of GD include a blunted antigen response to bacterial pathogens, which can improve with ERT (Marodi et al., 1995); increased incidence of B cell-related malignancies, such as lymphomas, leukemias, monoclonal gammopathies, and multiple myeloma (Camou and Viallard, 2012; Hawkesford et al., 2011; Ranade et al., 2010; Rosenbloom et al., 2009); and presence of autoantibodies (Shoenfeld et al., 1995). As lysosomal accumulation of substrate occurs in immune system cells, antigen presentation by HLA class I and class II molecules is also compromised (Balreira et al., 2005), and, as mentioned before, there is a decrease in the number of NK cells and impairment in their activity (Burstein et al., 1987). NK cells are important components of the innate immune system, as they are able to eliminate tumor cells and virus-infected cells (Leavy, 2012). The role of NK cells in the pathophysiology of and susceptibility to autoimmune disease has increasingly been the object of research (Jobim et al., 2008; Qin et al., 2011; Salim et al., 2010), due to the high allelic variability of NK cell receptors. It is also widely known that GD is associated with intra-familial variability (Amaral et al., 1994). All these factors together led us to believe that immune-related factors, including the *KIR* gene variants, may be phenotype modifiers in GD.

Comparison of the frequencies of the 15 known *KIR* genes between the GD and control groups revealed no significant differences. This was expected, in view of the ethnic homogeneity of both groups, or even because of the small size sample and the resulting insufficient statistical power. However, analysis of the potential associations between *KIR* variants and clinical, demographic, and laboratory parameters revealed some interesting data.

The KIR2DS2 or KIR2DL2/HLA-C1 combination appears to be associated with delayed symptom onset, as it was present in only 15.8% of patients whose symptoms first arose before the age of 18, whereas 58.3% of patients with adult-onset symptoms have these combinations. It bears stressing that the KIR2DS2 and KIR2DL2/HLA-C1 combination has been described as a protective factor against the development of chronic myeloid leukemia (CML) (Middleton et al., 2009). Patients with GD are at increased odds of developing CML (OR 3.4) (Landgren

Table 3
Frequencies (%) of C1 and C2 groups in Gaucher disease relative to the absence (17) and presence (14) of polyclonal/monoclonal bands.

Ligand groups	Polyclonal/monoclonal bands				p-Value	OR	95% CI
	Absent		Present				
	N	%	N	%			
C1C1	2	11.8	5	35.7	0.198	–	–
C2C2	1	5.9	8	57.1	0.007	21.3	2.18–208.7
C1C2	14	82.4	1	7.1	<0.001	0.016	0.002–0.179

C1C2: C1 and C2 group heterozygous;
C1 group: HLA-Cw 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04); C1C1: homozygous for C1 group;
C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18; C2C2: homozygous for C2 group.

et al., 2007). Furthermore, the KIR2DS2/DL2 genotype was recently associated with adult persistent immune thrombocytopenia (Nourse et al., 2012). Two of the three patients in our sample who exhibit persistent thrombocytopenia despite ERT have the KIR2DS2/DL2 genotype (data not shown); therefore, this variant may mask therapeutic failure.

The main finding of this study was the association between HLA-C and the presence of serum protein electrophoresis changes. Patients with the C2/C2 genotype had an OR of 21.3 for the development of monoclonal or polyclonal bands. Ninety-four percent of patients who did not develop gammopathy had at least one C1 allele, whereas 43% of patients who did develop gammopathy had at least one C1 allele. On the basis of these data, the presence of C1 appears to be a protective factor against the development of gammopathies. There is substantial interest in explaining why GD patients have a greater frequency of MM. The hypothesis that mutations in the *GBA* gene may be a risk factor was not confirmed in a study of 95 patients with MM (Rosenbloom et al., 2009). The only patient with suspected MM included in our sample, who presented with IgG kappa monoclonal pike in protein electrophoresis (data not shown), was found to be homozygous for the C2 allele, which leads us to believe that the C2/C2 genotype may be considered a risk factor for the development of this gammopathy.

This was the first study to analyze KIR/HLA variants in GD patients. Although limited in size, our sample exhibited broad variability of this gene system, with a variety of genotype profiles, and our findings suggest a potential association between *KIR* and *HLA* genes and GD, thus corroborating the importance of immune system involvement in this disorder. Further study of KIR/HLA variants in other patient cohorts is warranted, as they seem to be a phenotype-modifying factor in GD.

Conflicts of interest

The authors have no conflict of interest to declare.

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3.4) CAPÍTULO 4

Human leukocyte antigens and Gaucher disease

Título do manuscrito: *Human leukocyte antigens and Gaucher disease*

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Human leukocyte antigens and Gaucher disease

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ABSTRACT

Background: Gaucher disease (GD) is caused by the reduced activity of lysosomal enzyme glucocerebrosidase, which leads to the accumulation of glucocerebroside in macrophages and a chronic stimulation of the immune system. GD is divided into 3 main types according to the presence or absence of neurological involvement and to its presentation (acute or chronic). Gaucher cells show an increase in their expression of HLA-DR antigens on their surface, and there is an increase in levels of antigen-presenting molecules. Over 100 diseases have already been associated to HLA antigens; however, this association has never been studied in GD.
Objectives: To analyze the variability of HLA genes in a Southern Brazilian sample of GD patients, to compare it with controls, and to look for associations with clinical manifestations.
Methodology: Thirty-one GD patients (24 mild, 4 moderate, and 3 severe) were included in the study. They were typed for HLA A, B, and DR and compared to 250 healthy controls. The clinical data were obtained from the review of medical records.
Results/discussion: There was a significant difference in the frequency of B37 allele among patients when compared to controls ($p=0.011$, OR 13.28). An association was found between DR11 ($p=0.008$) and DR13 ($p=0.011$) alleles and the severity of the disease. DR11 allele seems to be associated to neurologic compromise, while DR13 seems to be associated to osteonecrosis.
Conclusion: Our data suggest a possible association of HLA variants and GD. The HLA variants must be further studied, for they seem to be a phenotype-modifier factor for GD.

Introduction

Gaucher disease (GD) is the most frequent of the lysosomal disorders with an estimated incidence of 1 in 57,000 live births worldwide [1]; however, in Ashkenazi Jews, its incidence reaches 1 in 400 live births [2].

GD is caused by mutations in both alleles of the *GBA* gene, which codifies the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45); this enzyme is responsible for the hydrolysis of glucocerebroside into glucose and ceramide [3]. Consequently, there is an accumulation of glucocerebroside in macrophages, mainly in the spleen, liver, bone marrow, and lungs, thus characterizing GD as a multisystemic disorder with phenotypic heterogeneity.

GD type I (MIM #230800) is the most prevalent form and is more frequent in the Ashkenazi Jewish population, although most patients with GD type I are not Jewish. GD affects children and adults of any age, and its typical clinical manifestations include hepatosplenomegaly, anemia, thrombocytopenia, and bone disease. GD types II and III are less frequent and occur in all ethnic groups. Type I differs from type II (MIM #230900) and type III (MIM #231000) for its lack of involvement of the central nervous system, although some studies report neurological characteristics in type I patients that are different from those seen in type II or type III patients [4]. Patients with GD who present neurological involvement (neuronopathic GD) are classified as being type II or type III, according, respectively, to the acute or chronic nature of their disease. According to the Brazilian Association of Patients with Gaucher Disease, there are over 600 patients diagnosed with GD in Brazil.

Despite the already well-characterized genetic alteration and biochemical pathways, the mechanisms by which the accumulation of substrate causes the clinical manifestations of GD have not yet been completely determined [5,6]. Several modifying genes, adjacent genes, transporting proteins, and environmental factors may

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influence the phenotype of patients with GD [7–9], and since different phenotypes have been described for monozygotic twins [10,11], the search for modifying factors is necessary.

Gaucher cells (cells that derivate from macrophages with lipid accumulation) are 20–100 μm in diameter, and present an eccentric nucleus, and a cytoplasm with striations. The detection of macrophage surface markers and an intense phagocytic activity confirm the ontogeny of mononuclear phagocytes. The accumulation of glucocerebroside in macrophages leads to a change in cellular phenotype [12]; macrophages show an increase in the expression of HLA-DR antigens on their surface [13]. It is speculated that alterations in the function of macrophages (due to the accumulation of glucocerebroside) in GD patients contribute to the disorder, especially to hepatosplenomegaly and bone compromise [14].

GD is the lysosomal disease that has the greatest involvement of the immune system, so much so that one of the existing hypothesis to explain the phenotypic expression of this disorder is the existence of a chronic stimulus of the immune system [15]. A piece of evidence is the presence of elevated levels of antigen-presenting molecules, including class II MHC molecules [16], which comprises the HLA class II.

The HLA (Human Leukocyte Antigen) system is divided into classes I, II, and III, located in region 6p21.3, and comprising more than 200 genes [17]. Class I antigens (HLA A, B, and C) are expressed in almost all cells except erythrocytes and trophoblasts. Class II antigens (HLA DR, DQ, DP) are expressed in antigen-presenting cells, such as monocytes and dendritic cells. The region of class III does not contain *HLA* genes, but several genes of immunological importance are located in this region as components of the complement system and cytokines [18,19].

The first associations of the HLA system with autoimmune diseases, such as HLA-B27 with ankylosing spondylitis [20] and HLA-DR4 with rheumatoid arthritis [21], date from over 30 years ago. Since then, more than 100 diseases have already been associated to *HLA* genes. Their polymorphisms are relevant to the auto and hetero recognition by T cells, besides the processing and presentation of infectious agents, infected cells, or cells with somatic mutations [22].

Methodology

The study sample comprised 31 patients (from 28 unrelated families) with a biochemical and molecular diagnosis of GD followed at the State Reference Center for Gaucher disease of Rio Grande do Sul, Brazil, since 2003. Of these 31 patients, 28 were receiving enzyme replacement therapy (ERT) with imiglucerase (n = 23) or taliglucerase alfa (n = 5). All patients were European descendants, had been born in the South region of Brazil, and denied Ashkenazi Jewish descent. Patients (or the legal guardians of patients under the age of 18) provided written informed consent for participation in the study, and clinical, biochemical, and radiological data were collected by means of a chart review. The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

The following clinical parameters were assessed: age at onset of symptoms (when available) or age at diagnosis; increased GD severity score index (SSI) during follow-up (use of this score was implemented at our service in 2007; therefore, patients diagnosed in previous years have no record of pretreatment SSI scores); presence of osteopenia or osteoporosis (as assessed by bone density scanning) or osteonecrosis (as diagnosed on plain radiographs) on at least one occasion; hyperimmunoglobulinemia; and presence of polyclonal or monoclonal bands on serum protein electrophoresis.

The control sample was obtained from 250 healthy bone marrow donors registered in the National Registry of Bone Marrow Donors (REDOME in the Portuguese abbreviation); controls were European descendants and from the state of Rio Grande do Sul, Brazil.

HLA typing

Leukocytes were isolated by centrifuging from the peripheral blood collected in a tube with EDTA. The DNA of the sample was extracted using the salting-out method [23]. DNA amplification was done using the polymerase chain reaction technique (PCR). For the HLA A, B, and DR typing, the PCR-SSO technique (Sequence Specific Oligonucleotides, *One Lambda Inc.*) and the automatized system with Luminex®technology [24] were used. Ambiguities were solved using the PCR-SSP technique (Sequence Specific Primers) [25].

Statistical analysis

Comparison of the *HLA A, B* and *DR* gene frequencies between the patient group and the control group was done by Pearson χ^2 with continuity correction, and Fisher's exact test was employed in a few where the expected difference between the two groups was small. Odds ratios (OR), confidence intervals (95% CI), and significance values ($p<0.05$) were calculated using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL).

Results

The clinical and demographic characteristics of patients are presented in Table 1. The comparison of frequencies of the *HLA* A (Table 2), *HLA* B (Table 3), and *HLA* DR (Table 4) genes between patients and controls showed a single significant difference in the frequency of allele HLA B37.

An association between HLA DR11 and HLA DR13 alleles and disease severity (Table 5) was found when the frequency of specific alleles of *HLA* genes in the group of patients with GD was compared with the clinical and demographic data.

The comparison of the remaining clinical and demographic variables with the frequencies of the *HLA* genes did not show any statistical significance.

Discussion

Gaucher disease is the most prevalent of the lysosomal disorders and the one that presents the greatest involvement of the immune system [26]. The main hypothesis is that the accumulation of glucocerebroside in the lysosomes of macrophages leads to a persistent inflammatory stimulation; however, the exact mechanism through which this happens remains unclear. One the factors possibly related to this is the compromise of the antigenic presentation of class I and class II HLA molecules that happens due to this accumulation [16].

We decided to analyze variants of *HLA* genes in our cohort of patients, given that there are descriptions of phenotypic variability in

Table 1
Clinical and demographic features of Gaucher disease patients included in the present study.

		Patients (n = 31)
Gender (female:male)		13:18
Mean age ± SD (years)		30.87 ± 15.3
Mean time of treatment ± SD (years)		9.0 ± 6.4
Mean age of onset of symptoms ± SD (years)[a]		20.9 ± 18.1
Disease severity (SSI)[b]	Mild (0–10)	24
	Moderate (11–25)	4
	Severe (>25)	3
Hyperimmunoglobulinemia		24
Monoclonal/Policlonal bands on protein electrophoresis		14
Osteopenia/Osteoporosis		18
Osteonecrosis		9

[a] When not available, age at diagnosis was considered.
[b] According to Zimran et al. 1992; SD: standard deviation.

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Table 2
Comparison of HLA-A allele frequencies in patients and controls.

	Controls (n=250)		Gaucher disease (n=31)	
	n	%	N	%
A*01	49	19.6	6	19.4
A*02	120	48.0	19	61.3
A*03	55	22.0	9	29.0
A*11	32	12.8	6	19.4
A*23	12	4.8	1	3.2
A*24	44	17.6	4	12.9
A*25	12	4.8	3	9.7
A*26	16	6.4	2	6.5
A*30	16	6.4	1	3.2
A*31	23	9.2	2	6.5
A*32	16	6.4	1	3.2
A*33	10	4.0	1	3.2

*Chi-Square Test or Fisher Exact Test, p values were not significant.

monozygotic twins with GD [10]. This led us to believe that genes related to the immune system may be phenotype modifiers in GD.

The frequency of allele HLA B37 found in our patients was 12 times higher when compared to controls. It should be pointed out that the HLA system is highly polymorphic and that many alleles present linkage disequilibrium; therefore, it cannot be inferred that this allele alone is associated to GD. This allele has already been associated to the development of psoriasis in young age patients in other populations [27]. So far, none of our patients have presented this disease.

One of our patients presented allele HLA B27, which is known to be related to ankylosing spondylitis. Studies show that up to 95% of the patients with this disorder carry this allele and they are diagnosed earlier [28]. Consequently, this patient will be regularly evaluated due to the possible ocular and vertebral complications caused by ankylosing spondylitis. So far, this patient has not presented any related symptoms.

An interesting finding of our study was the association of alleles DR11 and DR13 with the severity of GD. All our patients with GD type III (from 2 unrelated families — DR11 was the only allele shared by them), 1 patient with mild GD type I and possible multifactorial mild cognitive delay, as well as 1 patient with moderate GD type I who presented cerebellar symptoms and tremors as a side effect in an attempt to use substrate reduction therapy (an effect that has already been described in other patients) presented allele DR11. Therefore, one hypothesis is that this allele is associated to neurological compromise in patients with GD or to a predisposition to the development of neurological side effects with the use of substrate reduction therapy. This allele has already been related to a predisposition for the development of thyroid neoplasia [29], systemic sclerosis [30], and low production of antibodies against the hepatitis C virus [31], which none of this characteristics were found in our patients.

Three of 4 patients with moderate GD type I presented allele DR13. All patients presented osteonecrosis; 2 of them had already undergone arthroplasty, and one other presented significant functional limitations; therefore, this allele may be associated to the bone compromise seen in these patients. The other 3 patients who presented this allele had mild disease, presented symptoms before the age of 10 years, and started treatment early; as a result, this may have prevented the evolution of bone disease in these patients. DR13 allele has already been associated both to a better viral clearance for hepatitis B and C [32] and to the susceptibility to the development of uveitis in patients with juvenile rheumatoid arthritis [33]. This was important because one of the patients that had this allele did not serum convert for hepatitis B, even after 6 vaccination doses; this patient may be somehow protected against the virus.

This is the first study relating variants of *HLA* genes to GD. Despite the present limited patient sample and considering the great variability, with several genotypic profiles of this genic system, our data suggest the existence of a possible association between the *HLA* genes and the phenotypic variability of GD, thus corroborating to the significant immune involvement seen in this disorder. Therefore, they should be further studied in other patient cohorts, since they can be considered modifying factors of GD.

Table 3
Comparison of HLA-B allele frequencies in patients and controls.

	Controls (n=250)		Gaucher disease (n=31)	
	n	%	N	%
B*07	21	8.4	5	16.1
B*08	44	17.6	2	6.5
B*13	14	5.6	2	6.5
B*15	32	12.8	5	16.1
B*18	26	10.4	4	12.9
B*27	16	6.4	1	3.2
B*35	55	22.0	10	32.3
B*37#	2	0.8	3	9.7
B*38	17	6.8	1	3.2
B*39	8	3.2	3	9.7
B*40	17	6.8	1	3.2
B*44	47	18.8	7	22.6
B*45	14	5.6	1	3.2
B*48	2	0.8	1	3.2
B*49	21	8.4	1	3.2
B*50	9	3.6	2	6.5
B*51	55	22.0	4	12.9
B*52	2	0.8	1	3.2
B*55	10	4.0	1	3.2
B*57	20	8.0	1	3.2
B*58	7	2.8	2	6.5

*Chi-Square Test or Fisher Exact Test, #p=0.011; OR=13.28; 95%CI 2,12-82,9 the other p values were not significant.

Table 4
Comparison of HLA-DR allele frequencies in patients and controls.

	Controls (n=250)		Gaucher disease (n=31)	
	n	%	N	%
DRB1*01	35	14.0	5	16.1
DRB1*03	74	29.6	6	19.4
DRB1*04	77	30.8	6	19.4
DRB1*07	73	29.2	4	12.9
DRB1*08	22	8.8	3	9.7
DRB1*10	12	4.8	1	3.2
DRB1*11	52	20.8	8	25.8
DRB1*12	6	2.4	3	9.7
DRB1*13	66	26.4	6	20.0
DRB1*14	13	5.2	5	16.1
DRB1*15	27	10.8	7	22.6

*Chi-Square Test or Fisher Exact Test, p values were not significant.

Conflicts of interest

The authors state no conflicts of interest.

Table 5
Relation of disease severity, HLA DR11 and HLA DR13.

	Disease severity			p-Value*
	Mild (n=24)	Moderate (n=4)	Severe (n=3)	
HLA DR*11	16.7%	25.0%	100%	0.008
HLA DR*13	12.5%	75.0%	0%	0.011

*Chi-Square Test or Fisher Exact Test.

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3.5) CAPÍTULO 5

Brain-derived neurotrophic factor expression increases after enzyme replacement therapy in Gaucher disease

Título do manuscrito: *Brain-derived neurotrophic factor expression increases after enzyme replacement therapy in Gaucher disease*

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Short communication

Brain-derived neurotrophic factor expression increases after enzyme replacement therapy in Gaucher disease

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ABSTRACT

Mutations in the *GBA* gene are related to an increased risk of developing neurodegenerative diseases. The exact molecular mechanisms involved in the interaction between *GBA* and α-synuclein, a protein that has been associated with several neurological diseases, remain unsolved. Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is important for the normal development of the peripheral and central nervous system, and it plays a key role in neuronal survival and synaptic plasticity in the adult brain. A reduction in BDNF expression has been reported in patients with Parkinson's disease, Alzheimer's disease and dementia with Lewy bodies. We analyzed BDNF levels in the plasma of Gaucher Disease (GD) patients who were not being treated with enzyme replacement therapy (ERT) and then subsequently following ERT; we compared the levels to those of healthy controls. We demonstrated that BDNF levels were remarkably diminished in GD patients who were under no specific treatment and these levels increased following ERT. This is the first study that demonstrates a variation in the plasma levels of a neurotrophic factor in GD type 1 patients. Further studies are required to correlate BDNF level variations with the clinical findings and the response to therapy in GD patients. Low levels of BDNF are associated with neurodegenerative diseases; therefore, BDNF could provide a new therapeutic target for GD patients with neurological symptoms.

1. Introduction

Gaucher disease (GD, OMIM 230800) is one of the most prevalent lysosomal disorder, with an estimated worldwide incidence of 1 case per 57,000 live births (Meikle and Hopwood, 2003), although this rate can reach 1 in 850 among Ashkenazi Jews (Mistry et al., 2011).

GD is caused by mutations in both alleles of the *GBA* gene, which codes for lysosomal glucocerebrosidase (EC 3.2.1.45), an enzyme responsible for catalyzing the hydrolysis of glucocerebroside into glucose and ceramide (Beutler, 2006). Consequently, there is intracellular accumulation of the substrate in macrophages, particularly in the spleen, liver, bone marrow, and lungs. GD is therefore a multisystem disorder and exhibits phenotypic heterogeneity. Classically, GD is subdivided into three main forms (types I, II and III) defined by clinical characteristics, disease course, and ethnic prevalence. Nevertheless, there is a wide range of findings that overlap across the classical forms, which has led to a new assessment of GD as a continuous spectrum of disorders rather than a disease with three distinct subtypes (Beutler, 2006). Although the genetic changes and biochemical pathways that underlie GD have been well characterized, the mechanisms whereby the accumulation of glucocerebroside leads to clinical manifestations have yet to be fully determined (Jmoudiak and Futerman, 2005; Hughes and Pastores, 2010).

Mutations in the *GBA* gene have been associated with an increased risk of developing Parkinson's disease and dementia with Lewy body inclusions (Sidransky et al., 2009; Nalls et al., 2013). The exact molecular mechanisms involved in the interaction between *GBA* and α-synuclein, the main protein associated with these neurological diseases, remains unsolved.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, a group of neuronal growth factors including nerve growth factor (NGF), neutrotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Binder and Scharfman, 2004). These proteins are not only important for the normal development of the peripheral and central nervous system but they also play a key role in neuronal survival and synaptic plasticity in the adult brain (Arancio and Chao, 2007).

A reduction in BDNF expression has been reported in patients with Parkinson's disease, Alzheimer's disease and dementia with Lewy

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bodies (Murer et al., 2001; Imamura et al., 2005). Recently, increasing evidence suggests that alterations in the brain neurotrophic support and, in particular, in BDNF expression and signaling, might contribute to neurodegeneration (Balaratnasingam and Janca, 2012). Additionally, patients with mood disorders, such as major depression, present with decreased serum levels of BDNF, which increase following medication treatment (Lee and Kim, 2010; Bocchio-Chiavetto et al., 2010).

In this pilot study, we analyzed BDNF levels in the plasma of Brazilian adult type 1 GD patients during and after imiglucerase shortage which occurred in 2009–2010, and we showed the response of BDNF after the restart of enzyme replacement therapy.

2. Methodology

2.1. Study population

The study group was composed of 14 GD type 1 adult patients (7 males and 7 females, one splenectomized; median age = 35.7 years; median time of treatment pre-shortage = 5.8 years; median ERT dose pre-shortage = 25.7 UI/kg), 8 healthy controls (6 females and 2 males, median age = 37.1 years) and 5 male patients with Fabry disease on regular treatment (median age = 37.4 years).

We analyzed plasma samples from patients with a biochemical and molecular diagnosis of GD who were followed at the State Reference Center for Gaucher disease of Rio Grande do Sul, Brazil. Patients provided written informed consent for participation in the study, and clinical and biochemical data were obtained by chart review. The samples were obtained during the period of imiglucerase shortage (at least 4 months with no GD specific treatment) and after the shortage ended (at least 6 months on ERT). The control samples were obtained during routine blood work. All of the samples were collected, centrifuged in less than 1 h of collection and stored at −80 °C. The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

2.2. BDNF plasma assay

BDNF levels were determined using the MILLIPLEX® MAP system (HNDG3MAG-36K, Millipore Corporation, Billerica, Massachusetts) according to the manufacturer's protocol. The samples were analyzed in duplicate, and when the difference between the reads was less than 10%, the mean was used. When the difference was greater than 10%, the sample was not included in the analysis. BDNF levels were estimated using a 5-parameter polynomial curve. Values are expressed in pg/mL.

2.3. Statistical analysis

The null hypothesis considered in the study was the absence of a difference in the BDNF concentration among the groups studied. The values were compared using nonparametric tests as Wilcoxon signed rank test since the distribution was not normal. When comparing BDNF values during imiglucerase shortage and after the restart of ERT a related sample test was used. All the analyses were made with the software IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY). The statistical significance value was $p < 0.05$.

3. Results

Fig. 1 shows the median BDNF plasma levels of healthy controls (2019.5 pg/mL; minimum = 36, maximum = 5325, interquartile range = 4039.5), GD patients not receiving ERT (138.5 pg/mL; minimum = 51, maximum = 238, interquartile range = 92) and GD patients receiving ERT (395 pg/mL; minimum = 107, maximum = 1533, interquartile range = 248). The comparison among patients not receiving ERT, healthy controls and patients receiving ERT was significant ($p < 0.001$ and $p < 0.002$, respectively).

The BDNF levels in the plasma of five patients with Fabry disease receiving regular treatment (data not shown in figures) did not differ from controls (mean BDNF = 977 pg/mL; minimum = 415, maximum = 2301, interquartile range = 818.5; p value = 0.2).

4. Discussion

The typical classification of GD into three clinical phenotypes is being reassessed following the neurological findings in type 1 GD patients (Cherin et al., 2010) and the relationship between carriers of *GBA* mutations and the development of Parkinson's disease and dementia with Lewy bodies (Goker-Alpan et al., 2004; Ziegler et al., 2007; Tsuang et al., 2012). Studies in the brains of mouse models of neuronopathic GD detected the intracellular accumulation of glycosphingolipids, glucosylceramide and psychosine, leading to neurodegeneration (Farfel-Becker et al., 2014). Additionally, it has been reported that deficiencies of neurotrophic factors, including BDNF, result in massive cell death and neurodegeneration in the central nervous system of GD mouse models (Kim et al., 2006), however no patient studies to date have evaluated these neurotrophic factors.

We analyzed BDNF levels in the plasma of GD patients not receiving ERT and following ERT and compared these levels to healthy controls. We demonstrated that BDNF levels are remarkably diminished in GD patients receiving no specific treatment and increased by greater than 3 times following 6 months of ERT but yet different from healthy subjects. We also analyzed plasma samples from patients with Fabry disease since this is a lysosomal disorder with prominent neurological features. There were no differences between BDNF levels of treated patients with Fabry disease and healthy subjects, unlike the findings in patients with Gaucher disease.

The possibility of trafficking across the blood–brain barrier remains open; however, evidence suggests that the levels of BDNF in the blood reflect the levels in the central nervous system. This is noted particularly in studies related to major depression (Bocchio-Chiavetto et al., 2010; Castren and Rantamaki, 2010). Additionally, correlations were observed between serum BDNF levels and cerebral cortex integrity (Lang et al., 2007) and cognitive function (Gunstad et al., 2008) in healthy adults. No patients in this study presented with neurological impairment, parkinsonism, cognitive decline or major depression. However, we did not detect changes in the scores on the quality of life questionnaires because the time frame of this study spanned only a few months, and it is well established that ERT improves the health-related quality of life of GD patients following several years of treatment (Hayes et al., 1998; Masek et al., 1999; Oliveira et al., 2013).

Based on these results, we hypothesize that BDNF plays a neurochemical role leading to an improvement of the mental health of patients with GD disease.

BDNF is a potent inhibitor of apoptosis-mediated cell death and neurotoxin-induced degeneration of dopaminergic neurons, and there are some studies suggesting that it may be used in the development of neuroprotective therapies, especially for Parkinson's disease (Scalzo et al., 2010), and further translated to new therapeutic options for GD patients with neurological symptoms.

5. Conclusion

This is the first study that demonstrates a variation in the plasma levels of a neurotrophic factor in GD type 1 patients. Further studies are required to associate BDNF level variations with the clinical findings and the response to therapy in GD patients. A follow up of these levels through time is necessary to verify whether this increase is dose and/or time dependent. Because low levels of BDNF are related to neurodegenerative diseases, BDNF could be a new therapeutic target for GD patients with neurological symptoms.

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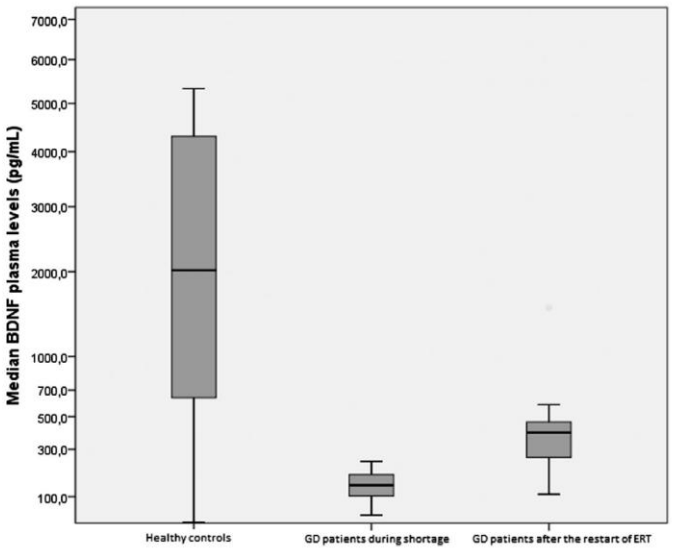

Fig. 1. Median BDNF values in plasma from healthy controls (n = 8) and Gaucher patients (n = 14) not receiving ERT (for at least 4 months) and receiving ERT (for at least 6 months). Healthy controls × Gaucher not receiving ERT, p value < 0.001, and Gaucher not receiving ERT × Gaucher receiving ERT, p value < 0.002.

Conflicts of interest

The authors have no conflict of interest to declare.

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Authorship

F. Vairo designed the research, performed the research, analyzed the data and wrote the paper. F. Sperb-Ludwig performed the research and analyzed the data M. Wilke performed the research and analyzed the data. K. Michellin-Tirelli performed the research and analyzed the data. C. Netto performed the research and analyzed the data. E.C. Neto performed the research and analyzed data. I. Schwartz designed the research, performed the research, analyzed the data and wrote the paper.

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3.6) CAPÍTULO 6

Osteopontin: a potential biomarker of Gaucher disease

Título do manuscrito: *Osteopontin: a potential biomarker of Gaucher disease*

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Osteopontin: a potential biomarker of Gaucher disease

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Abstract:	Gaucher disease (GD) is one of the most prevalent lysosomal storage disorder and the disorder that has the greatest immune system involvement. Pathologic lipid accumulation in macrophages accounts for a small amount of the additional tissue mass in the liver and spleen. The additional increase may be related to an inflammatory response because Gaucher cells secrete inflammatory mediators. Osteopontin (OPN) is a protein identified in cancer cells and in bone cells that is produced by several types of immune cells including T-cells and macrophages. We report here elevated OPN levels in the plasma of type 1 GD patients and its sensitive response to enzyme replacement therapy. The mean OPN value of GD patients receiving ERT was similar to the values of controls and patients with other lysosomal disorders. When comparing untreated and treated GD patients, the p value was <0.001. In GD, OPN appears to be more sensitive to ERT than chitotriosidase and can be used during the follow-up of patients who are chitotriosidase deficient. Additional extended studies are required to relate variations in the OPN levels to clinical findings and response to therapy in GD patients.

Osteopontin: a potential biomarker of Gaucher disease

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Running title:
Osteopontin and Gaucher disease

Abstract

Gaucher disease (GD) is one of the most prevalent lysosomal storage disorder and the disorder that has the greatest immune system involvement. Pathologic lipid accumulation in macrophages accounts for a small amount of the additional tissue mass in the liver and spleen. The additional increase may be related to an inflammatory response because Gaucher cells secrete inflammatory mediators. Osteopontin (OPN) is a protein identified in cancer cells and in bone cells that is produced by several types of immune cells including T-cells and macrophages. We report here elevated OPN levels in the plasma of type 1 GD patients and its sensitive response to enzyme replacement therapy. The mean OPN value of GD patients receiving ERT was similar to the values of controls and patients with other lysosomal disorders. When comparing untreated and treated GD patients, the p value was <0.001. In GD, OPN appears to be more sensitive to ERT than chitotriosidase and can be used during the follow-up of patients who are chitotriosidase deficient. Additional extended studies are required to relate variations in the OPN levels to clinical findings and response to therapy in GD patients.

Keywords:
Osteopontin; Gaucher disease; Biomarkers; Glucocerebrosidase deficiency

Introduction

Gaucher disease (GD, OMIM 230800) is one of the most prevalent lysosomal disorder, with an estimated worldwide incidence of 1 case per 57,000 live births (1), although this rate can reach 1 in 850 among Ashkenazi Jews.(2)

GD is caused by mutations in both alleles of the *GBA* gene, which encodes lysosomal glucocerebrosidase (EC 3.2.1.45), an enzyme that is responsible for catalyzing the hydrolysis of glucocerebroside into glucose and ceramide (3). Consequently, there is intracellular accumulation of glucocerebroside in macrophages, particularly in the spleen, liver, bone marrow, and lungs. GD is therefore a multisystem disorder and exhibits phenotypic heterogeneity. Classically, GD is subdivided into three main forms (types I, II and III), which are defined by clinical characteristics, disease course, and ethnic prevalence. Nevertheless, there is a wide range of phenotypes that overlap across the classical forms, which has led to a new assessment of GD as a continuous spectrum of disorders rather than a disease with three distinct subtypes (3). Although the genetic changes and biochemical pathways that underlie GD have been well characterized, the mechanisms through which the accumulation of glucocerebroside leads to clinical manifestations have yet to be fully determined (4, 5).

Gaucher cells (GCs, lipid-laden macrophage derivatives) have eccentric nuclei and cytoplasmic striations. Their expression of macrophage surface markers and marked phagocytic activity confirm the ontogeny of GCs as mononuclear phagocytes (6). In addition to lipid substrate buildup, the pathogenesis of GD can be explained by defects in enzyme conformation, endoplasmic reticulum stress (7), defects in calcium homeostasis (one of the mechanisms responsible for the neuropathology observed in patients with acute neuropathic GD) (8), increased sensitivity to oxidative stress (9), and changes in autophagy mechanisms (10).

Pathologic lipid accumulation in macrophages accounts for less than 2 percent of the additional tissue mass in liver and spleen (11). The additional increase may be related to an inflammatory response because Gaucher cells and others secrete cathepsins and inflammatory mediators such as interleukins 1ra, 6, 8, 10 and macrophage inflammatory proteins (MIPs) (12-14). It has been documented for some time that Gaucher patients have increased levels of certain proteins such as tartrate-resistant acid phosphatase (TRAP), angiotensin-converting enzyme (ACE), hexosaminidase and lysozyme (15). These proteins are used as surrogate biomarkers with some restrictions. None of these markers are a specific marker for pathological Gaucher cells, and their levels in Gaucher disease patient serum may overlap with those observed in healthy subjects. The detection of high chitotriosidase activity in

Gaucher patients' plasma has improved the follow-up and management of the disease (16). However, chitotriosidase activity is not a perfect biomarker because this enzyme may be increased in other diseases, though more modestly, and there is a high frequency of individuals who are chitotriosidase deficient. These reasons prompted research into other biomarkers such as PARC/CCL18 (17) and glucosylsphingosine (18).

Because enzyme replacement therapy is costly and may require time to observe clinical improvements, the identification of sensitive biomarkers is important to indicate a possible early response to treatment.

Osteopontin (OPN) is encoded by the gene *SPP1* (OMIM 166490) and is a secreted phosphorylated protein that has been identified in cancer cells (19) and in bone cells (20). It is also produced by several types of immune cells, including T-cells and macrophages (21). OPN is also overexpressed in human cancers and is correlated with poorer prognosis (22), which may be related to its ability to stimulate the migration of many cell types (23).

In vitro, monocytes express OPN at a low level; when they differentiate into macrophages, these levels increase (24). Several cytokines have been demonstrated to increase OPN expression, particularly INF-γ, IL-10, IL-6, TNF and IL-1β (25-27).

In vivo macrophage OPN expression has been reported in a variety of different models of tissue injury and pathology, specifically models associated with inflammation. There are reports of OPN affecting the gene expression of IL-12, IL-10 (28), CCL4 (29) and macrophage functions such as differentiation (30), migration (31), cell killing (32) and phagocytosis (33).

We report here elevated OPN levels in the plasma of type 1 GD patients and its sensitive response to enzyme replacement therapy, including a patient with chitotriosidase deficiency and a patient during pregnancy, in comparison with healthy controls, patients with Fabry disease (FD) and patients with Niemann-Pick type C disease (NPC).

Methodology

This is a cross-sectional and controlled study with convenience sampling. Regarding the comparison of GD patients on and off ERT and the evolution of OPN levels in pregnancy, the data are longitudinal.

Study population

The study sample comprised 14 GD type 1 adult patients (7 males and 7 females, mean age = 37.1 years), 8 healthy controls (mean age = 36 years), 5 FD male patients (mean age = 37.4 years, 1 at diagnosis and 4 on ERT) and 6 NPC patients (mean age = 9.2 years, two on miglustate treatment). We chose to compare patients with these lysosomal diseases to demonstrate that the OPN elevation observed in our GD samples is correlated with the very high chitotriosidase activity levels observed in GD patients (even during treatment) and is not correlated with the more moderate chitotriosidase activity elevation observed in NPC and FD patients (34)). Additionally, the evolution of OPN in pregnancy was examined in a pregnant GD patient, as previously reported (35).

Regarding GD, we analyzed plasma samples from patients with a biochemical and molecular diagnosis of GD who were followed at the State Reference Center for Gaucher disease of Rio Grande do Sul, Brazil. All patients were European descendants, had been born in the southern region of Brazil and were not of Ashkenazi Jewish descent. The samples were obtained during the period of imiglucerase shortage in 2010 (4 months with no GD specific treatment) and samples during regular treatment with imiglucerase (6 to 12 months on ERT). All of the samples were collected, centrifuged within less than 1 hour and stored at -80°C. The samples from the controls, FD and NPC patients were obtained during routine blood workups and followed the same procedure.

Patients provided written informed consent for participation in the study, and clinical and biochemical data were obtained by chart review. The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

Osteopontin plasma assay

The OPN levels were determined using the MILLIPLEX® MAP system (HBN1A-51K, Millipore Corporation, Billerica, MA, USA) according to the manufacturer's protocol. The samples were analyzed in duplicate, and when the difference between the reads was less than 10%, the mean value was used. When the difference was more than 10%, the sample was not used in the analysis. The levels were estimated using a 5-parameter polynomial curve. Values were expressed in pg/mL.

Statistical analysis

The null hypothesis considered in the study was the absence of difference in the OPN concentration among the groups studied. The values were compared using

nonparametric tests as Wilcoxon signed rank test since the distribution was not normal. When comparing OPN values of untreated and treated GD patients a related sample test was used. All the analysis were made with the software IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY). The statistical significance value was $p<0.05$.

Results

Figure 1 shows the median OPN plasma levels and range obtained for healthy controls, NPC patients, FD patients and GD patients not receiving ERT and receiving ERT. The median OPN values were as follows: healthy controls = 31,025 pg/mL (minimum=12,172; maximum=48,924; interquartile range=27,822.50), NPC patients = 11,572 pg/mL (minimum=3,638; maximum=37,871; interquartile range=26,256.50) and FD patients = 13,196 pg/mL (minimum=2,467; maximum=25,020; interquartile range=16,931). There was no significant difference among these values. The median OPN level of untreated GD patients was 68,656.16 pg/mL (minimum=26,927.24; maximum=125,635; interquartile range=64,155.54), where p=0.02 in comparison with healthy controls. The median OPN value of GD patients receiving ERT was similar to the values of controls, FD patients and NPC patients, with no significant differences observed. When comparing untreated GD patients and patients receiving ERT the p value was <0.001.

We had access to the values of chitotriosidase activity in plasma for 10/14 patients either during shortage of imiglucerase or during regular treatment. The median chitotriosidase of untreated GD patients was 9,245.50 nmol/h/mg prot (minimum=961; maximum=18,545; interquartile range=12,147.50), and the median chitotriosidase of GD patients receiving ERT was 4,404.50 nmol/h/mg prot (minimum=867; maximum=11,400; interquartile range=6,778). The p value was 0.03 (Figure 2).

Figure 3 shows the plasma OPN decrease in our patient who was homozygous for a *CHIT1* mutation (c.1049_1072dup24), which conferred chitotriosidase deficiency after 6 months on ERT with taliglucerase alpha.

Figure 4 shows the rapid OPN variation in a patient during pregnancy and lactation (she remained untreated for 2 months after delivery to care for her child).

Discussion

Gaucher disease (GD) is the most prevalent lysosomal storage disorder and the disorder that exhibits the greatest immune system involvement (36). The main hypothesis is that a buildup of glucocerebroside within macrophage lysosomes leads to persistent inflammatory stimulation, but the exact mechanism remains unclear.

We chose to analyze the OPN levels in GD patients' plasma because OPN is biosynthesized by many cell types, particularly osteocytes, osteoblasts and macrophages (37); these cell types are intrinsically involved in GD pathophysiology.

After comparisons with healthy volunteers, FD and NPC patients (who usually do not overexpress chitotriosidase as much as GD patients do), the OPN level increased in untreated adult GD type 1 patients and rapidly decreased after receiving ERT. It appears to be more sensitive than chitotriosidase because it varies more rapidly and more drastically (82% versus 52% of reduction) as shown in Figures 2 and 3. We confirmed this finding by analyzing plasma samples from a female patient during regular treatment before, during and after pregnancy. She ceased ERT during the first two months after delivery, and we noticed a rapid OPN increase during this period and a return to normal values after 6 months of ERT (imiglucerase, 30 UI/kg/infusion). The OPN level decrease observed in our patient throughout the pregnancy is consistent with the findings of previous studies because there is an anti-inflammatory status that induces maternal tolerance to the fetus (38). Regarding the other patient, OPN may be used as a biomarker for patients with chitotriosidase deficiency as well as PARC/CCL18, ACE and TRAP.

OPN participates in bone remodeling (39), which, in GD, was related to the overexpression of some pro-inflammatory cytokines such as IL-18, IL-8, MIP-1α, MIP-1β and TGF-β (14, 40). OPN acts as an immune modulator because it has chemotactic properties, which promote cell recruitment to inflammatory sites, mediates cell activation and cytokine production and promotes cell survival by regulating apoptosis (41). This is an important factor in the relationship of OPN and several types of cancers. OPN was reported to be up regulated in lung, breast, prostate, gastric, ovarian and other cancers (42). OPN has also been demonstrated to be an essential factor in tumor progression and metastasis and is considered a valuable prognostic factor (43). Interestingly, OPN is expressed in multiple myeloma (MM) cells and has a proliferative and migratory effect on these cells (44). Because it has been demonstrated that GD patients have an increased risk for developing MM, OPN may be involved in this relationship.

More recently, the OPN levels were observed to be increased in patients with neurodegenerative diseases such as Parkinson's disease (45), Lewy bodies disease (46) and Alzheimer's disease (47). In Parkinson's disease patients, higher OPN serum

levels were associated with more severe symptoms (45). The relationship of *GBA* mutations and synucleinopathies is well established, and multiple theories have been proposed, particularly those related to protein turnover pathways (48). A neuroinflammation linkage may exist, and OPN may be an essential participant in this phenomenon. A single-nucleotide polymorphism in *SPP1* gene was found to be more frequent in GD patients with Parkinson's disease (49) and may be a possible link between these conditions. Unfortunately, we did not follow any GD patient or relative with Parkinson's disease; therefore, we cannot infer a relationship regarding the OPN levels or in these patients.

Because OPN has the potential to regulate macrophage functions, it could be an interesting therapeutic target. The effect of OPN may be either advantageous (in the case of an infection where macrophage function is beneficial for the innate immune response) or detrimental (in the case of injury or autoimmune disease, where macrophage function can lead to a tissue damage) (37).

Conclusion

Twenty years after the report of elevated chitotriosidase levels in GD patient plasma (16), we pointed out a novel possible GD biomarker. OPN appears to be more sensitive to therapy than chitotriosidase and can be used for the follow-up of patients who are chitotriosidase deficient. Additional extended studies are required to relate the variations in the OPN levels to clinical observations and response to therapy in GD patients.

Conflicts of interest

The authors have no conflict of interest to declare.

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Authorship:

F. Vairo designed research, performed research, analyzed data and wrote the paper. F. Sperb-Ludwig performed research and analyzed data M. Wilke performed research, analyzed data. K. Michellin-Tirelli performed research, analyzed data. C. Netto performed research, analyzed data. E.C. Neto performed research, analyzed data. I. Schwartz designed research, performed research, analyzed data and wrote the paper.

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Figures

Figure 1: Osteopontin variation in plasma from healthy controls (n=8), Niemann-Pick C patients (n=6; two on miglustate therapy), Fabry patients (n=5; four on ERT), untreated Gaucher patients (for 4 months; n=14) and Gaucher patients receiving ERT (for 6 to 12 months). Untreated GD patients versus healthy controls – p=0.02. There was no significant difference between the other groups.

Figure 2: Chitotriosidase activity variation in plasma from untreated patients with Gaucher disease (n=10) and patients on enzyme replacement therapy. P value = 0.03.

Figure 3: Variation in the osteopontin plasma levels in a 65-year-old patient with Gaucher disease and chitotriosidase deficiency during the imiglucerase shortage (154,979 pg/mL) and after 6 months on enzyme replacement therapy with taliglucerase alpha (11,788 pg/mL).

Figure 4: Variation in the OPN plasma levels from a 20-year-old female before pregnancy, during pregnancy (first, second and third trimesters), after 2 months of ERT withdrawal and after 6 months on ERT with imiglucerase, 30 UI/kg/inf.

Figure 1:

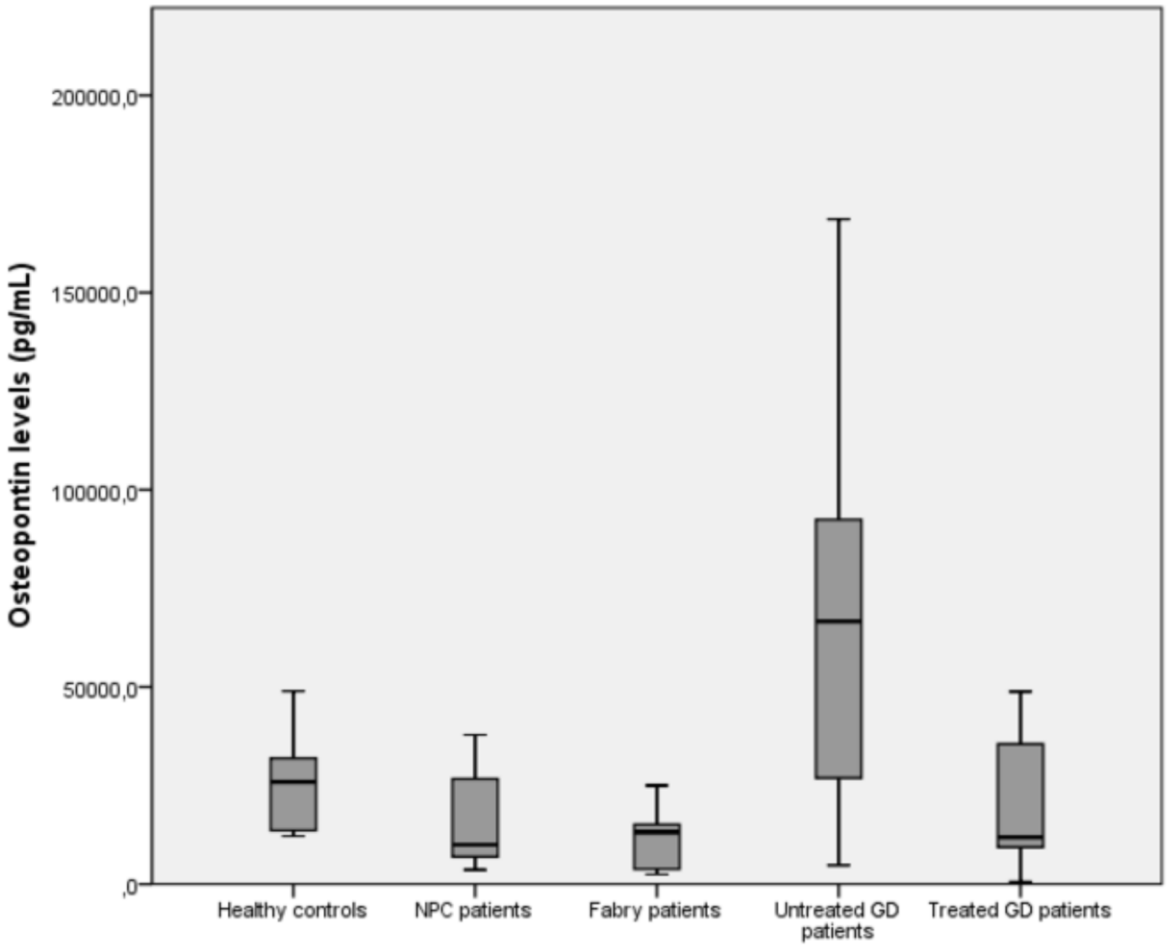

Osteopontin levels (pg/mL)
200000,0
150000,0
100000,0
50000,0
,0
Healthy controls
NPC patients
Fabry patients
Untreated GD patients
Treated GD patients

Figure 2:

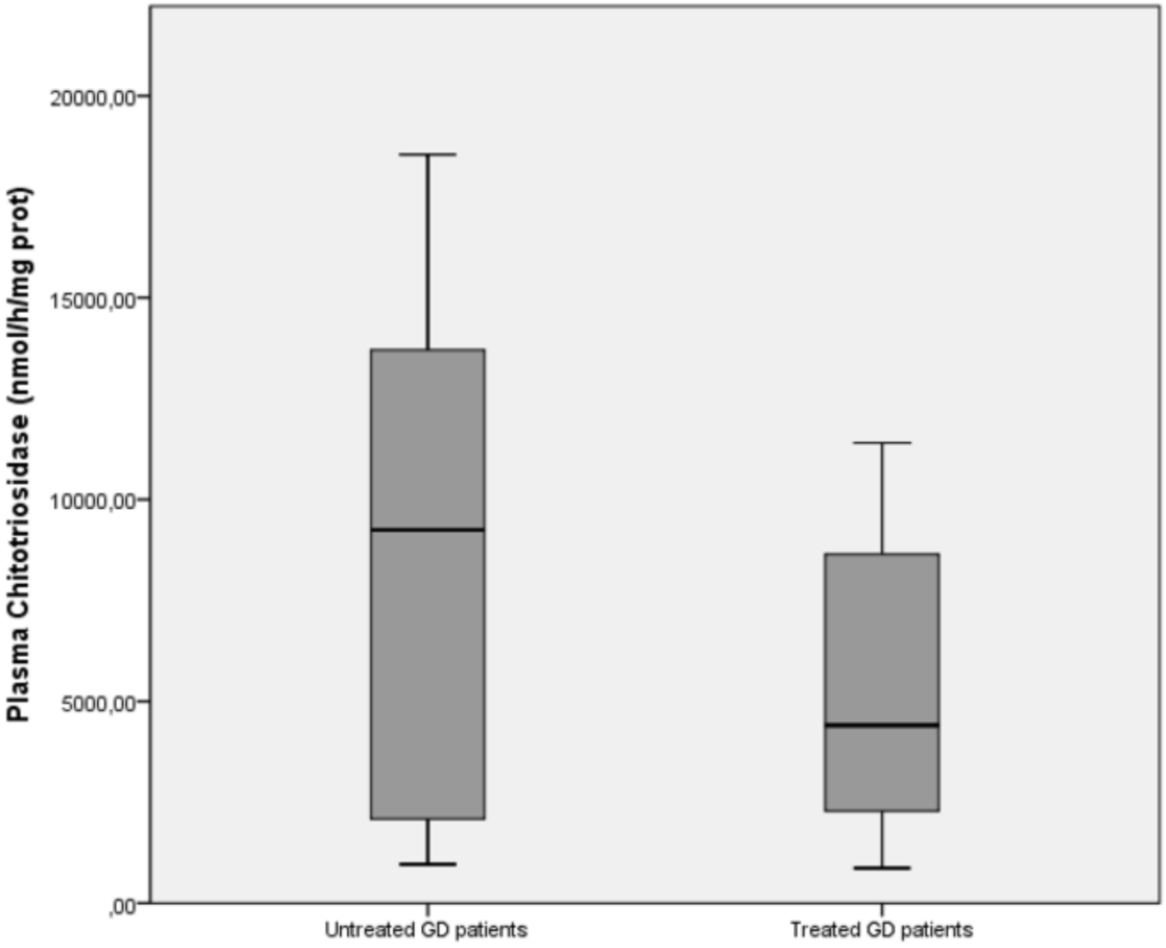

Plasma Chitotriosidase (nmol/h/mg prot)
20000,00
15000,00
10000,00
5000,00
,00
Untreated GD patients
Treated GD patients

Figure 3:

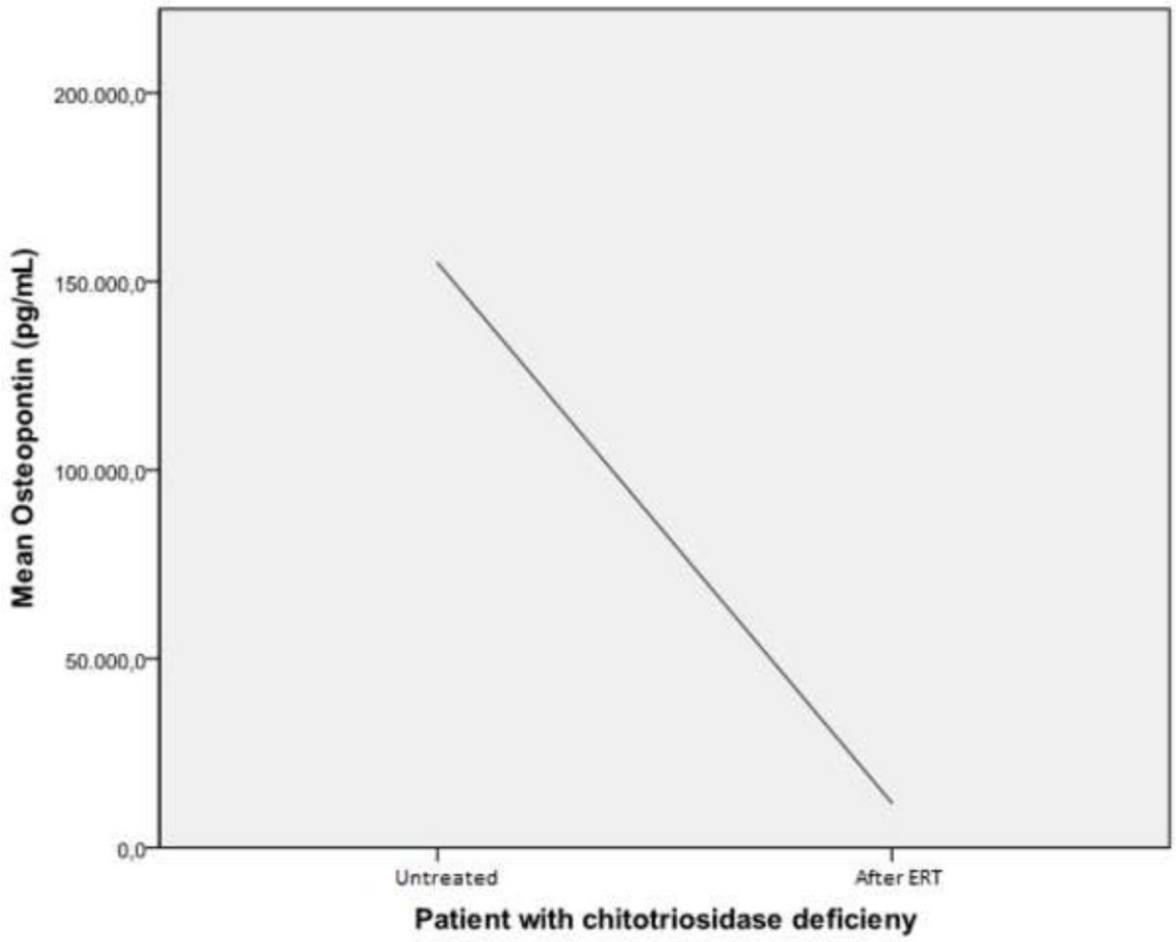
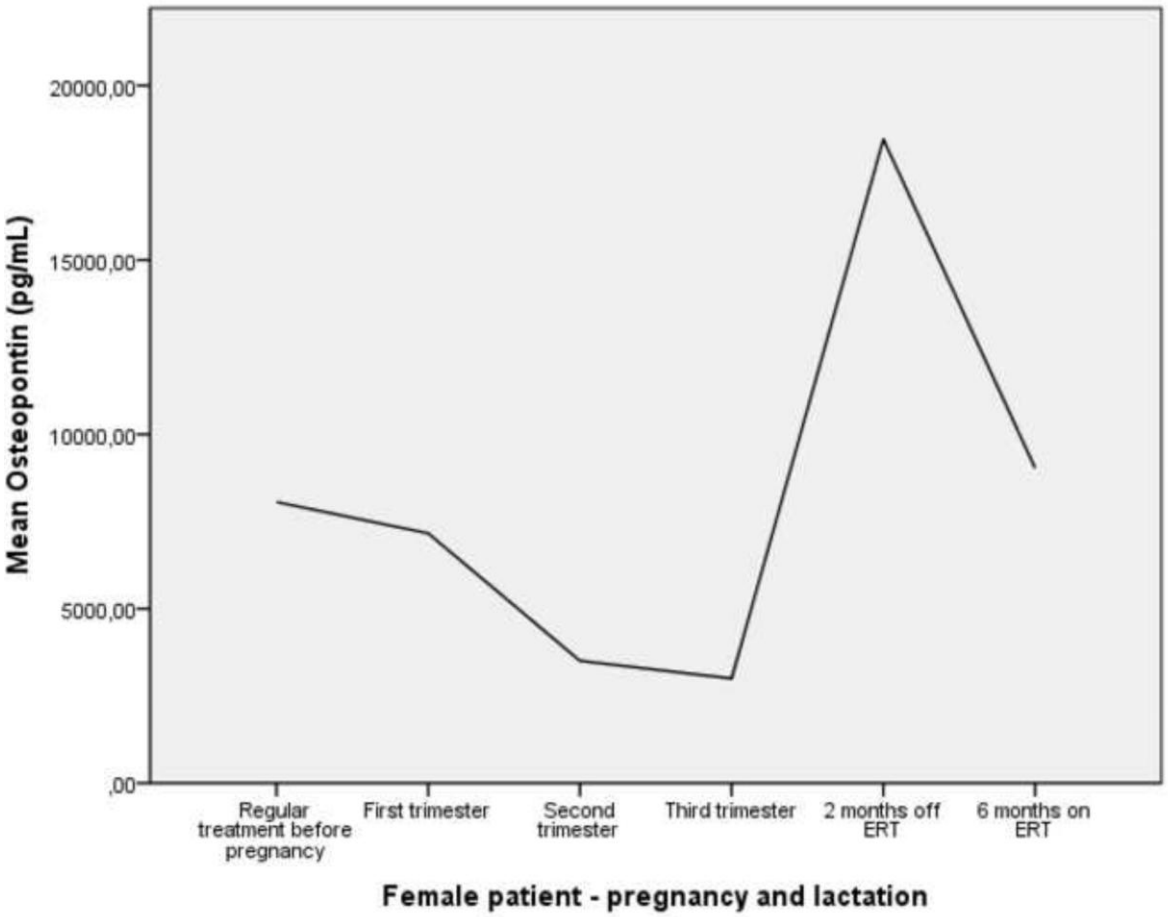

200.000,0
150.000,0
100.000,0
50.000,0
0,0
Mean Osteopontin (pg/mL)
Untreated
After ERT
Patient with chitotriosidase deficieny

Figure 4:


Mean Osteopontin (pg/mL)
20000,00
15000,00
10000,00
5000,00
,00
Regular treatment before pregnancy
First trimester
Second trimester
Third trimester
2 months off ERT
6 months on ERT
Female patient - pregnancy and lactation

3.7) CAPÍTULO 7

Cytokine profile of patients with Gaucher disease type I and its response to enzyme replacement therapy

Título do manuscrito: *Cytokine profile of patients with Gaucher disease type I and its response to enzyme replacement therapy*

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Cytokine profile of patients with Gaucher disease type I and its response to enzyme replacement therapy

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Keywords: Gaucher disease, Cytokines, Inflammation

Abstract

Gaucher disease (GD) is a lysosomal disorder that features extensive involvement of the immune system and is caused by the accumulation of glucocerebroside in macrophages. Although the macrophage is the most thoroughly studied cell type in the pathophysiology of GD, there is evidence for the involvement of other immunological cells. The chronic pro-inflammatory status of individuals with GD is thought to be one of the causes of their increased risk of developing malignancies. We studied the variation of thirty different cytokines in fourteen patients with GD type 1 during imiglucerase shortage and after restarting enzyme replacement therapy (ERT). We compared the changes in cytokine levels with clinical and biochemical data and observed correlations for a number of these cytokines. We found a decrease of TNF-α (29.9%), MIP-1α (46.1%), MIP-1β (23.2%) and MDC (18.9%) levels after the restart of ERT. There was an increase in GRO (55.3%), PAI-1 (79.8%) and leptin (21.5%) levels. In addition to corroborating previous findings, we obtained new data regarding endocrine, bone, and inflammatory cytokines that demonstrated that cytokine profiles might be useful to monitor therapeutic responses and to predict certain disease features in patients with GD.

Introduction

Gaucher disease (GD) is one of the most common lysosomal disorders with an estimated worldwide incidence of 1/57,000 live births (1), although this rate can reach 1/850 among Ashkenazi Jews (2). GD is caused by mutations in *GBA1* gene that lead to defective function of the glucocerebrosidase enzyme (EC 3.2.1.45), which is responsible for the metabolism of glucocerebroside into glucose and ceramide (3). Consequently, there is intralysosomal accumulation of glucocerebroside in macrophages, particularly in the spleen, liver, bone marrow and lungs. GD is therefore a multisystem disorder that exhibits phenotypic heterogeneity.

GD type I (MIM #230800) is the most prevalent form of GD and affects children and adults of all ages. This disease's typical clinical manifestations include hepatosplenomegaly, anemia, thrombocytopenia, and bone disease. GD type I differs from GD type II (MIM #230900) and GD type III (MIM #231000) in its lack of involvement of the central nervous system, although several studies have reported neurological phenotypes associated with type I that are different from those observed in type II or type III patients (4). Patients with GD who present with neurological involvement are typically classified as type II or type III according to the respective acute or chronic nature of their disease.

The treatment of GD is based mainly on enzyme replacement therapy (ERT). There are currently three recombinant enzymes on the market: imiglucerase (Genzyme Corp., Cambridge, MA, USA), taliglucerase alpha (Protalix Biotherapeutics, Carmiel, Israel) and velaglucerase alpha (Shire Human Genetic Therapies, Lexington, MA, USA). Another therapeutic strategy is substrate reduction therapy (SRT) by miglustat (Actelion Pharmaceuticals, Berne, Switzerland) or eliglustat (Genzyme Corp., Cambridge, MA, USA), which was recently approved by the FDA. From 2009 to 2010, there was an acute shortage of imiglucerase, which was then the most used recombinant enzyme, due to viral contamination of the production facility, thereby resulting in monitoring the follow-up and treatment of patients with GD worldwide.

Although the genetic changes and biochemical pathways underlying GD have been well-characterized, the mechanisms whereby accumulation of glucocerebroside leads to clinical manifestations have yet to be fully determined (5). Of lysosomal diseases, GD features the greatest involvement of the immune system; one hypothesis explaining this phenotypic manifestation of GD is that the immune system is chronically stimulated (6, 7). The accumulation of glucocerebroside in macrophages leads to an

“active” phenotype in these cells, known as Gaucher cells (8), that can contribute to some features of GD (9). Gene expression studies in animal models have shown increased expression of genes related to macrophage activation and immune response, especially those related to INFγ (e.g., TNFα and IL6) and IL4 cascades (10). Although the macrophage is thought to be the main player in the pathophysiology of GD, studies of the inflammatory secretome have implicated other immunological cells, such as monocytes, lymphocytes, natural killer cells, dendritic cells and neutrophils (11). Gaucher cells and other immunological cells secrete cathepsins and inflammatory mediators, such as the interleukins 1ra, IL-2, IL-6, IL-8, IL-10, and IL-18, macrophage inflammatory proteins (MIPs) and several chemokine ligands (10, 12-15).

The chronic pro-inflammatory status observed in GD is thought to contribute to the increased risk of developing malignancies, especially multiple myeloma (16). It is important to understand the mechanisms underlying this predisposition to propose new therapeutic approaches.

In this study, we studied the changes in thirty different cytokines - at least ten of which have never been studied in the context of GD - in fourteen patients with GD type 1 during imiglucerase shortage and following the restart of ERT. We compared the variations in cytokine levels with clinical and biochemical data, and we report several correlations among these cytokines that could aid in elucidating the efficacy of treatment on the inflammatory networks of patients with GD.

Methodology

This is a cross-sectional and controlled study with convenience sampling.

Study population

The study sample was comprised of 14 adult patients with GD type 1 (7 males and 7 females; one splenectomized; median age = 35.7 ± 14.8 years; median time of treatment pre-shortage = 5.8 ± 6.2 years; median ERT dosage pre-shortage = 25.7 ±10.8 UI/kg/inf). We analyzed plasma samples from patients with biochemical and/or molecular diagnoses of GD who were followed at the State Reference Center for Gaucher Disease of Rio Grande do Sul, Brazil. All patients were of European descent

and had been born in the southern region of Brazil and were not of Ashkenazi Jewish descent. Samples were obtained during a period of imiglucerase shortage in 2010 (median of 4 months with no GD-specific treatment) and during regular treatment with imiglucerase (6 to 12 months after the restart of ERT). All the samples were collected, centrifuged within 1 hour of collection and stored at -80°C. Patients provided written informed consent for participation in the study, and clinical and biochemical data were obtained by chart review. We compared hemoglobin, platelet, ferritin, chitotriosidase, immunoglobulin M (IgM), immunoglobulin G (IgG) and beta-2 microglobulin levels besides SSI (17) between the periods. The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

Cytokines plasma assays

The levels of adiponectin, leptin, osteocalcin (OC), osteoprotegerin (OPG), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 12 (IL-12), interleukin 17 (IL-17), soluble CD40L (sCD40L/CD154), epidermal growth factor (EGF), fibroblast growth factor (FGF 2), FMS-like tyrosine kinase 3 (Flt-3), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), growth regulated oncogene (GRO/CXCL1), interferon gamma (IFN γ), interferon gamma-induced protein 10 (IP10/CXCL10), monocyte chemotactic protein 1 (MCP1/CCL2), monocyte chemotactic protein 3 (MCP-3/CCL7), macrophage-derived cytokine (MDC/CCL22), macrophage inflammatory protein 1 alpha (MIP-1α/CCL3), macrophage inflammatory protein 1 beta (MIP-1β/CCL4), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), tumor necrosis factor beta (TNFβ); vascular endothelial growth factor (VEGF), plasminogen activator inhibitor 1 (PAI-1) and platelet-derived growth factor (PDGF) were determined using the MILLIPLEX® MAP system (HNDG3MAG-36, HCCBP1MAG-5 and HCYTOMAG-60 kits, Millipore Corporation, Billerica, MA, USA) according to the manufacturer's protocols. The samples were analyzed in duplicate, and when the difference between the reads was less than 10%, the mean value was used. When the difference was more than 10%, the sample was not used in the analysis. When we could not obtain reliable values for both periods, the cytokine was not analyzed. The levels of the cytokines were estimated using a 5-parameter polynomial curve. Values are expressed in pg/mL.

Statistical analysis

The null hypothesis considered in the study was the absence of difference in the levels of cytokines among the groups studied. The values were compared using a nonparametric related-samples test (the Wilcoxon signed-rank test) because the distribution was not normal and the number of samples was limited. Numeric variables are expressed as median and interquartile range and were assessed using the Mann-Whitney *U*-test and the Kruskal-Wallis test. We computed pairwise Spearman rank order correlations among the percentage of variation of cytokines and clinical and biochemical data between the untreated and post-treatment periods (Δ% = ((value during ERT - value off ERT) / value off ERT) x 100). All analyses were performed with the software IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY). Two-tailed *P* values below 0.05 were considered statistically significant.

Results

Fourteen adult patients with GD type I were included in this study. None of the patients presented with autoimmune disorders or had any alteration in protein electrophoresis or immunoglobulin titers during the periods with or without specific treatment. Hemoglobin, platelet, chitotriosidase and beta-2 microglobulin plasma levels changed significantly between the periods, while ferritin, IgM, IgG and SSI did not (Table 1). Of bone turnover-related cytokines, there was no significant difference in the levels of osteoprotegerin (p=0.16), osteocalcin (p=0.1) and IL8 (p=0.47) between the periods off treatment and during treatment. Interestingly, osteopontin (*paper in submission*), MIP-1α (untreated: median = 28.5 pg/mL [18-38.9]; treated: median = 21.6 pg/mL [18-25.8]; p = 0.008) and MIP-1β (untreated: median = 71.9 pg/mL [53.3-89.4]; treated: median = 52.3 pg/mL [42.9-58.9]; p = 0.01), which are potential biomarkers of osteonecrosis in GD (18), decreased after a few months of treatment. The two major adipocyte-derived hormones showed distinct variances. Leptin levels increased after treatment (untreated: median = 884.47 pg/mL [115.7-2840.1]; treated: median = 1165.48 pg/mL [117.5-3967.2]; p = 0.02) while adiponectin levels did not change (p = 0.2).

Of the known inflammatory markers, we analyzed more than 20 cytokines produced by macrophages, T-cells and other cells involved in several signaling

cascades. We showed that TNFα levels decreased after ERT (from 6.55 pg/mL [4.9-11.4] to 4.5 pg/mL [3.5-11.2]; p<0.001) while there was no significant difference in the levels of IL6 in our patients during ERT and in the off-treatment period. In addition to MIP-1α and MIP-1β, the macrophage cytokines GRO (CXCL1) and MDC (CCL22) changed significantly with ERT. GRO increased from 623 pg/mL [198.6-1630.7] to 1092.1 pg/mL [313.2-3195.9] with a p value = 0.003, while MDC decreased from 1053.2 pg/mL [315.6-2769] to 746.6 pg/mL [352.4-1561.9] with a p value = 0.01. PAI-1 increased after ERT (from 7732 pg/mL [4656-8731] to 13091 pg/mL [11391-17806]; p = 0.002). Other analyzed cytokines did not significantly change after treatment. Table 2 shows the percentage of variation (Δ%) of the cytokines after the initiation of ERT.

There were no strong correlations among the changes of cytokine levels and clinical and biochemical variables. There was a moderate negative correlation between the number of platelets and the SSI (rho = -0.67, p = 0.009). There were only moderate correlations between the variation in IgM level and Δ% GRO (rho = -0.7, p = 0.03) and Δ% TNFα (rho = 0.64, p = 0.04); variation of SSI with Δ% adiponectin (rho = -0.54, p = 0.04); variation of hemoglobin with Δ% G-CSF (rho = 0.65, p = 0.01), Δ% OPG (rho = 0.6, p = 0.02), Δ% GM-CSF (rho = 0.58, p = 0.04), and Δ% OC (rho = -0.58, p = 0.02).

Among the cytokines that exhibited a significant change between periods, there were some strong correlations with levels of other cytokines (Table 3). All correlations are shown in the Supplementary Table.

Discussion

It is well-established that the immune system plays an essential role in the pathophysiology of GD, but how each part of this broad network functions in GD remains unclear. For some time, the macrophage was the only immune component recognized for its role in GD, but the pathological lipid accumulation in Gaucher cells accounts for less than 2% of the additional tissue mass in liver and spleen, indicating that macrophages are not the only cells responsible (19). Currently, based on animal models and gene expression studies (11), monocytes, neutrophils and dendritic cells are emerging as additional immunological players. For example, INF-γ, IL-4 and MCSF, which are critical for the differentiation of monocytes, are increased in GD, as are some CXC chemokines involved in the activation and migration of neutrophils (20).

During imiglucerase shortage, some GD patients in Southern Brazil went untreated for several months. We analyzed plasma samples from these patients just before the restart of ERT and after 6 to 12 months of regular treatment and found a number of interesting results. Following this short window of treatment, we could detect changes in biomarkers and hematological parameters. Even SSI had not changed significantly due to the short period of time elapsed; we could detect an inverse correlation with the change in the platelet level, which makes sense because it is one of the parameters of the score. Regarding the other correlations between cytokines and biochemical parameters, we could not address any hypotheses because there was no significant variation.

The levels of cytokines related to bone resorption, such as OPG and OC, did not change between the periods. This may be because not enough time in treatment had elapsed, which would be consistent with the prolonged time needed for the recombinant enzyme to have a significant effect on bone, or because the months-long cessation of ERT was not enough time for a change to occur in the first place. Our findings regarding macrophage inflammatory protein 1α and 1β responses to treatment are consistent with the literature (14).

Leptin is one of the most important pro-inflammatory adipokines with a structure similar to those of IL-6 and G-CSF (21). In addition to its action in the neuroendocrine system, where it regulates appetite, food intake and energy expenditure (22), leptin induces monocytes and macrophages to produce IL-6 and TNFα (21) and stimulates hepatocytes to produce MCP-1 and VEGF (23). In our analysis, the increase of leptin after treatment correlated with the decrease of TNFα but did not correlate with IL-6, VEGF or MCP-1. There are some anecdotal reports about the influence of ERT on leptin levels in patients with GD. Usually, leptin levels correlate with body mass index (BMI) and ERT has no influence when comparing treated and untreated patients. In a recent report, leptin levels correlated with LDL, triglyceride and insulin levels as well as enzyme dosage in patients with GD (24). Leptin has a strict relationship with progesterone over the course of the menstrual cycle (25), which could be a confounding factor in our analysis. There was no difference in the mean BMI of our patients or their diets between periods and a decrease of pro-inflammatory cytokines was observed, so the change in leptin levels might be related to other factors and should be investigated. Our work is the first to evaluate the effect of ERT on leptin levels in the same group of patients.

Endothelial cells, megakaryocytes, fibroblasts, macrophages and other cell types produce PAI-1 which, once synthetized, is manly stored in platelets (26). IL-6, IL-1 and TNFα influence the release of PAI-1 (27), and it is reported to be elevated in a wide range of thrombotic conditions because it is related to hypofibrinolysis (28). ERT seems to diminish the prothrombotic state presented by patients with GD (29, 30). We hypothesized that the increase in PAI-1 levels after treatment may be related to megakaryocyte proliferation and an increase in platelet level because there was a decrease in the potential stimulatory pro-inflammatory cytokines.

Activated macrophages, monocytes, neutrophils, NK cells and T cells produce TNFα, which is induced by different stimuli, including INFγ (31). TNFα is involved in many inflammatory and autoimmune disorders (32) and is the target of several therapeutic agents aimed at ameliorating these diseases (33). Because TNFα is the central regulator of the immune system, it has been the subject of many studies of GD (34-38). We found a significant decrease in its level after the restart of ERT, which is consistent with other published results. Its levels strongly correlated with many macrophage-related cytokines, such as MIP-1α, MIP-1β and MDC, and with IP-10, which promotes chemoattraction in monocytes and macrophages as well as in other cells (39).

Interestingly, we found an increase of GRO/CXCL1 level after treatment. This cytokine mediates neutrophil recruitment in several inflammatory diseases (mainly infections) (40, 41). The activation of neutrophils links innate and adaptive immunities by generating chemotactic factors, which attract monocytes and macrophages as well as dendritic cells to the site of infection or inflammation (42). In corroboration, we found a strong correlation with MCP-1, which is a monocyte chemoattractant. After studying neutrophil chemotaxis in patients with GD, some authors have described a tendency toward infection, especially pyogenic, that decreased after ERT (43-45). Our findings may partially explain the recovery of normal neutrophil traffic in patients with GD.

We noticed a strong correlation among IL-8, MCP-1, MIP-1β and PDGF. The former three were previously described to be related to osteonecrosis in patients with GD (18). PDGF is a potent mitogen for mesenchymal cells and acts in several steps of bone formation. It stimulates local angiogenesis and upregulates the stimulation of osteogenic events (46). Based on the relationship that we observed, PDGF may be another biomarker of bone metabolism in GD.

Conclusions

Our study describes the behavior of several cytokines after ERT in a group of patients with GD. We corroborate the findings of several previous studies examining the immunological aspects in GD, and we present novel, interesting findings regarding endocrine, bone and inflammatory cytokines. A patient's cytokine profile may be useful for monitoring therapeutic responses and for predicting certain features of GD. We hope that these findings help to elucidate the influence of the immune system in GD.

Conflicts of interest

The authors have no conflict of interest to declare.

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Authorship

F. Vairo designed and performed the research, analyzed data and wrote the paper. F. Sperb-Ludwig, M. Wilke, K. Michellin-Tirelli, C. Netto and C. Netto performed the research and analyzed data. I. Schwartz designed and performed the research, analyzed data and wrote the paper.

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Table legends:

Table 1: Variation in clinical and biochemical parameters after ERT

Table 2: Median and interquartile range of the percentage of variation of different cytokines between the period of imiglucerase shortage and after the restart of ERT

Table 3: Correlations between cytokines that displayed a significant variation between the shortage period and after the restart of ERT with other cytokines

Supplementary table: Correlations between cytokine variation in the shortage period and during the ERT period

Table 1: Variation in clinical and biochemical parameters between the shortage period and after the restart of ERT

	GD patients (n=14)		
	Without ERT median [25th-75th]	With ERT median [25th-75th]	P value
Hemoglobin (g/dL)	12.75 [11.8-14]	13.65 [13.1-14.2]	0.037
Platelets (U/uL)	121500 [85250-134750]	133500 [118500-186250]	0.003
Chitotriosidase (nmol/mL/h)	5731 [2985-13321]	4404 [1974-8335]	0.002
Beta-2 microglobulin (ug/ml)	2414 [2045-2956]	1892 [1661-2119]	0.018
Ferritin (ng/mL)	352 [174-633]	267 [215-528]	0.365
IgM (mg/dL)	175 [135-240]	181 [147-195]	0.250
IgG (mg/dL)	1437 [1158-1822]	1634 [1220-1869]	0.193
Severity Score Index (SSI)	4.5 [2.25-6]	3 [1.25-5]	0.225

Mann-Whitney U-test; p values were considered significant when ≤0.05

Table 2: Median and interquartile range of the percentage of variation of different cytokines between the period of imiglucerase shortage and after the restart of ERT

Δ%	n	Median	IQ	p value
Adiponectin	14	4.75	[-1.43;15.33]	0.24
EGF	12	31.65	[-20.68;98.13]	0.23
Flt3	4	10.4	[-13.73;17.51]	0.62
G-CSF	13	-0.03	[-37.77;15.66]	0.89
GM-CSF	12	10.35	[-46.77;151.44]	0.85
GRO	13	55.36	[18.96-95.99]	**0.003**
IL-2	12	13.43	[-4.23;78.61]	0.23
IL-6	14	-4.35	[-31.25;3.33]	0.23
IL-8	12	-26.34	[-50.09;16.03]	0.47
IL-12	14	-11.32	[-51.00;6.52]	0.052
IL-17	10	-44.44	[-50.47;28.12]	0.19
INFγ	14	-19.30	[-32.05;12.48]	0.09
IP10	13	-19.27	[-34.33;13.02]	0.37
Leptin	14	21.52	[14.63;75.57]	**0.02**
MCP-1	14	-13.19	[-26.51;13.29]	0.15
MDC	13	-18.93	[-38.86;3.35]	**0.02**
MIP-1α	9	-46.04	[-52.75;-15.74]	**0.008**
MIP-1β	11	-23.27	[-40.29;-16.24]	**0.01**
OC	14	25.72	[6.45;62.04]	0.10
OPG	13	-10.51	[-21.7;3.21]	0.16
PAI-1	14	79.80	[35.02;248.20]	**0.002**
PDGF	14	-1.71	[-11.50;2.50]	0.26
sCD40L	13	45.46	[-9.07;238.19]	0.08
TGFα	12	-12.73	[-33.07;46.98]	0.67
TNFα	14	-29.91	[-35.90;-14.42]	**<0.001**
VEGF	13	-8.42	[-35.57;122.13]	0.97

Δ% = (value during ERT - value off ERT/ value off ERT) x 100

IQ: Interquartile range [25th;75th]
Mann-Whitney *U*-test; p values were considered significant when ≤0.05

Table 3: Correlations between cytokines that displayed a significant variation between the shortage period and after the restart of ERT with other cytokines

	Δ% GM-CSF	Δ% GRO	Δ% IL-8	Δ% IL-12	Δ% IL-17	Δ% IP10	Δ% leptin	Δ% MCP-1	Δ% MDC	Δ% MIP-1α	Δ% MIP-1β	Δ% OPG	Δ% PAI-1	Δ% TNFα
Δ% GRO	rho=-0.60 p=0.03		rho=0.65 p=0.02	rho=-0.69 p=0.82	rho=0.15 p=0.70	rho=0.39 p=0.18	rho=0.24 p=0.42	rho=0.70 p=0.007	rho=0.46 p=0.11	rho=0.55 p=0.12	rho=0.30 p=0.37	rho=-0.18 p=0.57	rho=0.69 p=0.008	rho=0.54 p=0.06
Δ% leptin	rho=-0.27 p=0.39	rho= 0.24 p=0.42	rho=0.46 p=0.13	rho=-0.47 p=0.08	rho=0.69 p=0.02	rho=0.31 p=0.29		rho=0.44 p=0.11	rho=0.18 p=0.54	rho=-0.23 p=0.54	rho=0.51 p=0.10	rho=0.41 p=0.16	rho=-0.16 p=0.56	rho=0.54 p=0.04
Δ% MDC	rho=-0.13 p=0.68	rho=0.46 p=0.11	rho=0.60 p=0.03	rho=-0.27 p=0.36	rho=0.40 p=0.28	rho=0.63 p=0.01	rho=0.18 p=0.54	rho=0.54 p=0.06		rho=0.75 p=0.02	rho=0.35 p=0.28	rho=0.35 p=0.26	rho=0.26 p=0.38	rho=0.74 p=0.004
Δ% MIP-1α	rho=-0.21 p=0.61	rho=0.55 p=0.12	rho=0.47 p=0.23	rho=-0.50 p=0.16	rho=-0.31 p=0.54	rho=0.88 p=0.002	rho=-0.23 p=0.54	rho=0.13 p=0.73	rho=0.75 p=0.02		rho=0.57 p=0.18	rho=0.16 p= 0.69	rho=0.41 p=0.26	rho=0.80 p=0.01
Δ% MIP-1β	rho=-0.33 p=0.34	rho=0.3 p=0.37	rho=0.70 p=0.01	rho=-0.72 p=0.01	rho=0.57 p=0.13	rho=0.73 p=0.01	rho=0.51 p=0.10	rho=0.44 p=0.17	rho=0.35 p=0.28	rho=0.57 p=0.18		rho=0.31 p=0.34	rho=-0.21 p=0.51	rho=0.73 p=0.01
Δ% PAI-1	rho=-0.47 p=0.11	rho=0.69 p=0.008	rho=0.04 p=0.89	rho=0.08 p=0.76	rho=-0.34 p=0.32	rho=0.08 p=0.77	rho=-0.16 p=0.56	rho=0.38 p=0.16	rho=0.26 p=0.38	rho=0.41 p=0.26	rho=-0.21 p=0.51	rho=-012 p=0.69		rho=0.29 p=0.31
Δ% TNFα	rho=-0.33 p=0.28	rho=0.54 p=0.06	rho=0.66 p=0.01	rho=-0.49 p=0.07	rho=-0.34 p=0.32	rho=0.83 p<0.001	rho=0.54 p=0.04	rho=0.53 p=0.04	rho=0.74 p=0.004	rho=0.80 p=0.01	rho=0.73 p=0.01	rho=0.56 p=0.04	rho=0.29 p=0.31	

Δ% = (value during ERT - value off ERT)/ value off ERT

Spearman rank order correlations ; p values were considered significant when ≤0.05

Dark grey = strong correlation (rho ≥ 0.7)

Blank = moderate, weak or no correlation

Supplementary table: Correlations between inflammatory cytokines variation in the shortage period and during the ERT period

Δ%	EGF	G-CSF	GM-CSF	GRO	IL-17	IL-6	IL-8	INFγ	IP10	MCP-1	MDC	MIP-1α	MIP-1β	OPG	PAI-1	PEDF	sCD40L	TGFα	TNFα	VEGF	IL-12
EGF		rho=-0.22 p=0.48	rho=0.00 p=1.00	rho=0.39 p=0.19	rho=0.71 p=0.04	rho=0.83 p=0.001	rho= 0.41 p=0.18	rho=0.39 p=0.20	rho=0.42 p=0.89	rho=0.68 p=0.01	rho=0.49 p=0.10	rho=0.00 p=1.00	rho=0.15 p=0.65	rho=0.06 p=0.85	rho=0.14 p=0.66	rho=0.39 p=0.20	rho=0.44 p=0.14	rho=0.75 p=0.007	rho=0.51 p=0.08	rho=0.49 p=0.10	rho=0.03 p=0.90
G-CSF			rho=0.84 p=0.001	rho=-0.54 p=0.06	rho=0.30 p=0.43	rho=-0.38 p=0.19	rho=-0.55 p=0.06	rho=0.56 p=0.04	rho=-0.12 p=0.68	rho=-0.47 p=0.10	rho=-0.21 p=0.48	rho=-0.16 p=0.66	rho=-0.48 p=0.13	rho=0.49 p=0.10	rho=-0.24 p=0.42	rho=0.20 p=0.42	rho=-0.68 p=0.01	rho=-0.29 p=0.35	rho=-0.21 p=0.48	rho=0.40 p=0.17	rho=0.24 p=0.42
GM-CSF				rho=-0.60 p=0.03	rho=0.09 p=0.82	rho=-0.19 p=0.54	rho=-0.55 p=0.07	rho=0.51 p=0.08	rho=-0.22 p=0.48	rho=-0.51 p=0.08	rho=-0.13 p=0.68	rho=-0.21 p=0.61	rho=-0.33 p=0.34	rho=0.43 p=0.18	rho=-0.47 p=0.11	rho=0.14 p=0.66	rho=-0.73 p=0.007	rho=0.01 p=0.95	rho=-0.33 p=0.28	rho=0.31 p=0.31	rho=0.36 p=0.24
GRO					rho=0.15 p=0.70	rho=0.40 p=0.17	rho=0.65 p=0.02	rho=0.13 p=0.65	rho=0.39 p=0.18	rho=0.70 p=0.007	rho=0.46 p=0.11	rho=0.55 p=0.12	rho=0.30 p=0.37	rho=-0.18 p=0.57	rho=0.69 p=0.008	rho=0.24 p=0.41	rho=0.42 p=0.15	rho=0.14 p=0.66	rho=0.54 p=0.06	rho=0.30 p=0.31	rho=-0.69 p=0.82
IL-17						rho=0.17 p=0.62	rho=0.76 p=0.02	rho=0.79 p=0.006	rho=0.13 p=0.73	rho=0.63 p=0.04	rho=0.4 p=0.28	rho=-0.31 p=0.54	rho=0.57 p=0.13	rho=0.45 p=0.18	rho=-0.34 p=0.32	rho=0.63 p=0.04	rho=0.00 p=1.00	rho=0.50 p=0.17	rho=0.55 p=0.09	rho=0.58 p=0.09	rho=-0.11 p=0.75
IL-6							rho= 0.51 p=0.08	rho=0.21 p=0.46	rho=-0.35 p=0.23	rho=0.54 p=0.04	rho=0.30 p=0.31	rho=-0.33 p=0.31	rho=-0.05 p=0.86	rho=-0.32 p=0.27	rho=0.01 p=0.95	rho=0.12 p=0.67	rho=0.65 p=0.01	rho=0.65 p=0.02	rho=-0.08 p=0.77	rho=0.19 p=0.52	rho=0.36 p=0.19
IL-8								rho=0.38 p=0.21	rho=0.47 p=0.11	rho=0.79 p=0.002	rho=0.60 p=0.03	rho=0.47 p=0.23	rho=0.70 p=0.01	rho=0.10 p=0.77	rho=0.04 p=0.89	rho=0.72 p=0.008	rho=0.43 p=0.15	rho=0.39 p=0.23	rho=0.66 p=0.01	rho=0.08 p=0.79	rho=-0.46 p=0.13
INFγ									rho=0.30 p=0.30	rho=0.20 p=0.47	rho=0.39 p=0.18	rho=0.06 p=0.86	rho=0.23 p=0.48	rho=0.54 p=0.06	rho=-0.18 p=0.52	rho=0.69 p=0.006	rho=-0.34 p=0.25	rho=0.17 p=0.58	rho=0.35 p=0.21	rho=0.64 p=0.01	rho=0.07 p=0.81
IP10										rho=0.24 p=0.41	rho=0.63 p=0.01	rho=0.88 p=0.002	rho=0.73 p=0.01	rho=0.43 p=0.15	rho=0.08 p=0.77	rho=0.33 p=0.26	rho=-0.23 p=0.44	rho=-0.32 p=0.30	rho=0.83 p<0.001	rho=0.17 p=0.56	rho=-0.53 p=0.06
MCP-1											rho=0.54 p=0.06	rho=0.13 p=0.73	rho=0.44 p=0.17	rho=-0.03 p=0.90	rho=0.38 p=0.16	rho=0.37 p=0.19	rho=0.53 p=0.06	rho=0.51 p=0.08	rho=0.53 p=0.04	rho=0.32 p=0.28	rho=-0.20 p=0.48
MDC												rho=0.75 p=0.02	rho=0.35 p=0.28	rho=0.35 p=0.26	rho=0.26 p=0.38	rho=0.48 p=0.09	rho=0.03 p=0.90	rho=0.00 p=0.98	rho=0.74 p=0.004	rho=0.42 p=0.14	rho=-0.27 p=0.36
MIP-1α													rho=0.57 p=0.18	rho=0.16 p=0.69	rho=0.41 p=0.26	rho=0.23 p=0.54	rho=-0.21 p=0.57	rho=-0.64 p=0.08	rho=0.80 p=0.01	rho=-0.08 p=0.83	rho=-0.50 p=0.16
MIP-1β														rho=0.31 p=0.34	rho=-0.21 p=0.51	rho=0.40 p=0.21	rho=-0.08 p=0.81	rho=0.22 p=0.50	rho=0.73 p=0.01	rho=-0.08 p=0.81	rho=-0.72 p=0.01
OPG															rho=-0.12 p=0.69	rho=0.45 p=0.11	rho=-0.51 p=0.09	rho=-0.20 p=0.52	rho=0.56 p=0.04	rho=0.52 p=0.08	rho=-0.40 p=0.17
PAI-1																rho=-0.06 p=0.82	rho=0.19 p=0.52	rho=-0.29 p=0.35	rho=0.29 p=0.31	rho=0.38 p=0.19	rho=0.08 p=0.76
PEDF																	rho=-0.08 p=0.78	rho=0.10 p=0.74	rho=0.48 p=0.08	rho=0.46 p=0.11	rho=-0.24 p=0.40
sCD40L																		rho=0.49 p=0.10	rho=0.08 p=0.77	rho=-0.20 p=0.50	rho=-0.04 p=0.89
TGFα																			rho=0.03 p=0.91	rho=0.06 p=0.84	rho=021 p=0.51
TNFα																				rho=0.45 p=0.12	rho=-0.49 p=0.07
VEGF																					rho=0.19 p=0.52
IL-12																					

Δ% = (value during ERT - value off ERT)/ value off ERT
Spearman rank order correlations ; p values were considered significant when ≤0.05
Dark grey = strong correlation (rho ≥ 0.7)
Blank = moderate, weak or or no correlation

4) DISCUSSÃO

A Doença de Gaucher é uma das mais frequentes doenças lisossomais, com aproximadamente 700 pacientes diagnosticados no país. Apesar do número limitado de pacientes, há grande interesse na avaliação e otimização do tratamento devido às comorbidades apresentadas e ao alto custo para o SUS de medicações específicas. Já está bem estabelecida a participação do sistema imune em muitos dos sinais e sintomas apresentados pelos pacientes, porém os mecanismos exatos ainda precisam ser elucidados.

Ao comparar a coorte de pacientes pediátricos com DG do Rio Grande do Sul com outras coortes internacionais nos deparamos com uma frequência maior de hiperimunoglobulinemia em nossos pacientes e uma resposta menos efetiva à terapia específica, apesar do uso de enzimas recombinantes, doses e tempo de tratamento, semelhantes. Ainda é incerta a significância clínica da manutenção de níveis elevados de imunoglobulinas circulantes por longos períodos, porém nosso trabalho chama a atenção para a possibilidade de individualização do acompanhamento e tratamento de pacientes com DG brasileiros, visto que tendemos a basear nossas ações em publicações e protocolos internacionais.

Com a publicação da primeira experiência internacional no tratamento de pacientes com DG tipo III com a mais nova das enzimas recombinantes, a alfavelaglicerase, não só demonstramos a dificuldade de manejo de pacientes com reações anafilactóides relacionadas às enzimas disponíveis, como atentamos para o fato de que, apesar das publicações tratarem as três enzimas como de mesma eficácia e segurança, o processo de fabricação pode estar envolvido em indução de resposta imune. O presente artigo teve repercussão internacional ao ser citado pelo comitê médico da *National Gaucher Foundation* dos Estados Unidos, em sua posição formal à *FDA*, publicado na *American Journal of Hematology*, em maio de 2014, a respeito das mudanças no fornecimento e escolha das enzimas recombinantes e manejo de pacientes naquele país. O uso de infusões domiciliares é uma realidade em vários países, inclusive no Brasil, e após a publicação da possibilidade de reação

anafiláctóide, mesmo após muitos anos de tratamento regular, essa forma de tratamento vem sendo discutida com parcimônia.

Há um crescente interesse na busca de fatores genéticos e ambientais que possam atuar como modificadores de fenótipo na DG. Apesar de publicações a respeito do envolvimento imune na DG datem de mais de 30 anos, os fatores imunológicos ficaram esquecidos por alguns anos. Após diversas publicações relacionando a DG a malignidades hematológicas, como o mieloma múltiplo e a doenças neurodegenerativas, como a doença de Parkinson, houve uma busca por associação dessas doenças com o gene *GBA1,* sendo encontrada apenas relação com doenças neurodegenerativas. Nosso trabalho traz uma possível associação de genes relacionados ao sistema imune com o aumento da frequência de alterações em imunoglobulinas, sabidamente predisponentes ao desenvolvimento do mieloma múltiplo. Além disso, demonstramos o papel do tratamento no *status* inflamatório apresentado pelos pacientes com DG, com diminuição de citocinas pró-inflamatórias importantes e aumento de outras, como a leptina, que exigirá maiores estudos para determinar o seu real papel na fisiopatogênese da DG. Com o ineditismo do estudo de diversas citocinas inflamatórias, ósseas, endócrinas e relacionadas à neurodegeneração, pudemos determinar a osteopontina como um biomarcador para acompanhamento de tratamento, inclusive mais sensível que a quitotriosidase, que vem sendo utilizada desde 1994, com o benefício de poder ser utilizado em pacientes com deficiência da atividade da quitotriosidase, o que pode ocorrer em até 25% da população de alguns países, como a Austrália, por exemplo.

5) CONCLUSÕES

As conclusões desse trabalho serão apresentadas separadamente para cada objetivo específico.

Objetivos específicos

1) *Avaliar a frequência de hiperimunoglobulinemia apresentada pelos pacientes com doença de Gaucher acompanhados no Centro de Referência do estado do Rio Grande do Sul e compará-la a outras coortes já publicadas.*

Foi possível descrever a frequência de hiperimunoglobulinemia em pacientes pediátricos com DG acompanhados no Centro de Referência Estadual do Rio Grande do Sul e compará-la a coortes internacionais. Determinamos que os nossos pacientes apresentam maior frequência de hipergamaglobulinemia G, M e E, mesmo após tempo e dose de tratamento similares a pacientes de outros países.

2) *Descrever o perfil imunogênico das três enzimas recombinantes disponíveis para tratamento da doença de Gaucher em um paciente com doença de Gaucher tipo III acompanhado no Centro de Referência do estado do Rio Grande do Sul.*

Foi possível descrever a dificuldade no manejo de reações anafilactóides em um paciente com DG tipo III, a utilização de diferentes enzimas recombinantes e publicar a primeira experiência internacional de sucesso em pacientes com DG tipo III com a enzima alfavelaglicerase.

3) *Verificar a existência de variantes de genes KIR e de combinações de variantes KIR-HLA associadas à gravidade do fenótipo clínico dos pacientes com doença de Gaucher do Centro de Referência Estadual do Rio Grande do Sul.*

Foi possível determinar variantes dos genes *KIR* relacionadas ao desenvolvimento tardio da doença, como as variantes KIR2DS2 e KIR2DL2 associadas ao seu ligante HLA-C1.

4) *Avaliar a relação da tipagem do MHC de classe I (HLA-A, HLA-B e HLA-C) e de classe II (HLA-DR) com características apresentadas pelos pacientes com doença de Gaucher do Centro de Referência Estadual do Rio Grande do Sul.*

Foi possível determinar que há variantes dos genes *HLA* relacionadas ao desenvolvimento de alteração em eletroforese de proteínas (HLA-C2), o que pode ser fator de risco para o desenvolvimento de mieloma múltiplo, uma doença com frequência elevada em pacientes com DG. A variante HLA DR11 foi associada ao comprometimento neurológico tanto em pacientes com DG tipo III, quanto em pacientes com DG tipo I leve com déficit cognitivo e a paciente que apresentou efeitos colaterais neurológicos na tentativa de utilização de terapia de redução de substrato. Pacientes com DG tipo I moderado e que apresentaram osteonecrose, com início tardio de terapia de reposição enzimática, apresentaram a variante HLA DR13, enquanto pacientes com DG tipo I leve, com início precoce de tratamento, não desenvolveram osteonecrose.

5) *Avaliar os níveis de diferentes citocinas inflamatórias, endócrinas, ósseas e relacionadas à neurodegeneração durante um período sem tratamento específico e após o reinício do tratamento em pacientes com doença de Gaucher tipo I acompanhados no Centro de Referência do*

estado do Rio Grande do Sul e compará-las com características clínicas e bioquímicas em busca de associações com a variabilidade fenotípica apresentada pelos pacientes.

Foram analisados os níveis de 30 diferentes citocinas e determinamos sua relação com o tratamento em pacientes com DG tipo I acompanhados no Centro de Referência Estadual do Rio Grande do Sul, em um período sem tratamento e após o reinício da TRE.

Foi possível determinar aumento significativo de níveis de BDNF após o tratamento, assim como diminuição importante de osteopontina, caracterizando-a como um possível biomarcador de acompanhamento de tratamento, inclusive para pacientes com deficiência de quitotriosidase.

Além disso, foram comparados os níveis de citocinas antes e durante tratamento regular com variáveis clínicas e bioquímicas e foram estabelecidas correlações entre as citocinas na tentativa de caracterizar o envolvimento das vias sinalizadoras e possíveis células envolvidas na fisiopatogênese da DG.

6) PERSPECTIVAS

Baseado em diferentes abordagens inovadoras foram obtidas dados interessantes a respeito do envolvimento do sistema imune na doença de Gaucher.

Com a demonstração da associação de variantes dos genes *KIR* e *HLA,* espera-se estimular a busca de novos genes modificadores de fenótipo, relacionados à imunidade. Pretende-se ampliar a amostra de pacientes com DG, possivelmente de outros centros no país e/ou exterior para corroborar nossos achados. Foi estabelecido convênio com o grupo liderado pelas Profa. Maria Clara Sá Miranda e Profa. Fátima Macedo do Instituto de Biologia Molecular e Celular (IBMC) no Porto, Portugal, fruto de um edital de missão internacional da UFRGS em 2011. Em virtude da hipótese de que variantes dos genes *HLA* possam estar envolvidas na patogênese do mieloma múltiplo, pretende-se estudar pacientes com essa doença em busca de sua associação com o grupo C2 do complexo HLA-C.

Com base em nossos estudos de citocinas, acredita-se que a determinação do aumento dos níveis de BDNF após o tratamento possa levar a pesquisas para novas abordagens terapêuticas para prevenção de manifestações neurológicas em pacientes com DG. Além disso, espera-se otimizar a análise de osteopontina em pacientes com DG, para monitorização de tratamento, especialmente os pacientes com deficiência de quitotriosidase e buscar associação com variáveis clínicas em coortes maiores e estudos a longo prazo.

Por fim, espera-se contribuir para o delineamento de estratégias baseadas em evidência para monitorização, acompanhamento e tratamento das comorbidades apresentadas pelos pacientes.

7) APÊNDICES

Apêndice 1 – Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

- Projeto: **Estudo abrangente sobre o envolvimento do sistema imune na doença de Gaucher: análise de citocinas, de variantes dos genes *HLA* e *KIR* e de sua associação com o Mieloma Múltiplo**
- Pesquisador Responsável: Ida Vanessa D. Schwartz, Departamento de Genética da Universidade Federal do Rio Grande do Sul, e Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos 2350, Porto Alegre-RS. Tel: 51-33598011.

Paciente: _____

Prezado paciente ou responsável,

Esta pesquisa tem por objetivo a obtenção de informações relativas ao sistema imunológico de pessoas com Doença de Gaucher e às alterações presentes nesse sistema. Nossa intenção é ajudar a entender porque acontecem certas complicações nessa doença.

Para que estas informações sejam obtidas será necessário que você (paciente) seja submetido à coleta de:

- 5 mL de sangue para a análise de dosagem de citocinas

- 5 mL de sangue para a análise de genes envolvidos na regulação do sistema imunológico;

Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). O desconforto e os riscos associados a estas avaliações serão minimizados pela realização da coleta por profissional treinado.

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

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

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

Cabe salientar que esta pesquisa não tem como finalidade imediata uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento desta doença, o qual, no futuro, pode levar ao desenvolvimento de uma terapia mais efetiva. Não existe um prazo exato ou estipulado para que você receba os resultados dos exames realizados nesta pesquisa, mas estes lhes serão informados assim que estiverem disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, pode contatar o pesquisador responsável por esta pesquisa, no endereço e telefone que constam no início deste Termo de Consentimento.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

Você tem direito à privacidade. Os resultados deste estudo poderão ser publicados, mas o seu nome não será revelado e todo esforço será feito que a sua identidade não seja revelada. Por meio deste termo, você autoriza que os pesquisadores envolvidos neste estudo pesquisem os seus registros médicos a fim de obter as informações necessárias para a realização desta pesquisa.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará em nada o seu tratamento no seu hospital. A sua participação pode também ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado (a).

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

Data: ___/___/_____

Paciente/assinatura: _____

Responsável legal/assinatura: _____

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

Data: ____/_____/_____

Nome/assinatura:_____

Apêndice II – Ficha de coleta de dados

Data de preenchimento: _____

Responsável:_____
Família:_____
Genótipo *GBA*:_____
Genótipo quitotriosidase: _____
Tipo de DG:_____

Paciente: _____
Data de nascimento : _____/_____/_____
Sexo: ( ) masculino
( ) feminino
Naturalidade: _____
Ascendência: _____

História familial (construir heredograma no verso, incluindo informações sobre história de D. Parkinson, mieloma múltiplo e outras neoplasias na família):

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

-Outros afetados na família?
( ) Sim. Número e grau parentesco:
_____
_____
_____
( ) Não
( ) Não informado

Idade de início da sintomatologia: _____

Manifestações clínicas iniciais:
_____
_____
_____
_____
_____

Desenvolvimento neuropsicomotor:

	Idade de aquisição	**Idade de perda**
sustento cefálico		
sentar sem apoio		
caminhar sem apoio		

palavras com no mínimo duas sílabas		
formar frases		

Escolaridade/ profissão:

_____
_____
_____

Internações hospitalares (data, motivo):

_____
_____
_____
_____
_____
_____

Procedimentos cirúrgicos (data, procedimento, intercorrências):

_____
_____
_____
_____
_____
_____

Doenças crônicas? Sim ( ) Quais?_____

Não ( )

Faz uso de alguma medicação? Qual? Data de início e motivo

_____

_____

Data de diagnóstico: _____
Atividade da glicocerebrosidase: em leucócitos_____VR:_____
Em fibroblastos_____VR:_____

Tratamento específico

( ) Terapia de reposição enzimática com imiglucerase

Idade de início: _____

Dose inicial: _____

Dose atual: _____

Intercorrências: ( ) Não

( ) Sim

_____
_____
_____

( ) Terapia de inibição de síntese de substrato. Nome:_____

Idade de início: _____

Dose inicial: _____

Dose atual: _____

Intercorrências: ( ) Não

( ) Sim

_____
_____
_____

( ) Outro

Idade de início: _____

Dose inicial: _____

Dose atual: _____

Intercorrências: ( ) Não

( ) Sim

_____
_____
_____

Último exame físico:

-data: _____
-idade: _____

-peso: _____

-comprimento/altura: _____

-dor? _____

-epistaxe, equimoses? _____

-hepatomegalia? _____

-esplenomegalia? _____

-alterações neurológicas?_____

-tremor? _____

-estrabismo?_____

-cifose?_____

-marcha:_____

-outros:
_____
_____
_____
_____

Preencher planilhas das avaliações laboratoriais

Planilha 1 – Avaliação hematológica/bioquímica

Data	Leuc	Neutr	Lfct	Plaq	Ht	Hb	Ferritina	AST	ALT	GGT	FA	LDH	Bil Direta	Bil Ind.	TP atividade	Vit B12	Quito	Tanner	Dose U/kg	Escore de gravidade

Planilha 2 – Avaliação imunoglobulinas/eletrofores proteínas

Data Exame	IgA	IgE	IgG	IgM

	Eletroforese de proteínas						Alteração
Data El. Prot	Proteína total	Albumina	Alfa1	Alfa 2	Beta	Gama	

Planilha 3 – Densitometria óssea

Data	Região	Valor (g/cm^2)	Escore T *

Planilha 4 – Imagem do abdômen

Data	Eco ou Tomo?	Fígado (cm3)	Volume Fígado	Laudo Exame	%peso	Baço(cm3)	Volume baço	Laudo exame	%peso	Peso (Kg)

Planilha 5 – RX de ossos

Data / n° RX	Local RX	Regiões Anatômicas	Densidade Óssea	Osteólise medular e ou cortical endosteal	Fratura atual	Fratura passada	Necrose Asséptica da cabeça do Fêmur atual	Necrose Asséptica da cabeça do Fêmur passada	Outras Osteonecrose epifiária	Infarto Medular	Osteomielite	Expansão metafisiária femoral	Expansão metafisiária outros ossos	Prótese articular	Vértebra biconcava	Vértebra cuneiforme