Universidade Federal do Rio Grande do Sul

O fenótipo hepático da doença de Gaucher e dos distúrbios congênitos da glicosilação

Rodrigo Tzovenos Starosta

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para obtenção de título de Doutor em Genética e Biologia Molecular.

Orientadora: Profa. Dra. Ida Vanessa Doederlein Schwartz

Coorientadora: Dra. Marina Siebert

Porto Alegre

Maio/2020

Agradecimentos

Agradeço primeiramente a minha orientadora, a professora dra. Ida Vanessa Doederlein Schwartz, que me deu a oportunidade de realizar esse doutorado e que me ensinou muitíssimo sobre pesquisa, ciência, genética básica e genética clínica. Agradeço também a minha coorientadora, a dra. Marina Siebert, que foi fundamental para a realização de partes experimentais e para a discussão de conceitos durante o desenvolvimento dos projetos de pesquisa que compuseram essa tese. Minha coorientadora no exterior, professora dra. Eva Morava-Kozicz, merece atenção especial nesse agradecimento por ter me recebido de braços abertos em seu laboratório e me ensinado imensamente durante 6 intensos meses muito sobre genética médica, sobre o trabalho em um laboratório, e sobre tratar pacientes com afeto e profissionalismo, além de tudo que sei sobre distúrbios congênitos da glicosilação. Por fim, sou extremamente grato ao "seu" Elmo Jurandir Cardoso, que me guiou com calma e bom-humor através todas as etapas burocráticas que se interpuseram durante o doutorado.

São mais que dignos de nota os pesquisadores, cientistas e seres humanos que me auxiliaram e ensinaram ao longo desses quatro anos: professor dr. Filippo Pinto e Vairo; professor dr. Carlos Thadeu Schmidt Cerski; professor dr. Mário Reis Álvares-da-Silva; dra. Fernanda Sperb-Ludwig; professor dr. Tamas László Kozicz; dr. Fabiano de Oliveira Poswar; dra. Kimiyo Raymond; professor dr. Ian Robert Wanless; professora dra. Eve Roberts. Um também muito obrigado a meus colegas e ex-colegas: dra. Lívia D'Ávila Paskulin, dra. Suélen Basgalupp, dra. Vitória Schütt Zizemer, dra. Gabriela Bolzan, dra. Alícia Dorneles Dornelles, dr. Graeme Preston, Silvia Slobodan Radenkovic, e Anna Ligezka; e às alunas de iniciação científica Ana Paula Pizzio Becker e Lethicia Campos Ferraro.

Finalmente, agradeço também a minha mãe Maria Margarida, meu irmão Gabriel, minha cunhada Roberta, meu sobrinho Arthur, e meu gato Monet pela paciência de me aturarem durante os diversos momentos intensos desse doutorado.

Lista de abreviaturas, símbolos e unidades

AFP: α-fetoproteína

ALG8-CDG: deficiência de α-1,3-glicosiltransferase

ALT: alanina-transaminase

APRI: *AST-to-platelets ratio index*

AST: aspartato-transaminase

BARD: *body mass index, AST/ALT ratio, diabetes*

BH: biópsia hepática

BMP: proteína morfogênica óssea

CCDC115-CDG: deficiência de proteína contendo domínio em mola 115

CG: complexo de Golgi

Cp: ceruloplasmina

CYBRD1: citocromo B redutase duodenal 1

dB: decibel

DCG: distúrbio congênito da glicosilação

DDL: doença lisossômica

DG: doença de Gaucher

DG1: doença de Gaucher tipo 1

DG2: doença de Gaucher tipo 2

DG3: doença de Gaucher tipo 3

DHGNA: doença hepática gordurosa não-alcoólica

dL: decilitro

DMT1: transportador de metais bivalentes 1

EHNA: esteato-hepatite não-alcoólica

EHPAC: elastografia hepática com parâmetro de atenuação controlada

EIM: erro inato do metabolismo

ETH: elastografia transitória hepática

FA: fosfatase alcalina

FPN: ferroportina

GCase: beta-glicosidase ácida

GCase2: beta-glicosidase não-lisossômica

GGT: γ-glutamiltransferase

GlcCer: glicosilceramida

GlcSph: glicosilesfingosina

HC: hepatocarcinoma

HCPA: Hospital de Clínicas de Porto Alegre

HDL: lipoproteína de alta densidade

HFE: regulador homeostático do ferro

HJV: hemojuvelina

IEF: isoeletrofocalização

IMC: índice de massa corporal

Kg: quilograma

kPa: quilopascal

m: metro

mg: miligrama

mL: mililitro

MPI-CDG: deficiência de fosfomanose-isomerase

NeuAc: ácido siálico

NFS: *non-alcoholic fatty liver disease fibrosis score*

NPCRS: *Nijmegen Paediatric CDG Rating Scale*

PGM1-CDG: deficiência de fosfoglicomutase-1

PMM2-CDG: deficiência de fosfomanomutase-2

RE: retículo endoplasmático

RNM: ressonância nuclear magnética

TC: tomografia computadorizada

TCTH: transplante de células-tronco hematopoiéticas

Tf: transferrina

TfR1: receptor de transferrina 1

TfR2: receptor de transferrina 2

TRE: terapia de reposição enzimática

TRS: terapia de redução de substrato

UI: unidade internacional

US: ultrassonografia

VB: vesícula biliar

Lista de figuras e tabelas

Este trabalho foi parcialmente desenvolvido em Porto Alegre, RS, Brasil: no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (HCPA); no laboratório BRAIN (*Basic Research and Advanced Investigations in Neuroscience*) do Centro de Pesquisa Experimental do HCPA; no Centro de Pesquisa Clínica do HCPA; e no Serviço de Patologia Cirúrgica do HCPA. Este trabalho foi complementado durante período sanduíche em Rochester, MN, Estados Unidos da América: no Morava-Kozicz Laboratory da Mayo Clinic Rochester; no Department of Clinical Genomics da Mayo Clinic Rochester; no Biochemical Genetics Laboratory da Mayo Clinic Rochester. Nada desse doutorado poderia ter sido realizado sem o apoio financeiro da Coordenaria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), que, através de dois editais (PBE Doutorado Médico 88887.144663/2017-00 e Doutorado-Sanduíche PRINT 88887.363709/2019-00) possibilitou que eu tivesse o privilégio de me dedicar à pesquisa e ao aprendizado com estabilidade financeira. O Fundo de Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA) também foi essencial para o financiamento dos projetos de pesquisa 2018-0639 e 2018-0654.

Resumo

Introdução: A doença de Gaucher (DG) e os distúrbios congênitos da glicosilação (DCGs) são erros inatos do metabolismo que apresentam fenótipo amplo e variável. Ainda pouco elucidado tanto na DG quanto nos DCGs, é o conjunto das manifestações patofisiológicas de origem no tecido hepático – o fenótipo hepático. **Objetivos:** 1) descrever e analisar o fenótipo hepático da DG; 2) descrever e analisar o aspecto clínico do fenótipo hepático dos DCGs. **Métodos:** Para o objetivo 1), foram realizadas as seguintes etapas: 1) descrição e análise do fenótipo hepático da DG a nível clínico em um estudo de coorte com coleta de dados clínicos, laboratoriais, imaginológicos, histológicos e genético-moleculares; 2) descrição e análise quantitativa o fenótipo hepático da DG a nível histológico, com desenvolvimento de um novo método de análise computadorizada (histomorfometria) de núcleos hepatocelulares e canalículos biliares em biópsias hepáticas; 3) investigação de fatores de modificação do fenótipo da DG, com sequenciamento de nova geração de genes candidatos, com correlação dos achados moleculares com achados clínicos (estudo de segregação de variantes com fenótipo) e dados populacionais (teste de carga), utilizando-se as bases de dados populacionais gnomAD e ABraOM; 4) investigação da prevalência de fibrose hepática na DG, e de métodos não-invasivos de baixo custo para a sua detecção, através da realização de elastografia transitória hepática (ETH) e o cálculo dos escores APRI FIB-4 e NFS; para o objetivo 2) foi realizado um estudo de coorte de pacientes com DCG, com obtenção de dados clínicos, laboratoriais, imaginológicos, histológicos e genético-moleculares. **Resultados:** Objetivo 1), etapa 1) foram incluídos 42 pacientes no estudo de coorte de DG. Foram encontrados: alta prevalência de alterações em marcadores de dano hepatocelular e biliar antes e durante o tratamento; aumento significativo da prevalência de esteatose após início do tratamento com terapia de reposição enzimática (TRE); indícios de hemossiderose, colestase canalicular, e fibrose hepática em uma proporção alta de pacientes investigados para tal; e presença de esteato-hepatite em 2 pacientes, o que não havia sido relatado anteriormente em associação com DG. Etapa 2) No estudo histomorfométrico de pacientes com DG, foram incluídas biópsias hepáticas de 4 pacientes e 4 controles saudáveis.

Pacientes com DG tiveram diferença significativa de parâmetros nucleares hepatocelulares (área nuclear 28,16 ± 9,45 *vs* 26,88 ± 9,42 μm², p=0,006; dimensão fractal média 1,11 ± 0,06 *vs* 1,09 ± 0,09, p=0,005; e absorbância 4,88 ± 2,52 *vs* 7,68 ± 4,60, p=0,014) e de parâmetros canaliculares (absorbância 88,24 ± 59,80 *vs* 173,52 ± 76,78, p=0.001; relação perímetro-Feret 2,70 ± 0,56 *vs* 2,79 ± 0,66, p=0,006). Etapa 3) 33 pacientes tiveram 19 genes sequenciados, com identificação de 95 variantes (sendo 4 novas). Foi encontrada segregação de variante com fenótipo em 3 genes (*A2M*, *CYBRD1* e *TF*), e diferenças no teste de carga na comparação com duas bases de dados populacionais em 5 genes (*CP*, *CYBRD1*, *PSAP*, *TF* e *TFR2*). A variante *CYBRD1* rs10455 foi associada a uma diminuição de chance de osteonecrose (razão de chances de 0,08, intervalo de confiança 0,01 – 0,64, valor p de 0,004) no teste de segregação e com uma menor gravidade geral de doença no teste de carga. Etapa 4) Doze pacientes foram incluídos no estudo com ETH, com elastografia média de 5,25 kPa; aproximadamente metade dos pacientes com DG apresentaram algum grau de fibrose; o escore APRI teve uma área abaixo da curva de 0,701, sendo o mais acurado dos 3 escores testados. Esse teste mostrou sensibilidade 100% e especificidade 100% para detecção de pacientes com fibrose F2 ou superior utilizando-se os pontos de corte 0,201 e 0,604. Objetivo 2) foram incluídos 42 pacientes no estudo de coorte de pacientes com DCGs. Foram encontrados: grande aumento dos marcadores de dano hepatocelular, mas não biliar, com tendência à normalização após os 5 anos de idade nos pacientes com DCGs tipo I, mas não naqueles com tipo II; todos os tipos de esteatose e todos os graus de fibrose, sendo que 3 pacientes apresentaram cirrose; à pesquisa histológica em pacientes com cirrose, houve completa prevalência de acúmulo hepatocelular de glicogênio e completa ausência de hemossiderose. **Conclusão:** o fenótipo hepático da DG e dos DCGs é amplo e variado, com diversos pontos convergentes e divergentes entre essas doenças. Os DCGs de tipos I e II têm evoluções similares internamente, mas distintas entre si. A histomorfometria foi capaz de identificar alterações morfológicas canaliculares e hepatocelulares previamente não-descritas em pacientes com DG e que podem ajudar a explicar o fenótipo desses pacientes. A variante *CYBRD1* rs10455 mostrou-se promissora como modificadora da DG. O escore APRI possui acurácia adequada ao seu uso clínico como preditor de fibrose na DG.

Abstract

Introduction: Gaucher disease (GD) and the congenital disorders of glycosylation (CDGs) are inborn errors of metabolism with a broad and highly variable phenotype. Still understudied in GD and the CDGs is the set of pathophysiological manifestations that arise from the liver tissue – the hepatic phenotype. **Objectives:** 1) to describe and analyze the hepatic phenotype of GD; 2) to describe and analyze the clinical hepatic phenotype of the CDGs. **Methods:** for objectives 1), the following steps were performed: 1) describing and analyzing the hepatic phenotype of GD at a clinical level in a cohort study with acquisition of clinical, laboratorial, imaging, histological, and molecular genetic data; 2) describing and analyzing quantitatively, at the histological level, the hepatic phenotype of GD with the development of a new computerized analysis method (histomorphometry) of hepatocellular nuclei and biliary canaliculi in tissue in liver biopsy samples; 3) investigation of modifier factors of GD – candidate genes were sequenced by next-generation sequencing, with correlation of molecular findings with clinical (phenotypic segregation analysis) and populational (burden test) data using the populational databases gnomAD and ABraOM; step 4), transient hepatic elastography (THE) was used and the APRI, FIB-4, and NFS scores calculated for patients with GD. For objective 2), a cohort of patients with CDGs was used, with acquisition of clinical, laboratorial, imaging, histological, and molecular genetic data. **Results:** Objective 1), step 1) forty-two patients were included in the GD cohort study. Findings included: a high prevalence of abnormalities in hepatocellular and biliary markers both before and during treatment; a significant increase in the prevalence of steatosis after initiation of treatment with enzyme replacement therapy (ERT); evidence for hemosiderosis, canalicular cholestasis, and liver fibrosis in a high proportion of investigated patients; and the presence of steatohepatitis in 2 patients (a previously undescribed feature of GD). Step 2) At the histomorphometric study of patients with GD 4 liver biopsy samples of GD patients and 4 of healthy controls were included. GD patients had significant differences in hepatocellular nuclear parameters (nuclear area 28.16 ± 9.45 *vs* 26.88 ± 9.42 μm², p=0.006; average fractal dimension 1.11 ± 0.06 *vs* 1.09 ± 0.09, p=0.005; and corrected absorbance

4.88 ± 2.52 *vs* 7.68 ± 4.60, p=0.014) and in canalicular parameters (absorbance 88.24 ± 59.80 *vs* 173.52 ± 76.78, p=0.001; perimeter-to-Feret ratio 2.70 ± 0.56 *vs* 2.79 ± 0.66, p=0.006). Step 3) 33 patients were sequenced for 19 genes, identifying 95 variants (4 of which were novel). Variants in 3 genes were positive for phenotypic segregation (*A2M*, *CYBRD1*, and *TF*), and burden test was positive for both populational databases in 5 genes (*CP*, *CYBRD1*, *PSAP*, *TF*, and *TFR2*). The *CYBRD1* rs10455 variant was associated with a decreased chance of osteonecrosis in the segregation analysis (odds ratio = 0.08, confidence interval 0.01-0.64, p-value = 0.004) and with a decreased overall disease severity in the burden test, being a strong candidate for a modifier variant. Step 4) Twelve patients were included in the study, with a mean elastography results of 5.25 kPa; approximately half of the DG patients had some degree of liver fibrosis; the APRI score had an area under the curve of 0.701, being the most accurate of the tested scores. This score was 100% sensitive and 100% specific for the detection of patients with an F2 or higher degree of fibrosis at the cut-off respectively of 0.201 and 0.640. Objective 2) Forty-two patients were included the CDG cohort study. Findings included: a stark increase in hepatocellular, but not biliary, markers with a trend towards normalization after 5 years of age in type I CDG patients but not in type II patients; all types of steatosis and all degrees of fibrosis were present, with cirrhosis in 3 patients; at histology of patients with cirrhosis, all patients had hepatocellular glycogen build-up, and none had hemosiderosis. **Conclusion:** the hepatic phenotype of GD and the CDGs is broad and varied, with many converging and diverging features between those pathologies. CDGs types I and II are internally similar but externally different regarding the hepatic natural history. Histomorphometry was able to identify canalicular and hepatocellular features which had not been described previously in GD, and which may help to explain some aspects of their clinical phenotype. The *CYBRD1* rs10455 variant is promising as a putative modifier of GD. The APRI score has adequate accuracy for a clinical use as a fibrosis predictor in GD.

Sumário

A. Introdução

1. Anatomia, fisiologia e fisiopatologia do fígado

1.1. Anatomia e histologia hepáticas

A manutenção da vida humana é uma tarefa de equilíbrio entre processos internos e externos, forças construtivas e forças destrutivas, síntese e degradação, informação e entropia. Enquanto seja necessário consumir nutrientes para a manutenção das funções orgânicas, faz-se também necessário gerenciar, transformar e excretar substâncias de maneira eficiente para que estas não interfiram com os mesmos processos que as geraram. Esse processo – a homeostase – é altamente regulado por diversos tecidos do corpo humano e tem no seu centro o fígado. O fígado é o maior órgão interno no corpo humano, com aproximadamente 1,5 Kg de massa em um homem adulto de estatura mediana (CHEDID; CHEDID, 2005). É um dos poucos órgãos humanos com um sistema de dupla vascularização, sendo suprido pela veia porta – que aporta sangue venoso rico em nutrientes – e pela artéria hepática – que aporta sangue arterial rico em oxigênio (SCHULZE; SCHOTT; CASEY; TUMA *et al.*, 2019). Esse sistema permite que ambos os suprimentos sanguíneos sejam misturados, gerando um grande volume de sangue rico em nutrientes e em oxigênio, que corre através dos sinusoides hepáticos até ramos da veia hepática conhecidos como veias centrolobulares. Entre os sinusoides, existem fileiras de células epiteliais especializadas: os hepatócitos (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018). Devido à fenestração dos sinusoides, há preenchimento do espaço entre o estes e os hepatócitos (o espaço de Disse) com sangue sinusoidal, ocorrendo extenso contato entre o sangue e a superfície sinusoidal (basolateral) dos hepatócitos, o que permite a captação de certas substâncias para o hepatócito e secreção de outras para a corrente sanguínea. Há três principais destinos para as substâncias captadas pela membrana hepatocelular basolateral: a modificação e secreção de volta à corrente sanguínea através da mesma membrana basolateral; o armazenamento dentro do próprio hepatócito; ou a excreção biliar através da membrana apical. A membrana apical é uma estrutura de membrana altamente especializada

que tridimensionalmente forma o espaço intercelular entre os hepatócitos, delimitando o canalículo biliar (KANEL; KORULA, 2011c). É através desta membrana que são secretados ácidos biliares, lipídeos complexos (sais biliares), e compostos endo- ou xenobióticos lipossolúveis (StatPearls, 2020). Os canalículos biliares comunicam-se aos ductos biliares, formados por colangiócitos, que conduzem a bile pela árvore biliar até o intestino delgado (KANEL; KORULA, 2011c).

Histologicamente, o fígado é organizado em lóbulos (Figura 1). Cada lóbulo caracteriza-se por inúmeros cordões de hepatócitos distribuídos radialmente em torno de uma veia centrolobular, atingindo diversos "espaços-porta" (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018). Um espaço-porta é definido como a área fibrosa contendo a "tríade portal" – um ramo da veia porta, um ramo da artéria hepática, e um ramo dos ductos biliares. Além dos hepatócitos, podem-se observar dentro dos lóbulos hepáticos outros dois tipos celulares: as células de Kupffer, também conhecidas como macrófagos hepáticos, e as células de Ito, também conhecidas como células estreladas hepáticas.

As células de Kupffer, devido ao grande tamanho do fígado, formam a maior população de macrófagos residentes no corpo humano (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018). São células grandes, de formato irregular, que se localizam no lúmen dos sinusoides hepáticos, com extensão para o espaço de Disse. Devido à alta atividade fagocítica destas células, as mesmas são ricas em lisossomos, correspondendo a aproximadamente 25% da quantidade de lisossomos em um fígado sadio (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018). Em resposta a diferentes estímulos fisiológicos e patológicos, as células de Kupffer podem adotar diferentes fenótipos, geralmente divididos entre o chamado "M1", de característica pró-inflamatória, e diversos "M2", de características não-inflamatórias (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017).

As células estreladas hepáticas têm seu corpo celular localizado majoritariamente no espaço de Disse, com longos e finos processos citoplasmáticos que envolvem externamente os sinusoides hepáticos. São miofibroblastos modificados que, em estados fisiológicos, se encontram principalmente em estado quiescente, com função de estoque de retinol, regulação hemodinâmica, e secreção e manutenção da matriz extracelular hepática (KANEL; KORULA, 2011c). Em resposta a citocinas pró-inflamatórias, as células estreladas hepáticas modificam seu fenótipo, transformando-se em miofibroblastos ativos, com aumento de sua contratilidade e secreção de elementos patológicos de matriz, levando à fibrose hepática (KANEL; KORULA, 2011c).

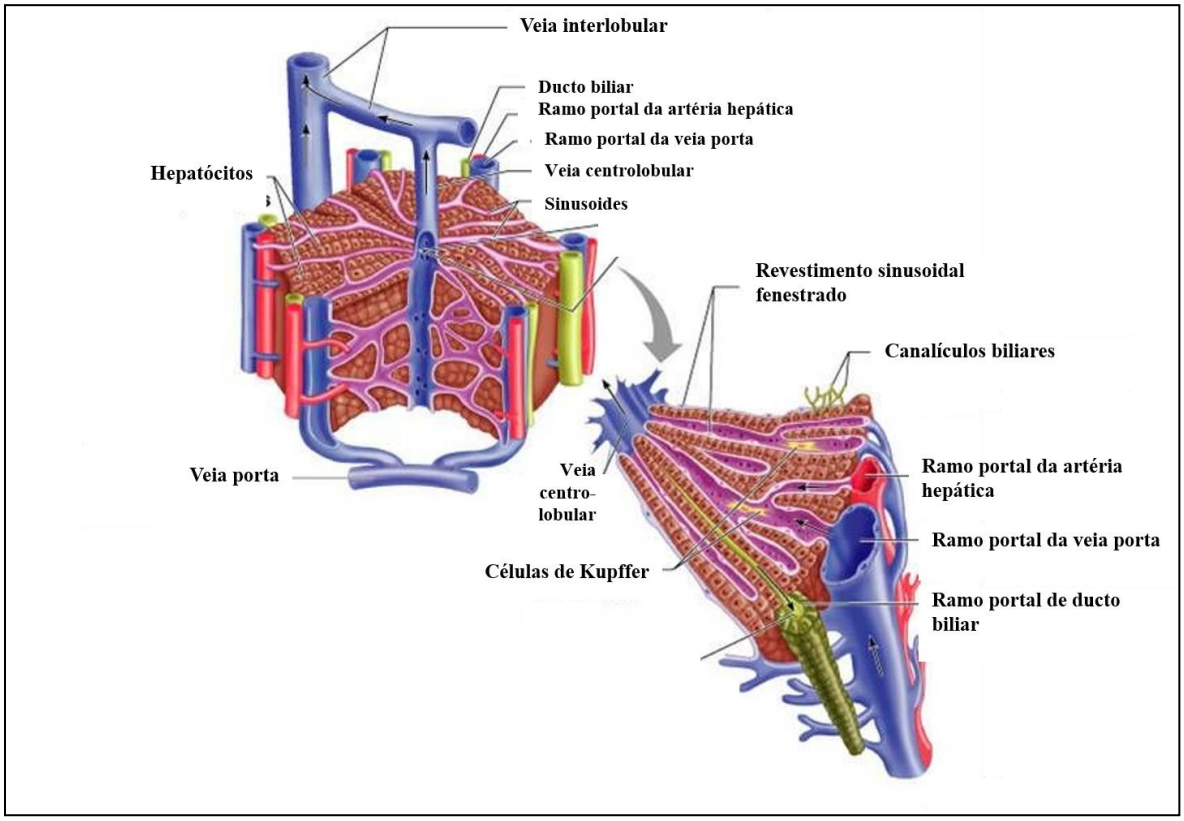


Figura 1. O lóbulo hepático histológico humano Esquema de um lóbulo hepático em destaque, com identificação de seus principais componentes celulares e de sua vascularização única. Imagem adaptada da página virtual (https://www.austincc.edu/apreview/PhysText/Digestive.html), acesso em 11 de maio de 2020.

1.2. Fisiologia do metabolismo do ferro

O ferro é um elemento essencial para o funcionamento de muitos processos fisiológicos, servindo como aceptor, cofator, e parte constituinte de diversos compostos, especialmente os citocromos, a hemoglobina e a mioglobina (KANEL; KORULA, 2011b).

O metabolismo do ferro, resumido na figura 2, inicia na sua absorção a partir de componentes da dieta na região apical da borda em escova do intestino delgado, particularmente na sua primeira porção, o duodeno (PIETRANGELO; TORBENSON, 2018). Neste local, ocorre sua primeira modificação, a redução de íons férricos (Fe^{+3}) a íons ferrosos (Fe^{+2}) mediada pela enzima ferriredutase (também conhecida como citocromo B redutase duodenal 1, ou CYBRD1), codificada pelo gene *CYBRD1* (MCKIE; BARROW; LATUNDE-DADA; ROLFS *et al.*, 2001) (MIM: 605745). O ferro reduzido é então transportado do lúmen duodenal para o meio intracelular dos enterócitos (células epiteliais intestinais) através do transportador de metais bivalentes 1 (DMT1), codificado pelo gene *SLC11A2* (HUBERT; HENTZE, 2002) (MIM: 600523). Quando o ferro da dieta está ligado ao heme (composto central da hemoglobina e da mioglobina), a estrutura completa do heme é transportada para o interior dos enterócitos, e o ferro é dissociado em sua forma já reduzida pela enzima heme oxigenase. O Fe^{+2} é então transportado ativamente para a circulação sistêmica através da membrana basolateral enterocelular pelo transportador ferroportina (FPN), codificado pelo gene *SLC40A1* (PIETRANGELO; TORBENSON, 2018)(MIM: 604653), ou estocado no interior dos enterócitos em ligação com a ferritina até sua posterior liberação sistêmica. A ferritina, codificada pelos genes *FTL* (MIM: 134790) e *FTH1* (MIM: 134770), é uma proteína multimérica presente no citoplasma de múltiplos tipos celulares, onde se liga a íons de ferro para o seu armazenamento. Em situações fisiológicas, há pouca secreção de ferritina para o meio extracelular (plasma) – no entanto, quando há excesso de ferro ou estímulo inflamatório, a sua produção aumenta e não pode ser contida no meio intracelular, extravasando em quantidades maiores para o plasma (LEFEBVRE; REIHANI; DAHER; DE VILLEMEUR *et al.*, 2018).

Uma vez exportado do enterócito, o ferro é novamente oxidado a Fe^{+3} pela enzima hefestina, uma ferroxidase codificada pelo gene *HEPH* (MIM: 300167). O Fe^{+3} se liga às proteínas transportadoras transferrina (Tf) – codificada pelo gene *TF* (MIM: 190000) – e ceruloplasmina (Cp) – codificada pelo gene *CP* (MIM: 117700). A Tf é uma glicoproteína plasmática secretada pelo fígado, capaz de transportar dois íons férricos com alta afinidade, impedindo o ferro de exercer sua função oxidativa fora de seu contexto e assim protegendo diversos tecidos da toxicidade gerada pelo alto potencial oxidativo desse metal (WINTERBOURN, 1995). A Tf, uma vez saturada, liga-se aos receptores de Tf (TfR1 e TfR2, codificados respectivamente pelos genes *TFRC* (MIM: 190010) e *TFR2* (MIM: 604720)) presentes na membrana plasmática de todas as células nucleadas, mas de concentração especialmente elevada em células cujas atividades necessitam um alto aporte de ferro – isto é, hepatócitos, células de Kupffer, e precursores hematopoiéticos da medula óssea – e sofre endocitose, liberando no compartimento lisossômico, devido ao pH ácido dessa organela, seus átomos de ferro (PIETRANGELO; TORBENSON, 2018). O TfR1 é ubíquo e controlado pelo *status* do ferro, enquanto o TfR2 é presente somente em hepatócitos e não é regulado pelo *status* dos estoques de ferro (FLEMING; MIGAS; HOLDEN; WAHEED *et al.*, 2000). A Cp é uma proteína de tripla função: ao mesmo tempo em que é a principal proteína carreadora de cobre no plasma, tem atividade de ferroxidase similar à hefestina e contribui para o transporte de ferro do plasma para os tecidos sistêmicos (HARRIS, 2019; ROESER; LEE; NACHT; CARTWRIGHT, 1970). Dos lisossomos, o ferro é transportado para o citoplasma novamente pelo DMT1, e lá é utilizado para funções biológicas ou armazenado em ligação com a ferritina.

A regulação do metabolismo do ferro é feita principalmente pela hepcidina, um peptídeo de 25 aminoácidos codificado pelo gene *HAMP* (MIM: 606464) e produzido pelos hepatócitos em resposta a níveis elevados de ferro intracelular e plasmático, os quais são monitorados pelo "sistema de monitoramento de ferro" (PIETRANGELO; TORBENSON, 2018). Esse sistema consiste em uma série de sinalizadores humorais, entre os quais encontram-se as proteínas morfogênicas ósseas (BMPs). Essa proteínas, especialmente a BMP6 – codificada pelo gene *BMP6* (MIM: 112266) – são secretadas em resposta à presença

de ferro hepatocelular e ao aumento da saturação da Tf plasmática, e ligam-se a receptores como a hemojuvelina (HJV), codificada pelo gene *HJV* (MIM: 608374) e estabilizada na membrana pela proteína neogenina (codificada pelo gene *NEO1* (MIM: 601907)), que são por sua vez modulados pelo regulador homeostático do ferro (HFE), codificado pelo gene *HFE* (MIM: 613609). O HFE é uma proteína de membrana similar ao complexo maior de histocompatibilidade tipo I, e conecta o sinal produzido pela BMP6 e pela HJV ao TfR1, levando à produção de hepcidina. A perda de função do HFE leva à diminuição de níveis de hepcidina e à doença autossômica recessiva hemocromatose hereditária tipo I. O sistema de monitoramento de ferro é, por sua vez, contrarregulado pela enzima serina-protease transmembrana 6, codificada pelo gene *TMPRSS6* (MIM: 609862), que degrada a HJV e impede a propagação excessiva do sinal indutor da produção de hepcidina (PIETRANGELO; TORBENSON, 2018).

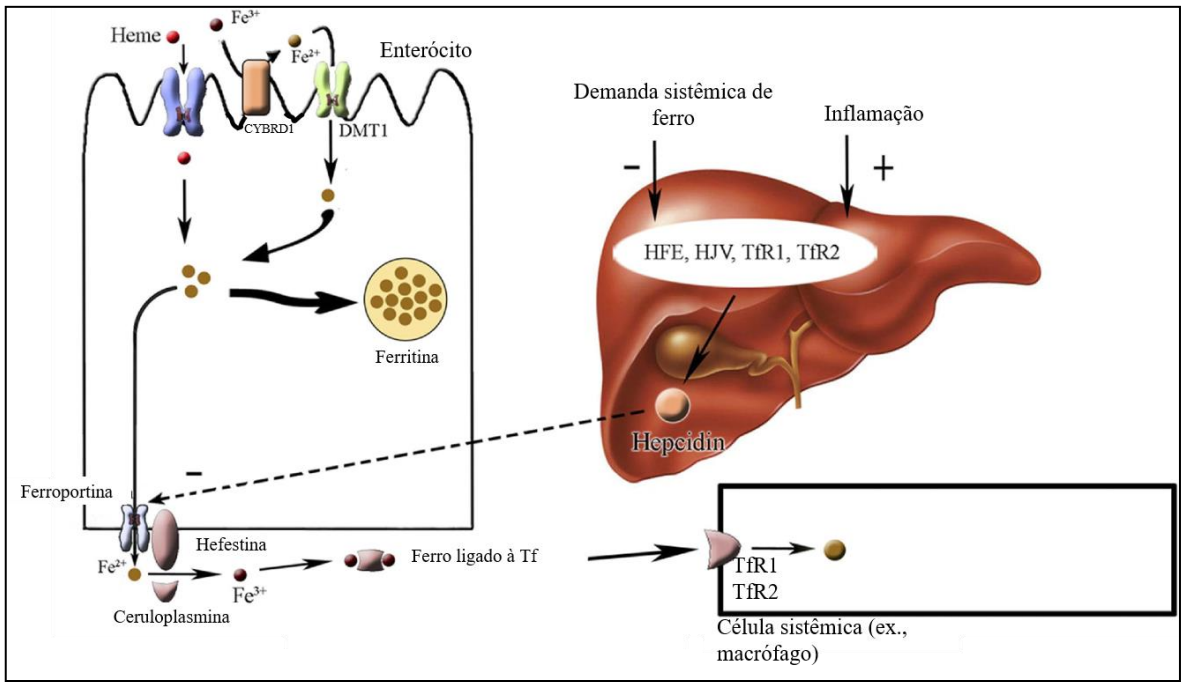


Figura 2. Principais componentes do metabolismo do ferro. A figura ilustra, em linhas gerais, o processo de absorção do ferro da dieta até a sua distribuição para os tecidos, onde exerce suas funções biológicas. Há papel de destaque para a regulação do metabolismo do ferro pela hepcidina, produzida pelo fígado, que age inibindo a FPN. Figura adaptada de (GUJJA; ROSING; TRIPODI; SHIZUKUDA, 2010).

Outro importante regulador dos níveis de hepcidina é a inflamação: citocinas pró-inflamatórias como as interleucinas 1 e 6, entre outros fatores, são potentes estimulantes para a produção de hepcidina, ligando-se a um sítio de ativação na região promotora a 5' do gene *HAMP* (LEE; BEUTLER, 2009). Estados de inflamação sistêmica crônica são caracterizados por níveis aumentados de hepcidina, o que leva a processos patológicos como a anemia da doença crônica (LEE; BEUTLER, 2009; WEISS; GANZ; GOODNOUGH, 2019).

Quando a hepcidina é produzida e secretada pelos hepatócitos na circulação sistêmica, liga-se ao DMT1 e leva à sua internalização e degradação. Isso faz com que o ferro absorvido no duodeno não seja liberado na circulação sistêmica, diminuindo os níveis plasmáticos de ferro e a saturação da Tf; há também inibição do uso hepatocelular do ferro, já que esse processo depende da liberação de ferro do lisossomo para o citoplasma pelo mesmo transportador; e inibição da liberação dos estoques de ferro em hepatócitos e macrófagos para a circulação sistêmica, através do mesmo mecanismo (LEE; BEUTLER, 2009).

1.3. Padrões fisiopatológicos hepáticos

1.3.1. Dano hepatocelular inespecífico

Existem diversas situações em que pode haver dano hepatocelular sem que haja características específicas que sugiram uma etiologia ou um processo fisiopatológico comum. Em um contexto de dano isquêmico ou hipóxico, químico, ou mesmo infeccioso, uma das primeiras manifestações nos hepatócitos é a degeneração hidrópica (também conhecida como edema intracelular), em que há perda da capacidade celular de prover energia adequada para o funcionamento do transportador de membrana sódio-potássio-ATPase, levando ao desbalanço hidroeletrolítico entre os meios intra- e extracelular e a entrada de água nas células por osmose (THEISE, 2015). Em casos de injúria estabelecida, o aumento de volume intracelular leva à vacuolização do citoplasma e aglutinação dos filamentos intermediários do citoesqueleto – com consequente perda de forma da membrana

plasmática –, sendo chamado o processo de “balonização” (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018).

Clinicamente, o dano hepatocelular inespecífico é detectado por dosagem laboratorial de dois marcadores, a alanina-transaminase (ALT, previamente conhecida como transaminase glutâmico-pirúvica, ou TGP) e a aspartato-transaminase (AST, previamente conhecida como transaminase glutâmico-oxalacética, ou TGO). A ALT é uma enzima citoplasmática presente em grande quantidade no citoplasma dos hepatócitos, e em menor quantidade no citoplasma de células de outros órgãos; a AST é uma enzima mitocondrial presente em grande quantidade em hepatócitos, em miócitos e em células da linhagem eritropoética (CONSORTIUM, 2019). Em situações de dano celular, ambas as enzimas são liberadas na circulação sistêmica e seu aumento pode ser detectado em amostras de sangue periférico, sendo que a ALT apresenta maior especificidade para o dano hepatocelular (ALMEIDA; MONTEIRO, 2018). Elevações das transaminases ALT e AST (chamadas erroneamente por alguns autores como “transaminite”) são comuns em praticamente todas as formas de dano hepático, incluindo doenças infecciosas (hepatites virais, leptospirose, toxoplasmose e outras), autoimunes (hepatite autoimune, cirrose biliar primária, colangite esclerosante), metabólicas (doenças esteatóticas – ver seção 1.3.2 –, doenças genéticas como hemocromatose, doença de Wilson, deficiência de alfa-1-antitripsina e outras), hepatotoxicidade por compostos exógenos, entre outras causas (ALMEIDA; MONTEIRO, 2018). Desse modo, a detecção de aumento de transaminases é um marcador sensível, mas pouco específico, de dano hepatocelular que deve ser investigado de forma mais aprofundada para esclarecimento de sua etiologia, como discutido nas próximas seções.

1.3.2. Esteatose

Esteatose é o acúmulo de lipídeos em vacúolos no citoplasma dos hepatócitos (THEISE, 2015). Pode ser dividida primariamente em macrogoticular, na qual há um vacúolo grande no citoplasma do hepatócito com deslocamento do núcleo para a periferia da célula; ou microgoticular, na qual há múltiplos vacúolos periféricos ao núcleo, com manutenção da posição central deste (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU,

2018). Existem diversos processos fisiopatológicos associados à formação de esteatose: a esteatose macrogoticular associa-se à síndrome metabólica (doença hepática gordurosa não-alcoólica, ou DHGNA, e esteato-hepatite não-alcoólica, ou EHNA), à toxicidade a medicamentos específicos como os bloqueadores de canais de cálcio e os hormônios esteroides, assim como a alguns erros inatos do metabolismo (EIMs) como galactosemia, intolerância hereditária à frutose, citrulinemia tipo II, defeitos do ciclo da ureia, doença de Wilson, e outras causas (QUAGLIA; ROBERTS; TORBENSON, 2018). Quando pouco prevalente entre os hepatócitos do fígado, a esteatose macrogoticular pode ser também consequência do envelhecimento normal, não tendo significado patológico (QUAGLIA; ROBERTS; TORBENSON, 2018). A esteatose microgoticular associa-se geralmente a processos tóxicos, como a doença hepática alcoólica, a toxicidade a medicamentos como antirretrovirais, anti-inflamatórios, antibióticos e outros, a doenças de acometimento mitocondrial ou que levem à ineficiência da beta-oxidação, a EIMs como a deficiência de lipase ácida lisossômica, e outras causas (KRISTIANSEN; VEIDAL; CHRISTOFFERSEN; JELSING *et al.*, 2019; QUAGLIA; ROBERTS; TORBENSON, 2018).

Clinicamente, a maioria dos pacientes com esteatose hepática não apresenta sintomas devido à esteatose em si, e a minoria apresenta sintomas leves e inespecíficos como mal-estar, fadiga, ou desconforto no quadrante superior direito do abdômen (PARISE; BORGES, 2018). Muitos pacientes com DHGNA ou EHNA apresentam critérios para o diagnóstico concomitante de síndrome metabólica, isto é, presença de ao menos três das seguintes manifestações: aumento de circunferência abdominal; concentração sérica de triglicerídeos igual ou superior a 150 mg/dL; concentração sérica de lipoproteína de alta densidade (HDL) inferior a 40 mg/dL para homens ou 50 mg/dL para mulheres; pressão arterial sistólica igual ou superior a 130 mmHg ou pressão arterial diastólica igual ou superior a 85 mmHg; resistência à insulina detectada por glicemia de jejum igual ou superior a 100 mg/dL ou índice HOMA-IR (HOMA-IR = [insulinemia em jejum] x [glicemia em jejum]/405) igual ou superior a 2,5 (PARISE; BORGES, 2018). Muitos pacientes apresentam aumento de marcadores de dano hepatocelular inespecífico como a ALT e a AST (PARISE; BORGES, 2018).

A detecção de esteatose hepática pode ser feita por modalidades de imagem, sendo a ultrassonografia (US) abdominal o exame mais utilizado para esse fim devido a ser não-invasiva, de baixo custo, e com acurácia adequada (MASHAV; SHIBOLET, 2015); com maior precisão, porém maior custo, há a ressonância nuclear magnética (RNM) e a espectroscopia com detecção de lipídeos (MASHAV; SHIBOLET, 2015). O padrão-ouro para o diagnóstico de esteatose é a detecção em biópsia hepática (BH) de uma fração de área ocupada por lipídeos igual ou superior a 5% da área de tecido (BEDOSSA, 2017) – no entanto, por ser um exame invasivo, com riscos associados ao procedimento, a BH raramente é realizada apenas para a confirmação diagnóstica de esteatose (BEDOSSA, 2018). Por outro lado, o único modo de diferenciar entre a DHGNA e a EHNA, assim como entre formas macrogoticulares e microgoticulares – todas percebidas não-invasivamente simplesmente como "esteatose" – é através da BH, sendo essa uma indicação para a realização desse exame (BEDOSSA, 2018). Uma modalidade emergente no diagnóstico de esteatose hepática é a elastografia hepática com parâmetro de atenuação controlada (EHPAC), realizado de forma não-invasiva com o uso de um transdutor de elastografia hepática (ver seção 1.3.6) mas medindo, ao invés de rigidez hepática, a atenuação de sinal ultrassonográfico após o impulso mecânico fornecido pela elastografia (LIPIŃSKI; SZYMAŃSKA-ROŻEK; SOCHA; TYLKI-SZYMAŃSKA, 2019; SASSO; TENGHER-BARNA; ZIOL; MIETTE *et al.*, 2012). A EHPAC fornece medidas de esteatose em dB/m, e pode classificar a esteatose em graus: S0 corresponde a esteatose ausente ou presente em menos de 10% dos hepatócitos; S1, a esteatose presente em 11 a 33% dos hepatócitos; S2, a esteatose presente em 34 a 66% dos hepatócitos; e S3, a esteatose presente em 67% ou mais dos hepatócitos (SASSO; TENGHER-BARNA; ZIOL; MIETTE *et al.*, 2012). Assim como os outros métodos não-invasivos, a EHPAC não é capaz de diferenciar esteatose simples da esteato-hepatite.

Apesar de ser geralmente considerada como uma alteração potencialmente benigna, a esteatose pode levar a consequências importantes. Estima-se que aproximadamente 20% dos pacientes com esteatose sem comorbidades venham a progredir para a esteato-hepatite (ver seção 1.3.3), e que 20% desses venham a ter graus significativos de fibrose, com progressão à cirrose se o processo patológico não for interrompido (EDMISON;

MCCULLOUGH, 2007). Além das consequências hepáticas diretas, a esteatose é um fator de risco independente para desfechos cardiovasculares, presumivelmente devido à alteração no metabolismo hepatocelular de lipídeos que acarreta (DHITAL; TIROSH, 2015; TURPIN-NOLAN; BRÜNING, 2020).

1.3.3. Inflamação

A inflamação hepática ("hepatite") inclui diversos processos fisiopatológicos que levam à infiltração do lóbulo hepático por células inflamatórias, independentemente de etiologia. É descrita de acordo com o local do lóbulo hepático acometido; com o tipo de células inflamatórias infiltrantes; e com achados concomitantes à inflamação, como necrose ou formação de inclusões. A análise do padrão de inflamação é muitas vezes informativo sobre a natureza do dano hepático que esteja ocorrendo: por exemplo, nas hepatites virais agudas típicas, ocorre infiltração dos espaços-porta (e, em menor grau, do parênquima hepatocelular) por linfócitos em grande número, com achados de hiperplasia de células de Kupffer, necrose e apoptose de hepatócitos, e presença esparsa de plasmócitos. A presença de grande número de linfócitos denota a natureza viral da infecção, já que o mecanismo de citotoxicidade linfocítica é a principal defesa imunológica contra infecções virais intracelulares; os achados de necrose e apoptose refletem a citotoxicidade exercida pelos linfócitos; a hiperplasia de células de Kupffer, sem verdadeira infiltração do parênquima por macrófagos, é parte da resposta de "limpeza" dos debris celulares decorrentes da necrose e da apoptose; e a presença de plasmócitos denota a produção de imunoglobulinas contra antígenos virais, que podem ser detectados clinicamente nas hepatites virais (KANEL; KORULA, 2011d; THEISE; BODENHEIMER JR; GUIDO, 2018). A inflamação por EHNA, por outro lado, é centrada nos hepatócitos e de característica mista, com números equivalentes de neutrófilos e linfócitos ou leve predomínio dos primeiros, com achados de balonização e degeneração dos hepatócitos (mas sem apoptose ou necrose abundantes) e, em alguns casos, hepatócitos rompidos pelo conteúdo lipídico intracitoplasmático circundados por células inflamatórias ("satelitose"). A natureza mista do infiltrado inflamatório, com hepatócitos degenerados (com esteatose e balonização – ver seção 1.3.1) mas sem morte

celular dos mesmos, denota a natureza não-infecciosa, não-citotóxica da inflamação; a proximidade entre as células inflamatórias e os hepatócitos e a presença de satelitose é reflexo da indução da inflamação pelos próprios hepatócitos (KANEL; KORULA, 2011a; TINIAKOS; ANSTEE; BURT, 2018).

Clinicamente, não há marcadores específicos da inflamação hepática. Os marcadores de dano hepatocelular inespecífico tendem a se encontrar deveras aumentados em casos de processos inflamatórios intensos, mas podem estar dentro dos valores de referência ou apenas levemente aumentados em casos de inflamação crônica ou leve. Os exames não-invasivos de imagem não são capazes de detectar estados inflamatórios, e o padrão-ouro para o diagnóstico de um transtorno inflamatório do fígado é a BH (TINIAKOS; ANSTEE; BURT, 2018).

As consequências da inflamação hepática são variadas, e o prognóstico depende em grande parte da etiologia do processo. Processos inflamatórios crônicos, como alguns casos de hepatites virais por vírus B ou vírus C, e a EHNA, podem levar à fibrose, como descrito na seção 1.3.7.

1.3.4. Siderose

A siderose é o acúmulo de ferro no citoplasma de hepatócitos ou de células de Kupffer, geralmente ligado à ferritina ou a sua forma parcialmente degradada, a hemossiderina. Apesar de que os hepatócitos e os macrófagos sejam as principais células com estoque de ferro no organismo, em circunstâncias fisiológicas a quantidade absoluta de ferro no corpo humano é baixa – 3 a 5 gramas, sendo que aproximadamente metade desse valor encontra-se constantemente utilizado na forma de hemoglobina, e 1g na forma de mioglobina e de citocromos (KANEL; KORULA, 2011b; PIETRANGELO; TORBENSON, 2018); nos hepatócitos, nessas circunstâncias, há menos de 20 μmol de ferro por grama de tecido desidratado (DEUGNIER; TURLIN, 2007). Em um contexto de distúrbio primário (hemocromatose) ou secundário (hemossiderose) do metabolismo do ferro (ver seção 1.2), esse metal tende a se acumular nos tipos celulares em que se encontra normalmente, levando

à siderose. Quando se acumula primariamente em hepatócitos, diz-se que ocorre "siderose parenquimatosa"; em macrófagos, "siderose mesenquimal"; em ambos os tipos celulares, "siderose mista" (DEUGNIER; TURLIN, 2007). Enquanto as hemocromatoses tendem a causar principalmente siderose parenquimatosa, e as hemossideroses tendem a causar siderose mesenquimal, é possível ocorrer hemocromatose com siderose mesenquimal, assim como hemossiderose com siderose parenquimatosa (KANEL; KORULA, 2011b).

Clinicamente, a sobrecarga de ferro pode ser detectada através de diversas modalidades diagnósticas (FUCUTA; BILAR, 2018): exames laboratoriais de sangue podem medir os níveis plasmáticos de ferritina e a saturação plasmática de Tf (calculada a partir da fórmula: saturação de Tf = [Fe] x 71,2/[Tf]) com boa sensibilidade, mas especificidade baixa devido à possibilidade de alteração desses marcadores, especialmente da ferritina, por estados inflamatórios. Os métodos de mensuração direta de ferro hepático são mais específicos, embora não tão sensíveis: de forma não-invasiva, a RNM pode ser usada, conforme o protocolo da Universidade de Rennes (GANDON; OLIVIÉ; GUYADER; AUBÉ *et al.*, 2004); de forma invasiva, mas com maior acurácia, a BH permite a mensuração semiquantitativa de ferro pela coloração de azul da Prússia (coloração de Perls) (BEDOSSA; PARADIS; ZUCMAN-ROSSI, 2018) ou quantitativa por espectrometria de absorção atômica (PIETRANGELO; TORBENSON, 2018).

As consequências da deposição hepática de ferro advêm da toxicidade desse metal quando satura a capacidade de estoque da ferritina e passa a existir em sua forma livre como Fe^{+2} (GALARIS; BARBOUTI; PANTOPOULOS, 2019). O principal mecanismo de toxicidade do Fe^{+2} é a geração de radicais livres através da reação de Fenton, na qual $Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH\bullet$. Essa produção de radicais livres leva ao estresse oxidativo, com consequente peroxidação e tiooxidação de biomoléculas e dano celular (MEHTA; FARNAUD; SHARP, 2019). Cronicamente, o dano hepatocelular e macrofágico por estresse oxidativo leva à inflamação hepática e à ativação das vias de fibrose (ver seção 1.3.6).

1.3.5. Dano biliar

O dano aos colangiócitos do epitélio biliar pode ocorrer devido a diversos fatores, incluindo causas luminais – vindas da composição, da pressão, ou de infecção da bile – e causas extrabiliares – como processos autoimunes ou dano hepático generalizado. A arquitetura dos ductos biliares é de certa forma mais delicada que a dos hepatócitos, já que a árvore biliar é uma estrutura contínua e comunicante, com muitos elementos em série, enquanto os lóbulos hepáticos consistem em elementos repetidos em paralelo (figura 3). Isso faz com que a perda de arquitetura e da organização dos colangiócitos em um ponto da árvore biliar possa ter influência sobre grande parte, ou mesmo toda, a via biliar; a nível histológico, esse processo é geralmente visto como a resposta "ductopênica" (de ausência de ductos biliares) e consequentemente colestática (com acúmulo de bile em canalículos e hepatócitos devido à ausência de fluxo da bile) a montante de um dano biliar focal (ZEN; HÜBSCHER; NAKANUMA, 2018). A progressão à ductopenia ocorre de forma gradual, com degeneração hidrópica e atrofia de colangiócitos, perda da polaridade celular, regeneração desordenada com dilatação do lúmen do ducto, e por fim apoptose dos colangiócitos da parede dos ductos, com criação de espaços desordenados entre as células remanescentes que permitem o extravasamento de bile, levando à perda dos colangiócitos restantes devido ao dano químico promovido por esta (ZEN; HÜBSCHER; NAKANUMA, 2018).

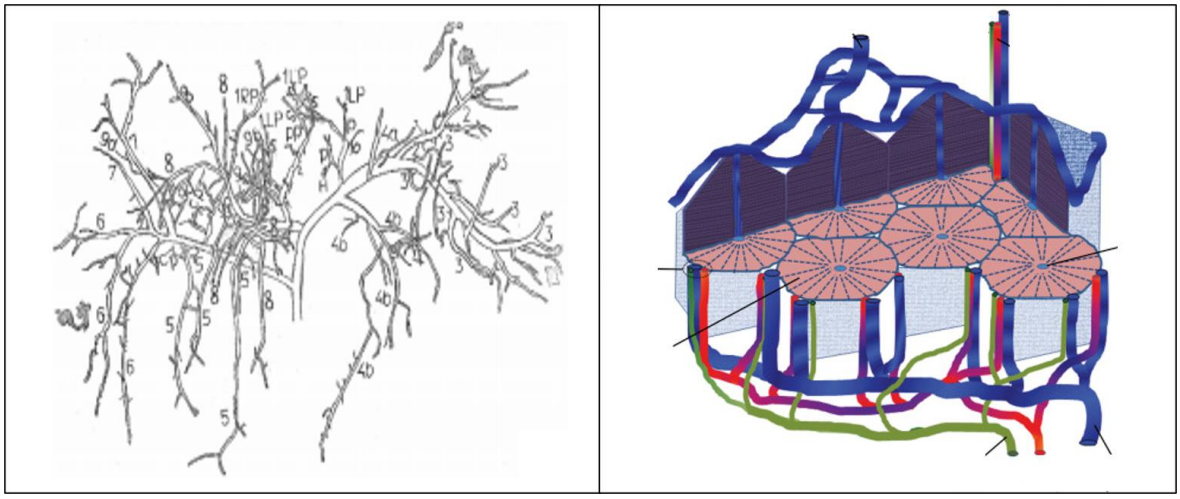


Figura 3. Comparação entre a organização em paralelo dos lóbulos hepáticos e a organização em série da árvore biliar. Painel da esquerda mostra desenho realizado a partir de molde *post-mortem* de árvore biliar humana em que se evidencia o caráter predominantemente em série da estrutura, o que é compatível com sua função de transporte mecânico da bile do fígado ao duodeno. Painel da direita mostra arranjo em paralelo dos lóbulo hepáticos, com divergência dos ramos vasculares a montante e convergência dos ramos vasculares e biliares a jusante. Imagens adaptadas de (DRAGICA; KOSTANDINA, 2013) e (FOSTER, 2018).

A vesícula biliar (VB) é uma víscera oca revestida por epitélio biliar e intimamente ligada ao fígado e à via biliar, comunicando com esta através do ducto cístico. Tem função de armazenamento e modificação química da bile. A principal doença da VB é a colelitíase (calculose) e as suas complicações (coledocolitíase, colestase extra-hepática, colangite). A colelitíase é prevalente, presente em aproximadamente 10% a 15% dos adultos da população em geral (PORTINCASA; MOSCHETTA; PALASCIANO, 2006). Pode ser dividida primariamente quanto à composição dos cálculos, sendo o tipo mais comum o cálculo de colesterol, e mais raros os cálculos de bilirrubina e de sais inorgânicos. Os primeiros são formados quando a bile se encontra supersaturada pelo colesterol (um de seus componentes emulsificantes), seja devido à presença aumentada desse componente ou à diminuição de outros componentes, como os fosfolipídeos e os lipídeos complexos, que aumentam a capacidade de suspensão do colesterol. (QUIGLEY; ADSAY, 2018). Os cálculos de bilirrubina e de sais inorgânicos geralmente ocorrem em condições de hemólise crônica ou

de infecção crônica das vias biliares, sendo raros na sociedade Ocidental, mas comuns em países do Extremo Oriente (QUIGLEY; ADSAY, 2018). Entre os fatores de risco para a colelitíase por cálculos de colesterol encontram-se: idade, sexo feminino, fertilidade, obesidade, perda rápida de massa adiposa, hipertrigliceridemia, uso de fármacos como anticoncepcionais orais, cirurgia de ressecção terminal do íleo, e síndromes genéticas como a síndrome de Gilbert (BEN HAROSH-KATZ; PATLAS; HADAS-HALPERN; ZIMRAN *et al.*, 2004). A colelitíase é uma doença benigna, não ocasionando sintomas a não ser que haja obstrução da via biliar pelos cálculos, com aumento da pressão a montante. Nesse caso, dano ao parênquima hepático e às vias biliares intra-hepáticas pode ocorrer, além de infecção bacteriana por translocação de bactérias do intestino delgado para a bile estática (GREENBERGER; PAUMGARTNER, 2015).

As enzimas fosfatase alcalina (FA) e γ-glutamiltransferase (GGT) são os principais marcadores bioquímicos do dano aos canalículos e ao epitélio biliar. A FA é uma enzima encontrada em diversas isoformas (“frações”) em diferentes tecidos, sendo que a fração hepática se encontra na membrana canalicular, onde sua função ainda não está completamente elucidada. A GGT, apesar de também presente em diversos órgãos, é produzida em quantidades significativas somente no fígado, estando presente na membrana canalicular e na porção luminal do epitélio biliar (colangiócitos). Tanto a FA quanto a GGT são marcadores sensíveis do dano canalicular e biliar, sendo que a fração hepática da FA é mais específica para o dano direto em canalículos, e a GGT para o dano colangiocelular. A medida isolada da fração hepática da FA, no entanto, é de difícil e onerosa mensuração, sendo mais comum a medida da FA total – isso representa um problema para a utilização da FA na prática clínica, especialmente em doenças com acometimento ósseo concomitante, já que a fração óssea da FA é mais abundante em termos absolutos que a fração hepática, o que faz com que aumentos da FA percam sua especificidade para o tecido hepático (ALMEIDA; MONTEIRO, 2018; LUM; GAMBINO, 1972).

1.3.6. Fibrose

A fibrose hepática é um processo parcialmente reversível caracterizado pela deposição imprópria de elementos de matriz extracelular no lóbulo hepático, levando à perda de função hepática. É a via comum de todos os processos patogênicos hepáticos crônicos. Em resposta ao dano celular agudo, hepatócitos e colangiócitos são capazes de promover a regeneração da população celular do lóbulo hepático, e os macrófagos e os fibroblastos hepáticos (células estreladas hepáticas e fibroblastos presentes no espaço-porta) orquestram uma resposta inflamatória aguda que organiza a matriz extracelular e "limpa" os debris celulares; no dano hepático crônico, no entanto, essa resposta torna-se redundante, levando ao aumento da deposição de colágeno primeiramente no espaço-porta e então no parênquima hepático; à deposição de proteínas componentes da membrana basal no espaço de Disse; e à perda de fenestrações no endotélio dos sinusoides (CRAWFORD; BIOULAC-SAGE; HYTIROGLOU, 2018). Esses processos levam à diminuição do contato do sangue com os hepatócitos, com perda da capacidade de troca de substâncias entre a circulação sistêmica e o fígado, e à distorção das vias biliares, levando à obstrução crônica ao fluxo biliar.

Para a graduação da fibrose hepática quanto a sua gravidade, o sistema mais utilizado é o METAVIR, desenvolvido inicialmente para uso em casos de hepatite por vírus C mas atualmente utilizado para diversas doenças hepatopatias (BEDOSSA; POYNARD, 1996). Nesse sistema (Figura 4), o estágio 0 (F0) representa a ausência de fibrose; o estágio 1 (F1), a presença de fibrose no espaço-porta, com alargamento e aumento da densidade celular do mesmo, mas sem fibrose dentro do parênquima hepático; o estágio 2 (F2) representa a presença de fibrose também no parênquima, na forma de septos fibrosos periportais (ao redor do espaço-porta) ou ligando dois espaços-porta ("ponte porta-porta"); o estágio 3 (F3) representa a presença de septos fibrosos conectando espaço-porta e veia centrolobular ("ponte porta-centro"), com consequente distorção da arquitetura do lóbulo hepático, mas ainda sem delimitação completa do lóbulo pela fibrose; o estágio 4 (F4) representa a cirrose hepática, com múltiplas pontes porta-porta e porta-centro causando distorção completa da arquitetura do lóbulo, com a delimitação de um nódulo hepatocelular cercado por septos

fibrosos. Por fim, um estágio de fibrose não contemplado pelo sistema METAVIR é a fibrose perissinusoidal (TINIAKOS; ANSTEE; BURT, 2018). Esse tipo de lesão é resultante do preenchimento do espaço de Disse por componentes fibróticos e de membrana basal sem que haja extensão da deposição de matriz extracelular para o espaço ocupado pelos hepatócitos. É um padrão associado à EHNA (ver seção 1.3.2) atual ou prévia, e estudos recentes mostram que a presença de fibrose perissinusoidal é um marcador independente de risco para desfechos cardiovasculares (ATHYROS; TZIOMALOS; KATSIKI; DOUMAS *et al.*, 2015).

Clinicamente, a fibrose pode ser detectada de diversas maneiras. Classicamente, é descrito que ao exame físico do abdômen, o fígado pode ter consistência endurecida e bordos afiados em casos de cirrose (CHEDID; CHEDID, 2005); no entanto, devido ao hábito corporal variado e ao advento de métodos mais avançados e mais precisos para a detecção de fibrose, o exame físico do abdômen tornou-se obsoleto para a detecção de fibrose. Exames laboratoriais de base sanguínea têm-se tornado cada vez mais importantes para esse diagnóstico, sendo utilizados diversos índices (escores), conforme a etiologia suspeitada: para pacientes com hepatite C, a estimativa laboratorial de fibrose pode ser feita com o índice APRI (*AST-to-Platelet Ratio Index*), que leva em conta o valor de AST e a contagem de plaquetas (BORSOI VIANA; TAKEI; COLLARILE YAMAGUTI; GUZ *et al.*, 2009); ou com o índice FIB-4, calculado com a idade do paciente, os valores de AST e ALT, e a contagem de plaquetas (VALLET-PICHARD; MALLET; NALPAS; VERKARRE *et al.*, 2007). Para pacientes com DHGNA ou EHNA, a estimativa pode ser feita com o escore NFS (*Nonalcoholic fatty liver disease Fibrosis Score*), que leva em conta a idade do paciente, o índice de massa corporal, a presença de diabetes ou resistência à insulina, os valores de AST e ALT, a contagem de plaquetas, e o nível sérico de albumina (ANGULO; HUI; MARCHESINI; BUGIANESI *et al.*, 2007); ou com o escore BARD (*Body mass index, AST/ALT Ratio, Diabetes*), que leva em conta o índice de massa corporal, os níveis de ALT e AST, e a presença de diabetes ou resistência à insulina (HARRISON; OLIVER; ARNOLD; GOGIA *et al.*, 2008). Cabe mencionar que, apesar de terem sido desenvolvidos para a detecção de fibrose em pacientes com doenças específicas, os índices são muitas vezes

utilizados por extensão em doenças hepáticas relacionadas, com valores variáveis de acurácia (CALÈS; OBERTI; MICHALAK; HUBERT-FOUCHARD *et al.*, 2005; CICHOŻ-LACH; CELIŃSKI; PROZOROW-KRÓL; SWATEK *et al.*, 2012; HAGSTRÖM; NASR; EKSTEDT; STÅL *et al.*, 2019). Em termos de métodos de imagem, podem ser realizadas técnicas morfológicas usuais – como a US e a RNM – que mostram contorno e densidade hepáticos; no entanto, esses métodos possuem baixa acurácia para a detecção da fibrose em todos os seus graus. As técnicas de mensuração de rigidez hepática, por outro lado, são exames não-invasivos de maior acurácia (que ainda assim é menor que a BH) que utilizam como base as técnicas de imagem morfológica, mas que avaliam parâmetros elastográficos. A modalidade de técnica de mensuração de rigidez hepática mais utilizada é a elastografia transitória hepática (ETH, conhecida comercialmente como "FibroScan"), a qual mede o coeficiente de propagação de uma onda mecânica aplicada sobre um tecido, com base no fato de que tecidos de diferentes composições possuem propriedades biomecânicas distintas. Com maiores graus de fibrose, a rigidez hepática é maior, e a transmissão da onda, medida em kPa, é proporcional (SANDRIN; FOURQUET; HASQUENOPH; YON *et al.*, 2003; WEBB; ZIMRAN; DINUR; SHIBOLET *et al.*, 2016).

As consequências da fibrose hepática são geralmente aparentes somente no seu grau mais avançado – isto é, a cirrose. Nesse estágio, há manifestações que ocorrem por três mecanismos: o primeiro diz respeito ao aumento da resistência vascular oferecida pelo fígado, devido à tortuosidade dos sinusoides e à deposição de matriz extracelular ao redor dos mesmos, o que faz com que a afluência venosa ao fígado através da veia porta seja submetida a pressões suprafisiológicas, estado denominado de "hipertensão portal". A afluência arterial do fígado não é significativamente afetada devido à pressão arterial sistólica ser já naturalmente superior à pressão sinusoidal, devido à bomba cardíaca. A hipertensão portal leva à retenção de sangue retrogradamente no sistema venoso esplâncnico que drena seu volume através do sistema portal: as veias esplênica (que drena sangue do baço, do estômago e do terço inferior do esôfago), mesentérica superior (que drena sangue de todo o intestino delgado, do pâncreas, do ceco e do cólon ascendente), e mesentérica inferior (que drena sangue dos cólons transverso, descendente e sigmoide, e do terço superior

do reto) (CHEDID; CHEDID, 2005); isso faz com que a pressão venosa em todos esses órgãos se eleve, levando à congestão crônica, ao extravasamento de plasma do espaço intravascular para o meio extracelular (levando ao edema tecidual e à ascite), e ao surgimento de varizes venosas no esôfago e no estômago com consequente hemorragia digestiva (uma das principais causas de morte em pacientes com cirrose) (TSOCHATZIS; BOSCH; BURROUGHS, 2014). O segundo mecanismo diz respeito à diminuição da função hepática em si, tanto graças à diminuição da massa de hepatócitos funcionantes, quanto à diminuição da área de contato entre sangue e hepatócitos pelo preenchimento fibrótico do espaço de Disse, o que faz que hepatócitos não consigam captar suficientemente substâncias circulantes para que sejam metabolizadas ou secretar adequadamente compostos para circulação sistêmica. Isso leva, entre outros efeitos, à diminuição nos níveis circulantes de albumina (a principal proteína plasmática, responsável por manter a pressão coloidal que impede o extravasamento espontâneo de líquido do espaço intravascular para o extravascular), o que contribui para a formação de edema e para o desenvolvimento de encefalopatia hepática (confusão mental, convulsões devido ao acúmulo de toxinas bacterianas provenientes do intestino, as quais o fígado cirrótico não consegue metabolizar) (TSOCHATZIS; BOSCH; BURROUGHS, 2014). O terceiro mecanismo diz respeito à perturbação ao fluxo biliar, devido à completa distorção dos canalículos e ductos biliares, o que leva à colestase (acúmulo de bile) e hiperbilirrubinemia por aumento da bilirrubina direta (conjugada). Clinicamente, esse processo se manifesta como icterícia (coloração amarelada de pele e mucosas), náuseas, prurido cutâneo e mal-estar (TAYLOR; WHEATLEY, 2018). Desse modo, a fibrose hepática é um processo de consequências multissistêmicas, com alta taxa de morbidade e letalidade. O único tratamento curativo para a cirrose é o transplante hepático (TSOCHATZIS; BOSCH; BURROUGHS, 2014).

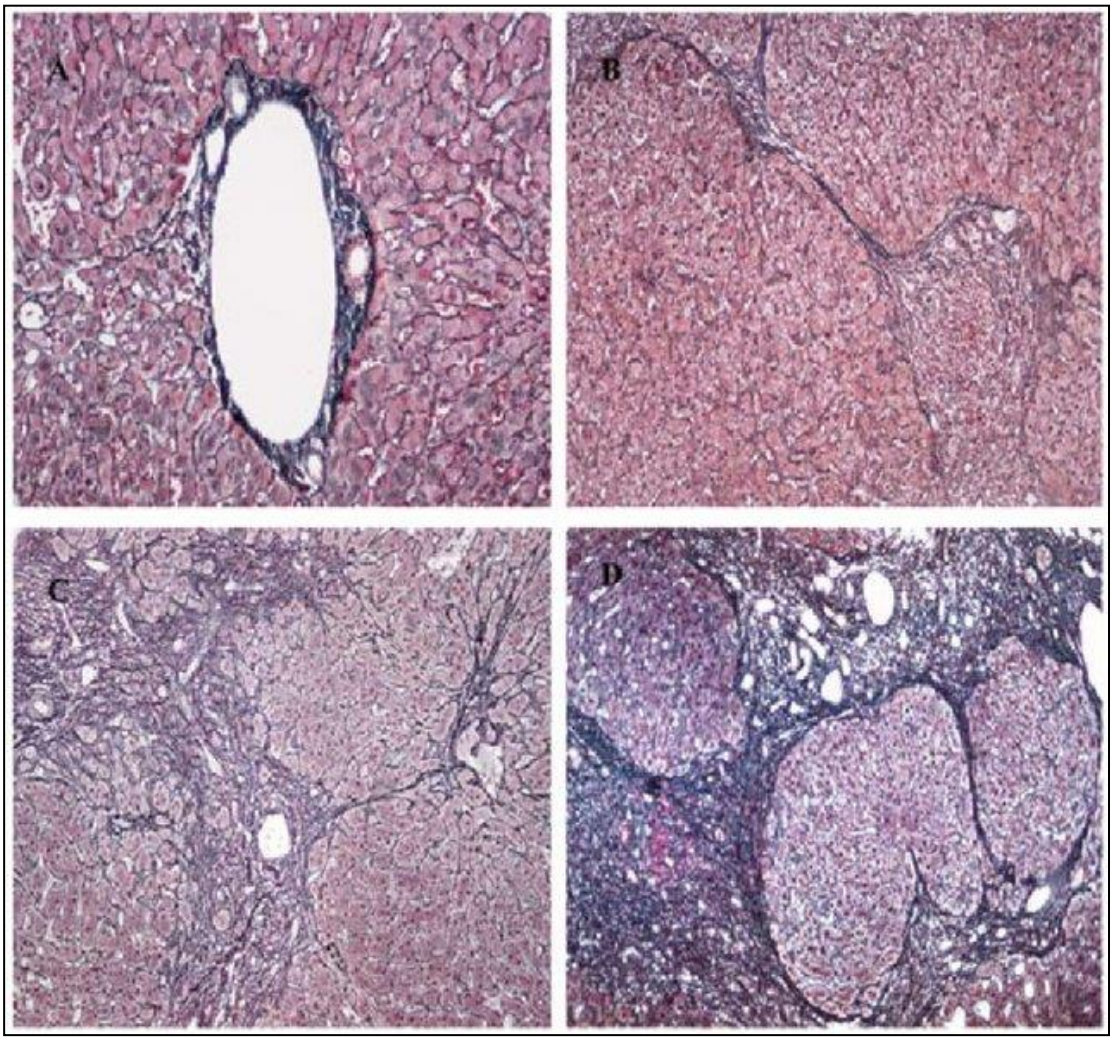


Figura 4. Sistema de classificação de graus de fibrose hepática METAVIR. Nessa figura estão representados os 4 graus de fibrose do sistema METAVIR. O painel A mostra F1, com fibrose ao redor da veia centrolobular. O painel B mostra F2, com presença de fona ponte fibrosa ligando um espaço-porta a uma veia centrolobular. O painel C mostra F3, com presença de pontes fibrosas de espessuras variáveis ligando diversos espaços-porta e veias centrolobulares, mas sem delimitação completa de nódulos hepatocelulares. O painel D mostra a presença de fibrose espessa delimitando completamente nódulos hepatocelulares, caracterizando cirrose. Figura retirada de (LI; QU; ZHENG; REN *et al.*, 2015).

2. Erros inatos do metabolismo

Os EIMs são um grupo de doenças individualmente raras causadas por defeitos no processamento de metabólitos, geralmente devido a uma atividade defeituosa de uma enzima. São doenças de origem genética, em sua maioria de padrão de herança autossômico recessivo, o que contribui para sua raridade (SAUDUBRAY; CAZORLA, 2016).

Os EIMs são comumente divididos em 3 grupos, conforme o processo fisiopatológico subjacente: doenças do grupo 1 são causadas por acúmulo de metabólitos tóxicos originários a montante do bloqueio da rota metabólica. O grupo 1 é caracterizado, em linhas gerais, pela ausência de manifestações durante o período embrionário-fetal e no período neonatal imediato, com um intervalo assintomático até o início da doença (que pode ser agudo ou crônico). São exemplos de doenças do grupo 1: defeitos do metabolismo de aminoácidos; acidúrias orgânicas; defeitos do ciclo da ureia e intoxicações metálicas. As doenças do grupo 2 são causadas por uma falha no metabolismo energético, gerando déficit de energia para o funcionamento fisiológico, especialmente em tecidos com alta demanda energética, como o fígado, o cérebro ou os músculos estriados. Diferentemente do grupo 1, as doenças do grupo 2 podem ter manifestações durante a vida intrauterina ou durante o período neonatal imediato, e levam a disfunções principalmente nos três tecidos acima mencionados. São exemplos de doenças do grupo 2: acidemias láticas congênitas; distúrbios do ciclo do ácido tricarboxílico; distúrbios da cadeia respiratória mitocondrial (SAUDUBRAY; CAZORLA, 2016).

As doenças do grupo 3 são os EIMs das moléculas complexas. São doenças que causam disfunção de diversas organelas – como lisossomos, retículo endoplasmático (RE) ou complexo de Golgi (CG) – e que são causadas por distúrbios da síntese, remodelagem, reciclagem, tráfego ou catabolismo de moléculas complexas como esfingolipídios (ou outros lipídios complexos), moléculas sinalizadoras, glicanos (e suas moléculas conjugadas, como as glicoproteínas). Todas as doenças lisossômicas (DLs) e os distúrbios congênitos da glicosilação (DCGs) são considerados doenças do grupo 3 (SAUDUBRAY; CAZORLA, 2016).

3. Doença de Gaucher

3.1. Via da beta-glicosidase ácida

A doença de Gaucher (DG) é um EIM do grupo 3 da classe das DDLs. Dentro destas, pode ainda ser classificada como uma esfingolipidose. Como todos os EIMs, é uma doença rara: sua prevalência é em torno de 1:40.000 a 1:60.000 na população geral (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). É causada pela diminuição da atividade enzimática da beta-glicosidase ácida (também conhecida como glicosilceramidase ou glicocerebrosidase; GCase) causada por variantes patogênicas em ambos os alelos do gene que a codifica, o *GBA*. A GCase é uma enzima que atua no lúmen lissossômico hidrolisando a ligação beta-glicídica entre a glicose e a ceramida do lipídio complexo glicosilceramida (ou glicocerebrosídeo; GlcCer) (figura 5) (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), o qual é proveniente da degradação de gangliosídeos de membranas celulares. A GCase necessita do co-ativador saposina C, proveniente da porção C do gene *PSAP*, para exercer sua atividade enzimática. Um substrato secundário da GCase é a glicosilesfingosina (GlcSph) (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), que é transformada em glicose e esfingosina.

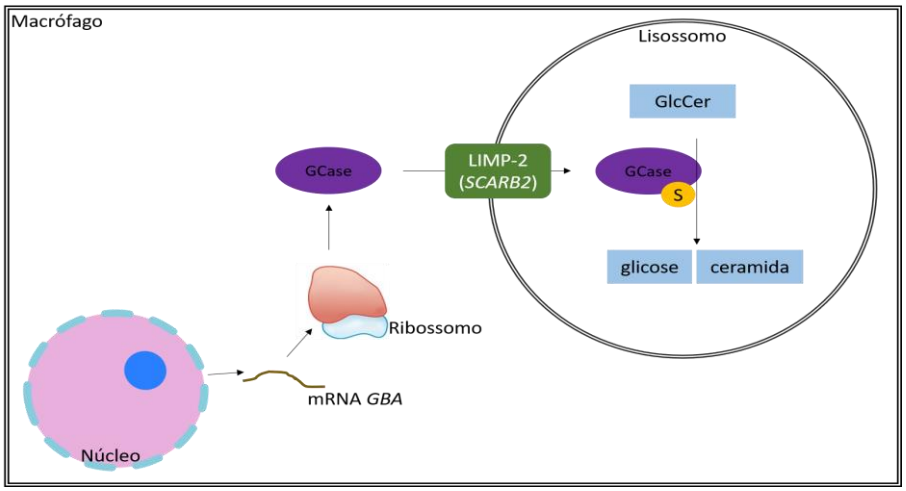


Figura 5. A via da GCase. Essa figura esquematiza simplificadamente a produção da GCase a partir do gene *GBA*, sua entrada no lisossomo através do transportador LIMP-2 (proteína integral da membrana lisossômica 2, na sigla em inglês), codificado pelo gene *SCARB2*, e hidrólise da GlcCer em glicose e ceramida com a co-ativação pela saposina C (círculo amarelo com "S"), codificada pela porção C do gene *PSAP*.

A via de degradação dos gangliosídeos é especialmente ativa em macrófagos devido a sua função de reciclagem celular que leva a um grande fluxo de material proveniente de membranas por seus lisossomos (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017; TAMÒ; HOCHULI; BEUSCHLEIN; NOWAK, 2018). Quando a atividade enzimática da GCase se encontra criticamente diminuída, há acúmulo lisossômico de GlcCer e GlcSph. Esse acúmulo de lipídios complexos torna os lisossomos ingurgitados e disfuncionais (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), levando à transformação de macrófagos em "células de Gaucher" (Figura 6) (as quais podem ser típicas – com 20-100 µm de diâmetro, núcleo excêntrico e citoplasma "enrugado" e basofílico, descrito como semelhante ao papel amassado, criado pelo acúmulo lamelar de GlcCer –, ou atípicas – geralmente maiores que as células típicas, com multinucleação e acúmulo vacuolar de GlcCer (IVANOVA; LIMGALA; CHANGSILA; KAMATH *et al.*, 2016)) (LEE, 1968) e aos efeitos patofisiológicos discutidos a seguir.

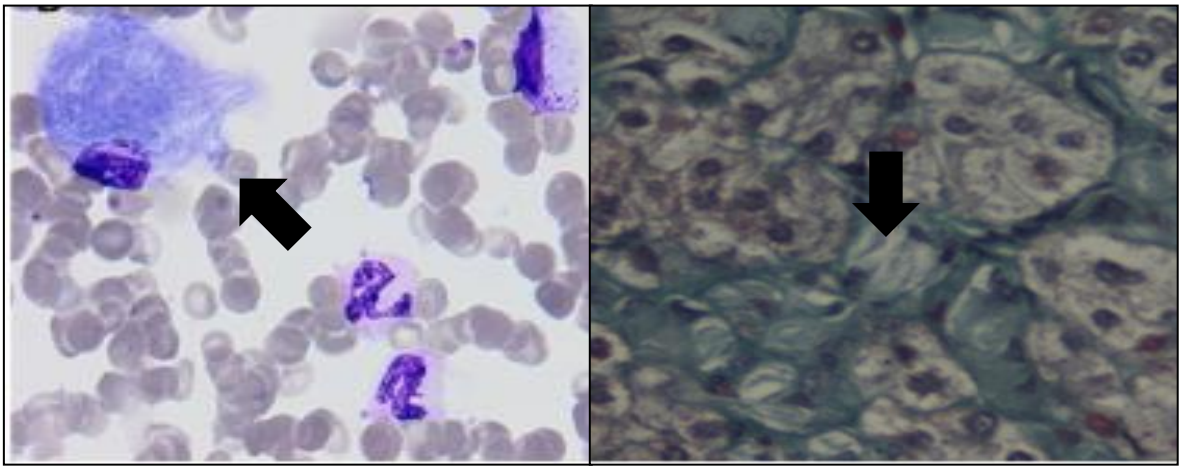


Figura 6. Células de Gaucher. No painel da esquerda, a seta preta aponta uma célula de Gaucher em um aspirado de medula óssea. No painel da direita, a seta preta aponta uma célula de Gaucher envolta em fibrose em uma BH. Pode-se notar a aparência ingurgitada, com citoplasma estriado e núcleo deslocado para a periferia celular em ambos os casos. Figuras adaptadas de (VUJOSEVIC; MEDENICA; VUJICIC; DAPCEVIC *et al.*, 2019) e (ESSABAR; MESKINI; LAMALMI; ETTAIR *et al.*, 2015)

3.2. Manifestações clínicas

A DG é uma doença sistêmica com uma miríade de manifestações clínicas possíveis. É classicamente dividida primariamente quanto à presença e à cronicidade de seu comprometimento neurológico: pacientes com DG tipo 1 (DG1) são descritos como possuindo fenótipo exclusivamente não-neurológico ("tipo não-neuronopático"), sendo aproximadamente 90% do número total de pacientes com DG no Ocidente (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Pacientes com DG tipo 2 (DG2) têm o fenótipo mais grave dentre os diferentes tipos, com manifestações neurológicas graves (encefalopatia, mioclonias e epilepsia) e óbito geralmente até o segundo ano de vida. Pacientes com DG tipo 3 (DG3) têm um fenótipo intermediário entre a DG1 e a DG2, com manifestações neurológicas insidiosas (epilepsia, atraso do desenvolvimento neuropsicomotor e anormalidades de movimentação ocular) e manifestações extra-neurológicas semelhantes à DG1. Em conjunto, a DG2 e a DG3 são chamadas de "tipos neuronopáticos". Hoje, no entanto, sabe-se que não há demarcações claras entre os diferentes tipos de DG, sendo a descrição mais aceita a de um espectro de manifestações, conforme mostrado na Figura 7. As manifestações extra-neurológicas da DG podem ser, por sua vez, estudadas conforme o sistema que afetam, sendo comumente classificadas em esqueléticas, hematológicas e viscerais (NGUYEN; STIRNEMANN; BELMATOUG, 2019; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017).

As manifestações esqueléticas da DG podem ser agudas ou crônicas. Em alguns pacientes, crises súbitas de dor óssea podem ocorrer, especialmente nos ossos da pelve ou dos membros inferiores (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), causadas pela oclusão (de etiologia incerta) do suprimento sanguíneo arterial para o tecido ósseo, levando à isquemia. Quando ocorrem nos ossos chatos ou nas diáfises ou metáfises dos ossos longos, esses eventos são chamados de "infartos ósseos"; quando ocorrem nas epífises dos ossos longos (como a cabeça do fêmur), são chamados de "osteonecrose avascular" (DRELICHMAN; FERNÁNDEZ ESCOBAR; BASACK; AVERSA *et al.*, 2016; KHAN; HANGARTNER; WEINREB; TAYLOR *et al.*, 2012;

STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Apesar dos diferentes nomes, a etiologia dos eventos é a mesma, isto é, a vaso-oclusão arterial súbita. Entre as manifestações esqueléticas crônicas da DG, encontram-se: deformações ósseas causadas pela infiltração da medula óssea por células de Gaucher, especialmente no fêmur – local onde levam à "deformidade em frasco de Erlenmeyer" (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017); diminuição da densidade mineral óssea, levando à osteopenia ou à osteoporose (NGUYEN; STIRNEMANN; BELMATOUG, 2019; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017); e infiltração difusa da medula óssea por células de Gaucher, levando à dor óssea crônica e a manifestações hematológicas (discutidas a seguir) (NGUYEN; STIRNEMANN; BELMATOUG, 2019; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017).

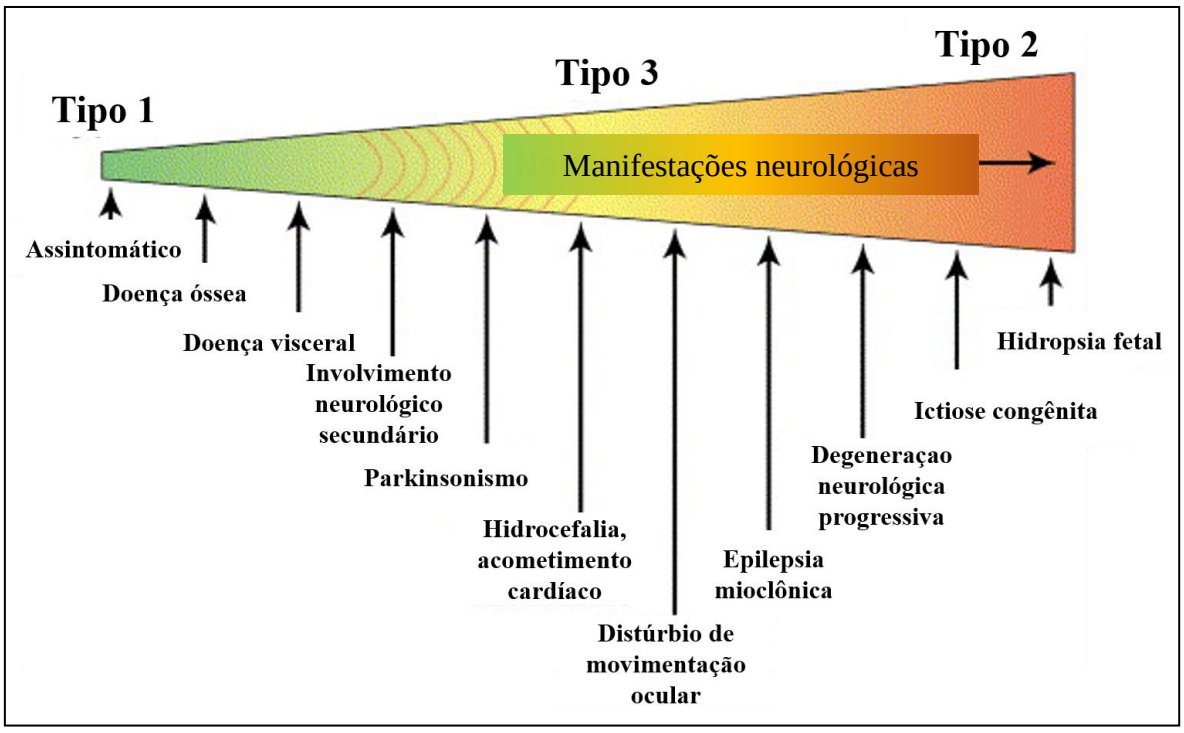


Figura 7. Espectro de manifestações da DG. Esquema mostrando a distribuição contínua das principais manifestações da DG, enfocando as manifestações neurológicas, conforme proposto por Sidransky. Modificado de (SIDRANSKY, 2004).

As manifestações hematológicas da DG são causadas pela secreção de moléculas inflamatórias pelas células de Gaucher (ver seção 3.2) (LINARI; CASTAMAN, 2016; NGUYEN; STIRNEMANN; BELMATOUG, 2019) e pela substituição da medula óssea por células de Gaucher infiltradoras (KLIMKOWSKA; MACHACZKA; PALMBLAD, 2018; NGUYEN; STIRNEMANN; BELMATOUG, 2019). São comuns a anemia e a plaquetopenia, que podem levar à fadiga crônica e a episódios de hemorragia patológica, respectivamente. A anemia está presente em 20% a 50% dos casos ao diagnóstico (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), e a plaquetopenia em 90% (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Tanto em paciente não-tratados quanto naqueles em tratamento, é comum o aumento no plasma das γ-globulinas, classe de proteínas dentre as quais se encontram as imunoglobulinas (ou anticorpos) (NGUYEN; STIRNEMANN; LAUTREDOUX; CADOR *et al.*, 2020); esse aumento pode ser encontrado na forma de uma hipergamaglobulinemia policlonal – quadro mais comum e mais benigno – ou monoclonal – menos comum, e condizente com um estado pré-patológico da neoplasia hematológica mieloma múltiplo, o qual também pode ser uma consequência da DG (NGUYEN; STIRNEMANN; LAUTREDOUX; CADOR *et al.*, 2020). Uma das prováveis razões deste estado de hipergamaglobulinemia é a inflamação crônica, que causa ativação constante de plasmócitos, os quais em resposta secretam imunoglobulinas (NGUYEN; STIRNEMANN; LAUTREDOUX; CADOR *et al.*, 2020); a consequência clínica da hipergamaglobulinemia na DG ainda não foi determinada. Além do mieloma múltiplo, discute-se o risco aumentado na DG de outras neoplasias hematológicas, especialmente a síndrome mielodisplásica e os linfomas não-Hodgkin (NGUYEN; STIRNEMANN; LAUTREDOUX; CADOR *et al.*, 2020; RUCHLEMER; MITTELMAN; ZIMRAN, 2020).

As manifestações viscerais da DG dizem respeito principalmente ao baço e ao fígado, sendo que este último será tratado em separado (ver seção 3.5). Há esplenomegalia em aproximadamente 90% dos pacientes ao diagnóstico (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017); além da compressão mecânica sobre outros órgãos e sobre a parede abdominal, que leva ao desconforto, a esplenomegalia pode levar ao

"sequestro esplênico" de elementos figurados do sangue, especialmente plaquetas, contribuindo para a plaquetopenia (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Em casos raros, a esplenomegalia leva à isquemia de regiões do parênquima esplênico e ao decorrente infarto esplênico, caracterizado por dor súbita e incapacitante no quadrante superior esquerdo do abdômen (VANIER; CAILLAUD; LEVADE, 2016); ou à ruptura esplênica, caracterizada por extensa hemorragia intra-abdominal com alta taxa de letalidade (STONE; GINNS; KRASNEWICH; SIDRANSKY, 2000). Outros órgãos viscerais com acometimento não tão frequente em pacientes submetidos a tratamentos, mas presentes na maioria dos pacientes não-tratados (GAWAD TANTAWY; MONEAM ADLY; MADKOUR; SALAH EL-DIN, 2020), são: os pulmões, com infiltração intersticial por células de Gaucher, levando à aparência radiológica de consolidação em vidro fosco (GAWAD TANTAWY; MONEAM ADLY; MADKOUR; SALAH EL-DIN, 2020) e subsequente hipertensão pulmonar, bronquiectasias e fibrose intersticial (GAWAD TANTAWY; MONEAM ADLY; MADKOUR; SALAH EL-DIN, 2020; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017); e o coração, com doença cardíaca restritiva ou valvar (ROGHI; POGGIALI; CASSINERIO; PEDROTTI *et al.*, 2017; SOLANICH; CLAVER; CARRERAS; GIRALDO *et al.*, 2012).

3.3. Inflamação crônica

Sabe-se, a partir de modelos experimentais, que a DG é associada à diferenciação pró-inflamatória de macrófagos com secreção de citocinas, ativação de inflamassomos (AFLAKI; MOAVEN; BORGER; LOPEZ *et al.*, 2016) e do sistema complemento (PANDEY; BURROW; RANI; MARTIN *et al.*, 2017; PANDEY; GRABOWSKI; KÖHL, 2018), o que é apenas atenuado pelos tratamentos existentes atualmente (PANDEY; BURROW; RANI; MARTIN *et al.*, 2017). Ao mesmo tempo em que essa ativação constante de rotas inflamatórias retroalimenta o acúmulo de GlcCer em macrófagos e outros tipos celulares (PANDEY; BURROW; RANI; MARTIN *et al.*, 2017), também leva a efeitos sistêmicos associados à inflamação crônica, como a ativação imprópria de células T e células dendríticas, o infiltrado inflamatório em tecidos à distância, e o consequente dano tecidual a

órgãos-alvos, dentre os quais inclui-se o fígado (PANDEY; BURROW; RANI; MARTIN *et al.*, 2017). Existem também evidências que apontam para a formação de autoanticorpos contra a GlcCer acumulada, que extravasa de lisossomos para o citoplasma e para o meio extracelular (ELLEDER, 2006; PANDEY; GRABOWSKI; KÖHL, 2018). Em conjunto com outros mecanismos fisiopatológicos descritos anteriormente e a seguir, a inflamação crônica provavelmente desempenha um papel no desenvolvimento do fenótipo hepático da DG.

3.4. Metabolismo do ferro na doença de Gaucher

O distúrbio no metabolismo do ferro no contexto da DG é conhecido há décadas: em estudos realizados na década de 1960, depósitos de ferro ligado à ferritina foram identificados em células de Gaucher (LORBER, 1960; LORBER; NEMES, 1967), e foi demonstrado que os estoques corporais globais de ferro são elevados em pacientes com essa doença (LEE; BALCERZAK; WESTERMAN, 1967). No entanto, foi somente nos anos mais recentes que a fisiopatologia desse depósito de ferro passou a ser investigada em mais detalhes. Em 2010, Stein e colaboradores (STEIN; YU; JAIN; MISTRY, 2010) descreveram uma coorte de pacientes com DG1 na qual, mesmo na vigência de tratamento com terapia de reposição enzimática (TRE) (ver seção 3.6: " Tratamento da doença de Gaucher"), os níveis séricos de ferritina foram em média 3,7 vezes acima do limite superior de normalidade. Nesse trabalho, foi também mostrado que pacientes que sofreram esplenectomia – os quais tendem a ter uma maior gravidade na sua DG – tiveram valores mais elevados de ferritina, com significância estatística. Na investigação de 3 pacientes com acúmulo de ferro suspeitado devido ao aumento de ferritina, saturação de Tf, e medição de ferro tecidual por RNM, BHs mostraram forte acúmulo de ferro no citoplasma de hepatócitos, células de Kupffer morfologicamente normais e células de Gaucher. Em 2013, Pilar e colaboradores (MEDRANO-ENGAY; IRUN; GERVAS-ARRUGA; ANDRADE-CAMPOS *et al.*, 2014) mostraram que o uso de agentes farmacológicos quelantes de ferro (que diminuem a quantidade de ferro biodisponível) em pacientes com DG levou a uma diminuição significativa dos níveis de ferritina, hepcidina, e ferro tecidual hepático, o que sugere que a ferritina seja dependente não somente do estado de inflamação crônica, mas

também da concentração e biodisponibilidade sistêmica e hepática de ferro; no entanto, não foi possível nesse estudo afastar um efeito modificador da biodisponibilidade de ferro para com o estado inflamatório crônico, o que limita as conclusões que daqui se possam tomar. Bohte e colaboradores (BOHTE; VAN DUSSEN; AKKERMAN; NEDERVEEN *et al.*, 2013) mostraram que pacientes que sofreram esplenectomia tendem a ter maior acúmulo de ferro que pacientes com baço íntegro, e que não há correlação entre a presença de fibrose hepática e o depósito de ferro em um pequeno estudo transversal.

Em 2017, Regenboog e colaboradores mostraram que, mesmo em pacientes com DG que realizaram tratamento regular por muitos anos, há deposição de ferro no fígado e na medula óssea (REGENBOOG; BOHTE; AKKERMAN; STOKER *et al.*, 2017). No mesmo trabalho, foi mostrado que há correlação entre deposição tecidual de ferro medida por RNM e os níveis de ferritina sérica, mas não com os níveis de atividade de quitotriosidase. Isso indica que a ferritina elevada durante o tratamento observada em grande parte dos pacientes pode refletir não a atividade residual da doença macrofágica – da qual a quitotriosidase é um marcador sensível – mas sim a alteração secundária no metabolismo do ferro que se mantém presente mesmo após a terapia. Uma explicação levantada para essas observações é a de que a inflamação crônica presente nos pacientes com DG leve, antes do início do tratamento, ao acúmulo e represamento de ferro dentro de macrófagos, devido à ativação de rotas envolvendo a hepcidina (ver seção 1.2); com o início do tratamento, a diminuição da inflamação levaria à diminuição da hepcidina, com consequente liberação dos estoques patológicos de ferro, que seriam então absorvidos por diferentes tecidos, especialmente aqueles com alto metabolismo de ferro como o fígado e a medula óssea. Essa hipótese foi investigada por Lefèbvre e colaboradores (LEFEBVRE; REIHANI; DAHER; DE VILLEMEUR *et al.*, 2018), que mostraram que, apesar da alta prevalência de hiperferritinemia, a saturação de Tf e os níveis séricos de hepcidina, além dos marcadores de inflamação sistêmica proteína C-reativa e interleucina 6, têm valores normais na maior parte dos pacientes, o que sugere que o acúmulo de ferro não é resultado da inflamação sistêmica. No mesmo trabalho, no entanto, foi-se demonstrado que a inibição farmacológica da GCase em macrófagos cultivados levou à inibição pós-traducional da FPN na membrana

plasmática macrofágica através da estimulação local da hepcidina, com consequente aumento dos níveis intracelulares de ferritina e ferro. Em um seguimento de pacientes após a introdução do tratamento com TRE, houve aumento nos níveis de saturação de Tf e diminuição de níveis de ferritina e do receptor solúvel de Tf, indicando maior disponibilidade sistêmica de ferro. Esses achados concordam com a hipótese anterior de Regenboog e colegas (REGENBOOG; BOHTE; AKKERMAN; STOKER *et al.*, 2017) de que há sequestro intracelular de ferro na DG, com liberação sistêmica após o início do tratamento, apesar de diferirem quanto à etiologia desse processo; e com as observações de Stein e colegas (STEIN; YU; JAIN; MISTRY, 2010) de que há acúmulo intracelular de ferro nestes pacientes, em hepatócitos (não estudados por Lefèbvre) e macrófagos.

3.5. Fígado na doença de Gaucher

As primeiras tentativas para a descrição sistemática de um fenótipo hepático da DG foram feitas por James e colaboradores em 1981 (JAMES; STROMEYER; CHANG; BARRANGER, 1981) em uma coorte de 25 pacientes com representação dos 3 tipos de DG. Nesse estudo, único na literatura devido a ter sido realizado antes do descobrimento da TRE e de que a esplenectomia agrava as manifestações da DG, todos os pacientes exceto um apresentaram hepatomegalia ao exame físico abdominal; 17 pacientes apresentaram aumento de pelo menos um marcador de dano hepatocelular (ALT ou AST), e 11 do marcador de dano canalicular e colangiocelular FA; 3 pacientes apresentaram complicações clínicas de cirrose hepática (sangramento de varizes esofágicas, icterícia e ascite); e, à BH, presença ubíqua de células de Gaucher no parênquima hepático – em alguns casos com compressão hepatocelular e substituição de hepatócitos por essas células – com níveis variados de fibrose (variando de fibrose no entorno das células de Gaucher a cirrose com septos espessos, e casos de gravidade intermediária) e presença de depósitos de ferro no citoplasma das células de Gaucher. A partir desse estudo, determinou-se que o fenótipo hepático da DG é resultante da infiltração do parênquima hepático pelas células de Gaucher, sendo hepatomegalia e fibrose hepática os principais achados.

Na década seguinte, dois estudos com RNM abdominal em coortes de pacientes com DG se seguiram: Hill e colaboradores (HILL; DAMASKA; LING; PATTERSON *et al.*, 1992) analisaram 46 pacientes com DG1 não-tratados, encontrando, além de hepatomegalia ubíqua, áreas de fibrose estrelada (com envolvimento de forma irregular de diversos segmentos hepáticos) ou segmental (fibrose focal em um segmento hepático) em 9 pacientes – 3 desses pacientes foram submetidos a BHs para avaliação das áreas fibróticas, com presença de células de Gaucher infiltrando espaços-porta e sinusoides e variados graus de fibrose, incluindo um paciente com cirrose. Os parâmetros técnicos da RNM desse estudo, no entanto, estão muito aquém dos padrões mínimos atuais, devendo-se considerar que a sensibilidade para lesões pequenas ou alterações mais sutis do parênquima hepático nesse estudo seja relativamente pequena. Por sua vez, Terk e colaboradores (TERK; ESPLIN; LEE; MAGRE *et al.*, 1995) realizaram, em uma coorte de 64 pacientes com DG1, o primeiro estudo de imagem abdominal após início de tratamento com TRE, utilizando RNM com parâmetros técnicos já mais compatíveis com os padrões atuais. Nesse estudo foram encontrados, além da hepatomegalia, nódulos hepáticos em 5 pacientes, sem esclarecimento de sua natureza por biópsia. Não há descrição de áreas fibróticas hepáticas nesse estudo, em contraste com o estudo de Hill *et al*, o que pode ser resultado de diferenças de composição genética da população estudada, efeito do tratamento, ou mesmo um viés de reportagem ou de metodologia.

No começo dos anos 2000, a TRE já estava estabelecida como tratamento padrão para a DG (ver seção 3.6) havia alguns anos, o que permitiu o estudo do fenótipo hepático de pacientes tratados a médio e longo prazo. Em 2000, Lachmann e colaboradores (LACHMANN; WIGHT; LOMAS; FISHER *et al.*, 2000) descreveram quatro pacientes com DG1 que desenvolveram cirrose hepática com hipertensão portal após esplenectomia, ajudando a estabelecer o elo entre a retirada cirúrgica do baço e a piora do acometimento hepático; 2 pacientes receberam TRE, com melhora parcial das complicações da cirrose após a implementação da terapia. À histologia hepática de todos os pacientes, foram encontradas grandes áreas de fibrose central acelular, correspondentes às áreas de fibrose estrelada identificadas por Hill em 1992 (HILL; DAMASKA; LING; PATTERSON *et al.*, 1992). Um

dos pacientes que não recebera TRE tinha sinais de colestase em canalículos biliares; o número de células de Gaucher no parênquima hepático dos pacientes que haviam recebido TRE era visivelmente menor que nos outros 2 pacientes, significando uma possibilidade de que a TRE revertesse a infiltração de células de Gaucher – em um dos pacientes, no entanto, mesmo sem a presença de células de Gaucher no parênquima, houve manutenção dos níveis elevados de marcadores de dano hepatocelular, sugerindo que não seria somente a presença dos macrófagos com acúmulo de GlcCer a causar dano hepático.

Com o avanço da tecnologia investigativa e com o controle de manifestações mais conspícuas e urgentes da DG (hepatoesplenomegalia, citopenias e crises ósseas) pela TRE, passou a ser possível investigar de maneira mais aprofundada o fenótipo hepático desses pacientes. A seguir, são apresentadas as manifestações de diferentes padrões fisiopatológicos hepáticos na DG. Para as manifestações de siderose, ver seção 3.4, "Metabolismo do ferro na doença de Gaucher".

3.5.1. Esteatose

Apesar de não descrita como uma manifestação da DG na era pré-TRE, estudos recentes em pacientes tratados mostraram a esteatose como uma complicação ou uma comorbidade emergente na DG. A esteatose hepática na DG é conhecida desde 2010, quando Hadas-Halpern e colaboradores (HADAS-HALPERN; DEEB; ABRAHAMOV; ZIMRAN *et al.*, 2010) descreveram sua presença, detectada por US abdominal, em 1,6% dos pacientes de uma coorte de 500 participantes, com predomínio em pacientes que estivessem recebendo TRE. Mais recentemente, Lipinski e colaboradores (LIPIŃSKI; SZYMAŃSKA-ROŻEK; SOCHA; TYLKI-SZYMAŃSKA, 2019) descreveram a presença de esteatose, detectada por EHPAC, em 23% dos pacientes de uma coorte com DG1 e 19% dos pacientes de uma coorte com DG3, com predomínio de esteatose em pacientes em uso de TRE. Uma possível explicação para a discrepância nas prevalências de esteatose entre os dois estudos é a diferença no tempo mediano de tratamento entre as duas coortes: enquanto que no último estudo o tempo mediano em tratamento com TRE foi de 13 anos, este foi o valor máximo de tempo em tratamento do primeiro estudo (medidas de tendência central não são relatadas

nesse estudo, não sendo possível a comparação direta). Uma hipótese levantada na literatura para a emergência de esteatose em coortes de pacientes tratados é de que a esteatose hepática seja um efeito adverso da administração a longo prazo de TRE, devido à indução de ganho de massa corporal e de síndrome metabólica (ADAR; ILAN; ELSTEIN; ZIMRAN, 2016; LANGEVELD; DE FOST; AERTS; SAUERWEIN *et al.*, 2008), os quais são os principais fatores para o desenvolvimento de DHGNA (ver seção 1.3.2); essa hipótese é corroborada pelo achado de que há uma correlação positiva, ainda que fraca a moderada, dos valores de EHPAC com o índice de massa corporal (IMC), uma medida de obesidade (LIPIŃSKI; SZYMAŃSKA-ROŻEK; SOCHA; TYLKI-SZYMAŃSKA, 2019).

3.5.2. Inflamação

Apesar do reconhecido envolvimento da inflamação crônica na patogênese da DG (ver seção 3.3), o dano hepático inflamatório na DG ainda não foi descrito em detalhe, e a presença de condições hepáticas inflamatórias secundárias como a esteato-hepatite não foi investigada por nenhum grupo de pesquisa. Sabe-se que inflamação crônica de baixo grau em espaços-porta pode ocorrer na DG, com um caso de paciente com DG1 e cirrose descrito (LOLLERT; HOFFMANN; LACHE; KÖNIG *et al.*, 2020). Hipoteticamente, a inflamação crônica hepática teria relação com a ativação crônica de macrófagos e contribuiria para o desenvolvimento de fibrose na DG (NASCIMBENI; DIONISI VICI; VESPASIANI GENTILUCCI; ANGELICO *et al.*, 2020) – no entanto, mais dados são necessários antes de que seja possível afirmar que tal relação existe, visto que não há estudos na literatura que comprovem essa causalidade.

3.5.3. Fibrose

Com o advento da era da TRE, casos de fibrose estrelada ou segmentar passaram a não ser mais observados em pacientes com DG, tendo sido o último caso descrito em 2002 (PEREL; BIOULAC-SAGE; CHATEIL; TRILLAUD *et al.*, 2002). No entanto, passaram-se a se observar processos crônicos de fibrose hepática progressiva em pacientes em tratamento, em muitos casos levando à cirrose (HADAS-HALPERN; DEEB;

ABRAHAMOV; ZIMRAN *et al.*, 2010), que pode ocorrer sem outros fatores de risco além da própria DG (REGENBOOG; VAN DUSSEN; VERHEIJ; WEINREB *et al.*, 2018). Em 2013, Bohte e colaboradores (BOHTE; VAN DUSSEN; AKKERMAN; NEDERVEEN *et al.*, 2013) mostraram que muitos pacientes com DG1 têm valores de rigidez hepática, uma medida indireta de fibrose realizada por ETH (ver seção 2.3.6), mais elevados que na população controle, especialmente em pacientes com esplenectomia prévia, e que há correlação da fibrose com biomarcadores de gravidade e atividade da doença (quitotriosidase e enzima conversora de angiotensina), mas não com parâmetros de acúmulo de ferro (ferritina, saturação de Tf, ou concentração hepática de ferro). Subsequentemente, foi-se demonstrado que os valores médios de rigidez hepática são significativamente maiores em geral nos pacientes com DG1 que na população saudável, situando a média de grau de fibrose nesses pacientes em F2, mas ainda abaixo dos valores encontrados em pacientes cirróticos (WEBB; ZIMRAN; DINUR; SHIBOLET *et al.*, 2016). Interessantemente, nesse estudo não houve correlação entre esplenectomia prévia e os valores de rigidez hepática.

Posteriormente, novos estudos aprofundaram o conhecimento da fibrose não-massiva durante a TRE em pacientes com DG1: Serai e colaboradores (SERAI; NAIDU; ANDREW BURROW; PRADA *et al.*, 2018) encontraram uma correlação positiva fraca, mas estatisticamente significativa, entre os valores de rigidez hepática e os valores de escores de gravidade global da DG, assim como entre a rigidez hepática e os volumes do fígado e do baço (os quais, por sua vez, são parte integrante dos escores de gravidade), sugerindo que a fibrose hepática seja devido à atividade de doença ainda presente em pacientes em tratamento, e não devido a fatores externos como TRE, apesar de mais estudos serem necessários para firmar-se essa hipótese. A correlação entre a gravidade geral da doença, medida por biomarcadores e escores clínicos de gravidade, assim como a esplenectomia prévia, com a rigidez hepática foi confirmada por Nascimbeni e colaboradores (NASCIMBENI; CASSINERIO; DALLA SALDA; MOTTA *et al.*, 2018); a correlação entre volumes de fígado e baço e a rigidez hepática, no entanto, não foi encontrada nesse estudo. Lollert e colaboradores (LOLLERT; HOFFMANN; LACHE; KÖNIG *et al.*, 2020), usando um método elastográfico de maior sensibilidade, mostraram que 70% dos pacientes

com DG1 em tratamento (com uma mediana de 13 anos de tratamento à testagem) têm um grau significativo de fibrose hepática (*i.e.*, igual ou superior a F2), novamente existindo uma correlação positiva entre o valor de escores de gravidade global da DG e a rigidez hepática, e contribuindo com o achado de uma correlação positiva com fatores constituintes da síndrome metabólica (ver seção 1.3.2, "Esteatose") – no entanto, não foi encontrada correlação da fibrose hepática com esplenectomia prévia, o que faz com que a afirmação de que a fibrose hepática não-massiva esteja associada à esplenectomia seja ainda uma questão em aberto. Por fim, Lipinski e colaboradores (LIPIŃSKI; SZYMAŃSKA-ROŻEK; SOCHA; TYLKI-SZYMAŃSKA, 2019), usando ETH, mostraram que a fibrose hepática é proporcional à idade dos pacientes, apesar de não ser proporcional à idade de início do tratamento, o que corrobora a ideia de que a fibrose durante a TRE é um processo crônico, ao contrário da fibrose massiva encontrada em pacientes de idade jovem antes da descoberta da TRE.

3.5.4. Dano biliar e canalicular

A associação da DG com dano biliar iniciou em 2002 (ROSENBAUM; SIDRANSKY, 2002), com a evidência de que a prevalência de colelitíase é 16 a 21% em uma amostra com média de idade de 39 anos, enquanto que uma população controle de idade similar, em um *quasi*-experimento, teve uma prevalência menor que 10,5%; e que pacientes com esplenectomia prévia teriam maior prevalência de colelitíase que pacientes com baço íntegro. Nesse mesmo estudo, foi levantada a hipótese de que GlcCer secretada na bile pelos hepatócitos – processo já demonstrado previamente (PENTCHEV; GAL; WONG; MORRONE *et al.*, 1981) – poderia contribuir para a litogenicidade (propensão à formação de cálculos) da bile nos pacientes com DG. Posteriormente, em um estudo realizado por Harosh-Katz e colaboradores (BEN HAROSH-KATZ; PATLAS; HADAS-HALPERN; ZIMRAN *et al.*, 2004) com uma grande coorte israelense de pacientes com DG, uma prevalência igualmente elevada de colelitíase (19,6% em pacientes do sexo masculino e 30,0% em pacientes do sexo feminino) foi encontrada, com confirmação estatística da correlação com esplenectomia prévia e descartando um efeito modificador de um

polimorfismo do gene *UDPGT1*, envolvido no metabolismo biliar e causador da síndrome de Gilbert.

Em 2010, Taddei e colaboradores (TADDEI; DZIURA; CHEN; YANG *et al.*, 2010) realizaram um estudo para avaliação da prevalência e dos fatores predisponentes à colelitíase em 417 pacientes com DG1. Uma prevalência de colelitíase de 32% foi encontrada, maior em mulheres e com aumento com a idade (sendo que 42,5% dos pacientes com idade superior a 20 anos apresentaram evidência de cálculo biliar) e correlação com esplenectomia prévia, síndrome metabólica, e gravidade global da DG (o que pode ser considerado como um confundidor, haja vista que a esplenectomia e a síndrome metabólica podem agravar os sintomas da DG). Foi também analisada a composição da bile de 6 pacientes, com coleta durante a colecistectomia (cirurgia para remoção da VB): foi encontrada uma alta concentração de GlcCer em apenas um dos pacientes, que à análise mais detalhista se mostrou ser o paciente com maior gravidade da DG, e composição normal de lipídeos complexos na bile dos outros pacientes analisados – no entanto, não houve análise de GlcSph na bile de nenhum dos pacientes, o que limita as conclusões que daí podem ser tomadas sobre a composição da bile na DG. Os achados de fatores de risco para o desenvolvimento de colelitíase na DG foram confirmados por Zimmermann e colaboradores (ZIMMERMANN; POPP; AL-KHZOUZ; BUCERZAN *et al.*, 2016) em uma coorte de 61 pacientes com GD1, na qual encontraram uma alta prevalência (45,9%) de colelitíase, sem que houvesse análise da composição dos cálculos.

O dano canalicular na DG não foi estudado de forma específica até hoje. Há, no entanto, achados indiretos que mostram que os canalículos têm participação na patogênese dessa doença: em 2013, Yildiz e colaboradores (YILDIZ; HOFFMANN; VOM DAHL; BREIDEN *et al.*, 2013) mostraram que a atividade da beta-glicosidase não-lisossômica (GCase2, codificada pelo gene *GBA2*) – a qual é presente somente nos canalículos e ductos biliares e é responsável tanto pela hidrólise de O-glicosídeos dos sais biliares quanto pela hidrólise da GlcCer e da GlcSph presentes na bile – previne o acúmulo sistêmico de GlcCer, e que na DG essa enzima é estimulada como forma de compensação da perda da GCase.

Posteriormente, foi-se demonstrado (MISTRY; LIU; SUN; CHUANG *et al.*, 2014) que a deleção do gene *GBA2* em um modelo animal leva à diminuição da gravidade da DG1 – incluindo de suas manifestações hepáticas – e ao aumento da concentração de esfingosina no plasma, presumivelmente devido à degradação de GlcSph na bile. Esses achados permitem que se levante a hipótese de que a presença de GlcCer e GlcSph na bile pode levar a algumas das manifestações da DG e potencialmente ao dano de canalículos e de epitélio biliar, devido à toxicidade celular e metabólica da GlcSph (LUKAS; COZMA; YANG; KRAMP *et al.*, 2017; REED; SCHIFFER; HEALES; MEHTA *et al.*, 2018; SCHUELER; KOLTER; KANESKI; BLUSZTAJN *et al.*, 2003).

3.5.5. Lesões focais: Gaucheroma

Gaucheromas são lesões pseudotumorais formadas por acúmulos de células de Gaucher; podem ocorrer em diversos órgãos, sendo um dos mais comuns o fígado (IVANOVA; LIMGALA; CHANGSILA; KAMATH *et al.*, 2016; KORULA; OWENS; CHARLTON; BHATTACHARYA, 2017). Essas lesões são comuns e devem ser consideradas na avaliação de qualquer paciente com DG, especialmente em pacientes com maior gravidade de doença (conforme mensurada por escores clínicos de gravidade global, biomarcadores como a quitotriosidase, ou ocorrência de outras complicações da DG (REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016)). Podem ter padrão hipoecoico ou hiperecoico, sólido ou cístico, à US (HADAS-HALPERN; DEEB; ABRAHAMOV; ZIMRAN *et al.*, 2010; KORULA; OWENS; CHARLTON; BHATTACHARYA, 2017); hipodensidade e captação variável de contraste (com algumas lesões formando um halo captante ao redor de um centro não-captante) à tomografia computadorizada (TC); intensidade variável à RNM, com uma proporção semelhante de lesões hiper- e hipodensas (PATLAS; HADAS-HALPERN; REINUS; ZIMRAN *et al.*, 2002; REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016). Aproximadamente 5% dos pacientes pediátricos (PATLAS; HADAS-HALPERN; ABRAHAMOV; ELSTEIN *et al.*, 2002) e 25% dos pacientes adultos (REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016) com DG têm lesões focais hepáticas com características de

Gaucheroma ao estudo com US abdominal, sem mudança de tamanho ou características das lesões após a introdução da TRE (ADAR; ILAN; ELSTEIN; ZIMRAN, 2016; KORULA; OWENS; CHARLTON; BHATTACHARYA, 2017; PATLAS; HADAS-HALPERN; ABRAHAMOV; ELSTEIN *et al.*, 2002; PATLAS; HADAS-HALPERN; REINUS; ZIMRAN *et al.*, 2002). À biópsia, consistem em agregados de células de Gaucher típicas ou atípicas, com características imuno-histoquímicas de macrófagos de diferenciação alternativa (anti-inflamatória) e sem capacidade de proliferação clonal, em um parênquima com diferentes graus de fibrose, com ou sem compressão por efeito de massa do parênquima adjacente (IVANOVA; LIMGALA; CHANGSILA; KAMATH *et al.*, 2016; PATLAS; HADAS-HALPERN; REINUS; ZIMRAN *et al.*, 2002).

3.5.6. Lesões focais: hepatocarcinoma

Uma causa importante de letalidade em pacientes com DG é o desenvolvimento de neoplasias malignas, especialmente o hepatocarcinoma (HC) e o mieloma múltiplo (DE FOST; VOM DAHL; WEVERLING; BRILL *et al.*, 2006). O HC é uma neoplasia maligna originada dos hepatócitos maduros, geralmente em um contexto de dano crônico ao fígado como ocorre na cirrose (EL-SERAG; MARRERO; RUDOLPH; REDDY, 2008). É identificado em exames de imagem como uma lesão sólida, com hipodensidade em T1 e hiperdensidade em T2 à RNM, e característica resposta ao contraste intravenoso de gadolínio (captação intensa na fase arterial e perda completa de captação na fase venosa) (EL-SERAG; MARRERO; RUDOLPH; REDDY, 2008). Apesar das múltiplas alternativas terapêuticas existentes atualmente – incluindo radioterapia, quimioterapia, quimio-embolização transarterial, e mesmo transplante hepático (EL-SERAG; MARRERO; RUDOLPH; REDDY, 2008) – o HC é ainda uma neoplasia maligna agressiva e de parco prognóstico. É conhecida a conexão entre DG e HC, com uma razão de chances de até 141,3 para o desenvolvimento de HC em pacientes com DG comparativamente à população em geral (DE FOST; VOM DAHL; WEVERLING; BRILL *et al.*, 2006). Apesar de que a maior parte dos pacientes com DG que desenvolvem HC tenha já desenvolvido cirrose ao surgimento do tumor, não é raro que o HC seja diagnosticado em um paciente com fígado não-cirrótico (DE

FOST; VOM DAHL; WEVERLING; BRILL *et al.*, 2006; REGENBOOG; VAN DUSSEN; VERHEIJ; WEINREB *et al.*, 2018; XU; MISTRY; MCKENNA; EMRE *et al.*, 2005). Em um recente estudo multicêntrico de HC em pacientes com DG1, foram identificados como fatores de risco definidos ou prováveis para o desenvolvimento de HC: cirrose; hepatites virais crônicas comórbidas; siderose hepática; esplenectomia prévia (REGENBOOG; VAN DUSSEN; VERHEIJ; WEINREB *et al.*, 2018).

Um ponto ainda não totalmente esclarecido na literatura é o diagnóstico diferencial na DG das lesões focais hepáticas, especialmente entre Gaucheromas e o HC. Devido a suas características de imagem semelhantes e à natureza ainda não completamente estudada das respostas dos Gaucheromas ao contraste intravenoso, essa diferenciação se torna muitas vezes difícil (REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016). Exames complementares, especialmente a mensuração da α-fetoproteína (AFP) plasmática – a qual encontra-se elevada em muitos casos de HC (DE LOPE; TREMOSINI; FORNER; REIG *et al.*, 2012) –, podem auxiliar na decisão de se considerar uma lesão como uma ou outra patologia; no entanto, não apresentam sensibilidade e especificidade suficiente para permitir o diagnóstico definitivo em muitos casos (por exemplo, a AFP pode encontrar-se elevada em pacientes com cirrose sem HC, ou pode encontrar-se dentro dos valores de referência em um caso de HC (DE LOPE; TREMOSINI; FORNER; REIG *et al.*, 2012; EL-SERAG; MARRERO; RUDOLPH; REDDY, 2008)). A BH, que é considerada o padrão-ouro para o diagnóstico diferencial de lesões focais hepáticas, não é recomendada em muitos dos casos suspeitos de HC devido aos riscos inerentes a ela, como hemorragia intra-abdominal de difícil controle (DE LOPE; TREMOSINI; FORNER; REIG *et al.*, 2012), sendo realizada somente em casos selecionados. Sendo assim, a mais recente recomendação clínica por consenso de especialistas sugere que a conduta médica para qualquer lesão hepática compatível com HC em um paciente com DG deva ser a conduta tomada para o tratamento de um HC, admitindo-se a chance de um tratamento desnecessário, em caso de tratar-se de um Gaucheroma (REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016).

3.6. Tratamento da doença de Gaucher

A DG, por ser a primeira DL descoberta e uma das mais prevalente dessa classe, é considerada como um modelo para o desenvolvimento de terapias para as DLs. Antes da descoberta da TRE em 1991 (BARTON; BRADY; DAMBROSIA; DI BISCEGLIE *et al.*, 1991), o único tratamento eficaz para a DG era o transplante de células-tronco hematopoiéticas (TCTH), com grande morbimortalidade associada devido à necessidade de imunossupressão e à ocorrência de complicações como infecções sistêmicas e doença do enxerto-contra-o-hospedeiro (KAYE, 1995). A partir do uso da TRE, o TCTH tornou-se obsoleto e contraindicado, com riscos superando os benefícios (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017).

A primeira TRE a ser criada foi a alglucerase, com uso de GCase purificada a partir de placentas humanas (BARTON; BRADY; DAMBROSIA; DI BISCEGLIE *et al.*, 1991). Apesar da grande eficácia clínica, com diminuição relativamente rápida da hepatoesplenomegalia e dos sintomas clínicos, além de aumento das contagens de hemoglobina e plaquetas (WHITTINGTON; GOA, 1992), a dificuldade técnica na produção (aproximadamente 20.000 placentas sendo necessárias para a extração de enzima para um paciente por ano) e a potencial presença de contaminantes (como príons responsáveis pela doença de Creutzfeldt-Jakob) levaram à necessidade do desenvolvimento de uma nova forma da enzima (ZIMRAN; ELSTEIN; LEVY-LAHAD; ZEVIN *et al.*, 1995). Em 1996, foi desenvolvida a imiglucerase (ZIMRAN; ELSTEIN; LEVY-LAHAD; ZEVIN *et al.*, 1995), uma enzima recombinante produzida em células cultivadas de ovário de hamster chinês, com glicosilação da GCase alterada a partir da conformação nativa para que houvesse exposição de seus resíduos de manose, levando à sua captação pelo transportador lisossômico de manose-6-fosfato, presente nos lisossomos de macrófagos (MISTRY; LOPEZ; SCHIFFMANN; BARTON *et al.*, 2017; REVEL-VILK; SZER; MEHTA; ZIMRAN, 2018; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). A imiglucerase logo substituiu a alglucerase, tornando-se a única TRE disponível até 2010, quando foi desenvolvida a alfavelaglicerase, enzima também recombinante, mas de estrutura secundária

idêntica à enzima nativa, produzida em culturas de células humanas. Assim como a imiglucerase, a alfavelaglicerase é direcionada a macrófagos devido ao seu perfil de glicosilação, e ambas enzimas têm eficácia similar (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017; ZIMRAN; ALTARESCU; PHILIPS; ATTIAS *et al.*, 2010). Em 2012, foi desenvolvida a terceira opção de TRE recombinante para a DG, a alfataliglicerase, produzida em cultura de células de raízes de cenouras e com um perfil de glicosilação diferenciado, mas também direcionado a macrófagos (ZIMRAN; BRILL-ALMON; CHERTKOFF; PETAKOV *et al.*, 2011). Hoje se é aceito que as três enzimas tenham eficácia similar, sendo sua escolha mais dependente de fatores mercadológicos que médicos ou biológicos (MISTRY; LOPEZ; SCHIFFMANN; BARTON *et al.*, 2017; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Cabe notar que a TRE é efetiva para o tratamento das manifestações viscerais, ósseas e hematológicas da DG (ver seção 3.2) mas, devido a não cruzar a barreira hematoencefálica, não age sobre o dano neurológico (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Outra limitação da TRE é a necessidade de infusões intravenosas a cada 2 semanas, com duração de 2 a 4 horas, o que acarreta transtornos à rotina dos pacientes, perda relativa de qualidade de vida e diminuição da adesão ao tratamento (ALIOTO; GOMEZ; MOSES; PATERNOSTRO *et al.*, 2020; OLIVEIRA; ALEGRA; DORNELLES; KRUG *et al.*, 2013).

Devido às limitações da TRE, Platt e Radin desenvolveram o conceito de terapia de redução de substrato (TRS), com o princípio de que, sendo o acúmulo de GlcCer a causa das manifestações da DG, seria tão lógico quanto aumentar a sua degradação (o que é feito pela TRE) diminuir a sua produção, através da inibição da enzima glicosilceramida-sintase (MISTRY; LOPEZ; SCHIFFMANN; BARTON *et al.*, 2017; WEINREB, 2017). Em 2002, a primeira TRS, miglustate, foi comercializada não como primeira linha terapêutica (devido a sua eficácia menor que a TRE com imiglucerase), mas como uma alternativa à TRE em pacientes com efeitos colaterais incapacitantes ou que, por motivos pessoais, decidissem não aderir à TRE (COX; LACHMANN; HOLLAK; AERTS *et al.*, 2000; HEITNER; ELSTEIN; AERTS; WEELY *et al.*, 2002; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Apesar de cruzar a barreira hematoencefálica, o miglustate não é eficaz para o

tratamento de manifestações neurológicas (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). Mais recentemente, uma nova TRS foi desenvolvida, com disponibilidade para uso clínico em 2015: o eliglustate, com eficácia similar à imiglucerase (COX; DRELICHMAN; CRAVO; BALWANI *et al.*, 2017; COX; DRELICHMAN; CRAVO; BALWANI *et al.*, 2015). O eliglustate atualmente é considerado em muitos países um fármaco de primeira linha para o tratamento da DG em pacientes de idade igual ou superior a 18 anos (REVEL-VILK; SZER; MEHTA; ZIMRAN, 2018).

No Brasil, o tratamento é guiado pelos Protocolos Clínicos e Diretrizes Terapêuticas (PCDT) do Ministério da Saúde (FIGUEIREDO; FIREMAN, 2017), tendo sido a edição mais recente elaborada em 2014. A primeira opção terapêutica é a TRE com alfataliglicerase, e, para o início do tratamento, o paciente deve ter positivos os dois critérios maiores (diagnóstico clínico de DG; diagnóstico laboratorial por medida de atividade enzimática da GCase ou por genótipo do gene *GBA*), além de um dos critérios menores (anemia; contagem de plaquetas abaixo de 50.000/mm³; sangramento espontâneo ou decorrente de trauma mínimo; fígado ou baço palpáveis abaixo da cicatriz umbilical; sinais radiológicos de acometimento esquelético reversível ao tratamento, como osteopenia, osteoporose, infiltração da medula óssea, ou deformidade em frasco de Erlenmeyer; sintomas gerais incapacitantes; crescimento inapropriado em pacientes menores de 19 anos de idade; acometimento cardíaco ou pulmonar; mieloma múltiplo; esplenectomia; DG3; acometimento hepatocelular). Para pacientes que apresentem hipersensibilidade à alfataliglicerase ou a algum de seus componentes, é autorizado o uso de imiglucerase ou de alfavelaglicerase para TRE.

O tratamento por TRS no Brasil é feito apenas com o uso de miglustate, devido ao fato de o PCDT ter sido formulado antes da aprovação do eliglustate. Para mudança de TRE para TRS, o paciente deve ter positivos os três critérios maiores (diagnóstico clínico de DG1 ou DG3; diagnóstico bioquímico de DG, conforme especificado acima; e idade igual ou superior a 18 anos), além de ao menos 1 critério menor (presença de condição médica que

contraindique a TRE; presença de quadro clínico estável durante administração de TRE, incluindo tamanho de baço e fígado, níveis de hemoglobina, e níveis de plaquetas).

4. Distúrbios congênitos da glicosilação

Os DCGs são um grupo de doenças metabólicas raras, a maioria de padrão de herança autossômico recessivo, que afetam o processo de síntese, remodelamento, e distribuição de cadeias complexas de carboidratos denominadas "glicanos" (JAEKEN; MORAVA, 2016). Existem aproximadamente 150 DCGs conhecidos até a presente data, cada um causado por um defeito enzimático distinto que afeta a formação e a distribuição dos glicanos (FRANCISCO; MARQUES-DA-SILVA; BRASIL; PASCOAL *et al.*, 2019). A função dos glicanos ainda não é completamente elucidada; sabe-se que a ligação dos glicanos com proteínas – formando glicoproteínas – estabiliza a conformação espacial dessas e permite o reconhecimento por diversos receptores e transportadores, auxiliando na sua compartimentalização e em atividades de sinalização (TAYLOR; DRICKAMER, 2011a; b; d). Devido à glicosilação ser um processo presente na maior parte dos tecidos humanos, com pouca variação de seu maquinário enzimático conforme sua localização, os DCGs tendem a ser patologias graves e de envolvimento multissistêmico (VERHEIJEN; TAHATA; KOZICZ; WITTERS *et al.*, 2020).

A classificação atual dos DCGs (figura 8) os divide primariamente de acordo com o tipo do sítio de ligação entre os glicanos e as cadeias polipeptídicas que está afetado: defeitos da N-glicosilação são causados por anormalidades nos glicanos ligados a proteínas através da porção N-terminal de resíduos de asparagina, sendo o tipo mais abundante e comum de DCG; defeitos da O-glicosilação são causados por anormalidades nos glicanos ligados a proteínas ou a outras cadeias carboidráticas através da porção O-terminal de resíduos de serina ou de treonina (PÉANNE; DE LONLAY; FOULQUIER; KORNAK *et al.*, 2018). Os defeitos da N-glicosilação são ainda subdivididos em defeitos do tipo I ou do tipo II: os primeiros são causados por alterações na síntese e no processamento de glicanos no

citoplasma ou no RE, e levam à diminuição no número de glicanos ligados a proteínas, mas não à presença de glicanos de composição anormal (“truncados”); os últimos são causados por alterações no processamento (remodelamento e distribuição) de glicanos no CG, levando à presença de glicanos truncados em proteínas plasmáticas (JAEKEN; MORAVA, 2016).

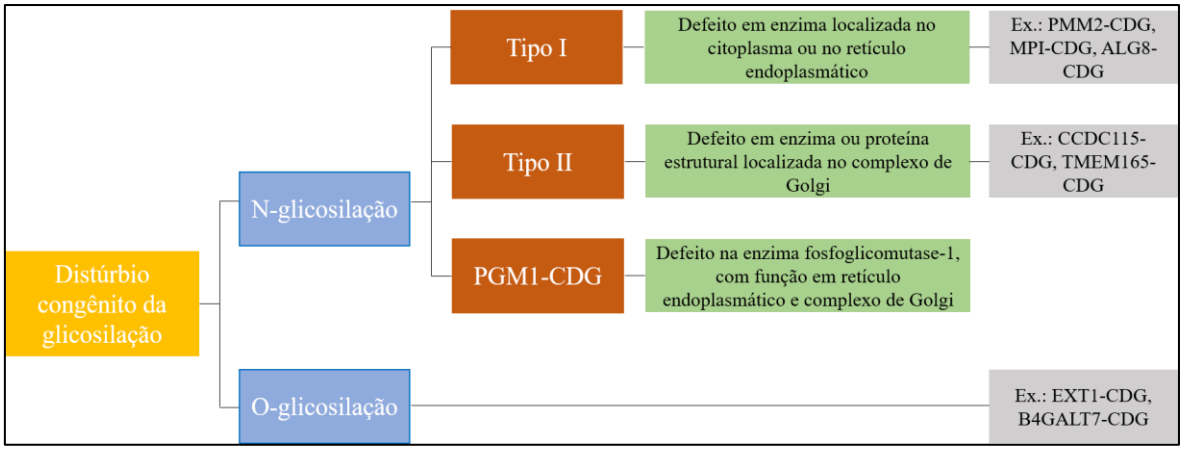


Figura 8. A classificação dos DCGs. Figura mostrando a classificação atual dos CDGs, primariamente conforme o tipo de sítio de glicosilação afetado e então a partir do comprometimento subcelular.

As manifestações clínicas dos DCGs são diversas e heterogêneas. Pontos em comum entre a maior parte dos DCGs do grupo I são o comprometimento neurológico levando a epilepsia e atraso do desenvolvimento neuropsicomotor, devido à abundância de proteínas glicosiladas no desenvolvimento extrauterino do sistema nervoso central (JAEKEN, 2013); o início precoce, por vezes neonatal, de dano hepatocelular inespecífico, com características inicialmente sugestivas de hepatites, mas com progressão rápida à fibrose (ver seção 1.3) devido ao fígado ser o principal órgão secretor de proteínas glicosiladas para circulação sistêmica, especialmente durante o início da vida extrauterina (VERHEIJEN; TAHATA; KOZICZ; WITTERS *et al.*, 2020); e coagulopatias com aumento simultâneo do risco hemorrágico e do risco trombótico, devido à glicosilação da maioria dos fatores pró- e anticoagulantes (FERREIRA; ALTASSAN; MARQUES-DA-SILVA; FRANCISCO *et al.*, 2018; TAYLOR; DRICKAMER, 2011c; VERHEIJEN; TAHATA; KOZICZ; WITTERS *et*

al., 2020). O DCG mais comum e que serve de protótipo para o estudo da classe dos defeitos da glicosilação é a PMM2-CDG (deficiência de fosfomanomutase-2), com mais de mil casos relatados no mundo até hoje (ALTASSAN; PÉANNE; JAEKEN; BARONE *et al.*, 2019). Essa doença é causada por variantes patogênicas bialélicas no gene *PMM2*, cuja enzima é responsável por transferir o grupo fosfato do carbono 6 para o carbono 1 da manose-6-fosfato, produzindo manose-1-fosfato, a qual é utilizada para a produção dos glicanos iniciais nas porções externa e interna da membrana do RE (ADAM; ARDINGER; PAGON; WALLACE *et al.*, 1993). A diminuição da atividade da PMM2 leva à produção insuficiente de manose-1-fosfato, e assim à diminuição na quantidade de glicanos produzidos em toda a cadeia. As manifestações típicas da PMM2-CDG, que iniciam nos primeiros anos de vida, são: estrabismo, epilepsia, ataxia, atraso do desenvolvimento neuropsicomotor, hipotonia, coagulopatia, e dano hepático metabólico, com progressão de alguns pacientes à fibrose hepática e à cirrose (MARQUES-DA-SILVA; DOS REIS FERREIRA; MONTICELLI; JANEIRO *et al.*, 2017; SCHIFF; RODA; MONIN; ARION *et al.*, 2017; WITTERS; HONZIK; BAUCHART; ALTASSAN *et al.*, 2019). Não existe tratamento específico para a PMM2-CDG, apesar de inúmeros compostos estarem sendo testados; a letalidade nos primeiros 5 anos de vida é de aproximadamente 25% (IYER; SAM; DIPRIMIO; PRESTON *et al.*, 2019; MARTÍNEZ-MONSENY; BOLASELL; CALLEJÓN-PÓO; CUADRAS *et al.*, 2019; VERHEIJEN; TAHATA; KOZICZ; WITTERS *et al.*, 2020).

O diagnóstico dos DCGs pode ser complexo e difícil (figura 9). O exame laboratorial mais comum para o rastreamento de DCG em pacientes com alta probabilidade pré-teste é a isoeletrofocalização (IEF) de proteínas plasmáticas (LEFEBER; MORAVA; JAEKEN, 2011). A IEF detecta a presença de proteínas de mesma estrutura primária, mas com pesos moleculares diferentes, o que é explicado por diferenças quantitativas e qualitativas em seus glicanos (LEFEBER; MORAVA; JAEKEN, 2011). A Tf, por ser uma proteína plasmática abundante e com 2 sítios de glicosilação bifurcantes terminados em ácido siálico (NeuAc) (o que lhe confere a presença de 4 NeuAcs), é a proteína mais comum a ser estudada por esse método, fornecendo dois padrões de resultados: padrões do tipo I são aqueles com aumento das formas da Tf com apenas dois NeuAcs (significando a perda do glicanos em

um dos sítios de glicosilação) e com nenhum NeuAc (significando a perda dos glicanos em ambos os sítios de glicosilação), característicos dos DCGs de N-glicosilação do tipo I. Os padrões do tipo II são aqueles com aumento das formas da Tf com um, três, ou cinco NeuAcs (significando a presença de glicanos estruturalmente anormais), característicos dos DCGs de N-glicosilação do tipo II (JAEKEN, 2013; LEFEBER; MORAVA; JAEKEN, 2011).

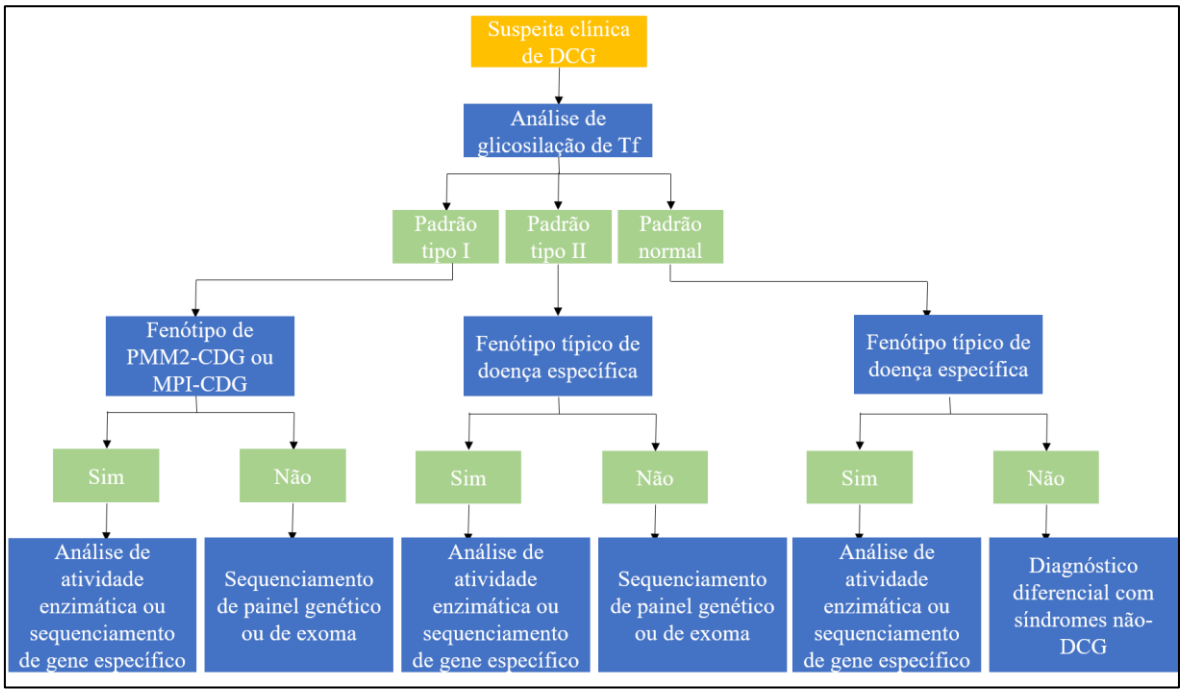


Figura 9. Algoritmo diagnóstico para a abordagem inicial de um paciente com suspeita de DCG. Algoritmo de abordagem inicial de um paciente que tenha fenótipo clínico sugestivo de um DCG. A análise de glicosilação de Tf pode ser realizada por IEF ou por espectrometria de massa. Adaptado de (FRANCISCO; MARQUES-DA-SILVA; BRASIL; PASCOAL; *et al*, 2019).

O fenótipo hepático dos DCGs é de difícil definição, devido à presença de múltiplas doenças heterogêneas incluídas nesse grupo. Alguns DCGs possuem fenótipo hepático grave que foi definido principalmente através de relatos de casos: a ALG8-CDG (deficiência de α-1,3-glicosiltransferase) é um DCG do tipo I que pode causar dano hepático com fibrose de início na infância ou na adolescência, levando à cirrose com hipertensão portal proeminente (MARQUES-DA-SILVA; DOS REIS FERREIRA; MONTICELLI; JANEIRO *et al.*, 2017);

a MPI-CDG (deficiência de fosfomanose-isomerase) é um DCG do tipo I que pode apresentar início neonatal ou mesmo intrauterino de perda de função hepática e fibrose hepática rapidamente progressiva, levando à cirrose e perda de função hepática (MARQUES-DA-SILVA; DOS REIS FERREIRA; MONTICELLI; JANEIRO *et al.*, 2017; MENTION; LACAILLE; VALAYANNOPOULOS; ROMANO *et al.*, 2008); a CCDC115-CDG (deficiência de proteína contendo domínio em mola 115) é um DCG do tipo II que apresenta início neonatal de hepatoesplenomegalia, colestase, e deposição de cobre nos hepatócitos, com progressão rápida à cirrose e à perda de função hepática (GIRARD; POUJOIS; FABRE; LACAILLE *et al.*, 2018; JANSEN; CIRAK; VAN SCHERPENZEEL; TIMAL *et al.*, 2016; MEDRANO; VEGA; NAVARRETE; ECAY *et al.*, 2019). Estudos sistemáticos do fenótipo hepáticos dos DCGs, são, no entanto, raros. Em 2017, Marques-da-Silva e colaboradores (MARQUES-DA-SILVA; DOS REIS FERREIRA; MONTICELLI; JANEIRO *et al.*, 2017) realizaram uma revisão sistemática da literatura científica, descrevendo achados hepáticos já relatados em 16 tipos de DCG, e dividindo os pacientes em dois grupos: os DCGs com envolvimento predominantemente hepático, e os DCGs com envolvimento hepático "associado". No entanto, exceto a tentativa de classificação, pouco foi abordado nesse estudo a identificação de padrões hepáticos comuns aos diferentes DCGs, que possam levar a um maior entendimento da história natural e da biologia dessas doenças e assim contribuir ao cuidado clínico que esses pacientes recebem.

Em 2019, Witters e colaboradores (WITTERS; HONZIK; BAUCHART; ALTASSAN *et al.*, 2019) publicaram um estudo de história natural de pacientes com PMM2-CDG, mostrando pela primeira vez de forma sistemática o comportamento dos marcadores de dano hepático inespecífico (ver seção 1.3.1) nessa doença, que segue um padrão de grande elevação até os 5 anos de idade, com melhora gradual após, atingindo níveis próximos à normalidade durante a adolescência na maioria dos pacientes. Esse estudo, no entanto, não analisou outros aspectos do fenótipo hepático – como esteatose, colestase, marcadores histológicos ou imaginológicos –, e restringiu o escopo do estudo aos pacientes com PMM2-CDG. Não há, assim, na literatura científica, estudo abrangente de fenótipo hepático em DCGs, especialmente considerando outros DCGs além da PMM2-CDG.

B. Justificativa

Tanto a DG quanto os DCGs são doenças genéticas que afetam múltiplos sistemas orgânicos e que causam grande impacto na vida dos pacientes afetados. São doenças monogênicas do metabolismo, mas que não incluem, em sua maioria, na sua descrição clássica a presença de alterações hepáticas complexas, sejam primárias ou secundárias, apesar de as rotas metabólicas que afetam estarem presentes em grande atividade no fígado. Essa falta se reflete na ausência de recomendações clínicas precisas que levem em conta o acometimento hepático nesses pacientes. Assim, assumindo-se que seja necessário compreender por completo e em detalhes o fenótipo de uma doença para que o paciente portador possa ser cuidado por completo, e para que tratamentos eficazes possam ser descobertos, faz-se premente a exploração aprofundada do fenótipo hepático da DG e dos DCGs.

Essa linha de pesquisa expande a linha de pesquisa do grupo da professora Dra. Ida Vanessa Doederlein Schwartz, que estuda, dentre outros tópicos relacionados, o fenótipo de pacientes com DG em múltiplos níveis, tendo trabalhos prévios explorado: as manifestações neurológicas; imunológicas; nutricionais e metabólicas; do metabolismo do ferro; e esqueléticas nessa doença. Esta é a primeira vez que o fenótipo hepático da DG, e que qualquer aspecto dos DCGs, são estudados neste grupo de pesquisa.

C. Objetivos

Objetivos gerais

1. Delinear o fenótipo hepático da doença de Gaucher;
2. Delinear o fenótipo hepático dos distúrbios congênitos da glicosilação.

Objetivos específicos

1. Descrever e analisar o aspecto clínico do fenótipo hepático da doença de Gaucher, com foco na história natural dos achados clínicos e em exames laboratoriais de prática clínica, e na resposta ao tratamento específico.

2. Descrever e analisar de forma quantitativa o fenótipo hepático da doença de Gaucher a nível histológico.

3. Propor fatores de modificação do fenótipo hepático da doença de Gaucher.

4. Investigar ativamente a presença de fibrose hepática em pacientes com doença de Gaucher, e estudar métodos não-invasivos e de baixo custo para a detecção dessa anormalidade nesses pacientes.

5. Descrever e analisar o aspecto clínico do fenótipo hepático dos distúrbios congênitos da glicosilação, com foco na história natural dos achados clínicos e em exames laboratoriais de prática clínica, e na resposta ao tratamento específico.

D. Métodos e Resultados

- Objetivo específico 1

Artigo 1: “Liver involvement in patients with Gaucher disease types I and III”. Rodrigo Tzovenos Starosta, Filippo Pinto e Vairo, Alícia Dorneles Dornelles, Suélen Porto Basgalupp, Marina Siebert, Maria Lúcia Alves Pedroso, Carlos Thadeu Schmidt Cerski, Mário Reis Álvares-da-Silva, Ida Vanessa Doederlein Schwartz.

Artigo publicado no periódico Molecular Genetics and Metabolism Reports, 2020; 22: 100564.

Molecular Genetics and Metabolism Reports 22 (2020) 100564

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

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

Liver involvement in patients with Gaucher disease types I and III

Rodrigo Tzovenos Starosta[a,b,*], Filippo Pinto e Vairo[c,d], Alícia Dorneles Dornelles[e], Suélen Porto Basgalupp[f], Marina Siebert[g,h], Maria Lúcia Alves Pedroso[i,j], Carlos Thadeu Schmidt Cerski[b,h,k], Mário Reis Álvares-da-Silva[b,h,l], Ida Vanessa Doederlein Schwartz[a,e,f,m]

[a] Postgraduate Programme in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] School of Medicine, UFRGS, Porto Alegre, Brazil
[c] Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA
[d] Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA
[e] Postgraduate Programme in Medical Sciences, UFRGS, Porto Alegre, RS, Brazil
[f] BRAIN Laboratory, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, RS, Brazil
[g] Unit of Laboratorial Research, Experimental Research Centre, HCPA, Porto Alegre, RS, Brazil
[h] Postgraduate Programme in Gastroenterology and Hepatology, UFRGS, Porto Alegre, Brazil
[i] Postgraduate Programme in Internal Medicine, Universidade Federal do Paraná (UFPR), Curitiba, Paraná, Brazil
[j] Gastroenterology Service, Complexo Hospital de Clínicas de Curitiba, UFPR, Curitiba, Paraná, Brazil
[k] Surgical Pathology Service, HCPA, Porto Alegre, RS, Brazil
[l] Gastroenterology Service, HCPA, Porto Alegre, RS, Brazil
[m] Clinical Research Center, HCPA, Porto Alegre, RS, Brazil

ARTICLE INFO

Keywords:
Gaucher disease
Enzyme replacement therapy
Liver steatosis
Hemosiderosis
Biopsy, large-core needle
Cholelithiasis

ABSTRACT

Background & aims: Gaucher disease (GD) is a multisystemic disease. Liver involvement in GD is not well characterised and ranges from hepatomegaly to cirrhosis and hepatocellular carcinoma. We aim to describe, and assess the effect of treatment, on the hepatic phenotype of a cohort of patients with GD types I and II.

Methods: Retrospective study based on the review of the medical files of the Gaucher Reference Centre of the Hospital de Clínicas de Porto Alegre, Brazil. Data from all GD types I and III patients seen at the centre since 2003 were analysed. Variables were compared as pre- ("baseline") and post-treatment ("follow-up").

Results: Forty-two patients (types I: 39, III: 3; female: 22; median age: 35 y; enzyme replacement therapy: 37; substrate reduction therapy: 2; non-treated: 3; median time on treatment-MTT: 124 months) were included. Liver enzyme abnormalities, hepatomegaly, and steatosis at baseline were seen in 19/28 (68%), 28/42 (67%), and 3/38 patients (8%), respectively; at follow-up, 21/38 (55%), 15/38 (39%) and 15/38 (39%). MRI iron quantification showed overload in 7/8 patients (treated: 7; MTT: 55 months), being severe in 2/7 (treated: 2/2; MTT: 44.5 months). Eight patients had liver biopsy (treated: 6; MTT: 58 months), with fibrosis in 3 (treated: 1; time on treatment: 108 months) and steatohepatitis in 2 (treated: 2; time on treatment: 69 and 185 months). One patient developed hepatocellular carcinoma.

Conclusions: GD is a heterogeneous disease that causes different patterns of liver damage even during treatment. Although treatment improves the hepatocellular damage, it is associated with an increased rate of steatosis. This study highlights the importance of a follow-up of liver integrity in these patients.

1. Introduction

Gaucher disease (GD) (OMIM #230800, #230900 and #231000) is an autosomal recessive disorder most frequently caused by biallelic pathogenic variants in the *GBA1* gene that codes for glucocerebrosidase (GCase). The impaired activity of GCase causes glucosylceramide (GlcCer) to build up into the lysosomes of the reticuloendothelial system cells, mainly macrophages that become engorged and dysfunctional being thus called "Gaucher cells" [1]. The incidence of GD ranges between 1:50,000 and 1:100,000 in the general population, and is about 1:855 in the Ashkenazi Jewish population [2]. GD is broadly categorised in three types, according to neurological manifestations:

* Corresponding author at. Medical Genetics Service, 3rd floor, 2350 Ramiro Barcelos street, Porto Alegre, RS 90035-007, Brazil.
E-mail address: rodrigo.starosta@ufrgs.br (R.T. Starosta).

https://doi.org/10.1016/j.ymgmr.2019.100564
Received 29 September 2019; Received in revised form 29 December 2019; Accepted 30 December 2019

type I, or "non-neuronopathic"; type II, or "acute neuronopathic"; and type III, or "chronic neuronopathic".

The manifestations of GD are multisystemic with a complex pathophysiologic process that arises from the infiltration of organs by Gaucher cells, the low-grade inflammation promoted by cells whose intracellular signalling is disrupted by the accumulation of GlcCer [3,4], and other factors such as aberrant complement activity [5,6] and dysfunctional autophagy [7,8]. The main signs and symptoms of GD include hepatosplenomegaly, anaemia, thrombocytopenia, bone deformities and pain, osteonecrosis, restrictive pulmonary disease, and neurological compromise in patients with GD type II and III [1,2] which cause significant impairment in life quality and reduction of life expectancy [9,10]. Treatment of GD is currently available in two modalities: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). The former is the most established treatment, consisting in the fortnightly infusion of recombinant GCase which is uptaken by the macrophages' lysosomes, decreasing the GlcCer build-up [1,2,11]. Imiglucerase (Sanofi Genzyme Corporation, Cambridge, MA, USA), taliglucerase alfa (Protalix Biotherapeutics, Carmiel, Israel), and velaglucerase alfa (Takeda Pharmaceutical Company, Tokyo, Japan) are the currently available enzymes with no detectable difference in efficacy or safety profile known between them [1,12, 13–16]. SRT is administered orally once or twice daily and works decreasing the production of GlcCer which consequently decreases its storage [17]. The currently SRT FDA-approved compounds are miglustat and eliglustat. ERT and/or SRT are not indicated for GD type II patients.

The extent of liver damage in GD is still subject of debate – first reports were limited to hepatomegaly, however it is currently known that patients are at increased risk for focal fibrosis, cholelithiasis, steatosis, haemosiderosis, overt cirrhosis, and hepatocellular carcinoma (HCC) [18,19]. Recent studies [20,21] have shown that liver stiffness is increased in a large proportion of patients with GD, suggesting that fibrosis may be a pervasive process even in patients with apparent controlled disease, and also that it is correlated to disease severity, making it an important cause of morbidity to be addressed in this population.

In this study, we aimed at characterising the liver involvement in a cohort of patients with GD type I and III, and the effect of ERT/SRT on those variables.

2. Methods

This is a retrospective study, based on the review of the medical records of the GD types I and III patients followed at the Gaucher Reference Centre of the Hospital de Clínicas de Porto Alegre, Brazil (GRC-HCPA) from 2003 to 2018. HCPA is a public, university hospital located in Southern Brazil. Inclusion criteria were: a) having biochemical or genetic diagnosis of GD; b) not having any other primary liver disease, as determined by clinical and laboratory features and serological screening for hepatitis B and C.

At the GRC-HCPA, patients have regular appointments every 3–4 months and most exams are made in an annual basis unless an acute event prompts a more frequent evaluation. The following exams were performed at baseline for most patients: complete blood count, chitotriosidase activity, aspartate-transaminase (AST), alanine-transaminase (ALT), and abdominal ultrasonography (US). The following exams are performed yearly: AST, ALT, γ-glutamyltransferase (γGT), direct bilirubin (DB), indirect bilirubin (IB), prothrombin time, alkaline phosphatase, total and fractional cholesterol, triglycerides, serum creatinine, blood urea, calcium, phosphorus, US, serum protein electrophoresis, serum immunoglobulins, transferrin saturation/iron-binding capacity, and serum iron. The following exams are performed every three months: complete blood count, serum ferritin, and chitotriosidase activity. All patients are tested for serological markers of viral hepatitis at initiation of treatment and again according to clinical indication. Alpha-foetoprotein (AFP) is not ordered for patients without cirrhosis due to its dubious efficacy as a screening test for hepatocellular carcinoma [22]. The presence of hepatomegaly was ascertained by US or by physical exam (when US was not available). The presence of steatosis was assessed by US. Elastography for fibrosis assessment is not routinely performed. Other exams are performed according to clinical indication [23]. All patients had genotyping of *GBA* and *HFE* by next-generation sequencing.

Immunological and iron metabolism findings of our cohort have already been described by Vairo et al. [24] and Koppe et al. [25], respectively.

Statistical analyses were performed using the SPSS software (IBM Inc., v.18); for comparison of frequencies of categorical variables, the χ^2 test was used. Patients were compared regarding the findings before the onset of treatment ("baseline" data points) and during treatment until last follow-up ("follow-up" data points). Findings were considered abnormal at baseline or at follow up if altered in at least two measurements for each datapoint, or one measurement when it was the only one available.

3. Ethics statement

This study was approved by the Institutional Review Board of HCPA (CEP/HCPA), Porto Alegre, RS, Brazil (projects #13–0537 and #15–0083). All studies were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all subjects or, when < 18 years-old, from their parents.

4. Results

4.1. Subjects

Forty-two patients were included (n = 39, type I; n = 3, type III; female = 22; median time on treatment: 124 months). One patient with GD type I (pt 26D) was excluded from the follow-up data analysis due to diagnosis of active chronic hepatitis B. One patient with GD type I (pt 26A) had serological evidence of spontaneously cured hepatitis B. No other patients had signs of other liver diseases, such as drug-related liver injury, autoimmune hepatitis, or viral hepatitis.

No patient had a history of blood transfusions in the past. A total of 36 patients had measurements of serum transferrin saturation after treatment; of these, 6 had decreased values and 5 had increased values (Supplementary Table 1). Four patients had used ferrous sulphate supplements in the past, one of them only during pregnancy (Supplementary Table). No patient was homozygous or compound heterozygous for pathogenic variants in the *HFE* gene, ruling out the concomitant diagnosis of *HFE*-associated haemochromatosis (MIM: #235200).

4.2. Laboratory findings

Laboratory findings of all patients are shown in Table 1

Out of the 28 patients with liver enzymes (AST, ALT, or γGT) data at baseline, 19/28 (68%) had abnormal liver enzymes in at least two measurements. At follow-up, 21/38 (68%) had abnormalities in at least one liver enzyme in at least two measurements. History was positive for excessive alcohol intake in two patients (19B and 26B).

Serum transferrin saturation, immunoglobulins, and serum protein electrophoresis results during treatment can be found in the supplementary table. Immunoglobulin measurements and serum protein electrophoresis results were available for 36 patients during treatment; of these, 26 had an abnormal serum immunoglobulin measurement at least twice and 20 had increased γ-globulins in serum electrophoresis at least twice.

Table 1
Liver enzymes in patients with Gaucher disease.

Patient	Age	Gender	GBA Genotype	Sx	Baseline					Time on treatment	Follow-up				
	(y)				AST	ALT	GGT	DB	IB	(months)	AST	ALT	GGT	DB	IB
1A	7	F	N370S/G202R	No	-	-	-	-	-	None	-	-	-	-	-
1B	22	M	N370S/G202R	No	-	-	-	-	-	I(168)	N	N	N	↑	N
2	20	M	L444P/L444P	No	↑	↑	↑	N	N	I(110) T(1) V(61)	↑	↑	↑	N	N
3	20	F	N370S/L444P	No	-	-	-	-	-	None	-	-	-	-	-
4A	22	F	N370S/L444P	No	N	N	N	N	N	I(68)	N	N	N	N	N
4B	36	F	N370S/L444P	No	N	↑	N	N	N	I(19) T(1) I(90)	N	N	N	N	N
5	23	F	N370S/RecNciI	No	↑	↑	-	-	-	I(184)	N	N	↑	N	N
6	23	F	N370S/L444P	No	↑	↑	↑	N	N	T(14)	↑	N	N	N	N
7	24	M	N370S/N370S	No	-	-	-	-	-	I(131)	N	N	N	↑	N
8	24	F	N370S/N370S	No	N	↑	N	-	-	I(156)	N	N	N	↑	↑
9	25	F	N370S/L444P	No	N	N	↑	↑	↑	I(113)	N	N	N	↑	↑
10	26	M	N370S/R120W	No	N	N	N	N	↑	I(202)	N	N	N	↑	↑
11A	26	M	L444P/L444P	Yes	-	-	-	-	-	I(176)	N	↑	N	↑	N
11B	29	F	L444P/L444P	Yes	-	-	-	-	-	I(175)	N	↑	N	N	N
12	27	M	N370S/c.1328+1G>A	No	N	N	N	↑	N	I(159)	N	N	N	↑	N
13	27	F	N370S/RecNciI	No	-	-	-	-	-	I(120) T(1) I(6) E(41) I(8) I(16)	N	N	N	N	N
14	27	M	N370S/L444P; c1483G>C	No	↑	↑	N	-	-	I(257) T(13)	↑	↑	N	↑	↑
15	28	F	N370S/L461F; c.1515+1G>T	No	-	-	-	-	-	I(211)	↑	↑	↑	N	N
16	28	M	N370S/L444R	No	-	-	-	-	-	A(31) I(227)	N	↑	N	N	N
17	31	M	N370S/RecNciI	No	-	-	-	-	-	I(109) T(2) I(9) T(80)	↑	↑	↑	N	N
18	33	F	N370S/L444P	No	N	N	-	-	-	M(7) I(115)	N	N	N	N	N
19A	35	M	N370S/L444P	No	↑	N	↑	N	N	I(75)	N	N	N	↑	N
19B	41	M	N370S/L444P	Yes	N	↑	-	↑	↑	I(74) T(3) I(88)	N	N	N	N	↑
19C	52	M	N370S/L444P	No	N	↑	↑	-	-	I(6) T(1) I(81)	N	N	↑	↑	↑
20	36	F	N370S/R163*	No	-	-	-	-	-	I(210)	↑	N	↑	N	N
21	36	F	N370S/RecNciI	Yes	-	-	-	-	-	A(25) I(28) T(3) I(53)	↑	↑	↑	N	N
22A	37	M	N370S/L444P	No	↑	↑	↑	N	N	I(5) T(81)	↑	N	↑	N	N
22B	39	F	N370S/L444P	No	N	N	N	N	N	I(22) T(2) I(14) E(77)	↑	N	N	N	N
23	38	F	N370S/RecNciI	No	↑	↑	↑	↑	↑	I(36)	↑	N	↑	N	N
24	45	M	N370S/RecNciI	No	N	N	-	N	N	I(102) T(3) I(29) T(50)	N	N	N	↑	N
25A	48	F	E249K/S366N	No	N	N	↑	N	N	M(11) T(1) I(62)	N	N	N	N	N
25B	52	F	E249K/S366N	No	N	N	N	N	N	M(30) T(14)	N	N	N	N	N
26A	53	M	N370S/RecNciI	No	N	↑	↑	↑	N	I(89)	N	N	N	↑	N
26B	57	M	N370S/RecNciI	No	↑	↑	N	N	N	I(79)	↑	↑	↑	↑	↑
26C	63	F	N370S/RecNciI	No	N	N	↑	↑	↑	I(73)	N	N	N	↑	↑
26D	51	F	N370S/RecNciI	Yes	-	-	-	N	N	I(96) T(5) I(13) T(79)	-	-	-	-	-
27	62	F	N370S/RecNciI	No	N	N	-	-	-	I(124) T(2) I(8) T(81)	↑	↑	↑	N	N
28	62	M	N370S/RecNciI	Yes	↑	↑	↑	↑	N	I(34) T(2) I(90)	↑	↑	↑	↑	↑
29A	64	M	N370S/N370S	No	N	N	↑	↑	↑	I(10) T(2) I(9) T(81)	N	N	↑	↑	↑
29B	65†	M	N370S/N370S	No	N	N	↑	N	N	I(19) T(2) I(8) T(14)	N	N	↑	N	↑
30	67	F	N370S/L444R	Yes	N	N	N	N	N	I(12) M(25) I(49)	N	N	N	N	N
31	62†	M	N370S/RecNciI	No	-	-	-	-	-	None	-	-	-	-	-

Values considered to be elevated were so in at least two measurements while on treatment. Patients sharing the same number are siblings, except for patient 26D who is a cousin of patients 26A, 26B, and 26C. Patients 5, 7, 8, 14, 19A, 19B, 19C, 21, 22A, and 24 have a low adherence to treatment (less than 75% of programmed yearly infusions performed). Sx = splenectomy; M = male; F = female; N = normal; A = alglucerase; I = imiglucerase; T = taliglucerase alfa; V = velaglucerase alfa; E = eliglustat; - = no value available. †age of death. Reference values: ALT < 34 U/L; AST < 33 U/L; GGT < 40 U/L; DB < 0.4 mg/dL; IB < 0.9 mg/dL.

Table 2
US findings from GD patients at baseline.

Patient	Age (y)	BMI	Steatosis	Hepatomegaly	Cholelithiasis	Ferritin (ng/dL)	MetS[a]	Liver biopsy
1A	7	15.9	No	No	No	378	No	No
1B	8	16.1	No	Yes	No	–	No	No
2	1	16.9	No	Yes	No	–	No	No
3	15	17.3	Yes	No	No	174.6	No	No
4A	16	21.8	No	No	No	284.8	No	No
4B	26	22.7	Yes	Yes	Yes	328.5	No	No
5	8	15.1	No	Yes	No	–	No	No
6	20	22.6	No	Yes	No	219.3	No	Yes
7	6	14.6	No	Yes	No	–	No	No
8	7	12	No	Yes	No	97.3	No	No
9	17	18.3	No	Yes	No	166	No	No
10	9	14.5	No	Yes	No	–	No	No
11A	–	14.1	–	Yes	No	–	No	No
11B	–	–	–	Yes	No	–	No	No
12	12	17.3	No	No	No	–	No	No
13	14	23	No	No	No	–	No	No
14	6	16.2	No	Yes	No	–	No	No
15	11	12.5	No	Yes	No	–	No	No
16	–	15.2	–	Yes	No	–	No	No
17	24	16.8	No	Yes	No	–	No	No
18	17	21.6	No	Yes	No	–	No	No
19A	39	30.2	No	Yes	Yes	758.3	No	No
19B	28	22.2	No	Yes	No	–	No	No
19C	43	26.5	No	No	No	951.8	No	No
20	18	21.9	No	Yes	No	–	No	No
21	13	16.6-	No	Yes	No	–	No	No
22A	30	24	No	Yes	No	469.6	No	No
22B	29	23.2	No	Yes	No	213.3	No	No
23	34	25.3	No	No	No	835	Yes	Yes
24	22	24.3-	No	No	No	–	No	No
25A	42	23.4	No	Yes	Yes	754.2	No	No
25B	43	30.2	Yes	Yes	Yes	860.7	No	No
26A	44	25	No	No	No	811	No	No
26B	50	31.4	No	No	No	1409	Yes	No
26C	57	23.5	No	No	No	1593	No	No
26D	34	19.3	No	Yes	No	–	No	No
27	44	–	No	Yes	No	–	No	No
28	52	24	No	Yes	No	3392	Yes	Yes
29A	55	28.5	No	No	Yes	1698	Yes	No
29B	61	27.2	No	No	Yes	778.2	No	No
30	60	29.8	No	Yes	No	1972	Yes	No
31	62	17.7	No	No	No	1343	No	No

y = years-old; US = ultrasonography; BMI = body-mass index; MetS = metabolic syndrome. Ferritin RV <150 ng/dL for women, <300 ng/dL for men.

[a] Metabolic syndrome is defined as the presence of at least three of the following: obesity, high triglycerides level, increased blood pressure, and elevated fasting blood glucose (reduced HDL level was not considered as a criterion because it is a feature of GD).

4.3. Liver ultrasound findings

Liver US reports were available from 39 patients (baseline = 39; follow-up = 38) (Tables 2 and 3). Hepatomegaly was present in 28/42 (67%) of patients at baseline and in 15/38 (39%) of patients at follow-up.

Steatosis was present in 3/39 (8%) of patients at baseline and in 15/38 (39%) at follow-up. In 6 patients, there was regression of steatosis within 2 years of US detection. Of these, none had any significant change in body-mass index (BMI) but two had changes in the ERT regimen (for patient 15, there was an increase in the imiglucerase dosage from 45 IU/Kg to 60 IU/Kg; for patient 19C, there was a switch from taliglucerase alfa to imiglucerase). Twelve out of the 16 patients (75%) with steatosis were overweight or obese, with 4 patients (two whose steatosis regressed, one that maintains the finding, and one that denied treatment and further follow-up) having a normal BMI. A significant difference was found between the frequency of overweight/obesity in patients with and without persistent steatosis (77.8% *vs* 40%, $p = .047$, Pearson's χ^2). Blood lipid levels were available for 7 of the 9 patients (78%) with non-regressing steatosis during treatment. All 7 patients had dyslipidaemia (four with high triglycerides, three with high total cholesterol and LDL, and five with low HDL). Levels were available for 31 patients without non-regressing steatosis – of these, 28 (90.3%) had dyslipidaemia (10 with high triglycerides, 5 with high total cholesterol and LDL, and 25 with low HDL). No significant difference was found between patients with and without non-regressing steatosis and the presence of dyslipidaemia ($p = .814$, Pearson's χ^2).

Twelve patients in the cohort had cholelithiasis, and 7 of them underwent cholecystectomy (pts. 13, 18, 19A, 20, 23, 25A, and 25B). Patient 23 had cholecystectomy before initiation of treatment for GD. Eight out of the patients with cholelithiasis were overweight or obese, but no significant difference in the prevalence of overweight/obesity was found between the patients with and without cholelithiasis (66.7% *vs* 40.7%, $p = .135$, Pearson's χ^2).

Other US findings observed in the cohort were: cysts, haemangioma, solid nodule compatible with an adenoma or a haemangioma, portal hypertension that resolved with initiation of ERT, and cirrhosis with HCC. The two cysts of unknown diagnosis were present in a pair of brothers with GD type I who also had steatosis (pts 29A and 29B). The older brother passed away at the age of 65 due to multiple myeloma. The cyst in the younger brother, now aged 65, is 5 mm in diameter and is stable since it was diagnosed 2 years ago. The patient with cirrhosis

Table 3
US from GD patients at follow-up.

Patient	Age (y)	Time on treatment (months)	Steatosis	Hepatomegaly	Cholelithiasis	BMI	Ferritin (ng/dL)	MetS during treatment[a]	Liver biopsy during treatment
1A	–	T(7)	–	No	–	17.4	308	No	No
1B	20	I(168)	No	No	No	28.6	485	No	No
2	20	I(110) T(1) V(81)	Yes[b]	Yes	Yes	21.7	254	No	No
3	–	–	–	–	–	–	–	–	–
4A	–	–	–	No	No	24.4	312.2	No	No
4B	36	I(19) T(1) I(89)	No	Yes	Yes	25.9	57.3	No	No
5	22	I(177)	No	No	No	22.7	84.7	No	No
6	23	T(33)	No	No	No	22.4	172.6	No	No
7	17	I(68)	No	No	No	19.2	885.3	No	No
8	25	I(156)	Yes[b]	Yes	No	20.5	508.3	No	No
9	26	I(112)	No	No	No	22.7	94.6	No	No
10	25	I(200)	No	Yes	No	24.7	547	No	No
11A	26	I(174)	Yes	Yes	Yes	18.8	242.6	No	No
11B	28	I(146)	No	Yes	No	27.8	285.9	No	No
12	25	I(126)	No	Yes	No	23.1	546.3	No	No
13	27	I(120) T(1) I(6) E(41) I(8) E(9)	No[c]	No	Yes	28.8	611.6	No	Yes
14	23	I(189)	No	Yes	No	24.9	619.9	No	No
15	29	I(216)	Yes[b]	Yes	No	26.6	311.1	No	No
16	25	A(31) I(222)	No	No	No	20.6	569.8	No	No
17	29	I(109) T(2) I(9) T(55)	No	No	No	30.6	325.8	No	No
18	33	M(7) I(111)	No	Yes	Yes	25.3	278.8	Yes	No
19A	45	I(67)	Yes[b]	No	Yes	32	66.5	No	No
19B	34	I(71)	No	No	No	27.8	585.3	No	No
19C	51	I(6) T(1) I(80)	Yes[b]	Yes	No	28.3	1084	Yes	No
20	36	I(207)	No	No	Yes	25.6	574.2	No	No
21	32	A(25) I(28) T(3) I(4)	No	No	No	22.7	415.7	No	No
22A	36	I(5) T(65)	No	Yes	No	26.8	463.8	No	No
22B	40	I(22) T(2) I(14) E(70)	Yes	No	No	30	32	Yes	No
23	38	I(35)	Yes[b]	No	Yes	30.7	427.2	No	No
24	44	I(102) T(3) I(29) T(38)	No	Yes	No	28.7	247.9	No	No
25A	48	M(11) T(1) I(55)	Yes	No	Yes	25.8	611.9	No	Yes
25B	51	M(30) T(10)	Yes	No	Yes	31.9	1053	No	Yes
26A	52	I(80)	No	No	No	24.1	480.6	No	No
26B	58	I(76)	Yes	No	No	32.2	1457	Yes	Yes
26C	62	I(66)	No	No	No	21.6	624.8	No	No
27	61	I(124) T(2) I(8) T(71)	Yes	No	No	26.6	1052	Yes	No
28	62	I(34) T(2) I(88)	No	No	No	22.3	543.2	No	Yes
29A	65	I(10) T(2) I(9) T(81)	Yes	No	Yes	34.5	670	Yes	No
29B	65	I(19) T(2) I(8) T(14)	Yes	Yes	Yes	29.7	686	Yes	No
30	67	I(12) M(25) I(47)	No	Yes	No	30.5	1103	Yes	Yes
31	–	–	–	–	–	–	–	–	–

y = years-old; BMI = body mass index; I = imiglucerase; T = taliglucerase alfa; V = = velaglucerase alfa; E = eliglustat; A = alglucerase; M = miglustat.

[a] Metabolic syndrome is defined as the presence of at least three of the following: obesity, high triglycerides level, increased blood pressure, and elevated fasting blood glucose (reduced HDL level was not considered as a criterium because it is a feature of GD).

[b] Steatosis regressed within two years of US detection.

[c] Steatosis at liver biopsy only.

(pt 28) is a 62-year-old male splenectomised GD type I patient described elsewhere [26].

4.4. Magnetic resonance iron quantification

Liver iron quantification by magnetic resonance had been performed in 7 patients with GD type I on treatment with ERT (Table 4). Iron overload was observed in 6/7 (85%) patients, ranging from 50 to 280 μmol/g (reference value (RV): <36). All the patients with iron overload had high ferritin values, ranging from 244 to 3011 ng/mL. Two patients had a high level of iron overload (>79 μmol/g [27]) – one was a 55-year-old male patient whose MRI

Table 4
Patients screened for hepatic iron overload with magnetic resonance.

Patient	Age (y)	Gender	*HFE* genotype	Ferritin (ng/dL)	Transferrin saturation	Treatment at exam	Time on treatment (months)	Iron concentration (μmol/g)
10	26	M	p.Cys282Tyr/wt	525	–	I	I(196)	70
15	24	F	wt/wt	298.4	30.7%	I	I(160)	50
23	34	F	p.His63Asp/wt	835	25%	None	–	5
25A	44	F	p.His63Asp/wt	937.1	50.8%	I	M(11) T(1) I(8)	55
26B	55	M	p.Cys282Tyr/wt	1813	47.2%	I	I(55)	280
27	59	F	wt/wt	347	44%	T	I(124) T(2) I(8) T(41)	65
30	63	F	wt/wt	3011	17.4%	M	I(12) M(22)	210

All patients are type I. Ferritin and transferrin saturation values given are approximately from the time of the MR iron quantification. Reference values: ferritin (males) <300 ng/mL; ferritin (females) < 150 ng/mL; transferrin saturation 25–45%; iron concentration < 6 μmol/g. y = years-old; *HFE* = homeostatic iron regulator gene; F = female; M = male; wt = wild-type; I = imiglucerase; T = taliglucerase; M = miglustat.

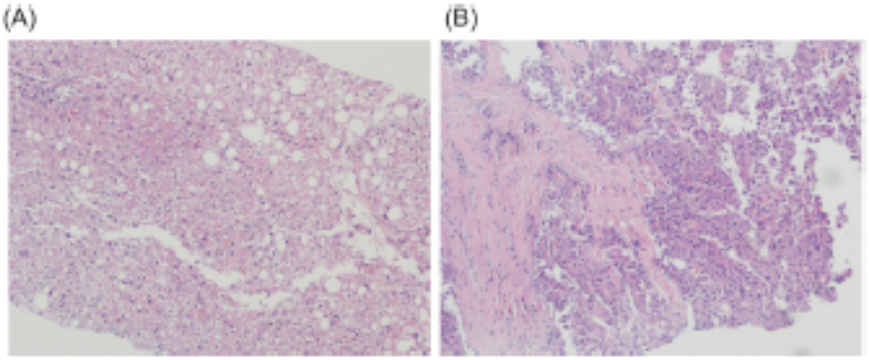


Fig. 1. A: Haematoxylin and eosin, 200× magnification. Liver biopsy of patient 26B showing macrovesicular steatosis in approximately 5% of hepatocytes, as well as a small focus of mixed inflammation (upper left corner). Fig. 1B: Haematoxylin and eosin, 400× magnification. Liver biopsy of patient 28 showing thick bridging fibrosis characteristic of cirrhosis, as well as substitution of the local hepatic parenchyma by moderately differentiated hepatocellular carcinoma cells.

Table 5
Findings in the liver biopsy.

Patient	Age (y) at biopsy	Treatment at biopsy (IU/Kg)	Time on treatment (months)	Inflammation	Steatosis	Siderosis	Gaucher cells	Fibrosis	Other findings
6	20	None	–	No	No	No	Yes	Pericinusoidal	No
13*	27	E	I(120) T(1) I(6) E(41) I(8) E (9)	Steatohepatitis		No	No	No	No
23*	34	None	–	No	No	No	Yes	Bridging	No
25A*	44	I [30]	M(1) T(1) I(14)	Mild	Mild	Mild	Yes	No	Cholestasis
25B*	49	M	M(11)	No	Mild	Mild	No	No	No
26B	56	I [30]	I(69)	Steatohepatitis		Severe	No	No	No
28	60	I [15]	I(34) T(2) I(72)	No	No	Severe	Yes	Cirrhosis	HCC
30*	63	M	I(12) M(22)	Mild	No	Moderate	Yes	No	Nuclear glycogenosis

y = years-old; Sx = splenectomy; E = eliglustat; I = imiglucerase; M = miglustat; HCC = hepatocellular carcinoma. In patient 28, HCC was noted only in a second biopsy, performed 9 months after the first one. *patients with normal liver enzymes; see Table 1.

showed a concentration of 280 μmol/g of iron in the liver, with ferritin at 1813 ng/mL and transferrin saturation at 47.3% (RV 20–45%), steatosis, and a heterozygous c.845G>A, p.Cys282Tyr variant in *HFE* gene. The other patient with high iron levels (210 μmol/g) is a 64-year-old female who had ferritin at 3011 ng/dL and low transferrin saturation at 17.4%. There were no signs of steatosis and she doesn't harbour any pathogenic variant in *HFE* gene.

4.5. Liver biopsy

Six patients with GD type I had a liver biopsy done (Fig. 1; Table 5) when on-treatment. One patient was found to have Gaucher cells in the liver parenchyma; One patient had atypical Gaucher cells in a cirrhotic parenchyma with severe iron overload in hepatocytes and Kupffer cells, and, in a subsequent biopsy, a moderately differentiated HCC [26]. Two patients who have had mild to moderate steatosis on ultrasound had a biopsy confirming macrovesicular steatosis – one also with evidence of cholestasis and a few foci of inflammation, and the other with mild haemosiderosis. Two patients had steatohepatitis with mild activity: a 27-year-old female with a BMI of 28.8 Kg/m^2 who did not show any sign of steatosis in the ultrasound, had normal serum blood glucose and lipid profile except for a low HDL (which is expected in GD) and that was on SRT with eliglustat at the time of the biopsy; and a 58 year-old man had moderate-to-severe haemosiderosis of hepatocytes and Kupffer cells, elevated triglycerides and total and LDL cholesterol, and low HDL, albeit a normal blood glucose, and signs of steatosis in the liver ultrasound, and that was on ERT at the time of biopsy.

Two patients with GD type I underwent liver biopsy before treatment initiation. A 34-year-old female's biopsy showed bridging (stage 3) fibrosis and scattered Gaucher cells; in the other, a 20-year-old woman, peri-sinusoidal fibrosis was noted together with high serum AST, ALT, and γGT, and a normal liver ultrasound.

5. Discussion

For the past few decades, the liver involvement in GD has become subject of great importance in the patients' management. It is now recognised that hepatomegaly is only one of the manifestations of hepatic compromise in GD, and more attention is needed to all the possible comorbidities that may arise from it. In our cohort, we observed that a significant number of patients have mildly increased markers of hepatic and biliary damage before the treatment initiation and throughout the clinical follow-up, indicating that a low-grade process of liver damage is not fully corrected by the treatment. This finding resembles the study by James et al. from when effective treatment for GD was not available [28], in which most patients with GD had mild-to-moderate transaminase elevations. In more recent cohorts, these alterations have also been found in a lesser proportion of patients [20,21]. However, the impact of these alterations is still unclear. Nascimbeni et al. have shown that levels of liver enzymes are not correlated with liver fibrosis [20]. The contribution of chronic liver damage to the development of other complications such as iron deposition, since chronic hepatitis and liver disease are strongly associated with hemosiderosis [29], has not been fully explored to date. The high frequency of patients with elevations in γGT may also be related to the known biliary alterations caused by GD [30] such as changes in bile composition, increased incidence of cholelithiasis, or with the chronic inflammatory process that happens in the disease [3,4,24] causing biliary damage.

A significant proportion of patients had bilirubin elevations, both before and during treatment. Most elevated bilirubin values corresponded to direct bilirubin, which points toward a biliary cause rather than overproduction (e.g., haemolysis). It is difficult to establish a clinical significance of this finding. It is known that GlcCer and glucosylsphingosine (GlcSph) [31,32] interact with a series of transporters of the ABC (ATP-binding cassette) family, including ABCB1 [33]; it is also known that the bile of patients with GD is different than in the general

population, being composed of lower total lipid concentration and, in some patients, high relative concentration of sphingolipids [30]; and finally, that ABC transporters such as ABCB1 are capable of transporting GlcCer and GlcSph [34] across cell membranes, and are modulated by these complex lipids [33]. ABCB1 is localized at the canalicular membrane contributing to the bile formation and xenobiotic excretion [35] – it is possible that, due to ABC-mediated efflux, the higher levels of GlcCer present in bile [36] lead to canalicular disturbances that may cause an impaired flow of bilirubin, leading to the slightly high levels of DB observed.

Iron homeostasis is being increasingly recognised as a key factor of GD's pathogenesis [25]. In a recent article by Lefèbvre et al. [37], it was reported that a local overstimulation of hepcidin related to the lower enzymatic activity of GCase causes iron to be sequestered within macrophages and other cell types, leading to a lower level of free iron, transferrin-bound iron and a higher production of ferritin by the liver. In our study, we observed that several patients with GD have high hepatic iron levels as measured by magnetic resonance, two of the tested patients with levels consistent with severe iron overload – whilst in one patient it may be caused by other risk factors such as alcoholism, steatohepatitis, and a pathogenic *HFE* variant, in the other patient the only obvious risk factor is obesity, and the low transferrin value with exceedingly high ferritin confirm the predictions by Lefèbvre et al. Other studies have observed increase liver iron concentration in GD patients [38], with a positive correlation with serum ferritin concentration. On liver biopsy, positive iron staining has been described extensively [28,39] both in Kupffer cells and in hepatocytes, similar to what was observed in our cohort. Data on pre- (median = 19%, $n = 8$ patients) and post-treatment (median = 28%, $n = 13$ patients) values for serum transferrin saturation in this cohort have been described by Koppe *et al* [25], with no significant difference ($p = .138$).

The main ultrasound finding in our cohort was steatosis, with predominance in overweight/obese patients. Our findings differ from the Israeli cohort, which has a much lower prevalence of fatty liver and a higher prevalence of focal lesions [40]. In the Israeli study, 500 patients were evaluated by US, of which 39 had ultrasonographic evidence of hepatic disease – of these, two-thirds were on ERT and one-fourth was splenectomised. ERT is a potent inducer of weight gain due to slowing the increased basal metabolic rate of patients before treatment [19,41]; thus, it may be difficult to establish whether the high prevalence of steatosis is a manifestation of GD itself, a complication of its treatment, or a comorbidity. A significant proportion of our patients had dyslipidaemia, which indicates that metabolic syndrome may play a role as a confounder in the development of steatosis in these patients [42]. Remarkably, a young patient being treated with eliglustat that had a hepatic biopsy done during cholecystectomy was diagnosed with steatohepatitis, regardless of having no signs of steatosis. This case raises two questions: whether ultrasound can be relied upon as a mean of screening for liver disease in GD patients; and whether steatohepatitis may be a manifestation of GD, since the only known risk factor that the patient had for steatohepatitis (a BMI of 28.8 Kg/m^2) is hardly considered enough for a sole causal factor; and, as the blood glucose and lipid levels of this patient were normal except for a low HDL, which is a marker of GD, dyslipidaemia and metabolic syndrome are not strongly suspected. Another possible cause for the steatohepatitis in this patient could be what is becoming known as "lean fatty liver disease" – that is, non-alcoholic steatosis (NAFLD) or steatohepatitis (NASH) in patients with few or no risk factors for such [43]. Although in the classical definition of "lean NASH" the patient's BMI is normally < 25 Kg/m^2 [43], despite some authors advocating for the use of a BMI of < 30 Kg/m^2 in Western populations [44], it is expected that patients with "non-lean NASH" are male, of older age, and have hypertension, insulin resistance, or hypercholesterolaemia - none of which is present in this patient [43]. It is speculated that lean NASH arises from "metabolic obesity" in non-obese people, which is reflected by the higher distribution of fat to the visceral intraabdominal organs [44,45], along with classical risk factors such as insulin resistance and hypercholesterolaemia [44] – none of which were present in this patient – and genetic predisposition due to polymorphisms in genes associated with lipid metabolism [44,46].

Liver fibrosis is shown to be increased in a significant proportion of patients [20], especially in those who were splenectomised [38], and it is a major risk factor for HCC [39]. Liver fibrosis is correlated with increased severity of GD [20], although its correlation with biomarkers of disease activity is still controversial [20,38]. In the pre-ERT era, when no specific treatment for GD was available, liver fibrosis was a common finding [28], and often culminated in a massive central area of hypocellular fibrotic tissue [47,48] that led to portal hypertension and other clinical manifestations of cirrhosis [28].

Cholelithiasis is a frequent comorbid process of GD with about 30–45% [49,50] lifetime incidence in these patients. Although the causes for this increased incidence are not completely elucidated, some authors speculate that the excretion of GlcCer in the bile may increase its lithogenicity, predisposing to the formation of gallstones [30,36,49]. In our cohort, we have observed a similarly increased prevalence of cholelithiasis in GD patients compared to the general population, with 12 patients affected in a total of 41.

6. Conclusion

In this study, we presented a comprehensive summary of the hepatic manifestations in a well-characterised cohort of patients with GD, showing that several patients have lingering alterations that may indicate a smouldering process of liver damage which is not completely avoided by standard therapy. It is also noticeable that many patients have liver steatosis or steatohepatitis, with a noticeable increase in prevalence during treatment with ERT, but it is still unclear whether it reflects a consequence from the treatment, a feature of the disease, or a coincidental finding.

Acknowledgements

We thank our undergraduate students Luciana Rizzon, Vitória Schütt Zitzmer, and Ana Paula Becker, who helped with keeping the Reference Centre database updated. We are also grateful to the staff of the Medical Genetics Service - Hospital de Clínicas de Porto Alegre, Brazil for the support.

Financial support

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) and by the Fundo de Apoio a Pesquisa e Eventos (FIPE) of the Hospital de Clínicas de Porto Alegre.

All authors disclose no potential personal or financial conflicts of interest regarding this publication

RTS and IVDS designed the study, reviewed the literature, collected and analysed the data, and wrote the manuscript. IVDS supervised the study and obtained funding. FPV, ADD and MRAS helped analysing the data and reviewed the manuscript. SPB and MS performed the molecular analyses and reviewed the manuscript. MLAP reviewed the manuscript. CTSC helped collecting and analysing the histological data and reviewed the manuscript.

Author statement

All authors (Rodrigo Tzovenos Starosta, Filippo Pinto e Vairo, Alícia Dorneles Dorneles, Suélen Porto Basgalupp, Marina Siebert, Maria Lúcia Alves Pedroso, Carlos Thadeu Schmidt Cerski, Mário Reis Álvares-da-Silva, Ida Vanessa Doederlein Schwartz) disclose no potential personal or financial conflicts of interest regarding this publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2019.100564.

References

[1] J. Stirnemann, N. Belmatoug, F. Camou, et al., A review of Gaucher Disease pathophysiology, clinical presentation and treatments, Int. J. Mol. Sci. 18 (2) (2017).
[2] P. GM, D.A.H. Gaucher Disease, Seattle, WA: GeneReviews, (2000).
[3] E. Aflaki, N. Moaven, D.K. Borger, et al., Lysosomal storage and impaired autophagy lead to inflammasome activation in Gaucher macrophages, Aging Cell 15 (1) (2016) 77–88.
[4] J.M. Mucci, M.F. Custilo, I. Kistnovsky, M. Larroude, M.V. Delpino, P.A. Rozenfeld, Proinflammatory and procatecolastogenic potential of peripheral blood mononuclear cells from Gaucher patients: implication for bone pathology, Blood Cells Mol. Dis. 55 (2) (2015) 134–143.
[5] M.K. Pandey, T.A. Burrow, R. Rani, et al., Complement drives glucosylceramide accumulation and tissue inflammation in Gaucher disease, Nature. 543 (7643) (2017) 108–112.
[6] M.K. Pandey, G.A. Grabowski, J. Kohl, An unexpected player in Gaucher disease: the multiple roles of complement in disease development, Semin. Immunol. 37 (2018) 30–42.
[7] K.J. Ringbom, A.M. Asghari, J.I. Castillo-Quan, The emerging role of autophagic-lysosomal dysfunction in Gaucher disease and Parkinson's disease, Neural Regen. Res. 12 (3) (2017) 380–384.
[8] E.A. Liu, A.P. Lieberman, The intersection of lysosomal and endoplasmic reticulum calcium with autophagy defects in lysosomal diseases, Neurosci. Lett. 697 (2018) 10–16.
[9] F.L. Oliveira, T. Alegra, A. Dornelles, et al., Quality of life of brazilian patients with Gaucher disease and fabry disease, JIMD Rep. 7 (2013) 31–37.
[10] M. Cerón-Rodríguez, E. Barajas-Colón, L. Ramírez-Devars, C. Gutiérrez-Camacho, J.L. Salgado-Loza, Improvement of life quality measured by Lansky Score after enzymatic replacement therapy in children with Gaucher disease type 1, Mol Genet Genomic Med. 6 (1) (2018) 27–34.
[11] L.L. Bennett, D. Mohan, Gaucher disease and its treatment options, Ann. Pharmacother. 47 (9) (2013) 1182–1193.
[12] A. Van Rooyen, M. Holsopple, Enzyme replacement or substrate reduction? A review of Gaucher Disease treatment options, Hosp. Pharm. 51 (7) (2016) 553–563.
[13] H. Ben Turkia, D.E. Gonzalez, N.W. Barton, et al., Velaglucerase alfa enzyme replacement therapy compared with imiglucerase in patients with Gaucher disease, Am. J. Hematol. 88 (3) (2013) 179–184.
[14] D. Elstein, A. Mehta, D.A. Hughes, et al., Safety and efficacy results of switch from imiglucerase to velaglucerase alfa treatment in patients with type 1 Gaucher disease, Am. J. Hematol. 90 (7) (2015) 592–597.
[15] H. Ida, A. Tanaka, T. Matsubayashi, et al., A multicenter, open-label extension study of velaglucerase alfa in Japanese patients with Gaucher disease: results after a cumulative treatment period of 24months, Blood Cells Mol. Dis. 59 (2016) 140–147.
[16] A. Zimran, M. Wajnrajch, B. Hernandez, G.M. Pastores, Taliglucerase alfa: safety and efficacy across 6 clinical studies in adults and children with Gaucher disease, Orphanet J Rare Dis. 13 (1) (2018) 36.
[17] P.K. Mistry, E. Lukina, H. Ben Turkia, et al., Outcomes after 18 months of eliglustat therapy in treatment-naive adults with Gaucher disease type 1: the phase 3 ENGAGE trial, Am. J. Hematol. 92 (11) (2017) 1170–1176.
[18] T. Adar, Y. Ilan, D. Elstein, A. Zimran, Liver involvement in Gaucher disease - Review and clinical approach, Blood Cells Mol. Dis. 58 (2016) 66–73.
[19] F. Nascimbeni, A. Dalla Salda, F. Carubbi, Energy balance, glucose and lipid metabolism, cardiovascular risk and liver disease burden in adult patients with type 1 Gaucher disease, Blood Cells Mol. Dis. 58 (2016) 74–80.
[20] F. Nascimbeni, E. Cassinerio, A. Dalla Salda, et al., Prevalence and predictors of liver fibrosis evaluated by vibration controlled transient elastography in type 1 Gaucher disease, Mol. Genet. Metab. 125 (1-2) (2018) 64–72.
[21] S.D. Serai, A.P. Naidu, T. Andrew Burrow, C.E. Prada, S. Xanthakos, A.J. Towbin, Correlating liver stiffness with disease severity scoring system (DSS) values in Gaucher disease type 1 (GD1) patients, Mol. Genet. Metab. 123 (3) (2018) 357–363.
[22] C.T. Fennette, A.J. Isaacson, I. Bargellini, S. Saab, A.G. Singal, A practical guideline for hepatocellular carcinoma screening in patients at risk, Mayo Clin Proc Innov Qual Outcomes. 3 (3) (2019) 302–310.
[23] FdA Figueiredo, Saúde Md (Ed.), Fleuman MAdA. PCDT - Protocolo de Diretrizes Clínicas e Terapêuticas para Doença de Gaucher, 2017, pp. 1–25.
[24] F. Vairo, T. Alegra, A. Dornelles, S. Mittelstadt, C.B. Netto, I.V. Schwartz, Hyperimmunoglobulinemia in pediatric Gaucher patients in Southern Brazil, Pediatr. Blood Cancer 59 (2) (2012) 339.
[25] T. Koppe, D. Doneda, M. Siebert, et al., The prognostic value of the serum ferritin in a southern Brazilian cohort of patients with Gaucher disease, Genet. Mol. Biol. 39 (1) (2016) 30–34.
[26] R.T. Starosta, E. Pinto, F. Vairo, A.D. Dornelles, C.T.S. Genkt, M.R. Álvares-da-Silva, I.V.D. Schwartz, Hepatocellular carcinoma in Gaucher disease: reinforcing the proposed guidelines, Blood Cells Mol. Dis. 74 (2019) 34–36.
[27] J.M. Aldrítza Echeverría, A. Castiella, J.I. Emparanza, Quantification of iron concentration in the liver by MRI, Insights Imaging. 3 (2) (2012) 173–180.
[28] S.P. James, F.W. Stromeyer, C. Chang, J.A. Barranger, Liver abnormalities in patients with Gaucher's disease, Gastroenterology. 80 (1) (1981) 126–133.
[29] Y. Deugnier, B. Turlin, Pathology of hepatic iron overload, World J. Gastroenterol. 13 (35) (2007) 4755–4760.
[30] T.H. Taddei, J. Dzira, S. Chen, et al., High incidence of cholesterol gallstone disease in type 1 Gaucher disease: characterizing the biliary phenotype of type 1 Gaucher disease, J. Inherit. Metab. Dis. 33 (3) (2010) 291–300.
[31] V. Murugesan, W.L. Chuang, J. Liu, et al., Glucosylsphingosine is a key biomarker of Gaucher disease, Am. J. Hematol. 91 (11) (2016) 1082–1089.
[32] J. Lukas, C. Cozma, F. Yang, et al., Glucosylsphingosine causes Hematological and visceral changes in mice-evidence for a pathophysiological role in Gaucher Disease, Int. J. Mol. Sci. 18 (10) (2017).
[33] V. Gousse-Andersson, M.C. Cabot, Glycosphingolipids and drug resistance, Biochim. Biophys. Acta 1758 (12) (2006) 2096–2103.
[34] J. Neumann, D. Rose-Sperling, U.A. Hellmich, Diverse relations between ABC transporters and lipids: an overview, Biochim. Biophys. Acta 1859 (4) (2017) 605–618.
[35] M.M. Pires, D.M. Emmert, J.A. Chmielewski, C.A. Hrycyna, ABCB1 and ABCG2: deciphering the role of human efflux proteins in cellular and tissue permeability, in: K.J. Linton, B. Holland (Eds.), The ABC Transporters of Human Physiology and Disease, First ed, 1 World Scientific, Singapore, 2011, pp. 94–103.
[36] P.G. Pentchev, A.E. Gal, R. Wong, et al., Biliary excretion of glycolipid in induced or inherited glucosylceramide lipidosis, Biochim. Biophys. Acta 665 (3) (1981) 615–618.
[37] T. Lefebvre, N. Reihani, R. Daher, et al., Involvement of hepcidin in iron metabolism dysregulation in Gaucher disease, Haematologica. 103 (4) (2018) 587–596.
[38] A.E. Sobin, L. van Dussen, E.M. Akkerman, et al., Liver fibrosis in type I Gaucher disease: magnetic resonance imaging, transient elastography and parameters of iron storage, PLoS One 8 (3) (2013) e57507.
[39] M. Regenboog, L. van Dussen, J. Verhoij, et al., Hepatocellular carcinoma in Gaucher disease: an international case series, J. Inherit. Metab. Dis. 41 (5) (2018) 819–827.
[40] I. Hadas-Halpern, M. Deeb, A. Abrahamov, A. Zimran, D. Elstein, Gaucher disease: spectrum of sonographic findings in the liver, J. Ultrasound Med. 29 (5) (2010) 727–733.
[41] D. Doneda, C.B. Netto, C.C. Moulin, I.V. Schwartz, Effects of imiglucerase on the growth and metabolism of Gaucher disease type I patients: a systematic review, Nutr. Metab. (Lond.) 10 (1) (2013) 34.
[42] D. Doneda, A.L. Lopes, B.C. Teixeira, S.D. Mittelstadt, C.C. Moulin, I.V. Schwartz, Ghrelin, leptin and adiponectin levels in Gaucher disease type I patients on enzyme replacement therapy, Clin. Nutr. 34 (4) (2015) 727–731.
[43] Z.M. Younossi, M. Stepanova, F. Negro, et al., Nonalcoholic fatty liver disease in lean individuals in the United States, Medicine (Baltimore) 91 (6) (2012) 319–327.
[44] J.J. Yoo, W. Kim, M.Y. Kim, et al., Recent research trends and updates on non-alcoholic fatty liver disease, Clin Mol Hepatol. 25 (1) (2018) 1–11.
[45] J. Wattacherril, A.J. Sanyal, Lean NAFLD: an Underrecognized outlier, Curr. Hepatol. Rep. 15 (2) (2016) 134–139.
[46] S. Sookoian, C.J. Pirola, Genetic predisposition in nonalcoholic fatty liver disease, Clin Mol Hepatol. 23 (1) (2017) 1–12.
[47] R.R. Glass, A.K. Poznanski, S. Young, M.A. Urban, Gaucher disease of the liver: CT appearance, Pediatr. Radiol. 17 (5) (1987) 417–418.
[48] R.H. Lachmann, D.G. Wight, D.J. Lomax, et al., Massive hepatic fibrosis in Gaucher's disease: clinico-pathological and radiological features, QJM. 93 (4) (2000) 237–244.
[49] H. Rosenbaum, E. Sidransky, Cholelithiasis in patients with Gaucher disease, Blood Cells Mol. Dis. 28 (1) (2002) 21–27.
[50] A. Zimmermann, R.A. Popp, C. Al-Khzouz, et al., Cholelithiasis in patients with Gaucher Disease type 1: risk factors and the role of ABCG5/ABCG8 gene variants, J Gastrointestin Liver Dis. 25 (4) (2016) 447–455.

Artigo 2: “Hepatocellular carcinoma in Gaucher disease: reinforcing the proposed guidelines”. Rodrigo Tzovenos Starosta, Filippo Pinto e Vairo, Alícia Dorneles Dornelles, Carlos Thadeu Schmidt Cerski, Mário Reis Álvares-da-Silva, Ida Vanessa Doederlein Schwartz.

Artigo publicado no periódico Blood Cells, Molecules, and Diseases, 2019; 74: 34-6.

Blood Cells, Molecules and Diseases 74 (2019) 34–36

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

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

Letter to the Editor

Hepatocellular carcinoma in Gaucher disease: Reinforcing the proposed guidelines

ARTICLE INFO

Editor: Narla Mohandas

To the Editor,

A recent international collaborative case series [1] of 16 patients with GD type 1 (GD1) showed a clear association of hepatocellular carcinoma (HCC) with splenectomy and liver cirrhosis. In a previous article [2], Regenboog and colleagues reported a high prevalence of focal hepatic lesions in GD1 patients (25%), with an apparent association with splenectomy. In that cohort, four patients were diagnosed with HCC, and eighteen with gaucheroma, making the diagnosis of the latter 4.5-fold more common than the former. However, there was one case in which the ultrasonographic image was suggestive of HCC (a hypoechoic and hypervascular nodule) but pathological examination confirmed that it was a gaucheroma, showing that it is possible for these tumours to emulate the imaging of HCC. Conversely, two confirmed cases of HCC did not present with the complete set of typical findings of arterial wash-in and venous wash-out on contrast-enhanced computed tomography (CECT). Based on these findings, the authors suggested an algorithm for the evaluation of focal hepatic lesions in GD1, in which splenectomized patients with lesions ≥ 1 cm presenting arterial wash-in and venous wash-out on CECT should be diagnosed immediately as having an HCC. In the Gaucher Reference Centre of the Hospital de Clínicas de Porto Alegre (HCPA), we follow a 62-year-old male patient of non-Jewish descent who was diagnosed with GD1 by enzymatic and genetic assays (genotype: N370S/RecNciI). He had hepatosplenomegaly and thrombocytopenia since age 42, and a splenectomy at age 49 yielded the suspicion of GD1 upon the histological identification of Gaucher cells. During that time, a liver biopsy showed micronodular cirrhosis (classified as Child-Pugh A). His first appointment at the Gaucher clinic was at 50 years of age, and he had no thrombocytopenia (231,000 platelets/mm^3, NRV 150,000–400,000) nor anaemia (Hb 14.7 g/dL, NRV 12.5–16.0), a mildly elevated AST (53 U/dL, NRV < 42), normal ALT and high γGT (363 U/dL, NRV < 40), chitotriosidase (7271 nmol/h/mL, NRV 8.8–132), and ferritin levels (3392 ng/dL, NRV 30–300). Enzyme replacement therapy (ERT) with imiglucerase was initiated at 15 IU/kg/2 weeks when he was 52. When he was 58, a routine abdominal ultrasonography (AUS) showed a liver nodule of 1.2 cm in diameter, which was confirmed by a CECT to have an arterial wash-in without venous washout pattern that disappeared two years later. After two years, another nodule seen on AUS followed by an abdominal CECT showed two liver nodules, the bigger measuring 1.8 × 1.4 cm with both arterial wash-in and venous washout, suggesting HCC. A CECT performed three months later did not detect the smaller nodule but confirmed the characteristics of the bigger one (Fig. 1). Meanwhile, the patient had unintentionally lost 3.1 kg (5.7% of his body weight), what was attributed to his recent diagnosis of symptomatic primary hypolactasia. By the time of the second CT, laboratory exams showed a mildly increased AST (57 U/dL), a highly increased γGT (196 U/dL), a slightly increased alpha-fetoprotein (11.5 pg/mL, NRV < 10), a normal transferrin saturation (36.8%, NRV 25–45%), and a high, although lower than at the diagnosis, serum ferritin level (627 ng/dL). Other exams, including a comprehensive viral hepatitides panel and genotyping for hereditary haemochromatosis, were negative. Due to the absence of tumour growth in the three months between the CTs and that liver gaucheromas may mimic HCC [2], we decided to perform a percutaneous core needle biopsy of the lesion.

The biopsy showed a cirrhotic liver with multiple engorged, micro vacuolar Gaucher cells, some clumped together in groups of approximately five cells (Fig. 2), similar, except for the microvacuolation, to the aspect described by Korula and colleagues [3]. Perls staining was strongly positive in hepatocytes and Gaucher cells. No neoplastic tissue was found. Based on these findings and a previous report of micro vacuolar Gaucher cells in an extraosseous gaucheroma [4], the lesion was diagnosed as gaucheroma, and imiglucerase dosage was increased to 30 IU/kg biweekly. In the subsequent months, the mass continued to grow, reaching 2.4 cm after 9 months. As alpha-fetoprotein also rose to 36.7 pg/mL, a new biopsy was performed which showed a moderately-differentiated HCC. The patient was staged with CTs and a bone scan which detected a subpleural nodule (diagnosed by biopsy as fungal granuloma) and a 1.4 cm intrahepatic metastasis. He has now successfully undergone transarterial chemoembolization and is listed for liver transplantation.

In a core needle liver biopsy, only about one-fifty-thousandth of the total liver volume is sampled [5], which implicates that inevitable sampling errors tend to occur. Because of this, we advocate that any nodule in patients with GD1 and at high risk of developing HCC, such as splenectomy and cirrhosis as in our patient's should be managed as HCC, regardless the possibility of being a gaucheroma. Therefore, we support the algorithm proposed by Regenboog and colleagues, hoping that it may help to avoid unnecessary delay in the management of such patients.

Declaration of interests

None.

https://doi.org/10.1016/j.bcmd.2018.10.004
Received 15 July 2018; Accepted 17 October 2018
Available online 18 October 2018

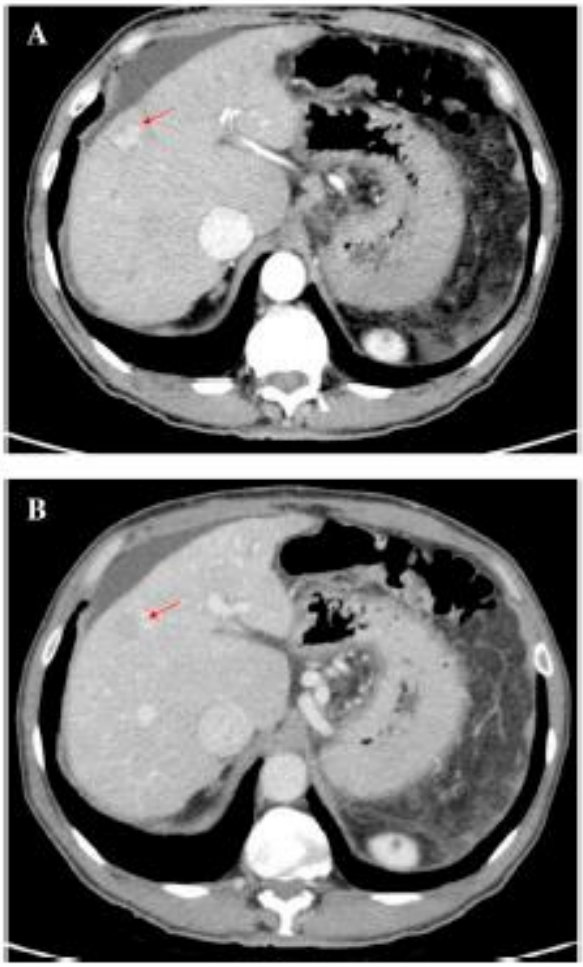


Fig. 1. CECT findings. A: arterial phase showing a discrete lesion in the left lobe of the liver (red arrow) with contrast wash-in. B: venous phase showing complete wash-out of contrast in the same lesion (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

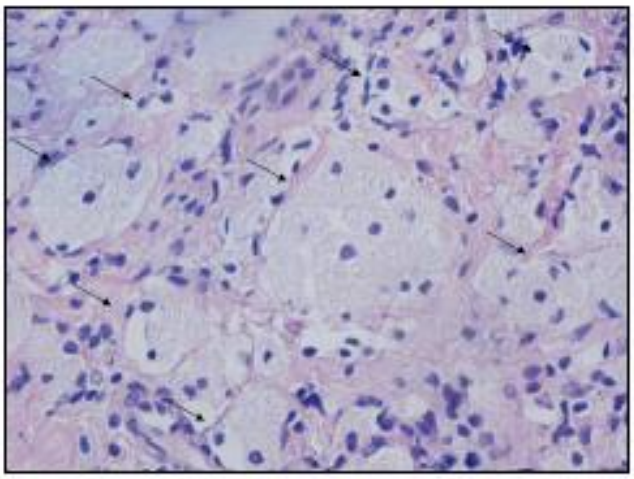


Fig. 2. Photomicrograph, haematoxylin and eosin, 400×. Fibrotic liver tissue with individual and grouped plump, microvacuolar macrophages (black arrows) diagnosed as atypical Gaucher cells in a gaucheroma.

Contribution

RTS, FPV, ADD and IVDS participated in the patient care in the Medical Genetics Service. RTS wrote the first version of the manuscript. FPV and IVDS reviewed the first version of the manuscript and co-wrote with RTS the next versions. CTSC performed the histological diagnoses and participated in the radiographical-pathological correlation. RTS and CTSC reviewed the literature on the liver histology of Gaucher disease. MRAS participated in the patient care at the Gastroenterology and Hepatology Service. MRAS, RTS, FPV, ADD, and IVDS reviewed the literature on hepatocellular carcinoma in Gaucher disease and on liver gaucheromas, and gathered to form a consensus opinion. ADD, CTSC, MRAS and IVDS reviewed the manuscript. All authors approve the final manuscript.

Acknowledgements

We are grateful to the patient and to the staff of the Hospital de Clínicas de Porto Alegre for all the support.

Funding source

None.

References

[1] M. Regenboog, L. van Dussen, J. Verheij, N.J. Weinreb, D. Santoro, S. Vom Dahl, D. Häussinger, M.N. Müller, A. Canbay, M. Rigoldi, A. Piperno, T. Dinur, A. Zimran, P.K. Mistry, K.Y. Salah, N. Belmatoug, D.J. Kuter, C.E.M. Hollak, Hepatocellular carcinoma in Gaucher disease: an international case series, J. Inherit. Metab. Dis. 41 (5) (2018) 819–827.

[2] M. Regenboog, A.E. Bohte, I. Somers, O.M. van Delden, M. Maas, C.E. Hollak, Imaging characteristics of focal splenic and hepatic lesions in type 1 Gaucher disease, Blood Cells Mol. Dis. 60 (2016) 49–57.

[3] S. Korula, P. Owens, A. Charlton, K. Bhattacharya, Rare case of hepatic Gaucheroma in a child on enzyme replacement therapy, JIMD Rep 32 (2017) 101–104.

[4] M. Ivanova, R.P. Limgala, E. Changotia, R. Kamath, C. Ioanou, O. Goker-Alpan, Gaucheromas: when macrophages promote tumor formation and dissemination, Blood Cells Mol. Dis. 68 (2018) 100–105.

[5] E.R. Tapper, A.S.F. Lok, Use of liver imaging and biopsy in clinical practice, N. Engl. J. Med. 377 (2017) 2296–2297.

Rodrigo Tzovenos Starosta[a,b], Filippo Pinto e Vairo[c,d,g,*], Alicia Dorneles Dornelles[c], Carlos Thadeu Schmidt Cerski[a,e], Mario Reis Álvares-da-Silva[a,f], Ida Vanessa Doederlein Schwartz[a,b,c]

[a] School of Medicine, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[b] Postgraduate Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, Brazil

[c] Medical Genetics Service, HCPA, UFRGS, Porto Alegre, RS, Brazil

[d] Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA

[e] Service of Surgical Pathology, HCPA, UFRGS, Porto Alegre, RS, Brazil

[f] Service of Gastroenterology, HCPA, UFRGS, Porto Alegre, RS, Brazil

[g] Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA

E-mail address: fvairo@hcpa.edu.br (F. Pinto e Vairo).

* Corresponding author at: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, 3° Andar, Porto Alegre, RS 90035-903, Brazil.

- Objetivo específico 2

Artigo 3: “Histomorphometric analysis of liver biopsies of treated patients with Gaucher disease type 1: new method for canalicular morphological quantification and evidence for canalicular injury in Gaucher disease”. Rodrigo Tzovenos Starosta, Marina Siebert, Filippo Pinto e Vairo, Bruno Lafaiete de Lima Costa, Christiano Tomaso Ponzoni, Ida Vanessa Doederlein Schwartz, Carlos Thadeu Schmidt Cerski.

Artigo submetido para o periódico American Journal of Clinical Pathology em 17 de abril de 2020.

AJCP

Histomorphometric analysis of liver biopsies of treated patients with Gaucher disease type 1: new method for canalicular morphological quantification and evidence for canalicular injury in Gaucher disease

Journal:	*American Journal of Clinical Pathology*
Manuscript ID	AJCP-2020-04-0181
Manuscript Type:	Original Article
Keywords:	Histomorphometry, Image cytometry, AP Hepatopathology, Canaliculus, Feret diameter

Abstract: Gaucher disease (GD) is an autosomal recessive lysosomal disorder caused by a disturbance in the metabolism of glucocerebroside in the macrophages. Most of its manifestations – hepatosplenomegaly, anemia, thrombocytopenia, and bone pain – are amenable to a macrophage-target therapy such as enzyme replacement. However, there is increasing evidence that abnormalities of the liver persist despite the specific GD treatment. In this work, we adapted histomorphometry techniques to the study of hepatocytes in GD using liver tissue of treated patients. We were able to find distinct absorbance and fractal dimension in hepatocellular nuclei, as well as thinner, less branching, and less abundant biliary canaliculi in GD patients when compared to healthy controls. This indicates that hepatocytes might be affected in GD even during treatment.

Key Points

Histomorphometry can provide a way of quantifying canalicular damage in IHC-stained liver biopsies.

Liver biopsy samples from treated patients with Gaucher disease type 1 show thinner, less branching, less abundant hepatic canaliculi than healthy controls.

Liver biopsy samples from treated patients with Gaucher disease type 1 show distinct hepatocellular nuclear characteristics than healthy controls.

Introduction

Gaucher disease (GD) is one of the most common lysosomal disorders with an estimated prevalence of 1:60,000 in the general population and 1:800 in the Ashkenazi Jewish population[1]. The pathophysiology of GD is classically defined as a disturbance in the processing of sphingolipids inside the macrophages[1,2], being the main manifestations of GD a function of dysregulated macrophagic activation[2,3] and invasion of tissues by Gaucher cells[4,5]. Macrophage-targeted therapy through infusions of recombinant glucocerebrosidase (GCase) which is uptaken via the mannose receptor pathway[6] has been successful in alleviating the key clinical findings in GD – anemia, thrombocytopenia, osteonecrosis, hepatosplenomegaly – and improving quality of life in these patients[7]. However, there are still features of GD that are not fully explained by the involvement of macrophages. It is known that patients with GD, even after long-term treatment, have a higher liver stiffness (which is a surrogate measurement for fibrosis) than controls[8-11]. Moreover, the biliary phenotype of GD has been subject of increasing focus: patients with GD have an increased incidence of gallstones[12-15] and an abnormal bile composition with increased level of glucocerebroside (glucosylceramide; GlcCer) and other sphingolipids such as glucosylsphingosine (GlcSph, alias lyso-GL1)[12,16]. Biliary excretion of GlcCer is being suggested as a protective factor against hepatocellular storage of this substance[16, 17]; however, this export of lysosomal GlcCer into the bile canaliculi may be one of the possible mechanisms of injury to the biliary system in GD through interference with the composition of bile and the function of transporters in the hepatocytic apical membrane. This interaction between GlcCer

and bile transporters has been demonstrated in studies of the same transporters in cancer multidrug resistance[18,19].

Histomorphometry, or histological morphometry, is the quantification of morphology in the tissue level. It is a well-established technique with applications in the research of many tissues, including the liver, where it has been used for the quantification of characteristics such as fibrosis[20,21], immunohistochemical markers[22], and steatosis[23]. It has also been used as a grading and prognostic marker in a variety of tumors[24-26]. Here, we adapted histomorphometry to the study the nuclear and canaliculi parameters in liver biopsies of patients with GD.

Methodology

Samples

We analyzed liver biopsies of patients with GD followed. The samples were preserved in paraffin and were retrieved from the archive of the Service of Surgical Pathology (SSP). The archive of the Service of Surgical Pathology was also searched for liver biopsy samples with a diagnosis of "healthy liver tissue" by an expert liver pathologist (CTSC).

Samples were processed and stained with hematoxylin and eosin (H&E) and with immunohistochemistry (IHC) for CD10 (to highlight the bile canaliculi) according to the SSP protocol. Briefly, samples were cut into 3 µm-thick sections in a microtome and then deparaffinized using the EZ Prep Solution (Ventana Medical Systems, Tucson, Arizona, USA) according to the manufacturer's protocol. Antigen recovery was performed with CC1 buffer at pH 9.0 and 95°C for 2 minutes followed by peroxidase blocking with OptiView Peroxidase Inhibitor (Ventana Medical Systems). The primary rabbit anti-human anti-CD10 monoclonal antibody (clone SP67; Roche Diagnostics, Tucson, Arizona, USA) was incubated for 28 minutes at 36°C. After primary antibody incubation, reaction was detected with the OptiView DAB IHC Detection Kit (Ventana Medical Systems) and slides were counterstained with hematoxylin and bluing reagent ($Li_2CO_3 + Na_2CO_3$).

Imaging

Stained and mounted slides were microphotographed on an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan) using Carl Zeiss lenses (Carl Zeiss AG, Oberkochen, Germany) and an Olympus DP73 microscope-attached camera (Olympus corporation) and the CellSens software (Olympus Corporation) at a magnification of 1000x. Each slide had 6 random high-power fields captured and saved as tagged image file format (.tiff) images to allow for better resolution.

Histomorphometry

Each H&E-stained image was converted from the native RGB color format to 8-bit using the ImageJ software[27]. The pixel-to-µm conversion was calculated from the scale provided by CellSens. For nuclear morphometric analysis, all hepatocytes nuclei in each image were selected

and saved as discrete regions of interest (ROIs). Each ROI was analyzed for nuclear area in μm², nuclear perimeter in μm, mean gray value (MGV), Feret diameter in μm, and, using the FracLac plugin (https://imagej.nih.gov/ij/plugins/fraclac), mean fractal dimension (Davg). For normalization of MGV, random background ROIs were selected in each image, averaged, and, after correction of MGV to absorbance (yielding the "corrected MGV", or "cMGV", variable) with the formula cMGV = 255 – MGV, were subtracted from cMGV to yield the "normalized cMGV" (n-cMGV) variable.

For canalicular histomorphometry, IHC-stained RGB images were treated with the IHC Toolbox plugin (https://imagej.nih.gov/ij/plugins/ihc-toolbox) to isolate IHC-positive areas (i.e., hepatic canaliculi). Images were then converted from native RGB color format to 8-bit. Manual thresholding was used to select all IHC-positive ROIs. ROIs with <10 pixels of area were considered to be artefactual and were excluded from the histomorphometric analysis. Each ROI was then analyzed for area in μm², MGV, perimeter in μm, Feret diameter in μm, and solidity. The perimeter-to-Feret ratio was calculated from obtained values and was used as a measure of canalicular branching, as detailed in Figure 1. MGV was corrected to absorbance (cMGV) with the formula cMGV = 255 – MGV. Due to the background correction obtained with the IHC Toolbox plugin, canalicular cMGV was not normalized. Because of the pattern similarity between the hepatic canaliculi and the bone trabeculae, we used the Map_BoneMicrostructure plugin (https://imagej.nih.gov/ij/plugins/microstructure) to obtain mean canaliculi thickness in μm for whole images.

Statistical analysis

For determination of parametricity, Q-Q plots were used. In the nuclear morphometric analysis, Davg values were found to follow a normal distribution. All other data (nuclear area, nuclear perimeter, Feret diameter, corrected-normalized mean gray value) were found to be non-parametric. All immunohistochemistry particle analysis canaliculi data obtained by particle analysis (*i.e.*, excluding the concatenated data obtained by Map_BoneMicrostructure) were found to be non-parametric. Descriptive statistics are expressed as median ± IQR for non-parametric variables and as mean ± SD for parametric variables. Differences were considered significant when $p<0.05$; p-value correction was performed as described below.

For comparison of continuous variables with dependent observations (*i.e.*, except the data obtained with the Map_BoneMicrostructure plugin), generalized linear models were generated using a gamma log-link distribution in a main effects model. The response was adjusted to account for intra-subject variation and the predictors were factored by group (Gaucher *vs* control).

Data obtained by Map_BoneMicrostructure are concatenated automatically before analysis, overcoming the problem of dependency of observations. These data were analyzed with a Student's T-test. Significance values were not corrected due to the small sample size.=

To account for variation of morphometry among GD patients, a pairwise analysis between controls and each GD patient was performed with Mann-Whitney U-test for non-parametric data and with Student T-test for parametric data. This comparison was not performed

for canaliculi data obtained using the Map_BoneMicrostructure plugin due to the small sample size. Significance values obtained in this analysis were corrected according to Bonferroni's post-hoc procedure.

Results

Patients

Liver biopsy samples of four GD patients were retrieved. Patient characteristics are displayed in Table 1.

Nuclear morphometry and canaliculi histomorphometry

Nuclear morphometry and canaliculi histomorphometry were performed on samples from four patients and four gender-matched healthy controls. There was a significant difference between patients and controls for nuclear area (respectively, in μm^2, 28.16 ± 9.45 *vs* 26.88 ± 9.42, p=0.006), Feret diameter (respectively, in μm, 6.58 ±1.08 *vs* 6.59 ± 1.17, p=0.004), Davg (respectively 1.11 ± 0.06 *vs* 1.09 ± 0.09, p=0.005), and n-cMGV (respectively 4.88 ± 2.52 *vs* 7.68 ± 4.60, p=0.014). No significant difference was observed in nuclear perimeter. When comparing each patient to the controls, both the nuclear Davg and the n-cMGV were significantly different in all comparisons with $p<0.005$.

There was a significant difference between patients and controls in canaliculi cMGV (88.24 ± 59.80 *vs* 173.53 ± 76.78, p=0.001) and perimeter-to-Feret ratio (2.70 ± 0.56 *vs* 2.79 ± 0.66, p=0.006). No difference was observed for canaliculi area, perimeter, Feret diameter, or solidity. The comparison between each patient and the control group is detailed in Table 2.

Discussion

Histomorphometry is a digital image analysis approach that relies on the identification and analysis of morphological elements in a histological section[28]. It is a technique used for diagnosis of neoplasms and to aid in tailoring cancer treatment[29]. We used histomorphometry to analyze hepatocytes, including nuclear and canaliculi parameters, in a population of patients with GD type 1.

In the nuclear analysis, we found that patients' cells had lower absorbance and fractal dimension indicating that there is more euchromatin and differences in the nuclei organization than healthy individuals. Since euchromatin is associated with higher transcriptional status, possible explanations may be the higher basal metabolic rate presented by GD patients in comparison to general population[30,31], as the liver is a known regulator of metabolism[32,33]; or the chronic low-grade inflammation that takes place in these patients changing hepatocyte transcriptional activity[2,34] – however, more specific experiments must be carried on before we can affirm that these events are truly correlated.

In the canaliculi analysis, we found that the patients with GD had thinner, less branching canaliculi than the controls. In contrast with the common cholestatic pattern of many diseases

which consists of canaliculi dilation[35], canaliculi thinning and reduced branching have not been studied as much due to the lack of methods to perform a detailed analysis of these structures. It is known that patients with GD have increased secretion of GlcCer in bile, leading to physiological changes such as upregulation of GBA2, a bile acid 3-O-glucosidase that can also metabolize GlcCer and GlcSph[36], producing toxic compounds such as sphingosine[37]. It is possible that this process leads to biliary injury, thus impacting on the normal canaliculi structure. Engorgement of hepatocytes could also be a potential cause for thinning of canaliculi; however, this was not assessed in this study.

A limitation of this study is the small sample size. GD is a rare metabolic disorder, and with the current technology available in clinical practice for follow-up of these patients – such as transient elastography and magnetic resonance imaging – liver biopsies are seldom performed because of the invasiveness of the procedure and the increased bleeding risk in this group.

In summary, this is the first report of the application of histomorphometry in the study of liver cells in a metabolic disorder. GD is commonly seen as a disorder associated with macrophages disturbance. Here, we provided evidence of the involvement of hepatocytes, with both nuclear and canaliculi changes. We hope that more studies will ensue from the development of this imaging technique in GD and other metabolic diseases so a better understanding of the pathophysiology of these diseases may arise.

Figure 1: The upper panel depicts a scheme of a less-branching canaliculus, for clarification of the perimeter-to-Feret ratio. The solid red line is the canaliculus perimeter (P_1). The solid green line represents the projection of the canaliculus on the Y axis of the image field, which is the Feret Y (Y_1) measure. The solid blue line represents the projection of the canaliculus on the X axis of the image field, which is the Feret X measure (X_1). The Feret diameter of a canaliculus (F_1) is the arithmetic mean of X_1 and Y_1. The lower panel depicts a more-branching canaliculus. The solid red line is the canaliculus perimeter (P_2). The projection of this canaliculus in the Y (Y_2) and X (X_2) axes are depicted as the solid green and blue lines, respectively, and the Feret diameter of this canaliculus (F_2) is the arithmetic mean of X_2 and Y_2. As shown, the increase in branching affects more the perimeter than the Feret diameter of a canaliculus: $P_1 < P_2$, $F_1 \approx F_2$. In this way, $P_1/F_1 < P_2/F_2$. These images are theoretical simplified schemes for clarification and are *per se* not representative of either group.

Table 1. Characteristics of GD patients

Patient	Sex	*GBA* Genotype	Age at biopsy (y)	Treatment at biopsy (months)	Biopsy diagnosis
1	F	p.Glu388Lys/p.Ser405Asn	48	Miglustat (11)	Macrovesicular steatosis
2	F	p.Asn409Ser/p.Leu483Arg	63	Miglustat (22)	Hemosiderosis, presence of Gaucher cells
3	M	p.Asn409Ser/Rec*Nci*I	56	Imiglucerase 30UI/Kg/biweekly (69)	Steatohepatitis with mild activity
4	M	p.Asn409Ser/Rec*Nci*I	61	Imiglucerase	Cirrhosis, hemosiderosis,

						30UI/Kg/biweekly (72)	presence of Gaucher cells

All patients are diagnosed with GD type 1. Y = years-old; F = female; M = male; Rec*Nci*I = recombinant allele with the *GBA1* pseudogene, includes the p.Leu483Arg, p.Ala495Pro, and p.Val499Val variants.

Table 2. Individual patient comparison of histomorphometric canaliculi variables obtained by particle analysis

Sample	Area (μm²)	cMGV	Perimeter (μm)	Feret diameter (μm)	Solidity	Perimeter-to-Feret ratio
Patient 1	0.24 ± 2.22[a]	79.69 ± 50.54	2.23 ± 6.95[a]	0.86 ± 2.16[a]	0.77 ± 0.15[f]	2.68 ± 0.62[a]
Patient 2	1.84 ± 3.19[d]	115.74 ± 38.91[a]	6.53 ± 6.94[c]	2.42 ± 2.57[a]	0.81 ± 0.13	2.70 ± 0.41[b]
Patient 3	0.62 ± 7.43	92.36 ± 68.25[b]	4.11 ± 13.52	1.42 ± 4.13	0.80 ± 0.14	2.77 ± 0.60
Patient 4	1.13 ± 8.72	86.23 ± 68.49	5.23 ± 14.49	1.81 ± 4.66	0.79 ± 0.17	2.72 ± 0.56[e]
Controls	0.68 ± 11.32	81.15 ± 77.93	4.00 ± 16.65	1.42 ± 5.28	0.79 ± 0.15	2.79 ± 0.66

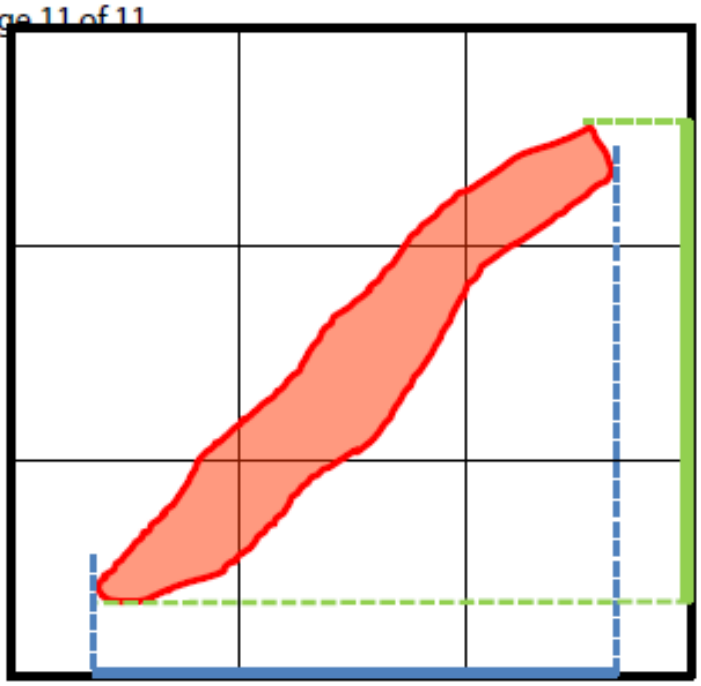
Values shown as median ± IQR. P-values are corrected according to Bonferroni's procedure and reflect the output of a Mann-Whitney U-test between the patient and the control group for the given variable. P-values above 0.05 are not shown in this table. [a]p≤0.006; [b]p=0.006; [c]p=0.018; [d]p=0.024; [e]p=0.03; [f]p=0.034. cMGV = corrected mean gray value.

References

1. Stirnemann J, Belmatoug N, Camou F, et al. A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. *Int J Mol Sci*. 2017;18(2).
2. Aflaki E, Moaven N, Borger DK, et al. Lysosomal storage and impaired autophagy lead to inflammasome activation in Gaucher macrophages. *Aging Cell*. 2016;15(1):77-88.
3. Boven LA, van Meurs M, Boot RG, et al. Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages. *Am J Clin Pathol*. 2004;122(3):359-369.
4. Adar T, Ilan Y, Elstein D, Zimran A. Liver involvement in Gaucher disease - Review and clinical approach. *Blood Cells Mol Dis*. 2016.
5. James SP, Stromeyer FW, Chang C, Barranger JA. Liver abnormalities in patients with Gaucher's disease. *Gastroenterology*. 1981;80(1):126-133.
6. Tekoah Y, Tzaban S, Kizhner T, et al. Glycosylation and functionality of recombinant β-glucocerebrosidase from various production systems. *Biosci Rep*. 2013;33(5).
7. Oliveira FL, Alegra T, Dornelles A, et al. Quality of life of brazilian patients with Gaucher disease and fabry disease. *JIMD Rep*. 2013;7:31-37.
8. Nascimbeni F, Cassinerio E, Dalla Salda A, et al. Prevalence and predictors of liver fibrosis evaluated by vibration controlled transient elastography in type 1 Gaucher disease. *Mol Genet Metab*. 2018.
9. Serai SD, Naidu AP, Andrew Burrow T, Prada CE, Xanthakos S, Towbin AJ. Correlating liver stiffness with disease severity scoring system (DS3) values in Gaucher disease type 1 (GD1) patients. *Mol Genet Metab*. 2018;123(3):357-363.
10. Alves Pedroso ML, Didoné Filho CN, Radunz V, de Barros JA. Liver damage in a patient with Gaucher's disease type 1 and alpha-1 antitrypsin deficiency: a potential epigenetic effect? *J Gastrointestin Liver Dis*. 2019;28(1):121-123.
11. Lipiński P, Szymańska-Rożek P, Socha P, Tylki-Szymańska A. Controlled attenuation parameter and liver stiffness measurements using transient elastography by FibroScan in Gaucher disease. *Mol Genet Metab*. 2019.
12. Taddei TH, Dziura J, Chen S, et al. High incidence of cholesterol gallstone disease in type 1 Gaucher disease: characterizing the biliary phenotype of type 1 Gaucher disease. *J Inherit Metab Dis*. 2010;33(3):291-300.
13. Zimmermann A, Popp RA, Al-Khzouz C, et al. Cholelithiasis in Patients with Gaucher Disease type 1: Risk Factors and the Role of ABCG5/ABCG8 Gene Variants. *J Gastrointestin Liver Dis*. 2016;25(4):447-455.
14. Rosenbaum H, Sidransky E. Cholelithiasis in patients with Gaucher disease. *Blood Cells Mol Dis*. 2002;28(1):21-27.
15. Starosta RT, Vairo FPE, Dornelles AD, et al. Liver involvement in patients with Gaucher disease types I and III. *Mol Genet Metab Rep*. 2020;22:100564.
16. Pentchev PG, Gal AE, Wong R, et al. Biliary excretion of glycolipid in induced or inherited glucosylceramide lipidosis. *Biochim Biophys Acta*. 1981;665(3):615-618.
17. Crawford JM. Role of vesicle-mediated transport pathways in hepatocellular bile secretion. *Semin Liver Dis*. 1996;16(2):169-189.
18. Lee WK, Kolesnick RN. Sphingolipid abnormalities in cancer multidrug resistance: Chicken or egg? *Cell Signal*. 2017;38:134-145.
19. Gouazé-Andersson V, Yu JY, Kreitenberg AJ, Bielawska A, Giuliano AE, Cabot MC. Ceramide and glucosylceramide upregulate expression of the multidrug resistance gene MDR1 in cancer cells. *Biochim Biophys Acta*. 2007;1771(12):1407-1417.

20. Stasi C, Leoncini L, Biagini MR, et al. Assessment of liver fibrosis in primary biliary cholangitis: Comparison between indirect serum markers and fibrosis morphometry. *Dig Liver Dis*. 2016;48(3):298-301.
21. Leite C, Starosta RT, Trindade EN, Trindade MRM, Álvares-da-Silva MR, Cerski CTS. Corrected integrated density: a novel method for liver elastic fibers quantification in chronic hepatitis C. *Surgical and Experimental Pathology*. 2020;3:7.
22. Estep M, Mehta R, Bratthauer G, et al. Hepatic sonic hedgehog protein expression measured by computer assisted morphometry significantly correlates with features of non-alcoholic steatohepatitis. *BMC Gastroenterol*. 2019;19(1):27.
23. Mendes LC, Ferreira PA, Miotto N, et al. Controlled attenuation parameter for steatosis grading in chronic hepatitis C compared with digital morphometric analysis of liver biopsy: impact of individual elastography measurement quality. *Eur J Gastroenterol Hepatol*. 2018;30(8):959-966.
24. Mendaçolli PJ, Brianezi G, Schmitt JV, Marques ME, Miot HA. Nuclear morphometry and chromatin textural characteristics of basal cell carcinoma. *An Bras Dermatol*. 2015;90(6):874-878.
25. Yang W, Tian R, Xue T. Nuclear shape descriptors by automated morphometry may distinguish aggressive variants of squamous cell carcinoma from relatively benign skin proliferative lesions: a pilot study. *Tumour Biol*. 2015;36(8):6125-6131.
26. Mello MR, Metze K, Adam RL, et al. Phenotypic subtypes of acute lymphoblastic leukemia associated with different nuclear chromatin texture. *Anal Quant Cytol Histol*. 2008;30(2):92-98.
27. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9(7):671-675.
28. Madabhushi A, Lee G. Image analysis and machine learning in digital pathology: Challenges and opportunities. *Med Image Anal*. 2016;33:170-175.
29. Barsoum I, Tawedrous E, Faragalla H, Yousef GM. Histo-genomics: digital pathology at the forefront of precision medicine. *Diagnosis (Berl)*. 2019;6(3):203-212.
30. Doneda D, Lopes AL, Oliveira AR, Netto CB, Moulin CC, Schwartz IV. Gaucher disease type I: assessment of basal metabolic rate in patients from southern Brazil. *Blood Cells Mol Dis*. 2011;46(1):42-46.
31. Doneda D, Vairo FP, Lopes AL, et al. Assessment of Basal Metabolic Rate and Nutritional Status in Patients with Gaucher Disease Type III. *JIMD Rep*. 2014;14:37-42.
32. Paglialunga S, Dehn CA. Clinical assessment of hepatic de novo lipogenesis in non-alcoholic fatty liver disease. *Lipids Health Dis*. 2016;15(1):159.
33. Reddavide R, Cisternino AM, Inguaggiato R, et al. Non-Alcoholic Fatty Liver Disease is Associated with Higher Metabolic Expenditure in Overweight and Obese Subjects: A Case-Control Study. *Nutrients*. 2019;11(8).
34. Pandey MK, Burrow TA, Rani R, et al. Complement drives glucosylceramide accumulation and tissue inflammation in Gaucher disease. *Nature*. 2017;543(7643):108-112.
35. Li MK, Crawford JM. The pathology of cholestasis. *Semin Liver Dis*. 2004;24(1):21-42.
36. Yildiz Y, Hoffmann P, Vom Dahl S, et al. Functional and genetic characterization of the non-lysosomal glucosylceramidase 2 as a modifier for Gaucher disease. *Orphanet J Rare Dis*. 2013;8:151.

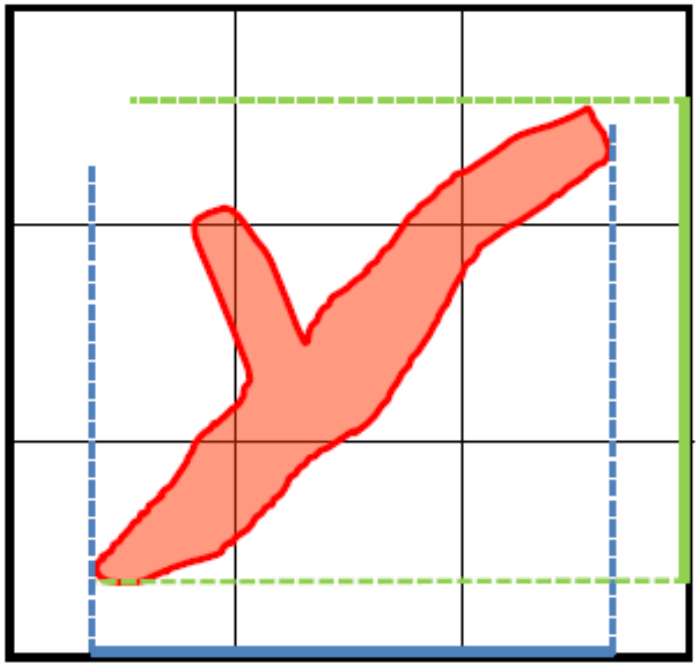
37. Mistry PK, Liu J, Sun L, et al. Glucocerebrosidase 2 gene deletion rescues type 1 Gaucher disease. *Proc Natl Acad Sci U S A.* 2014;111(13):4934-4939.



Perimeter (P_1)

Feret Y (Y_1)

Feret X (X_1)



Perimeter (P_2)

Feret Y (Y_2)

Feret X (X_2)

- Objetivo específico 3

Artigo 4: “Identification of putative modifier genes in Gaucher disease by targeted genetic sequencing”. Rodrigo Tzovenos Starosta, Suélen Porto Basgalupp, Filippo Pinto e Vairo, Fernanda Sperb-Ludwig, Marina Siebert, Ida Vanessa Doederlein Schwartz.

Artigo em elaboração, a ser submetido para o periódico American Journal of Medical Genetics Part A.

Identification of putative modifier genes in Gaucher disease by targeted genetic sequencing

Rodrigo Tzovenos Starosta[1], Suélen Porto Basgalupp[2], Filippo Pinto e Vairo[3,4], Fernanda Sperb-Ludwig[1,5], Marina Siebert[5,6,7], Ida Vanessa Doederlein Schwartz[1,5,8]

1 – Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

2 – Hospital Moinhos de Vento, Porto Alegre, RS, Brazil

3 – Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA

4 – Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA

5 – BRAIN Laboratory, Center for Experimental Research, Hospital de Clínicas de Porto Alegre (HCPA), UFRGS, Porto Alegre, RS, Brazil

6 – Laboratorial Research Unit, Experimental Research Center, HCPA, Porto Alegre, RS, Brazil

7 - Graduate Program in Science in Gastroenterology and Hepatology, UFRGS, Porto Alegre, RS, Brazil

8 –Medical Genetics Service, HCPA, Porto Alegre, RS, Brazil

Abstract

Introduction: Gaucher disease (GD) is an inborn error of metabolism with multi-system pathology leading to visceral, skeletal, hematological, and neurological manifestations. It is caused by bi-allelic pathogenic variants in *GBA*, which codes for glucosylceramidase (GCase). The phenotype of GD is heterogeneous, with limited genotype-phenotype correlation, and a role for a disturbance in the metabolism of iron has been proposed as part of the pathogenic process in GD. There is a current need to investigate modifier factors of genetic and environmental origin that may account for the observed heterogeneity.

Objective: To identify modifying effects of variants in genes related to the metabolism of iron or to the direct functioning of GCase on the phenotype of GD patients.

Methods: A cross-sectional study with retrospective collection of clinical data. Patients were categorized into 12 categorical phenotypic clusters reflecting the phenotypical spectrum of GD. Sixteen genes associated with the metabolism of iron and 3 genes associated biologically with GCase were sequenced by next-generation sequencing with primers designed to include all exons and intron-exon borders. Variants that were in Hardy-Weinberg disequilibrium and private variants were excluded from further analysis. Allelic frequencies in the genes of interest were compared to the populational cohorts ABraOM and gnomAD and according to their segregation into the phenotypic clusters.

Results: Thirty-three patients (GD type I = 30; GD type III = 3) were included in the study. Median age was 37 years (range: 7-67 years), and 17 were male. Thirty-two patients were on treatment (enzyme replacement therapy = 30; eliglustat = 2). Ninety-five variants in the 19 sequenced genes were found (novel variants = 4). A significant difference in cluster segregation of osteonecrosis and in populational frequency was found for the *CYBRD1* rs10455 variant, with an odds ratio = 0.08 (95% confidence interval 0.01-0.64), implying a protective factor of this variant for osteonecrosis. The *A2M* rs3832852 and the *TF* rs12769 variants were significant for the cluster segregation but did not have a differential populational frequency in our cohort.

Conclusions: the *CYBRD1* rs10455 variant may show a protective effect against osteonecrosis in patients with GD, making it a putative modifier gene. Other variants were identified as segregating in phenotypic clustering but did not achieve differential populational significance.

Keywords: modifier genes; iron metabolism; phenotype; next-generation sequencing; CYBRD1.

Introduction

Gaucher disease (GD; OMIM #230800, #230900, #231000) is one of the most common lysosomal storage diseases, with an incidence of approximately 1:60,000 live births (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). It is caused by bi-allelic pathogenic variants in *GBA*, the gene responsible for encoding glucocerebrosidase (GCase; EC.3.2.1.45), leading to accumulation of glucosylceramide (GlcCer) inside the lysosomes of reticuloendothelial cells such as macrophages (AERTS; KUO; LELIEVELD; BOER *et al.*, 2019; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017). GD is characterized by hematological, visceral, skeletal, and neurological manifestations – however, there is a broad variability in phenotype among patients that is not fully explained by *GBA* genotype alone (DAVIDSON; HASSAN; GARCIA; TAYEBI *et al.*, 2018; RYAN; SEEHRA; SIDRANSKY, 2019). In this way, there is a search for factors that might explain and predict phenotypical variation in GD.

Modifier genes are defined as genes that do not directly cause a given disease but that contribute to the expression and quality of its manifestations through changes in the penetrance, expressivity, dominance, or pleiotropy (NADEAU, 2001). Several approaches can be used to search for modifier effects, each with its own advantages and shortcomings. Broad screening methods such as genome-wide association studies (GWAS) rely on searching for variants in a high number of genes and associating them to phenotypes – however, due to the huge amount of data that is generated by this method, statistical correction procedures are necessary, which decreases accuracy. On the other hand, directed methods such as gene panel testing rely on searching for variants in a small number of genes chosen by their biological significance in the pathway affected by the disease, allowing for greater specificity (DAVIDSON; HASSAN; GARCIA; TAYEBI *et al.*, 2018).

Several studies have indicated that disturbances in iron metabolism play a major role in the pathophysiology of GD: while ferritin has been known to be a biomarker of GD activity for a long time (BOHTE; VAN DUSSEN; AKKERMAN; NEDERVEEN *et al.*, 2013; KOPPE; DONEDA; SIEBERT; PASKULIN *et al.*, 2016), it was not until recently

that iron entrapment inside macrophages has come up as a consistent abnormality in GD patients (LEFEBVRE; REIHANI; DAHER; DE VILLEMEUR *et al.*, 2018; REGENBOOG; BOHTE; AKKERMAN; STOKER *et al.*, 2017). This points to a disturbance of iron metabolism in the pathological process of GD(LEFEBVRE; REIHANI; DAHER; DE VILLEMEUR *et al.*, 2018; REGENBOOG; BOHTE; AKKERMAN; STOKER *et al.*, 2017; REGENBOOG; VAN KUILENBURG; VERHEIJ; SWINKELS *et al.*, 2016) and therefore to a possible modification of phenotype by variants that affect the pathways involved in the processing of iron.

In this study, we aim at identifying putative modifier genes for GD through sequencing of genes related to iron metabolism and to the activity of GCase and the use of populational and clinical data.

Methodology

Patients

Patients from the cohort of the Gaucher Reference Centre of Hospital de Clínicas de Porto Alegre (GRC-HCPA) were invited to participate. All patients had a confirmed diagnosis of GD based on enzyme activity in leukocytes and sequencing of the *GBA* gene. All patient data were obtained from the patient's medical records.

Genotyping of candidate genes and variant curation

Blood samples were collected in EDTA vacuum container. DNA was extracted with Easy-DNA Purification kit (Invitrogen™). Samples quantification was performed in NanoDrop 1000 (Thermo Fisher Scientific) and Qubit dsDNA HS Assay Kit (Invitrogen™).

A customized gene panel amplicon-based was designed using Ion AmpliSeq Designer software (Thermo Fisher Scientific) and included the exons and flanking 40 bp into introns of 19 genes involved in GD and iron metabolism (*A2M*, *BMP6*, *CP*, *CYBRD1*, *FTL*,

GBA2, *HAMP*, *HEPH*, *HFE*, *HJV*, *NEO1*, *PSAP*, *SCARB2*, *SLC11A2*, *SLC40A1*, *TF*, *TFR2*, *TFRC*, and *TMPRSS6*). This panel includes 326 amplicons, which were divided in two pools.

Libraries were amplified using Ion AmpliSeq™ Library kit 2.0 (Thermo Fisher Scientific) according to the manufacturer's recommendations, starting from 10 ng of gDNA per pool using the customized AmpliSeq panel. After cycling, the samples were ligated with adapters using Ion Xpress™ Barcode Adapters kit (Thermo Fisher Scientific). Unamplified libraries were purified with Agencourt AMPure XP kit (Beckman Coulter) and the final product of library preparation was quantified using Qubit® dsDNA HS Assay kit in the Qubit® Fluorometer 2.0 (Thermo Fisher Scientific). All barcoded libraries were pooled in equimolar concentration (100 pM) and subsequently amplified through emulsion PCR using the Ion PGM Hi-Q Template kit (Thermo Fisher Scientific) in the OneTouch2™ Instrument (Thermo Fisher Scientific). Next, positive ISPs were enriched by the OneTouch™ ES Instrument (Thermo Fisher Scientific). Finally, enriched ISPs were loaded onto an Ion 316 chip v2 (Thermo Fisher Scientific) and sequenced using the Ion PGM Hi-Q Sequencing kit (Thermo Fisher Scientific) on the Ion Personal Genome Machine (Thermo Fisher Scientific), according manufacturer´s instructions. A minimal overall depth of 200x per sample was considered.

The sequencing raw data was processed by Torrent Suite Software Suite v5.0 (Thermo Fisher Scientific) and coverage analysis and variant caller (VC) v.5.0 plugins were used. Processed reads were aligned to the hg19 reference genome (GRCh37.p13).

The software Enlis Genome Research (LLC), Variant Effect Predictor (Ensembl) and Ion Reporter (Thermo Fisher Scientific) were used to detect and classify variants. Validations of next-generation sequencing (NGS) results were performed by Sanger sequencing in patients and in their parents when the sample was available. The unbiased capture and depth of coverage of each coding exon and adjacent intronic region of all genes in this panel ensures the accuracy of variant detection.

Variants that were in Hardy-Weinberg disequilibrium at $p<0.05$ and variants that were present in only one patient were excluded from further statistical analysis.

Phenotypic clustering

Based on a review of the patient's medical records, patients were classified as to twelve dichotomous phenotypical characteristics (presence/absence of characteristic): altered liver enzymes (high levels of aspartate-transaminase, alanine-transaminase, or gamma-glutamyltransferase for at least six months during treatment); evidence of iron overload (increased transferrin saturation during treatment or iron deposition on liver biopsy or MRI); steatosis (according to liver biopsy or abdominal ultrasonography); cholelithiasis; anemia during treatment; thrombocytopenia during treatment; hypergammaglobulinemia during treatment; bone marrow burden (BMB) score >8; osteonecrosis before or during treatment; cardiac hypertrophy during treatment; restrictive pulmonary disease during treatment; overall severe disease (DS3 >3 (WEINREB; CAPPELLINI; COX; GIANNINI *et al.*, 2010) or Zimran Severity Score >9 (ZIMRAN; KAY; GELBART; GARVER *et al.*, 1992)) during treatment. Differences in allelic frequencies of each variant between patients with presence or absence of each phenotypical characteristic (*i.e.*, segregation within phenotypic clusters) were assessed in a chi-square test and an odds ratio (OR) was calculated when the difference was significant. Because of the small sample size, differences were considered to be significant when $p<0.01$.

Comparison of populational frequency

Because GD is a disorder largely undiagnosed and is not regularly screened in neonates in most populations, it is estimated that the patients who are diagnosed tend to sit in the more severe end of the phenotypic spectrum. We assessed whether there are any variants more frequent in our cohort than in the general population that could be contributing towards a more severe phenotype. The genomic databases ABraOM (NASLAVSKY; YAMAMOTO; DE ALMEIDA; EZQUINA *et al.*, 2017) and gnomAD (https://gnomad.broadinstitute.org/) were used for obtaining populational allelic frequencies.

ABraOM ("Arquivo Brasileiro Online de Mutações") is a repository of variants found by whole-exome sequencing (WES) in a cohort of 609 healthy elderly people in Brazil. gnomAD ("Genome Aggregation Database") is a repository of variants found by WES and whole-genome sequencing (WGS) in 141,456 individuals of several ethnicities. A Fisher's exact test was used to compare the allelic frequency of each SNP obtained through NGS to the two databases. For variants not reported in the ABraOM database, only the allelic frequency reported in the gnomAD database was used for statistical purposes. Novel variants were excluded from the burden test. Differences were considered significant when $p<0.0011$, according to Bonferroni's correction procedure. Variants with a significantly higher allelic frequency in our cohort than in the general population are hypothesized to contribute to a more severe phenotype, and variants with a significantly lower allelic frequency in our cohort are hypothesized to contribute to a less severe phenotype.

Ethical approval

This study was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre - number 2013-0537. All subjects or their tutors provided written consent.

Results

Thirty-three patients (GD type I = 30; GD type III = 3) were included in the study. Median age was 37 years (range: 7-67 years), and 17 were male. There were 6 sibling pairs and 1 family with three siblings and one cousin. All patients except one received treatment (enzyme replacement therapy (ERT) = 30; substrate reduction therapy (SRT-eliglustat) = 2). Patient characteristics have been recently described by Starosta *et al*. (STAROSTA; VAIRO; DORNELLES; BASGALUPP *et al.*, 2020).

Ninety-five variants were found in the cohort, including six novel variants (see Supplementary Table 1 for a complete list of the variants found in the cohort). The following variants were in Hardy-Weinberg disequilibrium and were excluded from further analysis:

GBA2 rs3833700 (p=0.006), *NEO1* rs1131854 (p=0.048), *TF* rs2692696 (p=0.002), and *TFRC* rs419059 (p=0.003). The following variants were not reported in the ABraOM database: *SLC11A2* rs17216051 and *TFR2* rs1478674823. The variant *TMPRSS6* rs60484081 was not reported in either ABraOM or gnomAD.

For the distribution of phenotypical characteristics, see Table 1. Three variants were found to have a significantly different distribution in patients according to the phenotypical characteristics: *A2M* rs3832852 (alias rs1799759) was only present in patients that had hypergammaglobulinemia (OR not calculable, p=0.008); *CYBRD1* rs10455 was present in 14.2% of the patients with osteonecrosis and in 75.0% of the patients without osteonecrosis (OR = 0.08, 95% confidence interval [95% CI] 0.01-0.64, p=0.004), appearing to be associated to protection against osteonecrosis; *TF* rs12769 was present in 80.0% of the patients with cardiac hypertrophy and in 37.5% of the patients without cardiac hypertrophy (OR = 7.54, 95% CI 2.20-25.86, p=0.002).

When comparing variant frequencies against public databases, eight variants had an allelic frequency significantly different in our cohort than in the reference population (Table 2). All variants found to have a significant difference had a lower frequency in our cohort than in the general population cohorts.

Discussion

GD, as many Mendelian disorders, has a wide variability of clinical manifestations, even in patients sharing the same *GBA* genotype. Modifier genes have been regarded as a potential explanation for phenotypical heterogeneity, and great effort has been put in the last decade in the search for candidate genes to fulfill this role. In the present study, we have investigated in a systematic way several genes related to iron metabolism or to the pathway of GlcCer degradation by GCase, and we have been able to identify variants that are candidate for modifiers of GD phenotype. One of the most promising genes identified is *CYBRD1*, which encodes cytochrome b reductase 1. Two variants in this gene (rs10455 and

rs3731976) had a significantly lower prevalence in our cohort than in the general population, indicating that these may confer a generally milder phenotype. The rs10455 also had a significantly lower prevalence in patients with osteonecrosis compared to patients with no history or signs of osteonecrosis in our cohort, indicating that it might be associated with protection against osteonecrosis. CYBRD1 regulates intestinal iron absorption by reducing non-absorbable Fe^{+3} to absorbable Fe^{+2} and is a known modifier of hereditary hemochromatosis type 1(CONSTANTINE; ANDERSON; VULPE; MCLAREN *et al.*, 2009; PELUCCHI; MARIANI; CALZA; FRACANZANI *et al.*, 2012). The rs10455 variant has been shown to increase CYBRD1 activity *in vitro* (SCHLOTTMANN; VERA-AVILES; LATUNDE-DADA, 2017) – this leads to the hypothesis that patients harboring this variant would have an increased absorption of iron. This might be protective against osteonecrosis in untreated patients with GD through reducing levels of anemia, which is the main risk factor for osteonecrosis in this population (KHAN; HANGARTNER; WEINREB; TAYLOR *et al.*, 2012).

Other candidate variants found in the differential phenotypical analysis were *A2M* rs3832851 and *TF* rs12769. The rs3832851 variant was associated with a higher prevalence of hypergammaglobulinemia in this cohort; this variant has been previously associated with a higher risk for Alzheimer disease (BLACKER; WILCOX; LAIRD; RODES *et al.*, 1998), but no functional evidence is available about it. It can be hypothesized that the association with hypergammaglobulinemia may be due to the protease inhibitor function of alpha-2-macroglobulin (A2M) (SOTTRUP-JENSEN, 1989) and its involvement with the regulation of several inflammatory processes (LARIONOV; DEDECK; BIRKENMEIER; THAL, 2007; LI; XIANG; WEI; SUN *et al.*, 2019) – however, it might be too early still to presume a causal link between this variant and hypergammaglobulinemia in GD. The rs12769 variant has been associated with lower serum iron levels in post-mortem samples of previously healthy subjects (FUJIHARA; YASUDA; KIMURA-KATAOKA; TAKESHITA, 2019). Although there have been studies associating anemia to cardiac remodeling and hypertrophy (HIGGINS; OTERO; JEFFERIS KIRK; PAK *et al.*, 2017; NAITO; SAWADA; OBOSHI; IWASAKU *et al.*, 2015), iron overload itself is a well-known cause of cardiac hypertrophy

(GUJJA; ROSING; TRIPODI; SHIZUKUDA, 2010; ROZWADOWSKA; RACZAK; SIKORSKA; FIJAŁKOWSKI *et al.*, 2019; SUKUMARAN; CHANG; HAN; MINTRI *et al.*, 2017). This paradoxical situation leads to the necessity of a deeper investigation of the role of the *TF* rs12769 variant in cardiac hypertrophy before it can be proposed as a modifier in GD.

Besides *CYBRD1*, we found variants in several genes that have a lower prevalence in our sample when compared to the general population. However, no phenotypical difference could be observed within our cohort in association with such variants. Due to the low specificity inherent to population prevalence comparisons, still more confirmation is necessary before suggesting a role of these variants as modifiers.

Two recent GWAS have explored genetic modifiers of GD. Zhang *et al*.(ZHANG; STEIN; LIU; WANG *et al.*, 2012) found that a *CLN8* variant correlated with overall disease severity in patients with GD. Klein *et al.*(KLEIN; FERREIRA; BEN-DOR; DUAN *et al.*, 2016) have performed GWAS in several mice strains treated with conduritol-β-epoxide (CBE) and identified variants in non-coding regions of 17 genes associated with lifespan, none of which was studied in our panel. There are several putative reasons for the lack of overlap of the variants found in our study and the ones identified by the GWAS: i) it is possible that the SNPs found in association with a differential phenotype in our study were not contemplated by the Zhang and the Klein studies; ii) due to the huge number of analysis in a GWAS, the value for significance according to Bonferroni's correction procedure is exceedingly low, which increases specificity but decreases sensibility, that being a major issue in studies with small populations as tend to be studies on rare diseases; iii) the biology of mice treated with CBE does not necessarily reflect the biology of patients with GD; iv) the genomic landscape of a Brazilian population is quite possibly different from the population in the Zhang study, potentially altering the effect of single variants.

An important limitation of this work is the lack of functional confirmation of the modifying effects found. This could be achieved by insertion of the variants in cellular and/or animal models of GD and studying of the differential phenotype. On the other hand, it is

important to note that *in vitro* or *in vivo* experimental studies also need confirmation of clinical relevance, which can only be demonstrated by patient-based studies as the one described in this article.

Conclusion

We have identified variants in several genes with a potential for a modifier effect in GD. An especially promising variant is *CYBDR1* rs10455, with a protective effect against osteonecrosis on the differential phenotypic analysis and a lower frequency in our GD cohort than in the general population. Studies in bigger populations and functional experiments need to be conducted in order to validate and further delineate these findings.

Acknowledgements

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundo de Apoio a Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA).

Table 1. Distribution of patients according to phenotypical characteristic.

Phenotypical characteristic	Presence (n)	Absence (n)	Not available (n)
Altered liver enzymes	17	15	1
Evidence of iron overload	9	23	1
Steatosis	14	18	1
Cholelithiasis	10	23	0
Anemia	8	22	3
Thrombocytopenia	15	17	1
Hypergammaglobulinemia	18	12	3
Severe BMB (>8)	16	13	4
Osteonecrosis	7	24	2
Cardiac hypertrophy	10	22	1
Restrictive pulmonary disease	5	27	1
Overall severe disease	8	21	4

Not available = number of patients which did not have available data on the characteristic.

Table 2. Comparison of variant frequencies identified in GD patients using the customized gene panel with NGS approach.

Gene	Variant	Cohort allelic frequency	ABraOM frequency	ABraOM (p-value)	gnomAD frequency	gnomAD (p-value)
A2M	rs2228222	6.06%	4.51%	0.5455	4.89%	0.6621
A2M	rs55761427	7.57%	2.62%	0.0119	0.000057%	**0.0000**
A2M	rs3832852	16.67%	15.35%	0.7671	13.90%	0.5161
A2M	rs669	34.84%	32.10%	0.6326	31.30%	0.5344
BMP6	rs17558	3.03%	4.59%	0.5431	3.92%	0.7075
BMP6	rs537332654	3.03%	1.94%	0.5236	0.000043%	**0.0000**
BMP6	rs61733611	4.54%	5.09%	0.8404	3.18%	0.5277
CP	rs16861582	3.03%	28.40%	$\mathbf{4.84 \cdot 10^{-6}}$	30.05%	$\mathbf{1.68 \cdot 10^{-6}}$
CP	rs61733458	3.03%	3.28%	0.9078	2.76%	0.8934
CP	rs1053709	10.60%	4.96%	0.0347	4.37%	0.0133
CYBRD1	rs3731976	3.03%	45.31%	$\mathbf{5.20 \cdot 10^{-12}}$	42.14%	$\mathbf{1.23 \cdot 10^{-10}}$
CYBRD1	rs10455	48.48%	68.71%	**0.0003**	64.92%	0.0051
FTL	rs2230267	68.18%	55.17%	0.0335	50.15%	0.0034
GBA2	rs34312177	4.54%	6.48%	0.5219	4.25%	0.9072
HFE	rs1800562	4.54%	1.97%	0.1322	3.37%	0.5992
HFE	rs1799945	9.09%	12.72%	0.3755	10.82%	0.6511
NEO1	rs2680348	6.06%	8.45%	0.4841	6.95%	0.7742
NEO1	rs3736510	54.54%	41.54%	0.0320	45.07%	0.1218
PSAP	rs4747203	4.54%	32.92%	$\mathbf{9.31 \cdot 10^{-7}}$	34.18%	$\mathbf{3.86 \cdot 10^{-7}}$
SLC11A2	rs17216051	4.54%	0.00%	N/A	0.24%	$\mathbf{1.25 \cdot 10^{-12}}$
SLC40A1	rs2304704	62.12%	56.32%	0.3421	61.52%	0.9200
TF	rs1049296	7.57%	14.12%	0.1267	15.71%	0.0693
TF	rs1130459	4.54%	59.03%	$\mathbf{2.24 \cdot 10^{-19}}$	59.77%	$\mathbf{5.73 \cdot 10^{-20}}$
TF	rs12769	25.75%	28.24%	0.6537	32.78%	0.2242
TF	rs1799852	21.21%	10.75%	0.0061	12.94%	0.0452
TF	rs1799899	3.03%	4.02%	0.6814	5.29%	0.4108
TF	rs8177232	3.03%	4.76%	0.5088	1.85%	0.4786
TF	rs8649	16.67%	23.97%	0.1643	22.31%	0.2708
TFR2	rs1478674823	3.03%	0.00%	N/A	$6.5 \cdot 10^{-6}$%	**0.0000**
TFR2	rs141968146	3.03%	0.04%	**0.0008**	0.01%	$\mathbf{2.96 \cdot 10^{-9}}$
TFR2	rs34242818	3.03%	1.88%	0.4954	1.98%	0.5426
TFRC	rs3817672	45.45%	46.71%	0.8372	44.66%	0.8966
TFRC	rs4130359	3.03%	0.90%	0.0677	0.60%	0.0114
TMPRSS6	rs115270691	4.54%	3.20%	0.5352	2.10%	0.1680
TMPRSS6	rs11704654	13.63%	16.17%	0.5755	16.27%	0.5621
TMPRSS6	rs2111833	37.87%	31.42%	0.0258	31.88%	0.2956
TMPRSS6	rs2235321	37.87%	38.09%	0.9711	36.50%	0.8160
TMPRSS6	rs2543519	15.15%	23.39%	0.1135	22.95%	0.1319
TMPRSS6	rs2543520	16.67%	21.61%	0.3285	21.04%	0.3833
TMPRSS6	rs4820268	66.67%	56.40%	0.0926	54.13%	0.0409
TMPRSS6	rs855791	57.58%	62.06%	0.4518	57.55%	0.9966
TMPRSS6	rs881144	13.63%	9.11%	0.2016	8.92%	0.1794
TMPRSS6	rs2235324	33.33%	38.34%	0.4027	38.37%	0.4001

N/A = not applicable. Bold values were significant considering the corrected statistical (Bonferroni procedure) significance level of $p < 0.0011$.

References

1. Stirnemann J, Belmatoug N, Camou F, et al. A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. *Int J Mol Sci.* 2017;18(2).
2. Aerts JMFG, Kuo CL, Lelieveld LT, et al. Glycosphingolipids and lysosomal storage disorders as illustrated by gaucher disease. *Curr Opin Chem Biol.* 2019;53:204-215.
3. Davidson BA, Hassan S, Garcia EJ, Tayebi N, Sidransky E. Exploring genetic modifiers of Gaucher disease: The next horizon. *Hum Mutat.* 2018;39(12):1739-1751.
4. Ryan E, Seehra GK, Sidransky E. Mutations, modifiers and epigenetics in Gaucher disease: Blurred boundaries between simple and complex disorders. *Mol Genet Metab.* 2019;128(1-2):10-13.
5. Nadeau JH. Modifier genes in mice and humans. *Nat Rev Genet.* 2001;2(3):165-174.
6. Bohte AE, van Dussen L, Akkerman EM, et al. Liver fibrosis in type I Gaucher disease: magnetic resonance imaging, transient elastography and parameters of iron storage. *PLoS One.* 2013;8(3):e57507.
7. Koppe T, Doneda D, Siebert M, et al. The prognostic value of the serum ferritin in a southern Brazilian cohort of patients with Gaucher disease. *Genet Mol Biol.* 2016;39(1):30-34.
8. Lefebvre T, Reihani N, Daher R, et al. Involvement of hepcidin in iron metabolism dysregulation in Gaucher disease. *Haematologica.* 2018;103(4):587-596.
9. Regenboog M, Bohte AE, Akkerman EM, Stoker J, Hollak CEM. Iron storage in liver, bone marrow and splenic Gaucheroma reflects residual disease in type 1 Gaucher disease patients on treatment. *Br J Haematol.* 2017;179(4):635-647.
10. Regenboog M, van Kuilenburg AB, Verheij J, Swinkels DW, Hollak CE. Hyperferritinemia and iron metabolism in Gaucher disease: Potential pathophysiological implications. *Blood Rev.* 2016;30(6):431-437.
11. Weinreb NJ, Cappellini MD, Cox TM, et al. A validated disease severity scoring system for adults with type 1 Gaucher disease. *Genet Med.* 2010;12(1):44-51.
12. Zimran A, Kay A, Gelbart T, et al. Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients. *Medicine (Baltimore).* 1992;71(6):337-353.
13. Naslavsky MS, Yamamoto GL, de Almeida TF, et al. Exomic variants of an elderly cohort of Brazilians in the ABraOM database. *Hum Mutat.* 2017;38(7):751-763.
14. Starosta RT, Vairo FPE, Dornelles AD, et al. Liver involvement in patients with Gaucher disease types I and III. *Mol Genet Metab Rep.* 2020;22:100564.

15. Constantine CC, Anderson GJ, Vulpe CD, et al. A novel association between a SNP in CYBRD1 and serum ferritin levels in a cohort study of HFE hereditary haemochromatosis. *Br J Haematol.* 2009;147(1):140-149.
16. Pelucchi S, Mariani R, Calza S, et al. CYBRD1 as a modifier gene that modulates iron phenotype in HFE p.C282Y homozygous patients. *Haematologica.* 2012;97(12):1818-1825.
17. Schlottmann F, Vera-Aviles M, Latunde-Dada GO. Duodenal cytochrome b (Cybrd1) ferric reductase functional studies in cells. *Metallomics.* 2017;9(10):1389-1393.
18. Khan A, Hangartner T, Weinreb NJ, Taylor JS, Mistry PK. Risk factors for fractures and avascular osteonecrosis in type 1 Gaucher disease: a study from the International Collaborative Gaucher Group (ICGG) Gaucher Registry. *J Bone Miner Res.* 2012;27(8):1839-1848.
19. Blacker D, Wilcox MA, Laird NM, et al. Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat Genet.* 1998;19(4):357-360.
20. Sottrup-Jensen L. Alpha-macroglobulins: structure, shape, and mechanism of proteinase complex formation. *J Biol Chem.* 1989;264(20):11539-11542.
21. Larionov S, Dedeck O, Birkenmeier G, Thal DR. Expression of alpha2-macroglobulin, neutrophil elastase, and interleukin-1alpha differs in early-stage and late-stage atherosclerotic lesions in the arteries of the circle of Willis. *Acta Neuropathol.* 2007;113(1):33-43.
22. Li S, Xiang C, Wei X, et al. Early supplemental α2-macroglobulin attenuates cartilage and bone damage by inhibiting inflammation in collagen II-induced arthritis model. *Int J Rheum Dis.* 2019;22(4):654-665.
23. Fujihara J, Yasuda T, Kimura-Kataoka K, Takeshita H. Association of SNPs in transferrin and transferrin receptor genes with blood iron levels in human. *Leg Med (Tokyo).* 2019;36:17-20.
24. Naito Y, Sawada H, Oboshi M, et al. Cardiac remodeling in response to chronic iron deficiency: role of the erythropoietin receptor. *J Hypertens.* 2015;33(6):1267-1275.
25. Higgins D, Otero J, Jefferis Kirk C, et al. Iron Laboratory Studies in Pediatric Patients With Heart Failure from Dilated Cardiomyopathy. *Am J Cardiol.* 2017;120(11):2049-2055.
26. Sukumaran A, Chang J, Han M, Mintri S, Khaw BA, Kim J. Iron overload exacerbates age-associated cardiac hypertrophy in a mouse model of hemochromatosis. *Sci Rep.* 2017;7(1):5756.
27. Gujja P, Rosing DR, Tripodi DJ, Shizukuda Y. Iron overload cardiomyopathy: better understanding of an increasing disorder. *J Am Coll Cardiol.* 2010;56(13):1001-1012.
28. Rozwadowska K, Raczak G, Sikorska K, Fijałkowski M, Kozłowski D, Daniłowicz-Szymanowicz L. Influence of hereditary hemochromatosis on left ventricular wall thickness: does iron overload exacerbate cardiac hypertrophy? *Folia Morphol (Warsz).* 2019.
29. Zhang CK, Stein PB, Liu J, et al. Genome-wide association study of N370S homozygous Gaucher disease reveals the candidacy of CLN8 gene as a genetic

modifier contributing to extreme phenotypic variation. *Am J Hematol.* 2012;87(4):377-383.
30. Klein AD, Ferreira NS, Ben-Dor S, et al. Identification of Modifier Genes in a Mouse Model of Gaucher Disease. *Cell Rep.* 2016;16(10):2546-2553.

Supplementary table 1. All variants found by NGS of the target genes.

Gene	Variant	c.DNA impact	Protein impact	Allele count†
CYBRD1	rs10455	c.797G>A	p.Ser266Asn	39
	rs3731976	5' UTR	N/A	3
SLC40A1	rs2304704	c.663T>C	p.Val221=	46
	rs768744143	c.993C>T	p.Tyr331=	1
	rs1453956325	c.657G>A	p.Glu219=	1
TF	rs12769	c.624G>A	p.Ser208=	17
	rs2692696	c.1342A>G	p.Ile448Val	60
	rs1049296	c.1765C>T	p.Pro589Ser	6
	rs1799852	c.739C>T	p.Leu247=	14
	rs8649	c.1572G>C	p.Leu524=	11
	rs1799899	c.829G>A	p.Gly277Ser	2
	rs8177232	c.804T>C	p.His268=	2
	rs1130459	5' UTR	N/A	3
CP	rs61733458	c.1652C>T	p.Thr551Ile	3
	rs701753	c.1632A>T	p.Glu544Asp	64
	rs1053709	c.1950A>C	p.Gly650=	8
	novel	**c.37_38insA**	**p.Leu12***	**1**
	rs16861582	splicing	N/A	2
	rs139633388	c.2684G>C	p.Gly895Ala	1
	rs34394958	c.2991T>G	p.His997Gln	1
	rs56033670	c.2522C>G	p.Thr841Arg	1
	rs1250117082	c.2075G>C	p.Glu692Asp	1
	rs757188970	5' UTR	N/A	1
	rs17847017	splicing	N/A	1
TFRC	rs3817672	c.424G>A	p.Gly142Ser	33
	rs1805051	c.2124G>A	p.Thr708=	8
	rs41301359	c.312C>T	p.Thr104=	2
	rs386670122	splicing	N/A	4
	rs419059	splicing	N/A	4
SCARB2	rs143655258	c.475A>G	p.Met159Val	1
	rs143558324	c.382C>T	p.Pro128Ser	1
BMP6	rs17557	c.1104G>C	p.Val368=	32
	rs17558	c.1062C>T	p.Asp354=	2
	rs537332654	c.335_337del	p.Gln118del	2
	rs61733611	c.1029C>T	p.Ala343=	3
HAMP	rs146776859	c.92C>T	p.Thr31Met	1
HFE	rs2071303	splicing	N/A	2
	rs1800562	c.845G>A	p.Cys282Tyr	3
	rs1799945	c.187C>G	p.His63Asp	6
HJV	rs782681137	c.305C>T	p.Ala102Val	1

TFR2	**novel**	**c.1606-8T>G**	**N/A**	**1**
	rs2075674	c.1851C>T	p.Ala617=	13
	rs748376968	c.2277C>T	p.Pro759=	1
	rs41295942	c.2255G>A	p.Arg752His	1
	novel	**c.2086C>A**	**p.Ser695Leu**	**2**
	rs141968146	c.135G>T	p.Ala45=	2
	rs139178017	c.1473G>A	p.Glu491=	1
	rs1478674823	c.2092A>G	p.Arg698Gly	2
	rs34242818	c.714C>G	p.Ile238Met	2
GBA2	rs3833700	splicing	N/A	8
	novel	**c.32C>A**	**p.Thr11Asn**	**1**
	novel	**c.34G>T**	**p.Gly12Cys**	**1**
	rs754147042	splicing	N/A	1
	rs779358452	splicing	N/A	1
	rs150861266	c.964C>T	p.Leu322=	1
	rs34312177	c.33C>T	p.Thr11=	2
PSAP	rs759556759	c.354G>C	p.Leu118=	1
	rs762050153	c.337A>C	p.Tyr113Asp	1
	rs1407593392	5’ UTR	N/A	1
A2M	rs669	c.2998A>G	p.Ile1000Val	24
	rs226405	c.1915G>A	p.Asp639Asn	62
	rs369574498	c.521G>T	p.Arg174Leu	1
	rs3832852	splicing	N/A	11
	rs2228222	c.1296C>T	p.Tyr432=	6
	rs761812812	splicing	N/A	1
	rs55761427	c.2531C>T	p.Ala844Val	3
	rs2229298	c.1079G>A	p.Arg360Gln	1
	novel	**c.2370C>G**	**p.Gln790His**	**1**
SLC11A2	rs161044	splicing	N/A	12
	rs1048230	c.1341T>C	p.Ile447=	11
	rs17216051	c.980T>C	p.Ile219Thr	3
	rs146202526	c.1584C>A	p.Val528=	1
	rs115874705	c.712G>A	p.Ala238Thr	1
NEO1	rs3736510	c.1719A>G	p.Lys573=	36
	rs2623989	splicing	N/A	8
	rs1131854	c.2943C>T	p.Ser981=	39
	rs118087147	c.3388C>T	p.Arg1130Cys	1
	rs2680348	c.2676C>T	p.Thr892=	4
FTL	rs2230267	c.163T>C	p.Leu55=	45
TMPRSS6	rs2235321	c.2256C>T	p.Tyr752=	25
	rs855791	c.2246T>C	p.Val749Ala	41
	rs200434923	splicing	N/A	16
	rs4820268	c.1536C>T	p.Asp512=	43

	rs881144	c.1227C>T	p.Tyr409=	9
	rs2111833	c.1056G>C	p.Ser352=	22
	rs2235324	c.730A>G	p.Lys244Glu	21
	rs11704654	c.72G>A	p.Pro24=	8
	rs115270691	c.15C>T	p.Phe5=	3
	rs2543519	splicing	N/A	10
	rs2543520	splicing	N/A	11
	rs1261398165	splicing	N/A	1
	rs201148397	c.838G>T	p.Val280Leu	1
HEPH	rs5919015	c.1167T>A	p.Val39Glu	35
	rs806607	c.2457T>C	p.Tyr819=	22
	rs809363	c.2541A>G	p.Thr847=	22

All description of impact based on the canonical transcript, except rs115270691. †n=66 except for *HEPH*, which is in the X chromosome, having a n=49. Novel variants are highlighted in bold.

- Objetivo específico 4

Artigo 5: “The use of transient hepatic elastography to validate prediction scores for liver fibrosis in treated patients with Gaucher disease type 1.”. Rodrigo Tzovenos Starosta, Lethicia Campos Ferraro, Fabiano de Oliveira Poswar, Mário Reis Álvares-da-Silva, Ida Vanessa Doederlein Schwartz.

Artigo em elaboração, a ser submetido para o periódico Molecular Genetics and Metabolism.

The use of transient hepatic elastography to validate prediction scores for liver fibrosis in treated patients with Gaucher disease type 1.

Rodrigo Tzovenos Starosta[1,2], Lethicia Campos Ferraro[3], Fabiano de Oliveira Poswar[4], Mário Reis Álvares-da-Silva[5,6], Ida Vanessa Doederlein Schwartz[1,2,4]

1 – Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

2 – BRAIN Laboratory, Hospital de Clínicas de Porto Alegre (HCPA), UFRGS, Porto Alegre, RS, Brazil

3 – School of Medicine, UFRGS, Porto Alegre, RS, Brazil

4 – Service of Medical Genetics, HCPA, UFRGS, Porto Alegre, RS, Brazil

5 – Graduate Program in Gastroenterology and Hepatology, UFRGS, Porto Alegre, RS, Brazil

6 – Service of Gastroenterology, HCPA, UFRGS, Porto Alegre, RS, Brazil

Abstract

Gaucher disease (GD) is a rare genetic disorder with multi-system involvement. One of its long-term consequences, even during treatment, is liver fibrosis, which may progress to cirrhosis and end-stage liver disease. The detection of liver fibrosis in this population is commonly performed by liver biopsy or by elastography – however, because of the risks associated with the former and the inter-observer variability and lack of global availability, fibrosis detection is oftentimes hindered in patients with GD. In this study, we aim at assessing the validity of fibrosis-predicting scores developed for hepatitis C and non-alcoholic liver disease (NAFLD) for the use in GD, using transient elastography as a gold-standard. We enrolled patients with GD type 1 who had been on treatment for a minimum of 1 year on enzyme replacement therapy or substrate reduction therapy and who had no evidence of any other liver disease except GD or NAFLD, which is a common comorbidity of GD. Laboratory tests necessary to calculate the fibrosis prediction scores APRI, FIB-4, and NFS were performed. Twelve patients were enrolled, and, after one exclusion due to consent withdrawal, eleven patients were analyzed for the fibrosis prediction scores. The score with a higher AUC for detection of significant fibrosis (F2 or higher) was APRI, with 0.701. This score showed 100% sensitivity for significant fibrosis at the cut-off of 0.201 and 100% specificity for significant fibrosis at the cut-off of 0.604. In this way, we recommend the use of the APRI score for screening of fibrosis in treated patients with GD type 1.

Keywords: liver fibrosis; cirrhosis; APRI; Gaucher disease; transient hepatic elastography

Introduction

Gaucher disease (GD) is an inborn error of metabolism characterized by a broad and variable phenotype of multi-systemic organic compromise. It is caused by bi-allelic pathogenic variants in *GBA* (STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017) or in the saposin C portion of *PSAP* (TYLKI-SZYMAŃSKA; GROENER; KAMIŃSKI; ŁUGOWSKA *et al.*, 2011), leading to insufficient activity of the glucosylceramidase enzyme (GCase). GD manifestations include mainly four systems: hematological (anemia, thrombocytopenia, hypergammaglobulinemia, predisposition to develop multiple myeloma) (LINARI; CASTAMAN, 2016; NGUYEN; STIRNEMANN; LAUTREDOUX; CADOR *et al.*, 2020; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), visceral (hepatosplenomegaly, liver fibrosis, biliary damage, pulmonary fibrosis, cardiac valve calcification and cardiac hypertrophy, predisposition to develop hepatocellular carcinoma) (LINARI; CASTAMAN, 2016; STAROSTA; VAIRO; DORNELLES; BASGALUPP *et al.*, 2020; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), skeletal (bone pain and crises, skeletal deformations, osteonecrosis, scoliosis) (KHAN; HANGARTNER; WEINREB; TAYLOR *et al.*, 2012; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), and neurological (epilepsy, oculomotor apraxia, dysphagia, peripheral neuropathy, predisposition to develop Parkinson disease) (GARY; RYAN; STEWARD; SIDRANSKY, 2018; SCHIFFMANN; SEVIGNY; ROLFS; DAVIES *et al.*, 2020; STIRNEMANN; BELMATOUG; CAMOU; SERRATRICE *et al.*, 2017), leading to a phenotype continuum that can be categorized as GD type 1 (with no overt neurological symptoms), GD type 3 (chronic, slowly progressive neurological symptoms such as myoclonic epilepsy and motor dysphagia), and the extremely severe GD type 2 (acute neurological symptoms leading to death before 2 years of age) (SCHIFFMANN; SEVIGNY; ROLFS; DAVIES *et al.*, 2020; SIDRANSKY, 2004).

Two types of liver fibrosis have been described in GD. In the pre-treatment era, a relatively common complication of GD was the rapid development of massive hepatic areas

of confluent acellular fibrosis mostly in the central parts of the liver, which became known as “stellate fibrosis” (HILL; DAMASKA; LING; PATTERSON *et al.*, 1992; JAMES; STROMEYER; CHANG; BARRANGER, 1981; LACHMANN; WIGHT; LOMAS; FISHER *et al.*, 2000). With the advent of enzyme replacement therapy (ERT) – and later substrate reduction therapy (SRT) – no new cases of stellate fibrosis have been reported, the last one described being in 2002 (PEREL; BIOULAC-SAGE; CHATEIL; TRILLAUD *et al.*, 2002). However, after almost two decades in which ERT had changed the common phenotype of GD towards a more chronic and benign disorder, reports of increased liver stiffness (a surrogate measure of fibrosis) in treated patients with GD as measured by transient elastography (TH) (BOHTE; VAN DUSSEN; AKKERMAN; NEDERVEEN *et al.*, 2013; NASCIMBENI; CASSINERIO; DALLA SALDA; MOTTA *et al.*, 2018), with correlations to markers of disease severity such as chitotriosidase levels (BOHTE; VAN DUSSEN; AKKERMAN; NEDERVEEN *et al.*, 2013; NASCIMBENI; CASSINERIO; DALLA SALDA; MOTTA *et al.*, 2018) and severity scores (NASCIMBENI; CASSINERIO; DALLA SALDA; MOTTA *et al.*, 2018; SERAI; NAIDU; ANDREW BURROW; PRADA *et al.*, 2018) that suggest that an indolent pathogenic process of liver injury still happened. Strikingly, a recent study (LOLLERT; HOFFMANN; LACHE; KÖNIG *et al.*, 2020) using acoustic radiation force impulse point shear-wave elastography (which is more sensitive to fibrosis than TH) showed that up to 70% of patients with GD type 1 have fibrosis of METAVIR (BATTS; LUDWIG, 1995) stage F2 or higher. In this way, measurements of liver fibrosis are becoming standard practice in the routine follow-up of patients with GD. However, elastographic techniques are subject to inter-observer, positional, and technical differences (SHIHA; EL-ETREBY; BAHGAT; HAMED *et al.*, 2018; ZELBER-SAGI; YESHUA; SHLOMAI; BLENDIS *et al.*, 2011; ZELESCO; ABOTT; O'HARA, 2017); furthermore, this technology may not be available in resource-limited settings such as developing countries.

Clinical-laboratorial scores for the prediction of liver fibrosis have been proposed and used for many liver pathologies. For people with hepatitis C the APRI (AST-to-platelets ratio index) (WAI; GREENSON; FONTANA; KALBFLEISCH *et al.*, 2003) and the FIB-4

(Fibrosis 4) (VALLET-PICHARD; MALLET; NALPAS; VERKARRE *et al.*, 2007) have high sensitivity (at a lower cut-off value) and high specificity (at a higher cut-off value) for fibrosis of METAVIR grade F3 or higher (*i.e.*, with bridging fibrotic bands or cirrhosis), although they are unreliable to detect patients with lesser grades of fibrosis. Similarly, for people with non-alcoholic fatty liver disease (NAFLD), the NFS (NAFLD fibrosis score)(ANGULO; HUI; MARCHESINI; BUGIANESI *et al.*, 2007) can distinguish with high accuracy patients with higher degrees of fibrosis by using a sensitive lower cut-off value and a specific higher cut-off value. GD, despite being a cause of liver fibrosis and its complications (STAROSTA; PINTO E VAIRO; DORNELLES; CERSKI *et al.*, 2019; STAROSTA; VAIRO; DORNELLES; BASGALUPP *et al.*, 2020), has not seen the development of a disease-specific fibrosis score; moreover, all existent scores use in their equations the platelet count, which is lower in patients with GD independently of liver disease.

In this article, we aim at investigating the validity of existent liver fibrosis scores for the use in treated patients with GD.

Methods

Subjects

Patients with GD with follow-up at the Gaucher Reference Center (GRC) of the Hospital de Clínicas de Porto Alegre (HCPA) were enrolled according to the significant inclusion criteria: diagnosis of GD by leukocyte enzyme activity and sequencing of *GBA* with identification of biallelic pathogenic variants; treatment with ERT or SRT for at the least 12 months; written consent to participate in the study. Exclusion criteria were: younger than 18 years-old; presence of concomitant, unrelated liver diseases such as viral hepatitis, hypothyroidism, or alpha-1-antitripsin disease. NAFLD was not considered an exclusion criteria because of its high prevalence in patients with GD (STAROSTA; VAIRO; DORNELLES; BASGALUPP *et al.*, 2020).

Laboratory tests and anthropometric variables

Serum laboratory tests were performed in the same day as TH after a minimum 3 hour fast and consisted in: alanine-transaminase (ALT), aspartate-transaminase (AST), albumin, alpha-1-antitrypsin, anti-HBs IgM and IgG, anti-HCV IgG, transferrin saturation, serum free iron, total and fractional cholesterol, ferritin, γ-glutamyltransferase (GGT), glucose, insulin, hepatitis B surface antigen (HBsAg) , thyroxine (T4), thyroid-stimulating hormone (TSH), triglycerides, platelets, mean platelet volume. All tests were performed in the HCPA as per standard protocol. Height, weight, and age from the last consultation at the GRC, which was a maximum of three months. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters.

Transient elastography

TH was performed by an experienced investigator (MRAS, >500 valid tests performed) with a FibroScan machine (Echosens, Paris, France) and an M (medium) probe. Ten measurements were performed by patient, with the probe located on the 5^{th} intercostal space at the mid-clavicular line. Patients with an interquartile range (IQR) higher than 30% were considered to have low-quality scores and were excluded from further analysis. Values lower than 5.3 kPa were considered as F0; values of 5.3 to 6.9 kPa were considered as F1; values of 7.0 to 13.9 kPa were considered as F3; values of 14.0 kPa or higher were considered as F4.

Statistical analysis

Descriptive statistics were described as mean ± standard deviation. TH was used as a gold-standard for liver fibrosis for the calculation of receiver-operator characteristics (ROC) curves for the APRI, FIB-4, and NFS scores, with estimation of area under the curve (AUC) for each ROC curve. All statistics were performed in SPSS v.18.0 (SPSS Inc., IBM).

Ethical considerations

This study was approved by the Institutional Review Board (Comitê de Ética em Pesquisa) of HCPA, number 2018-0639. All subjects provided written consent.

Results

Twelve patients were included in the study (mean age = 37 ± 11.2 years; female = 8). Eleven patients were on ERT (imiglucerase = 8; taliglucerase alfa = 3) and one patient was on SRT with eliglustat. One patient withdrew consent for a blood draw and was excluded from the score analysis. No patient was positive for viral hepatitis, hypothyroidism, or alpha-1-antitrypsin disease.

Mean liver stiffness measured by TH was 5.25 kPa (range: 4.3 – 22.0 kPa). Six patients had scores in the F0 range; three patients had scores in the F1 range; two patients had scores in the F2 range; and one patient had a score in the F4 range. IQRs ranged from 5% to 23%. The patient with an F4 score was the only one splenectomized.

The APRI, FIB-4, and NFS were calculated for 11 patients. Mean APRI score was 0.293 ± 0.203, ranging from 0.09 to 0.818 (lower cut-off = 0.500; higher cut-off = 1.500). Mean FIB-4 score was 0.836 ± 0.677, ranging from 0.361 to 2.814 (lower cut-off = 1.450; higher cut-off = 3.25). Mean NFS was -2.392 ± 0.686, ranging from -3.568 to -1.442 (lower cut-off = -1.455; higher cut-off = 0.675). For the detection of fibrosis stage F2 or higher ("significant fibrosis"), the AUCs were respectively 0.708, 0.625, and 0.375 for APRI, FIB4, and NFS, as shown in the ROC curve in figure 1. For the detection of significant fibrosis using the APRI score, which showed the higher AUC, a lower cut-off value of 0.201 had a sensitivity of 100% and a higher cut-off value of 0.649 had a specificity of 100%.

Discussion

Liver fibrosis is an important complication of GD, leading to cirrhosis, portal hypertension, and hepatocellular carcinoma (ADAR; ILAN; ELSTEIN; ZIMRAN, 2016; REGENBOOG; VAN DUSSEN; VERHEIJ; WEINREB *et al.*, 2018; STAROSTA; VAIRO; DORNELLES; BASGALUPP *et al.*, 2020). Although there is not to date a therapy to reverse fibrosis, understanding when it arises is vital in the follow-up of a patient with GD because it may allow for early interventions that decrease its symptoms and its complications, such as prevention of variceal bleeding by the use of beta-blocking medications (TSOCHATZIS; BOSCH; BURROUGHS, 2014) or the screening of hepatocellular carcinoma (KANWAL; SINGAL, 2019).

Traditionally, liver biopsy was the only reliable method for detection of liver fibrosis (StatPearls, 2020); it is, however, subject to sampling mistakes, and may cause complications such as intra-abdominal bleeding and pain (BOYD; CAIN; CHAUHAN; WEBB, 2020). With the advent of elastography (SANDRIN; FOURQUET; HASQUENOPH; YON *et al.*, 2003) it became possible to detect fibrosis non-invasively, improving quality of care for patients with liver diseases. Nonetheless, elastography is operator-dependent (SHIHA; EL-ETREBY; BAHGAT; HAMED *et al.*, 2018), and because of the elevated price of the equipment and the necessity of a trained operator it is not available in all contexts. In this article, we have tested three common non-invasive, inexpensive scores of liver fibrosis for the use in patients with GD. We have found that the APRI score, developed initially for the use in patients with hepatitis C, shows a good accuracy for screening for significant fibrosis (F2 or higher) in treated patients with GD – however, with a substantial difference from the cut-off values used for hepatitis C patients. This difference probably stems from the unique pathophysiological aspects of both diseases, which show little overlap.

The major limitation of this study is the limited sample size, which limits the fine-tuning of the cut-off points and tends to distort the estimates of AUC in the ROC curves. We expect to counter this limitation by the expansion of the sample size. A second limitation is

the use of elastography instead of liver biopsy as the gold standard – liver biopsy being the most accurate measurement, it is possible that the limitations of elastography are a source of bias. However, since liver biopsy presents significant risks (including bleeding, to which patients with GD are already prone), we believe that elastography is the only viable solution in the current technological development.

Conclusion

A significant proportion of patients with GD have detectable liver fibrosis by elastographic investigation. The APRI score is accurate for the screening of significant liver fibrosis in treated patients with GD when the adequate cut-off values are used.

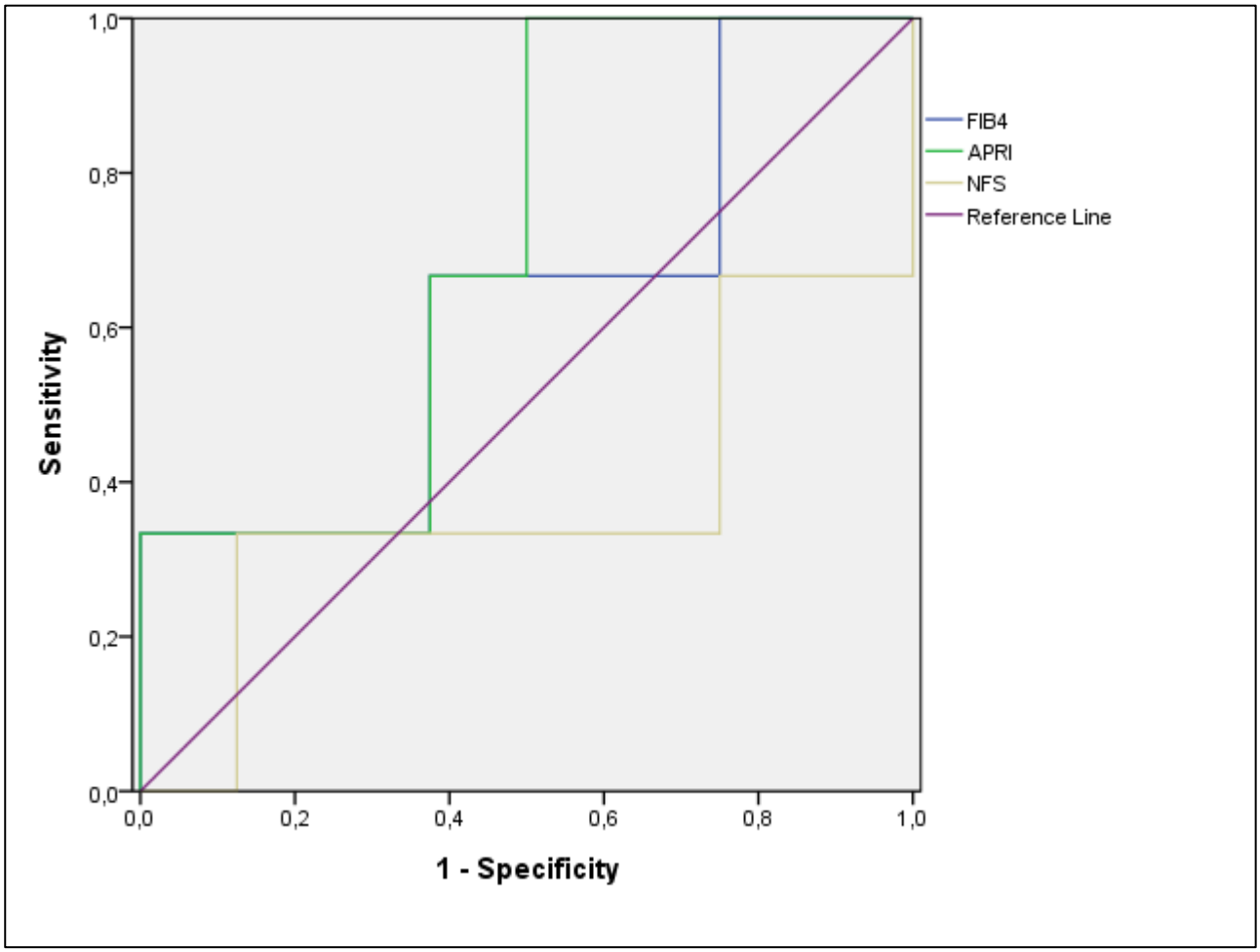


Figure 1. ROC curve showing accuracy for the APRI (green line), FIB-4 (blue line), and NFS (yellow line) scores.

References

1. Stirnemann J, Belmatoug N, Camou F, et al. A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. *Int J Mol Sci.* 2017;18(2).
2. Tylki-Szymańska A, Groener JE, Kamiński ML, Ługowska A, Jurkiewicz E, Czartoryska B. Gaucher disease due to saposin C deficiency, previously described as non-neuronopathic form--no positive effects after 2-years of miglustat therapy. *Mol Genet Metab.* 2011;104(4):627-630.
3. Linari S, Castaman G. Hematological manifestations and complications of Gaucher disease. *Expert Rev Hematol.* 2016;9(1):51-58.
4. Nguyen Y, Stirnemann J, Lautredoux F, et al. Immunoglobulin Abnormalities in Gaucher Disease: an Analysis of 278 Patients Included in the French Gaucher Disease Registry. *Int J Mol Sci.* 2020;21(4).
5. Starosta RT, Vairo FPE, Dornelles AD, et al. Liver involvement in patients with Gaucher disease types I and III. *Mol Genet Metab Rep.* 2020;22:100564.
6. Khan A, Hangartner T, Weinreb NJ, Taylor JS, Mistry PK. Risk factors for fractures and avascular osteonecrosis in type 1 Gaucher disease: a study from the International Collaborative Gaucher Group (ICGG) Gaucher Registry. *J Bone Miner Res.* 2012;27(8):1839-1848.
7. Schiffmann R, Sevigny J, Rolfs A, et al. The definition of neuronopathic Gaucher disease. *J Inherit Metab Dis.* 2020.
8. Gary SE, Ryan E, Steward AM, Sidransky E. Recent advances in the diagnosis and management of Gaucher disease. *Expert Rev Endocrinol Metab.* 2018;13(2):107-118.
9. Sidransky E. Gaucher disease: complexity in a "simple" disorder. *Mol Genet Metab.* 2004;83(1-2):6-15.
10. James SP, Stromeyer FW, Chang C, Barranger JA. LIver abnormalities in patients with Gaucher's disease. *Gastroenterology.* 1981;80(1):126-133.
11. Hill SC, Damaska BM, Ling A, et al. Gaucher disease: abdominal MR imaging findings in 46 patients. *Radiology.* 1992;184(2):561-566.
12. Lachmann RH, Wight DG, Lomas DJ, et al. Massive hepatic fibrosis in Gaucher's disease: clinico-pathological and radiological features. *QJM.* 2000;93(4):237-244.
13. Perel Y, Bioulac-Sage P, Chateil JF, et al. Gaucher's disease and fatal hepatic fibrosis despite prolonged enzyme replacement therapy. *Pediatrics.* 2002;109(6):1170-1173.
14. Bohte AE, van Dussen L, Akkerman EM, et al. Liver fibrosis in type I Gaucher disease: magnetic resonance imaging, transient elastography and parameters of iron storage. *PLoS One.* 2013;8(3):e57507.
15. Nascimbeni F, Cassinerio E, Dalla Salda A, et al. Prevalence and predictors of liver fibrosis evaluated by vibration controlled transient elastography in type 1 Gaucher disease. *Mol Genet Metab.* 2018.
16. Serai SD, Naidu AP, Andrew Burrow T, Prada CE, Xanthakos S, Towbin AJ. Correlating liver stiffness with disease severity scoring system (DS3) values in Gaucher disease type 1 (GD1) patients. *Mol Genet Metab.* 2018;123(3):357-363.

17. Lollert A, Hoffmann C, Lache M, et al. Acoustic radiation force impulse point shear wave elastography of the liver and spleen in patients with Gaucher disease type 1: Correlations with clinical data and markers of disease severity. *Mol Genet Metab.* 2020;130(2):140-148.
18. Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol.* 1995;19(12):1409-1417.
19. Zelesco M, Abott S, O'Hara S. Pitfalls and sources of variability in two dimensional shear wave elastography of the liver: An overview. *Sonography.* 2017;5(1):9.
20. Zelber-Sagi S, Yeshua H, Shlomai A, et al. Sampling variability of transient elastography according to probe location. *Eur J Gastroenterol Hepatol.* 2011;23(6):507-514.
21. Shiha GE, El-Etreby S, Bahgat M, et al. Chronic Hepatitis C Patients with Obesity: Do we Need two Operators for Accurate Evaluation of Liver Stiffness? *Ann Hepatol.* 2018;17(5):795-801.
22. Wai CT, Greenson JK, Fontana RJ, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology.* 2003;38(2):518-526.
23. Vallet-Pichard A, Mallet V, Nalpas B, et al. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology.* 2007;46(1):32-36.
24. Angulo P, Hui JM, Marchesini G, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology.* 2007;45(4):846-854.
25. Starosta RT, Pinto E Vairo F, Dornelles AD, Cerski CTS, Álvares-da-Silva MR, Schwartz IVD. Hepatocellular carcinoma in Gaucher disease: Reinforcing the proposed guidelines. *Blood Cells Mol Dis.* 2019;74:34-36.
26. Adar T, Ilan Y, Elstein D, Zimran A. Liver involvement in Gaucher disease - Review and clinical approach. *Blood Cells Mol Dis.* 2016.
27. Regenboog M, van Dussen L, Verheij J, et al. Hepatocellular carcinoma in Gaucher disease: an international case series. *J Inherit Metab Dis.* 2018.
28. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet.* 2014;383(9930):1749-1761.
29. Kanwal F, Singal AG. Surveillance for Hepatocellular Carcinoma: Current Best Practice and Future Direction. *Gastroenterology.* 2019;157(1):54-64.
30. StatPearls. In:2020.
31. Boyd A, Cain O, Chauhan A, Webb GJ. Medical liver biopsy: background, indications, procedure and histopathology. *Frontline Gastroenterol.* 2020;11(1):40-47.
32. Sandrin L, Fourquet B, Hasquenoph JM, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol.* 2003;29(12):1705-1713.

- Objetivo específico 5

Artigo 6: “Liver manifestations in a cohort of 42 patients with congenital disorders of glycosylation: pin-pointing the characteristics of liver injury and proposing recommendations for follow-up.”. Rodrigo Tzovenos Starosta, Suzanne Boyer, Shawn Tahata, Kimiyo Raymond, Hee Eun-Lee, Ida Vanessa Doederlein Schwartz, Eva Morava.

Artigo submetido para o periódico Journal of Inherited Metabolic Disease em 20 de abril de 2020, aceito com revisões em 15 de maio de 2020.

Journal of Inherited Metabolic Disease

Liver manifestations in a cohort of 42 patients with congenital disorders of glycosylation: pin-pointing the characteristics of liver injury and proposing recommendations for follow-up.

--Manuscript Draft--

Manuscript Number:	BOLI-D-20-00150
Full Title:	Liver manifestations in a cohort of 42 patients with congenital disorders of glycosylation: pin-pointing the characteristics of liver injury and proposing recommendations for follow-up.
Article Type:	Original Article
Keywords:	CDG; fibrosis; aminotransferases; transient elastography; liver transplantation; liver biopsy
Manuscript Classifications:	130: Carbohydrate disorders; 260: Glycosylation disorders/CDG, protein modification disorders
Corresponding Author:	Rodrigo Tzovenos Starosta, M.D. Universidade Federal do Rio Grande do Sul Porto Alegre, RS BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidade Federal do Rio Grande do Sul
Corresponding Author's Secondary Institution:	
First Author:	Rodrigo Tzovenos Starosta, M.D.
First Author Secondary Information:	
Order of Authors:	Rodrigo Tzovenos Starosta, M.D. Suzanne Boyer, RDN-AP, CSP, LD, CNSC, FAND Shawn Tahata, M.D. Kimlyo Raymond, M.D. Hee Eun Lee, M.D., Ph.D. Ida Vanessa Doederlein Schwartz, M.D., Ph.D. Eva Morava, M.D., Ph.D.
Order of Authors Secondary Information:	
Funding Information:	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior — Dr. Rodrigo Tzovenos Starosta National Institutes of Health (U54 NS115198-01) — Dr. Eva Morava National Center for Advancing Translational Sciences — Dr. Eva Morava

Abstract:	The congenital disorders of glycosylation (CDG) are metabolic diseases with multi-system involvement. The liver phenotype of CDG varies according to the specific disorder, and from patient to patient. In this prospective study, we sought to identify common patterns of liver injury among patients with CDG, and to provide recommendations for follow-up in practice. Patients were enrolled to the Frontiers in Congenital Disorders of Glycosylation (FCDGC) natural history study. We analyzed clinical history, molecular genetics, serum markers of liver injury, liver ultrasonography and elastography, liver histopathology (when available), and clinical scores of 42 patients with 17 different CDG subtypes (PMM2-CDG, n=19), with a median age of 7 years (range: 10 months to 65 years). Our main findings are i) there is a clear pattern in the evolution of the markers of hepatocellular injury markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to age, especially in PMM2-CDG patients but also in other type I CDGs, and that the cholangiocyte injury marker gamma-glutamyltransferase (GGT) is not elevated in most patients, pointing to an exclusive hepatocellular origin of injury; ii) there is dissociation between liver ultrasound and transient elastography regarding signs of liver fibrosis; iii) histopathological findings in liver tissue of PMM2-CDG patients include cytoplasmic glycogen deposits, pointing to a disruption of glycogen metabolism in these patients; and iv) most CDGs manifest with more than one type of liver injury. Therefore, we recommend that all CDG patients have systematic, comprehensive screening for liver disease, including physical examination (hepatomegaly, signs of liver failure), laboratory tests (ALT, AST), ultrasound (steatosis, tumors), and elastography (fibrosis).
Suggested Reviewers:	

Cover Letter

April 20th, 2020

Rochester, MN, USA; Porto Alegre, RS, Brazil.

Dear Editor,

It is with pleasure that we submit our manuscript entitled "Liver manifestations in a cohort of 42 patients with congenital disorders of glycosylation: pin-pointing the characteristics of liver injury and proposing recommendations for follow-up" for publication in the Journal of Inherited Metabolic Diseases. This manuscript presents our research work to characterizing in detail the hepatic phenotype of congenital disorders of glycosylation (CDG) in a big cohort, outlining patterns that can help clinicians better predict the prognosis of their patients. We have also identified potential predictors of liver disease, as well as pitfalls in the follow-up of these patients – the most important being an ultrasound-elastography dissociation. Based on that, we propose recommendations for the follow-up patients with CDG that take in consideration the heterogeneity of manifestations in these diseases and the longitudinal experience summarized in this article.

We believe our manuscript would be a great addition to your journal for clarifying the hepatic phenotype of patients with CDG and providing evidence-based recommendations for their follow-up. The CDG being mostly ultra-rare disorders, there is an urging need for systematic studies that standardize the medical care provided. In using a big cohort with an extensive and comprehensive follow-up, we hope to contribute to fill this gap. We are grateful in advance for your time in reviewing this manuscript and are happy to answer any questions or concerns that may arise from it.

With our best regards,

The Authors

Liver manifestations in a cohort of 42 patients with congenital disorders of glycosylation: pin-pointing the characteristics of liver injury and proposing recommendations for follow-up.

Rodrigo Tzovenos Starosta[1,2], Suzanne Boyer[2], Shawn Tahata[3], Kimiyo Raymond[4], Hee Eun Lee[4], Ida Vanessa Doederlein Schwartz[1,5], Eva Morava[2,5,6].

1 – Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

2 – Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA

3 – Department of Internal Medicine, Mayo Clinic, Rochester, MN, USA

4 – Department of Laboratory Medicine and Pathology, Biochemical Genetics Laboratory, Mayo Clinic, Rochester, MN, USA

5 – Service of Medical Genetics, Hospital de Clínicas de Porto Alegre, UFRGS, Porto Alegre, RS, Brazil

6 – Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA

Word count (abstract): 286. Word count (text): 3107.

Number of tables: 4. Number of figures: 3.

A color picture that can be used for the front cover of the issue in which this article appears has been provided.

Abstract

The congenital disorders of glycosylation (CDG) are a heterogeneous group of rare metabolic diseases with multi-system involvement. The liver phenotype of CDG varies not only according to the specific disorder, but also from patient to patient. In this prospective study, we sought to identify common patterns of liver injury among patients with a broad spectrum of CDG, and to provide recommendations for follow-up in clinical practice. Patients were enrolled to the Frontiers in Congenital Disorders of Glycosylation (FCDGC) natural history study. We analyzed clinical history, molecular genetics, serum markers of liver injury, liver ultrasonography and transient elastography, liver histopathology (when available), and clinical scores of 42 patients with 17 different CDG subtypes (PMM2-CDG, n=19), with a median age of 7 years (range: 10 months to 65 years). Our principal findings are i) there is a clear pattern in the evolution of the markers of hepatocellular injury markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to age, especially in PMM2-CDG patients but also in other type I CDGs, and that the cholangiocyte injury marker gamma-glutamyltransferase (GGT) is not elevated in most patients, pointing to an exclusive hepatocellular origin of injury; ii) there is dissociation between liver ultrasound and transient elastography regarding signs of liver fibrosis; iii) histopathological findings in liver tissue of PMM2-CDG patients include cytoplasmic glycogen deposits, pointing to a disruption of glycogen metabolism in these patients; and iv) most CDGs manifest with more than one type of liver injury. Based on these findings, we recommend that all CDG patients have regular systematic, comprehensive screening for liver disease, including physical examination (for hepatomegaly and signs of liver failure), laboratory tests (ALT and AST), liver ultrasound (for steatosis and liver tumors), and liver elastography (for fibrosis).

Synopsis: Several congenital disorders of glycosylation have hepatic phenotypes involving more than one liver manifestation, warranting a regular multimodal follow-up with screening for hepatomegaly, hepatocellular damage, steatosis, and liver fibrosis.

Introduction

The congenital disorders of glycosylation (CDG) are a group of rare inherited metabolic diseases, mostly autosomal recessive, that affect the complex process of building, remodeling, and delivering glycan chains. There are approximately 150 known CDG to date, each caused by a deficiency of a different component of the glycan-assembly and remodeling pathways (Francisco et al. 2019). The current classification divides CDG according to which of the two main glycosylation sites in proteins or lipid is affected (Péanne et al. 2018): N-linked (glycosylation of asparagine residues) or O-linked glycosylation (glycosylation of serine and threonine residues). N-linked CDG are further subdivided based on whether the enzymatic defect impacts the assembly and transfer of primary glycan chains in the endoplasmic reticulum (type I) or the processing of glycan chains in the Golgi apparatus (type II). Because of the ubiquity of glycosylation in human physiology (Fisher et al. 2019), CDG tend to be multisystem diseases, affecting different organs and tissues in a heterogeneous way (Verheijen et al. 2020). One of the most commonly affected organs in many CDG is the liver, due to its central role in N-glycosylation (Verheijen et al. 2020); however, there is limited study of the liver phenotype of CDG beyond PMM2-CDG (Witters et al. 2019).

PMM2-CDG (MIM: #212065) is the most common CDG, with more than a thousand cases reported so far (Altassan et al. 2019). Most PMM2-CDG patients have mild liver dysfunction with increased aminotransferases alone, especially during the first 5 years of life, when all-cause lethality is also higher (Witters et al. 2019); however, a proportion of patients develops steatosis, fibrosis/cirrhosis, and may die from the complications of liver failure (Witters et al. 2019). Other CDG have more specific hepatic phenotypic characteristics. For instance, CCDC115-CDG (MIM: #616828) and MPI-CDG (MIM: #602579) have especially severe liver disease (Marques-da-Silva

et al. 2017). CCDC115-CDG presents with a Wilson disease-like phenotype with hepatosplenomegaly, elevated aminotransferases, neonatal jaundice, early cirrhosis, and elevated concentration of copper in liver tissue (Girard et al. 2018, Jansen et al. 2016, Medrano et al. 2019). MPI-CDG tends to present with severe liver dysfunction and rapidly progressive (sometimes congenital) fibrosis (Marques-da-Silva et al. 2017, Mention et al. 2008), although asymptomatic adults have been reported (Helander et al. 2014, de la Morena-Barrio et al. 2019). Other CDG, such as ALG8-CDG (MIM: #603147), can present with severe liver disease with cirrhosis and complications of portal hypertension, with high lethality (Marques-da-Silva et al. 2017).

The hepatic phenotype in CDG is complex and understanding the manifestations and the type of liver injury to be expected in a patient is critical for appropriate management. In this study, we prospectively evaluate a large cohort of patients with different CDG to systematically explore the hepatic phenotype of these disorders, seeking to identify common patterns of liver injury and to derive recommendations for clinical practice.

Methods

Subjects

Patients with a molecularly confirmed diagnosis of CDG and biochemical confirmation of the specific defect were prospectively recruited to the Frontiers of Congenital Disorders of Glycosylation (FCDGC) natural history study or to the CDG Nutritional Intervention study at Mayo Clinic. Retrospective data collection was part of the enrollment process, as the patients has been previously followed-up by standard of care. All patients provided signed informed consent.

This study was approved by the Institutional Review Board of Mayo Clinic under protocols #18-007276 and #19-005187.

Data extraction

The following variables for the composite categorical parameters on extra-hepatic phenotype were used, being considered as positive when at least one of the variables in each parameter was abnormal: hematological abnormalities (hemoglobin, white blood cell count, platelet count); coagulation abnormalities (prothrombin time, activated partial thromboplastin time, factor XI activity, antithrombin III activity, clinically significant bleeding or thrombosis); dyslipidemia (low-density lipoprotein, high-density lipoprotein, total cholesterol, triglycerides); hypothyroidism (thyroid-stimulating hormone); glucose homeostasis (blood glucose, documented hypoglycemia); seizures (history of seizures, diagnosis of epilepsy).

The Nijmegen Pediatric CDG Rating Score (NPCRS) was performed in every patient in the study by the same investigator (EM). The NPCRS is a clinical tool developed as a means of quantifying clinical severity of patients with CDG in a global and comprehensive way, and is validated for all age groups (Achouitar et al. 2011).

Statistical analysis

Categorical variables were compared using Fisher's exact test with a significance level of $p<0.05$. Q-Q plots were used to determine approximate parametricity. Parametric continuous variables were compared between groups using Student's *t*-test with a significance level of $p<0.05$. A logistic regression was used to identify correlations of continuous variables with categorical outcomes, with a significance level of $p<0.05$.

Results

Subjects

Forty-two patients were included in the study. Median age was 7 years (range: 10 months to 65 years), and 64.3% (27/44) were male (table 1). The most common diagnosis was PMM2-CDG (n=19); followed by ALG12-CDG (MIM: #607143), ALG13-CDG (MIM: #300884), DHDDS-CDG (MIM: #613861), PGM1-CDG (MIM: #614921), PIGT-CDG (MIM: #615398), and SLC35A2-CDG (MIM: #300896) (n=2 each); and ALG6-CDG, ALG8-CDG (MIM: #608104), CCDC115-CDG, DDOST-CDG (MIM: #614507), MPI-CDG, PIGV-CDG (MIM: #239300), SLC10A7-CDG (MIM: #618363), SLC35C1-CDG (MIM: #266265), SLC39A8-CDG (MIM: #616721), TMEM165-CDG (MIM: #614727), and VMA21-CDG (MIM: #310440) (n=1 each).

Clinical findings

Clinical descriptions were available for 42 patients. Six patients had hepatomegaly on physical exam (patients 2, 6, 8, 11, 23, and 36). Three patients had neonatal jaundice requiring phototherapy (patients 10, 12 and 26), but no patient had jaundice outside of the neonatal period. One patient had ascites (patient 6); no patient had other stigmata of liver disease (e.g., *caput medusae*, gynecomastia, or palmar erythema) on physical exam, although one patient (patient 7) had unexplained pruritus. Patient 6 underwent liver transplantation at 4 years of age because of liver failure (hyperammonemia, recurrent ascites).

Molecular findings

A total of 42 patients had genetically confirmed CDG genotypes. The most common genetic variant found in *PMM2* was c.422G>A (p.Arg141His), comprising 13/36 (36.1%) of alleles reported (table 1). The only homozygous *PMM2* variant found was c.357C>A (p.Phe119Leu). Two different *PMM2* variants were found to arise in the same nucleotide position: c.710C>G (p.Thr234Arg) and c.710C>T (p.Thr234Met).

Laboratory findings – liver markers

Liver markers for all patients are shown in detail in table 1. Alanine-aminotransferase (ALT) values were available for 41 patients: of these, 21 (51.2%) had at least one elevated value (normal <42 IU/L). Aspartate-aminotransferase (AST) values were available for 42 patients; of these, 32 (76.2%) had at least one elevated value (normal <41 IU/L). Elevations in both enzymes were found in 19 patients (48.7%). Gamma-glutamyltransferase (GGT) values were available for 15 patients; of these, 4 had at least one elevated value (patient 6: 157 IU/L; patient 7: 45 IU/L; patient 9: 57 IU/L; patient 27: 56 IU/L; normal <41 IU/L). Alkaline phosphatase (AlkP) values were available for 42 patients; of these, 7 (16.7%) had elevated values (normal <300 IU/L). Total bilirubin values were available for 41 patients; of these, only 2 had increased values (patient 34: 1.5 mg/dL, no direct/indirect differential; patient 42: 2.3 mg/dL, direct bilirubin 0.5 mg/dL; normal total bilirubin <1.3 mg/dL). Aminotransferase values according to age are displayed in figure 1.

In PMM2-CDG patients, no association was observed between the frequency of altered aminotransferase values and presence of the most common *PMM2* variant, c.422G>A (p.Arg141His) (ALT, p=0.604; AST, p=0.518).

Liver ultrasound and transient elastography (FibroScan) findings

Liver ultrasound results were available for 22 patients (table 2). The most common finding was coarse hepatic echotexture (7/22, 31.8%). Four patients (18.2%) had steatosis and two patients (9.1%) had focal lesions (one diagnosed with a solitary hemangioma of the liver; the other with multiple circumscribed echogenic lesions with no blood flow on color imaging, the largest being subcapsular and measuring 6 x 7 x 9 mm). Six patients had normal liver ultrasounds.

Four patients had transient hepatic elastography (table 2); of these, 3 had had also liver ultrasounds. One patient who had coarse liver parenchyma on ultrasound had a METAVIR score (Frulio and Trillaud 2013) of F0 on elastography, indicating no fibrosis. On the other hand, two patients with no signs of fibrosis on ultrasound had METAVIR scores of F1 and F2 on elastography, indicating incipient fibrosis.

Liver biopsy

Histopathological reports for liver tissue were available for three patients: two patients underwent liver biopsy and one had an explanted liver. The liver explanted from patient 6 (PMM2-CDG) showed micronodular cirrhosis on gross and microscopic examination (figure 2); there was mild macrovesicular steatosis and mild-to-moderate biliary ductular proliferation, as well as multiple foci of hepatocellular glycogen storage in the cytoplasm; iron staining was negative. Patient 7 (PMM2-CDG) had a liver biopsy at 3 years of age that showed broad, irregular bridging fibrosis with minimal mixed (macrovesicular and microvesicular) steatosis and increased hepatocellular glycogen, with no signs of biliary changes, iron overload, or hepatocellular regeneration. Patient 36 (CCDC115-CDG) had a liver biopsy at 2 years of age that showed cirrhosis with extensive portal and periportal bridging fibrosis, microvesicular steatosis, positive glycogen staining with fine hepatocellular cytoplasm vacuolization in a mosaic pattern, with no

signs of biliary changes or iron overload. All glycogen deposits were confirmed by positivity on periodic acid Schiff (PAS) staining, with attenuation after diastase treatment.

Treatment effects

Patient 26 with PGM1-CDG started treatment with oral galactose 0.5 g/kg/day at age 2 years, with a planned increase to 1 g/kg/day after 6 weeks of the escalating dose. ALT and AST were elevated before treatment, with a rapid decrease (3 weeks) after introduction of treatment (figure 3a). Patient 27 with PGM1-CDG started treatment with oral galactose 0.5 g/kg/day at age 27 years, with a significant decrease in AST and ALT within 6 months after introduction of treatment (figure 3b). Patient 30 with SLC35A2-CDG started treatment with oral galactose 1.5 g/kg/day at age 6 months. AST and GGT were elevated before treatment, which both normalized on treatment within 4 months, and additionally, significant improvement was observed in other clinical features such as seizure control and motor skills. Patient 31 with SLC35A2-CDG started treatment with oral galactose 1.5 g/kg/day at age 10 years. There was a mild transient elevation in ALT and AST levels after initiation of treatment (figure 3c). Although transaminases normalized, no significant improvement was observed in clinical features during the first 4 months of therapy, as described elsewhere (Witters et al. 2020). Patient 35 with MPI-CDG has been on treatment with oral mannose 1 g/kg/day divided in 6 doses at age 4 years for 2 years. High aminotransferase values were observed during the whole period of 2 years long treatment and the patient needed additional nutritional intervention with a complex carbohydrate diet. Patient 39 with SLC35C1-CDG started treatment with oral fucose 2 g/day at age 10 years, with an increase to 4g after 6 months of therapy and 8 g/day at age 11 years. There was no elevation in ALT, AST, or AlkP before or during treatment (figure 3d), although clinical parameters such as tendency to infections were improved. Patient 40 with SLC39A8-CDG started treatments with oral galactose 1g/kg/day

at age 2 years and escalating dose of manganese from 5 mg/day to 65 mg/day during a period of 6 months. No significant change was observed in ALT and AST levels after introduction of treatment; however, the patient had significant clinical improvement with respect to seizure control and motor development over the first 6 months of therapy. Patient 41 with TMEM165-CDG started treatment with oral galactose 1.5 g/kg/day at age 5 years. No pre-treatment laboratory results are available. High aminotransferase values were observed during treatment.

Nijmegen Pediatric CDG Rating Scale (NPCRS)

A NPCRS (Achouitar et al. 2011) score was available for 41 patients (median: 22.5, range: 3-40). A Q-Q plot determined the distribution of NPCRS values to be approximately normal. There was no difference in the NPCRS values between patients with elevated ALT and patients with normal ALT (Student's *t*-test, p=0.215). A significant difference was however found in the NPCRS scores between patients with elevated and normal AST values, with patients with elevated AST measurements having higher NPCRS values (respectively, 23.46 ± 8.04 *vs* 15.60 ± 6.83, p=0.009). A binary logistic regression between having high AST values and the tree sections of the NPCRS showed that this significant association is due to the first section ("current function") only, with a p=0.045, Nagelkerke's r^2=0.353.

Patients with a diagnosis of coarse liver parenchyma on ultrasound had a significantly higher NPCRS than patients who did not have this diagnosis (30.20 ± 7.66 *vs* 20.84 ± 6.79, p=0.022).

Extra-hepatic associations

Prevalence of extra-hepatic manifestations of CDG is displayed in table 3. A significant association was found in the co-distribution of high ALT levels and abnormal coagulation

parameters (85% of patients with high ALT levels had coagulation abnormalities *vs* 22.7% of patients with coagulation abnormalities with normal ALT, p=0.002) and hypothyroidism (all patients with hypothyroidism had high ALT values *vs* 41.3% of patients without hypothyroidism having high ALT values, p=0.004). No association was found in the co-distribution of high ALT levels and abnormal hematological parameters, dyslipidemia, glucose homeostasis, or seizures. For high AST values, a significant association was found for coagulation parameters (73.1% of patients with high AST levels had coagulation abnormalities *vs* 13.6% of patients who had normal AST values, p=0.026) and seizures (65.5% of patients with high AST levels had seizures *vs* 13.6% of patients who had normal AST values, p=0.037).

Discussion

The liver is one of the main sites of N-glycosylation in the body (Zhu et al. 2014) and is responsible for the attachment of glycans to most secreted proteins. CDG affect the liver in a number of ways. In this prospective study, we have described a cohort of patients with 17 different CDG and confirmed previous findings in the literature (Marques-da-Silva et al. 2017) that liver enzymes, which are common, reliable markers of hepatocellular injury, are mostly elevated during the first 5 years of life in most types of CDG, but improve significantly after this age. Exceptions to this in our study were ALG8-CDG, CCDC115-CDG, MPI-CDG, PGM1-CDG, and TMEM165-CDG patients. In contrast to aminotransferases, we found that few patients had an elevation of the cholangiocyte injury marker GGT, and that most of such elevations were mild. Normal GGT in the setting of elevated aminotransferases had previously been reported in isolated cases of CDG (Mention et al. 2008, Girard et al. 2018), but to the best of our knowledge this is the first time it

has been reported systematically. This highlights the hepatocellular, rather than global, nature of liver damage in this group of disorders.

The most common finding on liver ultrasound was a coarse liver parenchyma, which is usually associated with fibrotic changes. We have found an association of this finding with more severe disease, as measured by the NPCRS. However, we have observed a disparity between liver ultrasound and a fibrosis-directed measurement, transient elastography. Unfortunately, we only have data from a few patients on liver elastography, because elastography hasn't been considered as standard-of-care in pediatric patients with CDG, and these studies were performed prior to the study recruitment. Nonetheless, the disparity between elastography and liver ultrasound results, and medical literature on the efficacy of elastography in fibrosis (Frulio and Trillaud 2013) indicates that both liver ultrasound and elastography should be used for follow-up in patients in CDG.

When analyzing the relationship between elevations in liver enzymes and extra-hepatic manifestations, we found that both elevated ALT and AST are associated with the presence of coagulation abnormalities. Since most coagulation factors are glycosylated in the liver, these changes mirror the degree of glycosylation abnormality. This highlights the necessity of screening for coagulopathy in patients with high aminotransferases in this population. Although we also found an association of ALT with hypothyroidism and of AST with seizure, the lack of a direct biological link makes it probable that this association comes from patients with more severe disease having elevated liver enzymes as well as more extra-hepatic manifestations.

With advances in imaging technology, liver biopsy in metabolic patients is becoming less common. We had access to specimens of liver tissue from 3 patients. In the two PMM2-CDG

patients, there was increased cytoplasmic glycogen deposits; although in one of these patients this findings was in the context of liver cirrhosis and may be considered as a part of the cirrhotic phenotype (Bannasch et al. 1997), the presence of scattered hepatocytes with foci of glycogenosis in the other suggests that a disturbance of hepatic glycogen breakdown may be part of the pathophysiology of PMM2-CDG.. In the biopsy of the patient with CCDC115-CDG we could not observe any signs of cholestasis, foamy histiocytes, or necrotic lesions, as previously described in the literature (Girard et al. 2018, Jansen et al. 2016). This highlights the heterogeneity of manifestations within a single subtype of CDG, underscoring the need for pathologists and clinicians to be aware that findings described in previous patients are not necessarily required for diagnosis.

We have observed different effects of short-term monosaccharide treatment according to the subtype of CDG. For PGM1-CDG, there was a rapid improvement in aminotransferase levels after introduction of galactose therapy, as expected (Wong et al. 2017, Radenkovic et al. 2019, Witters, Cassiman, and Morava 2017). For SLC35A2-CDG, although we did notice improvement in neurological features as expected (Dörre et al. 2015, Witters, Cassiman, and Morava 2017), there was no significant change detected in aminotransferase levels. For MPI-CDG and TMEM165-CDG, it has been described that mannose and galactose supplementation, respectively, do not always fully correct or prevent liver damage (Mention et al. 2008, Janssen et al. 2014, Morelle et al. 2017), and our observations match this expectation. SLC35C1-CDG affects primarily leukocytes and the central nervous system (Dauber et al. 2014), and therapeutic goals with fucose supplementation involve restoration of immune function and neurological function (Brasil et al. 2018). SLC39A8-CDG affects primarily bone development and central nervous

system function, without significant hepatic findings (Park et al. 2018), in line with our laboratory findings.

Recently, Ferreira et al. proposed a categorization of metabolic liver disease according to type of liver manifestation (Ferreira, Cassiman, and Blau 2019) including diseases with hepatomegaly, hepatocellular disease with elevation of aminotransferases or liver failure, cholestasis, steatosis, fibrosis, and liver tumors. According to this classification, most CDGs span more than one category (table 4). This warrants the recommendation that every patient with CDG be systematically evaluated for different types of liver disease with a combination of physical exam, laboratory testing, ultrasonography, and elastography.

In summary in our prospective data set of CDG patients we detected spontaneous improvement of hepatocellular injury markers AST and ALT in type I CDG, but not in type II CDG. There was dissociation between liver ultrasound and transient elastography results in several patients necessitating prospective liver elastography in CDG patients to survey for possible liver fibrosis. The cytoplasmic glycogen deposits found in liver biopsy specimens suggest a disturbance of glycogen metabolism and needs more dietary attention/intervention. Based on our findings, we recommend, that all CDG patients have regular systematic, comprehensive screening for liver disease, including clinical, laboratory and imaging techniques that detect both steatosis and fibrosis.

Acknowledgements

RTS was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Programa Institucional de Internacionalização (CAPES/PRINT) #002-2019, Brazil. This work

was supported by National Institutes of Health grant 1 U54 NS115198–01 and the Rare Disease Clinical Research Network (RDCRN) by the National Center for Advancing Translational Sciences, NIH, USA.

Table 1. Patient characteristics and markers of liver injury.

Patient	Age (Y)	Sex	CDG	Genotype	Protein change	ALT range	AST range	AlkP range
1	1	M	PMM2	c.44G>C; c.422G>A	p.Gly15Ala; p.Arg141His	15-145	10-147	172-301
2	1	M	PMM2	c.357C>A; c.422G>A	p.Phe119Leu; p.Arg141His	103-571	93-829	172-304
3	2	M	PMM2	c.357C>A; c.422G>A	p.Phe119Leu; p.Arg141His	53-1595	54-1222	222-464
4	3	M	PMM2	c.422G>A; c.691G>A	p.Arg141His; p.Val231Met	48-66	54-57	173-182
5	5	M	PMM2	c.422G>A; c.548T>C	p.Arg141His; p.Phe183Ser	34-41	23	186
6	5	F	PMM2	c.338C>T; c.710C>G	p.Phe113Leu; p.Thr234Arg	8-745	13-678	17-283
7	6	M	PMM2	c.357C>A; c.422G>A	p.Phe119Leu; p.Arg141His	25-1366	25-1986	115-1083
8	6	F	PMM2	c.415G>A; c.422G>A	p.Glu139Lys; p.Arg141His	19-31	31-48	157-250
9	6	M	PMM2	c.338C>T; c.422G>A	p.Pro113Leu; p.Asp148Asn	32-2524	26-4789	60-292
10	6	M	PMM2	c.422G>A; c.647A>T	p.Arg141His; p.Asn216Ile	37-71	45-69	104-155
11	7	F	PMM2	c.563A>G; c.691G>A	p.Asp188Gly; p.Val231Met	38-556	31-457	228-299
12	7	M	PMM2	unknown	unknown	28	42	225-242
13	8	M	PMM2	c.98A>C; c.140C>T	p.Gln33Pro; p.Ser47Leu	15-20	29-34	139-149
14	11	M	PMM2	c.422G>A; c.722G>C	p.Arg141His; p.Cys241Ser	17-18	25-26	154-172
15	12	M	PMM2	c.470T>C; c.710C>T	p.Phe157Ser; p.Thr237Met	44-68	46-58	187-240
16	15	M	PMM2	c.422G>A; c.458T>C	p.Arg141His; p.Ile153Thr	30-117	30-244	94-215
17	23	M	PMM2	unknown	unknown	19-25	21-27	61-75
18	27	M	PMM2	c.357C>A; c.422G>A	p.Phe119Leu; p.Arg141His			
19	33	F	PMM2	c.357C>A; c.357C>A	p.Phe119Leu; p.Phe119Leu	14-164	21-327	44-48
20	31	M	ALG12	c.671C>T; c.1001delA	p.Thr224Met; p.Asn334ThrfsX15	18	23	61
21	46	M	ALG12	c.671C>T; c.1001delA	p.Thr224Met; p.Asn334ThrfsX15	15	22	121
22	1	F	ALG13	c.320A>G	p.Asn107Ser	10-18	40-49	102-165
23	4	F	ALG13	c.320A>G	p.Asn107Ser	17-34	28-51	118-405

24	59	M	DHDDS	c.124A>G; c.124A>G	p.Lys42Glu; p.Lys42Glu	25-27	37-38	77-82
25	63	F	DHDDS	c.124A>G; c.124A>G	p.Lys42Glu; p.Lys42Glu	20-27	28-33	69-82
26	2	M	PGM1	c.265G>A; c.988G>C	p.Gly89Arg; p.Gly330Arg	25	122	168
27	27	F	PGM1	c.206T>C; c.313A>T	p.Met67Arg; p.Lys105X	26-61	46-236	53-61
28	1	F	PIGT	c.1582G>A; c.1730dup	p.Val528Met; p.Leu578ThrfsX35	33	44	197
29	7	F	PIGT	c.1582G>A; c.1730dup	p.Val528Met; p.Leu578ThrfsX35	18-26	34-45	145-167
30	1	F	SLC35A2	c.340A>T	p.Lys114X	8-35	33-78	83-235
31	12	F	SLC35A2	c.815G>A	p.Trp272X	12-87	17-61	147-177
32	21	F	ALG6	c.998C>T	p.Ala333Val	12-50	17-51	81-275
33	8	M	ALG8	c.584T>C; c.1334T>C	p.Leu195Pro; p.Leu445Pro	20-165	14-148	73-275
34	65	F	DDOST	c.20C>G; c.1325T>A	p.Ala7Gly; p.Phe442Tyr	16-33	13-21	66-123
35	6	M	MPI	c.488-1G>C; c.656G>A	IVS4-1G>C; p.Arg219Gln	141-237	69-90	141-210
36	3	M	CCDC115	unknown	unknown	117-204	129-319	1071-1459
37	11	M	SLC10A7	Whole gene deletion (biallelic)		23	50-56	161-203
38	12	F	SLC35C1	c.503_505del TCT; c.942C>G	p.Phe168del; p.Tyr314X	17-29	19-29	211-273
39	2	M	SLC39A8	c.802C>T; c.802C>T	p.His268Tyr; p.His268Tyr	19-23	40-45	200-233
40	14	M	TMEM165	c.151C>T; c.725C>A	p.Gln51X; p.Thr242Lys	51-60	252-309	158-219
41	42	M	VMA21	c.52A>G	p.Arg18Gly*	24-48	39-62	113-160
42	2	M	PIGV	c.649C>T; C.1022C>A	p.Gln217X; p.Ala341Glu	15-21	69	2300

Patients 20-21; 24-25; 28-29 are siblings. Y = years-old; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AlkP = alkaline phosphatase; M = male; F = female. Age displayed is the current age.

Table 2. Patients that underwent liver ultrasound or transient elastography.

Patient	CDG	Age at ultrasound (Y)	Ultrasound findings	Transient elastography result
1	PMM2	1	Coarse liver parenchyma	F0 (3.8 kPa)
2	PMM2	1	Coarse liver parenchyma	-
3	PMM2	1	Steatosis[a]	-
6	PMM2	4	Coarse liver parenchyma	-
7	PMM2	5	Coarse liver parenchyma	-
8	PMM2	6	Central intrahepatic bile duct dilation, otherwise normal	F2 (8.4 kPa)
9	PMM2	6	Normal	-
10	PMM2	1	Normal	-
11	PMM2	5	Hemangioma, otherwise normal	F1 (7.3 kPa)
13	PMM2	6	"Starry sky" parenchyma	-
14	PMM2	10	Normal	-
16	PMM2	15	Steatosis	
18	PMM2	28	Coarse liver parenchyma	-
22	ALG13	1	Geographic steatosis	-
23	ALG13	4	Multiple well-circumscribed echogenic lesions	-
24	DHDDS	63	Normal	-
27	PGM1	2	Increased echogenicity	
29	SLC35A2	4 days	Normal	-
32	ALG8	6	Steatosis	-
35	MPI	-	-	F2 (8.0 kPa)
36	CCDC115	2	Mildly increased echogenicity	-
39	SLC39A8	2	Coarse liver parenchyma	-
40	TMEM165	12	Normal	-
41	VMA21	34	Normal	-

[a]Resolved subsequently.

Table 3. Prevalence of extra-hepatic manifestations in CDG patients.

CDG	Hematological abnormalities	Coagulation abnormalities	Dyslipidemia	Hypothyroidism	Glucose homeostasis	Seizures
PMM2	3/15	12/15	4/11	6/15	8/13	8/16
ALG6	N/A	1/1	0/1	0/1	0/1	1/1
ALG8	0/1	1/1	N/A	0/1	0/1	1/1
ALG12	N/A	2/2	0/2	0/2	0/2	0/2
ALG13	0/2	0/2	1/1	0/2	0/2	2/2
CCDC115	0/1	0/1	1/1	N/A	0/1	0/1
DDOST	0/1	0/1	1/1	0/1	1/1	0/1
DHDDS	1/2	0/2	2/2	0/2	2/2	1/2
MPI	0/1	1/1	N/A	1/1	1/1	1/1
PGM1	1/2	2/2	0/1	1/2	2/2	0/2
PIGT	0/2	N/A	N/A	N/A	1/2	2/2
PIGV	0/1	N/A	0/1	0/1	1/1	1/1
SLC10A7	0/1	1/1	1/1	0/1	0/1	0/1
SLC35A2	1/2	0/2	1/1	0/2	1/2	2/2
SLC35C1	0/1	N/A	N/A	0/1	0/1	0/1
SLC39A8	0/1	0/1	N/A	0/1	0/1	1/1
TMEM165	0/1	0/1	1/1	0/1	0/1	1/1
VMA21	0/1	0/1	1/1	0/1	0/1	0/1

Values are displayed as the number of patients with the manifestation divided by the number of patients for whom there was available data regarding the manifestation.

Table 4. Distribution of liver manifestations per CDG in this study.

CDG	Hepatomegaly	Hepatocellular disease	Cholestasis	Steatosis	Fibrosis	Liver tumor
PMM2	+	+	+	+	+	+
ALG6	-	+	-	N/A	N/A	-
ALG8	-	+	-	+	-	-
ALG12	-	-	-	N/A	N/A	-
ALG13	+	+	-	+	-	+
CCDC115	+	+	-	+	+	-
DDOST	-	-	+	N/A	N/A	-
DHDDS	-	-	-	-	-	-
MPI	-	+	-	N/A	+	-
PGM1	-	+	-	N/A	N/A	-
PIGT	-	-	+	N/A	N/A	-
PIGV	-	+	+	N/A	N/A	-
SLC10A7	-	+	-	N/A	N/A	-
SLC35A2	-	+	-	-	-	-
SLC35C1	-	-	-	N/A	N/A	-
SLC39A8	-	-	-	-	-	-
TMEM165	-	+	-	-	-	-
VMA21	-	+	-	-	-	-

Classification adapted from Ferreira *et al.* (Ferreira, Cassiman, and Blau 2019). Hepatomegaly was defined as an enlarged liver on physical exam or ultrasonography. Hepatocellular disease was defined as elevated ALT or AST. Cholestasis was defined as jaundice, elevated bilirubin, or histopathological evidence of bile accumulation. Steatosis was defined as ultrasonographic or histopathological evidence of hepatocellular lipid accumulation. Fibrosis was defined as transient elastography score equal or greater than F1 or histopathological evidence of fibrosis. Liver tumor was defined as any imaging evidence of tumoral growth (neoplastic or non-neoplastic) in the liver.

Figure 1: Aminotransferase values in all CDG patients according to subtype (PMM2-CDG; Type I, Non-PMM2; Type II). There is a notable inflexion point around 5 years of age in the PMM2 ALT and AST values, after which most values tend to be normal or near-normal. A less defined inflexion point vcan be noted in the Type I, Non-PMM2, and in the Type II ALT and AST curves at around 8 years of age, although there are still many patients with elevated values after this age.

Figure 2: A – liver, hematoxylin and eosin, 20x. Cirrhotic liver tissue with extensive bridging and enlarged portal tract. There is focal glycogen deposition in the cytoplasm of hepatocytes of a nodule (black arrow). B – liver, hematoxylin and eosin, 200x. Hepatocellular nodule with cytoplasmic glycogen deposition (black arrow). C – liver, hematoxylin and eosin, 200x. Hepatocellular nodule with discrete macrovesicular steatosis.

Figure 3: evolution of ALT and AST values in treated patients. Red line = onset of treatment; green line = upper limit of normal. A – improvement after initiation of oral galactose therapy in a patient with PGM1-CDG; B – mild transient elevation of aminotransferase values after initiation of oral galactose therapy in a patient with SLC35A2-CDG, with rapid normalization; C – absence of significant change from a normal baseline after initiation of oral fucose therapy in a patient with SLC35C1-CDG; D – absence of significant change from a near-normal baseline after initiation of oral galactose and manganese therapy in a patient with TMEM165-CDG.

References

Achouitar, S., M. Mohamed, T. Gardeitchik, S. B. Wortmann, J. Sykut-Cegielska, R. Ensenauer, H. O. de Baulny, K. Ounap, D. Martinelli, M. de Vries, R. McFarland, D. Kouwenberg, M. Theodore, F. Wijburg, S. Grunewald, J. Jaeken, R. A. Wevers, L. Nijtmans, J. Elson, and E. Morava. 2011. "Nijmegen paediatric CDG rating scale: a novel tool to assess disease progression." *J Inherit Metab Dis* 34 (4):923-7. doi: 10.1007/s10545-011-9325-5.

Altassan, R., R. Péanne, J. Jaeken, R. Barone, M. Bidet, D. Borgel, S. Brasil, D. Cassiman, A. Cechova, D. Coman, J. Corral, J. Correia, M. E. de la Morena-Barrio, P. de Lonlay, V. Dos Reis, C. R. Ferreira, A. Fiumara, R. Francisco, H. Freeze, S. Funke, T. Gardeitchik, M. Gert, M. Girad, M. Giros, S. Grünewald, T. Hernández-Caselles, T. Honzik, M. Hutter, D. Krasnewich, C. Lam, J. Lee, D. Lefeber, D. Marques-de-Silva, A. F. Martinez, H. Moravej, K. Ounap, C. Pascoal, T. Pascreau, M. Patterson, D. Quelhas, K. Raymond, P. Sarkhail, M. Schiff, M. Seroczyńska, M. Serrano, N. Seta, J. Sykut-Cegielska, C. Thiel, F. Tort, M. A. Vals, P. Videira, P. Witters, R. Zeevaert, and E. Morava. 2019. "International clinical guidelines for the management of phosphomannomutase 2-congenital disorders of

glycosylation: Diagnosis, treatment and follow up." *J Inherit Metab Dis* 42 (1):5-28. doi: 10.1002/jimd.12024.

Bannasch, P., U. Jahn, H. Hacker, Q. Su, W. Hoffmann, R. Pichlmayr, and G. Otto. 1997. "Focal hepatic glycogenosis." *Int J Oncol* 10 (2):261-8. doi: 10.3892/ijo.10.2.261.

Brasil, S., C. Pascoal, R. Francisco, D. Marques-da-Silva, G. Andreotti, P. A. Videira, E. Morava, J. Jaeken, and V. Dos Reis Ferreira. 2018. "CDG Therapies: From Bench to Bedside." *Int J Mol Sci* 19 (5). doi: 10.3390/ijms19051304.

Dauber, A., A. Ercan, J. Lee, P. James, P. P. Jacobs, D. J. Ashline, S. R. Wang, T. Miller, J. N. Hirschhorn, P. A. Nigrovic, and R. Sackstein. 2014. "Congenital disorder of fucosylation type 2c (LADII) presenting with short stature and developmental delay with minimal adhesion defect." *Hum Mol Genet* 23 (11):2880-7. doi: 10.1093/hmg/ddu001.

de la Morena-Barrio, M. E., E. Wypasek, D. Owczarek, A. Miñano, V. Vicente, J. Corral, and A. Undas. 2019. "MPI-CDG with transient hypoglycosylation and antithrombin deficiency." *Haematologica* 104 (2):e79-e82. doi: 10.3324/haematol.2018.211326.

Dörre, K., M. Olczak, Y. Wada, P. Sosicka, M. Grüneberg, J. Reunert, G. Kurlemann, B. Fiedler, S. Biskup, K. Hörtnagel, S. Rust, and T. Marquardt. 2015. "A new case of UDP-galactose transporter deficiency (SLC35A2-CDG): molecular basis, clinical phenotype, and therapeutic approach." *J Inherit Metab Dis* 38 (5):931-40. doi: 10.1007/s10545-015-9828-6.

Ferreira, C. R., D. Cassiman, and N. Blau. 2019. "Clinical and biochemical footprints of inherited metabolic diseases. II. Metabolic liver diseases." *Mol Genet Metab* 127 (2):117-121. doi: 10.1016/j.ymgme.2019.04.002.

Fisher, P., J. Thomas-Oates, A. J. Wood, and D. Ungar. 2019. "The." *Front Cell Dev Biol* 7:157. doi: 10.3389/fcell.2019.00157.

Francisco, R., D. Marques-da-Silva, S. Brasil, C. Pascoal, V. Dos Reis Ferreira, E. Morava, and J. Jaeken. 2019. "The challenge of CDG diagnosis." *Mol Genet Metab* 126 (1):1-5. doi: 10.1016/j.ymgme.2018.11.003.

Frulio, N., and H. Trillaud. 2013. "Ultrasound elastography in liver." *Diagn Interv Imaging* 94 (5):515-34. doi: 10.1016/j.diii.2013.02.005.

Girard, M., A. Poujois, M. Fabre, F. Lacaille, D. Debray, M. Rio, F. Fenaille, S. Cholet, C. Ruel, E. Caussé, J. Selves, L. Bridoux-Henno, F. Woimant, T. Dupré, S. Vuillaumier-Barrot, N. Seta, L. Alric, P. de Lonlay, and A. Bruneel. 2018. "CCDC115-CDG: A new rare and misleading inherited cause of liver disease." *Mol Genet Metab* 124 (3):228-235. doi: 10.1016/j.ymgme.2018.05.002.

Helander, A., J. Jaeken, G. Matthijs, and G. Eggertsen. 2014. "Asymptomatic phosphomannose isomerase deficiency (MPI-CDG) initially mistaken for excessive alcohol consumption." *Clin Chim Acta* 431:15-8. doi: 10.1016/j.cca.2014.01.018.

Jansen, J. C., S. Cirak, M. van Scherpenzeel, S. Timal, J. Reunert, S. Rust, B. Pérez, D. Vicogne, P. Krawitz, Y. Wada, A. Ashikov, C. Pérez-Cerdá, C. Medrano, A. Arnoldy, A. Hoischen, K. Huijben, G. Steenbergen, D. Quelhas, L. Diogo, D. Rymen, J. Jaeken, N. Guffon, D. Cheillan, L. P. van den Heuvel, Y. Maeda, O. Kaiser, U. Schara, P. Gerner, M. A. van den Boogert, A. G. Holleboom, M. C. Nassogne, E. Sokal, J. Salomon, G. van den Bogaart, J. P. Drenth, M. A. Huynen, J. A. Veltman, R. A. Wevers, E. Morava, G. Matthijs, F. Foulquier, T. Marquardt, and D. J. Lefeber. 2016. "CCDC115 Deficiency Causes a Disorder of Golgi Homeostasis with Abnormal Protein Glycosylation." *Am J Hum Genet* 98 (2):310-21. doi: 10.1016/j.ajhg.2015.12.010.

Janssen, M. C., R. H. de Kleine, A. P. van den Berg, Y. Heijdra, M. van Scherpenzeel, D. J. Lefeber, and E. Morava. 2014. "Successful liver transplantation and long-term follow-up in a patient with MPI-CDG." *Pediatrics* 134 (1):e279-83. doi: 10.1542/peds.2013-2732.

Marques-da-Silva, D., V. Dos Reis Ferreira, M. Monticelli, P. Janeiro, P. A. Videira, P. Witters, J. Jaeken, and D. Cassiman. 2017. "Liver involvement in congenital disorders of glycosylation (CDG). A systematic review of the literature." *J Inherit Metab Dis* 40 (2):195-207. doi: 10.1007/s10545-016-0012-4.

Medrano, C., A. Vega, R. Navarrete, M. J. Ecay, R. Calvo, S. I. Pascual, M. Ruiz-Pons, L. Toledo, I. García-Jiménez, I. Arroyo, A. Campo, M. L. Couce, M. R. Domingo-Jiménez, M. T. García-Silva, L. González-Gutiérrez-Solana, L. Hierro, E. Martín-Hernández, M. Martínez-Pardo, S. Roldán, M. Tomás, J. C. Cabrera, F. Mártinez-Bugallo, L. Martín-Viota, I. Vitoria-Miñana, D. J. Lefeber, M. L. Girós, M. Serrano Gimare, M. Ugarte, B. Pérez, and C. Pérez-Cerdá. 2019. "Clinical and molecular diagnosis of non-phosphomannomutase 2 N-linked congenital disorders of glycosylation in Spain." *Clin Genet* 95 (5):615-626. doi: 10.1111/cge.13508.

Mention, K., F. Lacaille, V. Valayannopoulos, S. Romano, A. Kuster, M. Cretz, H. Zaidan, L. Galmiche, F. Jaubert, Y. de Keyzer, N. Seta, and P. de Lonlay. 2008. "Development of liver disease despite mannose treatment in two patients with CDG-Ib." *Mol Genet Metab* 93 (1):40-3. doi: 10.1016/j.ymgme.2007.08.126.

Morelle, W., S. Potelle, P. Witters, S. Wong, L. Climer, V. Lupashin, G. Matthijs, T. Gadomski, J. Jaeken, D. Cassiman, E. Morava, and F. Foulquier. 2017. "Galactose Supplementation in Patients With TMEM165-CDG Rescues the Glycosylation Defects." *J Clin Endocrinol Metab* 102 (4):1375-1386. doi: 10.1210/jc.2016-3443.

Park, J. H., M. Hogrebe, M. Fobker, R. Brackmann, B. Fiedler, J. Reunert, S. Rust, K. Tsiakas, R. Santer, M. Grüneberg, and T. Marquardt. 2018. "SLC39A8 deficiency: biochemical correction and major clinical improvement by manganese therapy." *Genet Med* 20 (2):259-268. doi: 10.1038/gim.2017.106.

Péanne, R., P. de Lonlay, F. Foulquier, U. Kornak, D. J. Lefeber, E. Morava, B. Pérez, N. Seta, C. Thiel, E. Van Schaftingen, G. Matthijs, and J. Jaeken. 2018. "Congenital disorders of glycosylation (CDG): Quo vadis?" *Eur J Med Genet* 61 (11):643-663. doi: 10.1016/j.ejmg.2017.10.012.

Radenkovic, S., M. J. Bird, T. L. Emmerzaal, S. Y. Wong, C. Felgueira, K. M. Stiers, L. Sabbagh, N. Himmelreich, G. Poschet, P. Windmolders, J. Verheijen, P. Witters, R. Altassan, T. Honzik, T. F. Eminoglu, P. M. James, A. C. Edmondson, J. Hertecant, T. Kozicz, C. Thiel, P. Vermeersch, D. Cassiman, L. Beamer, E. Morava, and B. Ghesquière. 2019. "The Metabolic Map into the Pathomechanism and Treatment of PGM1-CDG." *Am J Hum Genet* 104 (5):835-846. doi: 10.1016/j.ajhg.2019.03.003.

Verheijen, J., S. Tahata, T. Kozicz, P. Witters, and E. Morava. 2020. "Therapeutic approaches in Congenital Disorders of Glycosylation (CDG) involving N-linked glycosylation: an update." *Genet Med* 22 (2):268-279. doi: 10.1038/s41436-019-0647-2.

Witters, P., D. Cassiman, and E. Morava. 2017. "Nutritional Therapies in Congenital Disorders of Glycosylation (CDG)." *Nutrients* 9 (11). doi: 10.3390/nu9111222.

Witters, P., T. Honzik, E. Bauchart, R. Altassan, T. Pascreau, A. Bruneel, S. Vuillaumier, N. Seta, D. Borgel, G. Matthijs, J. Jaeken, W. Meersseman, D. Cassiman, L. Pascale de, and E. Morava. 2019. "Long-term follow-up in PMM2-CDG: are we ready to start treatment trials?" *Genet Med* 21 (5):1181-1188. doi: 10.1038/s41436-018-0301-4.

Witters, P., S. Tahata, R. Barone, K. Õunap, R. Salvarinova, S. Gronborg, G. Hoganson, F. Scaglia, A. M. Lewis, M. Mori, J. Sykut-Cegielska, A. Edmondson, M. He, and E. Morava. 2020. "Clinical and biochemical improvement with galactose supplementation in SLC35A2-CDG." *Genet Med*. doi: 10.1038/s41436-020-0767-8.

Wong, S. Y., T. Gadomski, M. van Scherpenzeel, T. Honzik, H. Hansikova, K. S. B. Holmefjord, M. Mork, F. Bowling, J. Sykut-Cegielska, D. Koch, J. Hertecant, G. Preston, J. Jaeken, N. Peeters, S. Perez, D. D. Nguyen, K. Crivelly, T. Emmerzaal, K. M. Gibson, K. Raymond, N. Abu Bakar, F. Foulquier, G. Poschet, A. M. Ackermann, M. He, D. J. Lefeber, C. Thiel, T. Kozicz, and E. Morava. 2017. "Oral D-galactose supplementation in PGM1-CDG." *Genet Med* 19 (11):1226-1235. doi: 10.1038/gim.2017.41.

Zhu, J., Z. Sun, K. Cheng, R. Chen, M. Ye, B. Xu, D. Sun, L. Wang, J. Liu, F. Wang, and H. Zou. 2014. "Comprehensive mapping of protein N-glycosylation in human liver by combining hydrophilic interaction chromatography and hydrazide chemistry." *J Proteome Res* 13 (3):1713-21. doi: 10.1021/pr401200h.

Figure 1

Click here to download Figure CDG_Liver_figure_1.jpg

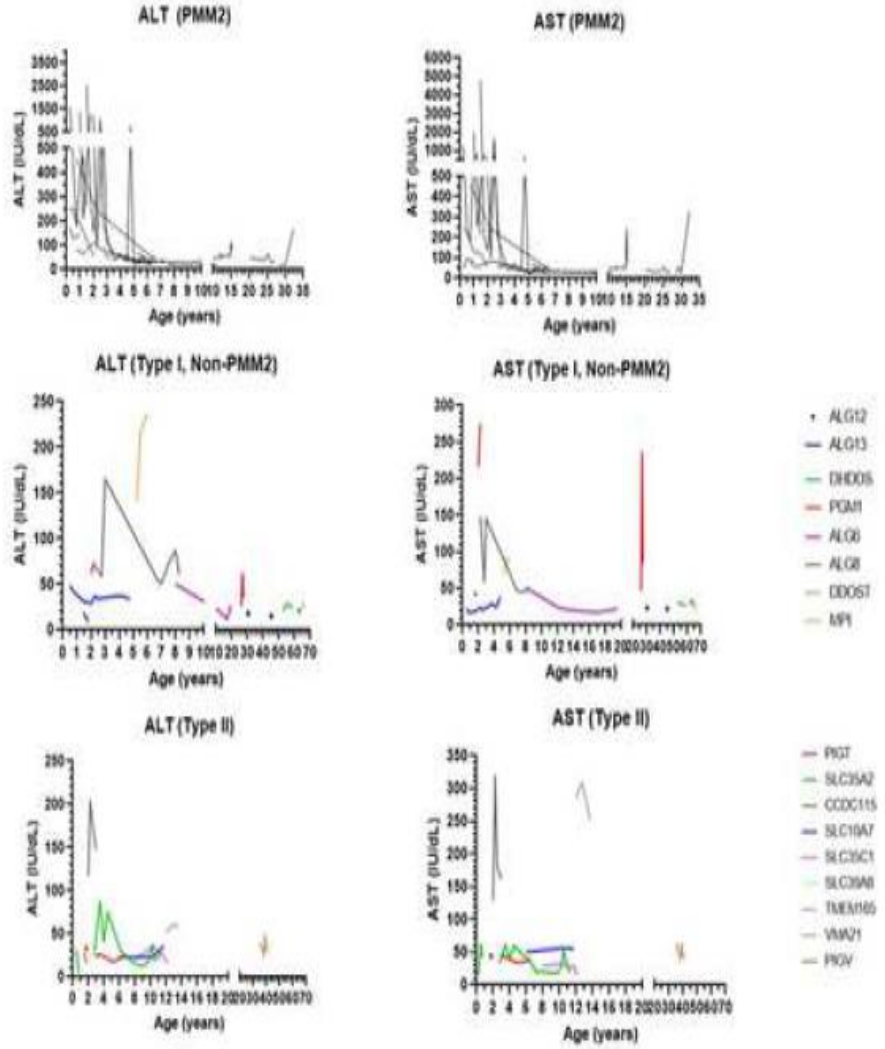


Figure 2

Click here to download Figure CDG_Liver_figure_2.jpg

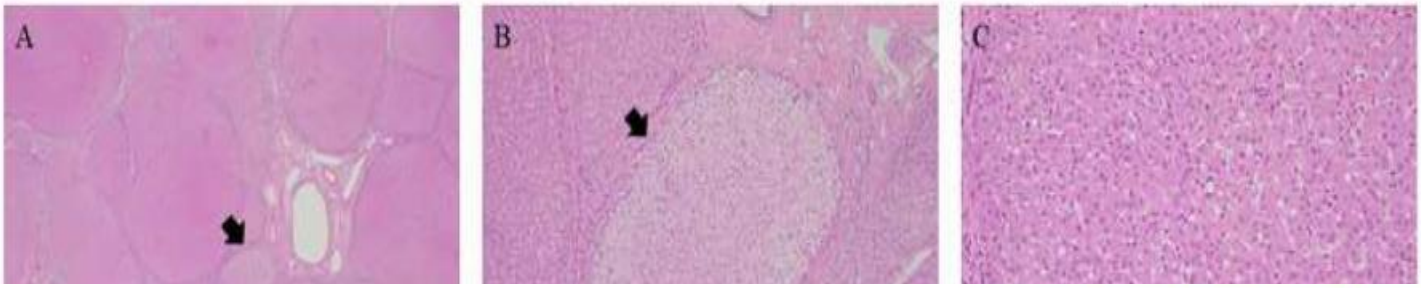
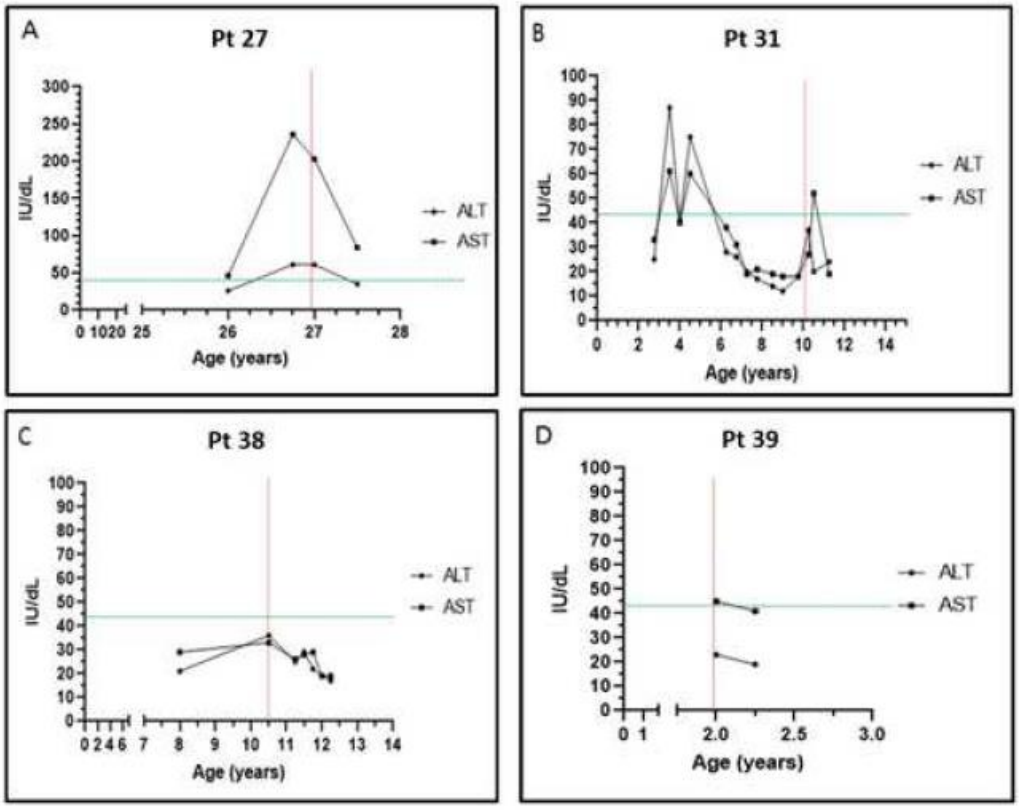


Figure 3

Click here to download Figure CDG_Liver_figure_3.jpg



E. Discussão

1. O que é um fenótipo?

Em 1911, o cientista dinamarquês Wilhelm Johannsen (JOHANNSEN, 2014) propôs que a base da hereditariedade de caracteres seria o *gene* – uma partícula que conteria, em porções segregáveis, a potencialidade do desenvolvimento do zigoto em questão; o conjuntos de todos os genes de um determinado zigoto seria o seu *genótipo* (do grego antigo, "aquilo que gera"), e o diferenciaria de outros potenciais zigotos. Ao se analisar um organismo, no entanto, Johannsen propõe que tudo que pode ser observado a seu respeito seja o *fenótipo* (do grego antigo, "aquilo que se mostra") – não sendo possível a observação de genes por sequenciamento como o é hoje, tendo sido seu artigo publicado 42 anos antes da descoberta do DNA por Franklin, Crick, Watson e Wilkins (WATSON; CRICK, 1953). Essa definição foi expandida por Richard Dawkins (DAWKINS, 1976), que definiu o fenótipo como o efeito diferencial de um determinado alelo em comparação com outros alelos que competem consigo no processo evolutivo. A partir dessa definição, pode-se aplicar a ideia de um fenótipo não somente a uma espécie ou a um organismo, mas também ao efeito diferencial exercido por variantes patogênicas sobre um sistema – isso é, à totalidade de diferenças encontradas na comparação de um organismo (ou mesmo de uma subunidade deste, como um órgão ou um tecido) que possam ser atribuídas a essa variante. Dawkins ainda estabelece o conceito de "fenótipo estendido" (DAWKINS, 1978), que nada mais é que a extensão da ideia de um fenótipo diferencial: o fenótipo referir-se-ia ao efeito de um alelo não somente sobre o que é diretamente afetado por si dentro do organismo que o porta, mas também às modificações causadas pelo organismo sobre o meio, pelo meio sobre o organismo, se essas não tivessem sido causadas em um contexto em que o alelo estivesse ausente.

Aplicando esse entendimento do que seja o fenótipo, e incluindo nessa definição o fenótipo estendido, pode-se passar para o seu estudo específico. Para o fenótipo hepático, é útil, a fins descritivos, que esse seja categorizado conforme o nível em que seja observado:

na “superfície”, de fácil observação devido à magnitude de seus fenômenos ser proporcional àquela de seu observador – isso é, do cientista – encontra-se o fenótipo clínico, que pode ser estudado por meios simples como a anamnese e o exame físico, e por meios indiretos como marcadores laboratoriais e imaginológicos. Mais profundamente, encontra-se o nível histológico, caracterizado pelo estudo do funcionamento de tecidos a um nível não-sistêmico, a partir principalmente de ferramentas morfológicas e funcionais. Por fim, encontra-se o nível celular e molecular, correspondendo à unidade básica de funcionamento de qualquer órgão – o fígado, no entanto, por possuir população celular heterogênea e dinâmica, deve ter seu fenótipo celular entendido como a soma e a cooperação entre os diferentes tipos celulares que o compõem, o que faz com que os níveis histológico e celular sobreponham-se em parte.

2. O fenótipo hepático da DG

2.1. Dano hepatocelular inespecífico

O dano hepatocelular inespecífico foi abordado no artigo 1, no qual foi mostrado que, mesmo após a introdução do tratamento (realizado principalmente com TRE), uma grande parte dos pacientes com DG tem indícios indiretos de dano hepático de natureza tanto hepatocelular quanto biliar através da medida com marcadores laboratoriais, significando que existe um processo patológico em atividade apesar da terapia. Esse achado destoa do conhecimento prévio da DG, que prega que o envolvimento hepático pela doença seria de natureza predominantemente infiltrativa, causando hepatomegalia devido ao número de células de Gaucher presentes no parênquima hepático, com cessação ou mitigação do processo após o início da TRE (a qual é direcionada aos macrófagos); mas pode explicar achados recentes de comprometimento hepático, como os valores de rigidez hepática aumentados durante o tratamento descritos em diversos estudos (ver Introdução, seção 3.5.3), já que é possível que essa fibrose hepática ocorra devido (ao menos em parte) ao dano hepático misto descrito no artigo 1.

A nível histológico, o dano hepatocelular foi abordado no artigo 3, que descreve a identificação histomorfométrica de alterações no núcleo dos hepatócitos de pacientes com DG comparados com controles saudáveis. Os achados estatisticamente significativos de maior área nuclear, maior dimensão fractal, e menor absorbância dos núcleos hepatocelulares dos pacientes com DG são indícios de que esses núcleos se encontram mais eucromáticos. O significado direto da presença de maior eucromasia hepatocelular é ainda pouco compreendido, devido à falta de estudos na área e à ainda parca utilização da histomorfometria como ferramenta de pesquisa e diagnóstico em hepatologia e hepatopatologia; sabe-se que a entropia (medida de textura de imagem proporcional à dimensão fractal (ZMESKAL; DZIK; VESELY, 2013)) do núcleo hepatocelular é maior em núcleos de características malignas (JAGOE; SOWTER; SLAVIN, 1984), mas há um silêncio na literatura sobre o significado de diferenças de tamanho e cromasia nuclear no fígado fora do contexto de lesões neoplásicas (HORAI; KAKIMOTO; TAKEMOTO; TANAKA, 2017) – são explicações possíveis que os núcleos hepatocelulares na DG1 reflitam o estado metabólico basal aumentado observado nesses pacientes (DONEDA; LOPES; OLIVEIRA; NETTO *et al.*, 2011; DONEDA; VAIRO; LOPES; REISCHAK-OLIVEIRA *et al.*, 2014) (já que o fígado é um dos reguladores do metabolismo sistêmico), ou que mostrem uma resposta à inflamação crônica de baixo grau (AFLAKI; BORGER; MOAVEN; STUBBLEFIELD *et al.*, 2016); no entanto, não há dados suficientes atualmente na literatura para apoiar quaisquer dessas hipóteses. Assim, a principal implicação desse achado no momento é desafiar a noção previamente reconhecida do envolvimento hepático na DG como exclusivamente macrofágico, trazendo atenção também para o envolvimento hepatocelular.

2.2. Esteatose

A esteatose foi abordada de forma clínica no artigo 1, que mostrou sua alta prevalência (estimada por US) em pacientes em tratamento, especialmente naqueles em uso de TRE, e a sua associação com a síndrome metabólica. A prevalência de esteatose é um assunto controverso na DG, com estudos conflitantes sobre a presença de uma prevalência

aumentada (ver Introdução, seção 3.5.1). É reveladora a diferença de prevalência de esteatose e de síndrome metabólica nos mesmos pacientes antes e depois da instituição da TRE, o que leva à reflexão de que possa haver alguma correlação causal entre esta e aquelas. Estudos prévios sugeriram que pacientes com DG que não recebem tratamento tenham um metabolismo basal aumentado, e que com o uso de TRE haveria redução da taxa metabólica novamente aos níveis normais ou mais próximos do normal (DONEDA; NETTO; MOULIN; SCHWARTZ, 2013; NASCIMBENI; DALLA SALDA; CARUBBI, 2016), levando a um aumento da prevalência da síndrome metabólica devido aos pacientes terem tido sua educação nutricional intuitiva no contexto de metabolismo aumentado e assim não se adaptarem completamente a um metabolismo a níveis mais baixos. A esteatose hepática, assim, seria uma consequência não do processo patológico primário da DG, mas da correção desse processo pela terapia, podendo ser considerada como parte do fenótipo estendido da DG.

Histologicamente, a esteatose na DG foi descrita em 4 pacientes no artigo 1. A esteatose foi em todos os casos do tipo macrogoticular, não coocorreu com fibrose, e não esteve presente em pacientes que não tivessem recebido tratamento, apesar de que devido ao baixo tamanho amostral de BHs não seja possível concluir correlações da esteatose com nenhum desses fatores; em dois casos, a esteatose fez parte de um quadro de esteato-hepatite (ver próxima seção).

2.3. Inflamação

A inflamação hepática foi abordada apenas de forma histológica, devido à impossibilidade de métodos clínicos usuais em detectar, qualificar ou quantificar a hepatite de etiologia não-viral (ver Introdução, seção 1.3.3). No artigo 1, foram descritos 4 pacientes com evidência de inflamação na BH, sendo dois com infiltração leve e difusa por células inflamatórias (polimorfonucleares e linfócitos) e dois com critérios para esteato-hepatite. De forma bastante interessante, enquanto foram encontradas células de Gaucher no parênquima hepático dos dois pacientes com inflamação leve, essas células não foram encontradas, mesmo após extensiva revisão por dois médicos patologistas, nas amostras dos pacientes

com esteato-hepatite. Isso faz com que seja necessário investigar causas concomitantes à DG que possam causar esse achado: em um dos pacientes, descrito no artigo 1 como "paciente 26B", foi encontrada siderose significativa (ver seção a seguir) tanto no estudo com RNM quanto na própria BH, além da presença de síndrome metabólica e consumo excessivo de álcool – causas possíveis de EHNA e de hepatite alcoólica que podem explicar a esteato-hepatite encontrada à BH. No entanto, a segunda paciente, descrita no artigo 1 como "paciente 13", não apresentava síndrome metabólica, marcadores de dano hepático alterados, siderose, ou consumo de álcool, não estando assim em risco para EHNA ou hepatite alcoólica – desse modo, deve-se considerar a possibilidade de que a esteato-hepatite encontrada na BH seja parte do fenótipo hepático da DG, como consequência direta do processo patológico dessa doença ou mesmo como parte do fenótipo estendido devido a efeito do tratamento, já que essa paciente estava em tratamento com a mais nova modalidade de TRS, o eliglustate (ver Introdução, seção 3.6).

É importante ressaltar que a inflamação hepática na DG é ainda pouco estudada devido à necessidade de BH para o sua evidenciação. O fato de termos identificado a presença de esteato-hepatite em dois pacientes submetidos à BH manda que se considere a possibilidade de que possivelmente alguns pacientes diagnosticados com esteatose por métodos não-invasivos (ver seção anterior) sejam na verdade acometidos por esteato-hepatite, já que métodos não-invasivos não podem diferenciar entre essas duas condições.

2.4. Siderose

A siderose foi abordada de forma clínica no artigo 1, que descreveu a presença de depósitos de ferro mensuráveis por RNM em pacientes com DG. Apesar de todos os pacientes com hemossiderose hepática terem tido valores elevados de ferritina sérica, os valores de saturação de Tf foram deveras variáveis, sem associação aparente com o grau de depósito de ferro ou com variáveis ligadas ao tratamento (como tempo de tratamento ou modalidade terapêutica). Uma possível influência modificadora de variantes do gene *HFE* foi também explorada no artigo 1, e de forma mais aprofundada no artigo 4 (ver seção 3),

porém sem achados que corroborem um efeito modificador desse gene no fenótipo hepático da DG.

De forma histológica, a siderose foi descrita em BHs de pacientes com DG no artigo 1: dos 8 pacientes com BHs disponíveis, foi observada siderose em 5, todos em biópsias realizadas durante o tratamento. Nos dois pacientes com siderose considerada grave (DEUGNIER; TURLIN, 2007), essa foi de distribuição mista (ver Introdução, seção 1.3.4), e outros achados de hepatopatia estavam presentes: um dos pacientes foi também diagnosticado com esteato-hepatite, o outro com cirrose e HC.

2.5. Dano biliar

O fenótipo biliar clínico foi abordado no artigo 1. A nível canalicular e colangiocelular, foi descrito aumento da GGT (marcador inespecífico de dano em ambas estruturas) e aumento leve dos valores séricos de bilirrubina direta. O aumento da GGT é de extremo interesse por sinalizar que na DG haja dano não só dos hepatócitos, mas também de canalículos e potencialmente do epitélio biliar, o que até hoje não foi estudado ou descrito. A presença de aumento da bilirrubina direta sinaliza, por sua vez, que possa haver comprometimento no fluxo biliar fisiológico, levando a um grau leve de colestase intra-hepática e ao consequente extravasamento de bilirrubina direta para o meio intravascular. Sabe-se que a colestase é uma consequência grave e rara da DG2 (ELIAS; JOHNSON; BOITNOTT; VALLE, 2012; SOUDEK; SIDDIQUI; GUERIN; SONDHEIMER *et al.*, 2020), o que significa que exista uma via fisiopatológica possível que conecte ambos processos patológicos. Desse modo, a presença de alterações biliares em pacientes com DG1 pode significar que o mesmo processo que leva à colestase na DG2 possa estar presente, porém em níveis proporcionais à gravidade da doença. Essa hipótese é corroborada pelos achados histológicos de dano biliar, discutidos a seguir.

A nível de VB, foi relatado no artigo 1 uma elevada prevalência de colelitíase, com aproximadamente 29% dos pacientes positivos. Esse achado corrobora os relatos recentes de uma prevalência aumentada de colelitíase em pacientes com DG (ver Introdução, seção

1.3.5); interessantemente, não foi encontrada uma correlação entre a presença de sobrepeso ou obesidade e a colelitíase, apesar de a obesidade ser um dos principais fatores de risco para o desenvolvimento de cálculos biliares.

Histologicamente, o fenótipo biliar da DG foi abordado no artigo 1 superficialmente, mas em detalhe no artigo 3. Colestase já fora relatada em pacientes com DG2, e foi observada na BH de uma paciente com DG1, relatada no artigo 1. Para melhor compreender o padrão do dano biliar, fez-se necessário estudar em mais detalhes os canalículos biliares, os quais são de difícil estudo devido a seu diminuto tamanho (aproximadamente 0,5µm de diâmetro) e a ausência de epitélio de revestimento próprio que os identifique. Estudos prévios realizaram medidas quantitativas de canalículos biliares com o uso de tecnologias como a microscopia eletrônica (HALL; LE; ARNOLD; BRUNTON *et al.*, 2018) ou a reconstrução tridimensional com microscopia confocal (DAMLE-VARTAK; BEGHER-TIBBE; GUNTHER; GEISLER *et al.*, 2019) – no entanto, essas não são técnicas viáveis para o estudo de espécimes histológicos humanos de forma retrospectiva, já que a preservação em parafina padrão para os procedimentos histológicos não permite que a amostra seja usada para técnicas de microscopia eletrônica e que as BHs realizadas por agulha grossa (a técnica mais comum e menos invasiva) não possuem espessura suficiente para a reconstrução; outrossim, ambas técnicas são de alto custo, não sendo aplicáveis de maneira fácil para a pesquisa científica no Brasil devido à exiguidade de recursos financeiros. Desse modo, uma técnica de baixo custo e fácil execução foi desenvolvida, conforme relatado no artigo 3, para a quantificação de parâmetros canaliculares: a histomorfometria (isso é, a quantificação de caracteres morfológicos a nível tecidual) de canalículos biliares com uso de imuno-histoquímica e de análise digital de imagem. Essa técnica mostrou-se bem sucedida em diferenciar canalículos biliares de pacientes com DG1 de pacientes saudáveis, e mostrou, através de parâmetros convencionais como largura e contagem e de um parâmetro novo, a ramificação canalicular (representada pela relação perímetro-Feret, conforme delineado no artigo 3), que pacientes com DG1 em tratamento têm canalículos menos ramificados e menos densos que suas contrapartes saudáveis, levando a crer que haja um processo de dano canalicular na DG1.

Uma hipótese a ser levantada para explicar as alterações biliares encontradas na DG é a da interação da GlcCer e da GlcSph com os transportadores da membrana canalicular. Os hepatócitos são células de alta atividade metabólica, com reciclagem constante de componentes de membranas utilizados em processos vesiculares como exocitose e transcitose (SCHULZE; SCHOTT; CASEY; TUMA *et al.*, 2019) – no entanto, até hoje não foi possível observar acúmulo de qualquer desses produtos em hepatócitos na DG. Foi-se proposto como explicação para essa ausência de acúmulo a excreção desses componentes para a bile através dos canalículos biliares, onde seriam metabolizados por enzimas como a GCase2 e excretados no lúmen intestinal, em consonância com achados de aumento de litogênese e diferente composição biliar já relatados (ver introdução, seção 3.5.4). Sabe-se que a GlcCer e a GlcSph são substratos do transportador ABCB1 (RAGGERS; VAN HELVOORT; EVERS; VAN MEER, 1999; RAGGERS; VOGELS; VAN MEER, 2002), presente na membrana canalicular, onde realiza a função de efluxo de xenobióticos (PIRES; EMMERT; CHMIELEWSKI; HRYCYNA, 2011); sabe-se também que esses lipídeos complexos interagem com os transportadores ABCB1 e ABCC1 (este último presente na membrana basolateral hepatocelular (PIRES; EMMERT; CHMIELEWSKI; HRYCYNA, 2011)), interferindo em suas distribuição na compartimentalização celular e na formação de jangadas lipídicas de membrana (LEE; KOLESNICK, 2017). Assim, é possível que o aumento da concentração de GlcCer e GlcSph leve a alterações ultraestruturais e funcionais dos canalículos biliares que participem da formação do fenótipo observado nos artigos 1 e 3.

2.6. Fibrose

A fibrose foi abordada de uma perspectiva clínica nos artigos 1, 2 e 5. Nos artigos 1 e 2, foi descrita a presença de cirrose em um paciente com DG1 com esplenectomia prévia, ressaltando a já descrita relação entre uma maior gravidade da doença após a remoção do baço (ver Introdução, seção 3.5.3). No artigo 5, ainda em elaboração, foi estudada a prevalência de fibrose na coorte de pacientes com DG do HCPA e explorada a capacidade de predição de fibrose por relações entre marcadores simples através de escores clínico-

laboratoriais (ver Introdução, seção 1.3.6). Uma alta prevalência de fibrose foi encontrada nessa coorte, apesar de que, devido aos números ainda incompletos de pacientes analisados, conclusões definitivas não possam ser tomadas a esse respeito atualmente. No entanto, os números encontrados estão em ressonância cos os mais recentes artigos, discutidos na seção 1.3.6 da Introdução, que mostram alta prevalência de fibrose hepática em pacientes tratados a longo prazo.

Apesar do recrutamento ainda incompleto para o estudo 5, essa amostra já se mostrou suficiente para a análise de preditores, com destaque do escore APRI para o rastreio de fibrose hepática nesses pacientes: para fibrose de grau F2 ou maior, houve sensibilidade de 100% com o ponto de corte de APRI = 0.201 e especificidade de 100% com o ponto de corte de APRI = 0.640. Desse modo, esse escore é promissor para o uso na prática clínica, potencialmente podendo suprir parte da demanda para uso de ETH, a qual não está disponível no momento no Brasil através do Sistema Único de Saúde, sendo um exame inacessível para a maior parte dos pacientes do país devido a seu elevado preço.

2.7. Lesões focais

Quanto às lesões focais hepáticas da DG, tanto o artigo 1 quanto o artigo 2 trazem à tona a importante questão da diferenciação das lesões hepáticas benignas – entre elas os Gaucheromas – e o HC. Devido à raridade da DG, e consequentemente dos Gaucheromas, há poucas evidências para a realização do diagnóstico diferencial entre essas duas lesões; no entanto, o correto diagnóstico é de vital importância, haja vista que o Gaucheroma seja uma lesão benigna e o HC uma lesão altamente maligna, de crescimento rápido e alta letalidade, com tratamento específico. O caso do paciente relatado em ambos os artigos (no artigo 1, como “paciente 28”) se desenrolou concomitantemente à publicação de um artigo de colaboração internacional em 2018 (REGENBOOG; VAN DUSSEN; VERHEIJ; WEINREB *et al.*, 2018), o qual sugere que todos os pacientes com DG sejam rastreados para a presença de fibrose, e que pacientes com esplenectomia prévia, cirrose hepática, hiperferritinemia grave, ou comorbidades hepáticas como hepatites virais crônicas fossem rastreados para a presença de HC – sendo portando aplicável ao caso do paciente em questão

a recomendação de rastreio. Esse caso também reforça com um exemplo real a recomendação recente feita em um artigo prévio do mesmo grupo (REGENBOOG; BOHTE; SOMERS; VAN DELDEN *et al.*, 2016) de que lesões hepáticas com características imaginológicas de HC devam ser tratadas como HC, mesmo que possam se tratar de um Gaucheroma – devido à possibilidade de erro amostral inerente à BH, e à gravidade do prognóstico da lesão maligna, a perspectiva de que seja tratada como um HC uma lesão que na verdade seja um Gaucheroma torna-se menos grave que a perspectiva inversa: de que seja tratada como Gaucheroma uma lesão que seja na verdade um HC. Tragicamente, o paciente relatado nesses artigos apresentou progressão rápida do HC para uma doença metastática e intratável após a publicação, e há aproximadamente 3 meses pereceu devido a ela.

3. A modificação de fenótipo na DG

As manifestações clínicas da DG são extremamente heterogêneas, com grande variabilidade mesmo entre pacientes com o mesmo genótipo *GBA*, o que faz com que devam existir fatores modificadores de fenótipo em ação, sejam esses de origem genética ou ambiental (DAVIDSON; HASSAN; GARCIA; TAYEBI *et al.*, 2018). Estima-se que os fatores modificadores genéticos (isso é, "genes modificadores") ajam através da modificação da penetrância, da expressividade, da dominância, ou da relação de pleiotropia dos genes causadores de uma determinada patologia (NADEAU, 2001). A busca por esses fatores é um tema de grande interesse atual no entendimento científico da DG, devido à possibilidade de que a sua identificação ajude para o estabelecimento de conduta terapêutica e traçado de prognóstico para os pacientes, individualizando o cuidado que esses recebem (RYAN; SEEHRA; SIDRANSKY, 2019). No entanto, a maior parte dos modificadores tem se mostrado elusiva, com poucos genes tendo sido identificados até hoje apesar do emprego de diferentes técnicas de análise como estudos de associação genômica ampla (GWAS, na sigla em inglês) – os quais são capazes de detectar variantes comuns – e estudos de sequenciamento de genes candidatos – os quais são capazes de detectar efeito modificador de variantes de efeito forte (DAVIDSON; HASSAN; GARCIA; TAYEBI *et al.*, 2018).

No artigo 4, foi utilizada uma técnica de identificação de modificadores genéticos por sequenciamento de genes candidatos envolvendo especificamente genes diretamente envolvidos no metabolismo do ferro (ver Introdução, seção 1.2), além dos genes *GBA2*, *SCARB2* e *PSAP*, os quais interagem diretamente com a função da GCase e já foram propostos anteriormente como modificadores (RYAN; SEEHRA; SIDRANSKY, 2019; TAMARGO; VELAYATI; GOLDIN; SIDRANSKY, 2012; VELAYATI; DEPAOLO; GUPTA; CHOI *et al.*, 2011; YILDIZ; HOFFMANN; VOM DAHL; BREIDEN *et al.*, 2013). Nesse artigo, a análise das variantes encontradas foi baseada na sua segregação conforme o fenótipo (tanto de características hepáticas quanto extra-hepáticas) e no conceito de "teste de carga" (*burden test*, em inglês), o qual consiste na comparação de frequências alélicas entre a população de interesse e a população em geral com a finalidade de identificar diferenças na frequência (denominadas "cargas") de determinados alelos (ou variantes) que possam explicar a diferença na frequência de fenótipos (GUO; PLUMMER; CHAN; HIRSCHHORN *et al.*, 2018). Essa abordagem nos levou à identificação de 3 variantes com distribuição significativamente distinta de acordo com o fenótipo (isso é, com segregação fenotípica): *CYBRD1* rs10455, *A2M* rs3832852, *TF* rs12769; e de 5 variantes com diferença de carga estatisticamente significativa na comparação de nossa coorte com duas populações de referência: *CYBRD1* rs3731976, *CP* rs16861582, *PSAP* rs4747203, *TF* rs1130459, *TFR2* rs141968146, sendo que a variante *CYBRD1* rs10455 teve diferença significativa com uma das populações de referência, e quase-significativa com a outra população (o que deve ser levado em consideração, já que o método estatístico de correção de significância pelo procedimento de Bonferroni foi aplicado, levando ao aumento do limiar de detecção de diferenças estatisticamente significativas). Na intersecção entre as duas análises (segregação e teste de carga), dois genes despontam: *CYBRD1* e *TF*, sendo a variante *CYBRD1* rs10455 especialmente promissora como um modificador devido a estar presente em níveis significativos nos resultados de ambos os testes, enquanto que as variantes *CYBRD1* rs3731976, *TF* rs12769 e *TF* rs1130459 foram significativas somente em um teste cada. Qualitativamente, a *CYBRD1* rs10455 mostrou-se mais presente em pacientes sem episódios prévios de osteonecrose, e uma frequência 25,32% a 29,44% menor na coorte de pacientes

com DG comparativamente às duas populações gerais estudadas. A frequência menor na coorte é condizente com o aparente efeito protetor para osteonecrose, com base no fato de que a DG é bastante subdiagnosticada no Brasil (FERREIRA; DA SILVA; ARAÚJO; TANNÚS *et al.*), e que pacientes com DG sem osteonecrose têm menor chance de serem diagnosticados devido à osteonecrose ser um dos principais sintomas que levam pacientes a serem testados para essa doença (BOUAYADI; LYAGOUBI; AARAB; LAMRABAT *et al.*, 2019; MEHTA; BELMATOUG; BEMBI; DEEGAN *et al.*, 2017). O mecanismo proposto para a proteção à osteonecrose promovida pela variante *CYBRD1* rs10455 é o aumento da função da ferriredutase do epitélio intestinal, com maior absorção de ferro (SCHLOTTMANN; VERA-AVILES; LATUNDE-DADA, 2017) – em um paciente com DG1 não tratado, o ferro tende a acumular-se intracelularmente (ver Introdução, seção 3.4), levando à anemia e, consequentemente, à estimulação da eritropoiese na medula óssea, com aumento do volume ocupado por células de progenitores eritrocíticos. No contexto da DG1, essas células competem por espaço com as células de Gaucher, levando ao aumento da infiltração da medula óssea e assim à osteonecrose (HUGHES; MIKOSCH; BELMATOUG; CARUBBI *et al.*, 2019). O papel protetor de um ganho de função da ferriredutase seria dado através de diminuir a necessidade de eritropoiese, por aumentar os níveis circulantes de ferro. Essa hipótese é corroborada pelo fato de a anemia ser o principal fator de risco para o desenvolvimento de osteonecrose em pacientes com DG (KHAN; HANGARTNER; WEINREB; TAYLOR *et al.*, 2012) – no entanto, mais dados experimentais são necessários para comprovar a associação entre *CYBRD1* e osteonecrose, e entre *CYBRD1* e predisposição à anemia em pacientes com DG antes do início do tratamento, o que não foi investigado nesse estudo.

Apesar do envolvimento direto do fígado com o metabolismo corporal do ferro, não foi encontrada nenhuma associação entre componentes do fenótipo hepático da DG e genes participantes das rotas de metabolismo do ferro. Uma explicação imediata para a ausência de achados se encontra nas limitações desse estudo: apesar de ter sido utilizada uma coorte relativamente grande de pacientes com DG, o número absoluto de pacientes na amostra ainda é pequeno comparado com as populações de referência, o que limita a sensibilidade da

análise estatística. É possível também que, mesmo que haja envolvimento do metabolismo do ferro na modificação de fenótipo da DG, esse processo aconteça não a nível gênico, mas sim a níveis de maior complexidade, como por exemplo por modulação epigenética ou por fatores ambientais ou nutricionais. Para a elucidação completa dessa influência, fazem-se necessários mais estudos que investiguem a nível experimental e clínico aspectos finos da deposição hepática de ferro e da modulação das funções hepáticas por níveis de ferro hepatocelulares e macrofágicos na DG.

4. O fenótipo hepático dos DCGs

No artigo 6, o fenótipo hepático de 42 pacientes com 18 diferentes DCGs foi delineado de forma longitudinal e comparativa. Primariamente, os dados dos pacientes foram divididos conforme o grupo de DCG (ver Introdução, seção 4), devido à diferença na biologia subjacente ao processo patológico nesses pacientes. Os pacientes do grupo I, conforme mostrado na figura 1 do artigo, tiveram de modo geral grande aumento dos marcadores hepatocelulares inespecíficos nos primeiros anos de vida, com declínio gradual após, excetuando-se os pacientes portadores de MPI-CDG e PGM1-CDG (deficiência de fosfoglicomutase-1) – a MPI-CDG é um dos poucos DCGs com acometimento predominantemente hepático e gastrointestinal, conhecida por levar precocemente à cirrose hepática (ČECHOVÁ; ALTASSAN; BORGEL; BRUNEEL *et al.*, 2020) mesmo na vigência de tratamento específico com suplementação de manose (MENTION; LACAILLE; VALAYANNOPOULOS; ROMANO *et al.*, 2008), apesar de que o mecanismo para esse dano hepático ainda não seja elucidado; a PGM1-CDG, apesar de ter sido incluída nos DCGs de grupo I, é na verdade o único CDG de acometimento tanto do RE quanto do CG, e com achados também característicos de doenças de depósito de glicogênio, o que faz com que sua não-adequação ao padrão observado na maior parte dos DCGs do tipo seja esperado (MORAVA, 2014; WONG; GADOMSKI; VAN SCHERPENZEEL; HONZIK *et al.*, 2017). Quanto aos DCGs do grupo II, não foi possível observar padrão comum na evolução dos marcadores de dano hepático, refletindo a natureza diversa das doenças que fazem parte

desse grupo. Interessantemente, não foram observados aumentos consistentes dos marcadores canaliculares (FA e GGT) em pacientes de qualquer dos grupos, apontando que o fenótipo hepático dos DCGs é predominantemente hepatocelular.

Ainda no nível de análise do fenótipo clínico dos DCGs, são de grande interesse os dados obtidos por US abdominal: um achado comum nos pacientes com DCG foi a heterogeneidade de ecotextura hepática (*coarse liver parenchyma*), especialmente em pacientes diagnosticados com PMM2-CDG. Apesar de ser geralmente um achado ultrassonográfico associado à fibrose hepática (PARTANEN; PIKKARAINEN; PASANEN; ALHAVA *et al.*, 1990), foi observada uma dissociação completa entre esse achado e os resultados da ETH; com a devida ressalva de que o tamanho amostral de pacientes com US e ETH foi bastante baixo, esse descompasso indica que processos não-fibróticos que alterem macroscopicamente a estrutura hepática possam estar ocorrendo, sendo necessários estudos com enfoque histológico e experimental para a determinação de sua natureza. Outrossim, esses achados reforçam o fato de que a acurácia da US é baixa para a fibrose hepática, e que técnicas específicas como a ETH devem ser utilizadas rotineiramente em pacientes com doenças que aumentem o seu risco – como os DCGs.

A análise histológica do fenótipo hepático dos DCGs, por sua vez, foi limitada pelo baixo número amostral: conforme descrito no artigo 6, apenas 3 pacientes tinham resultados histológicos disponíveis, sendo 2 pacientes com BHs prévias e 1 paciente com um explante hepático total. Em todos esses pacientes, fibrose avançada (F3 ou F4) foi detectada, indicando que eles estivessem na parte mais grave do espectro fenotípico hepático. Focos de glicogênio hepatocelular foram detectados nos 3 pacientes, sugerindo que um distúrbio secundário do metabolismo do glicogênio possa estar em curso, potencialmente contribuindo com a patogênese da hepatopatia conforme já descrito por Choi e colaboradores (CHOI; WOO; CHOE; PARK *et al.*, 2015).

É de grande interessante também a identificação de fatores associados com um fenótipo hepático mais grave: houve correlação do primeiro domínio (que trata do quadro atual do paciente) da escala de gravidade NPCRS (*Nijmegen Paediatric CDG Rating Scale*,

na sigla em inglês) com o marcador de dano hepatocelular inespecífico AST. Isso pode significar que há contribuição do dano hepatocelular em curso para a gravidade geral da doença, possivelmente através da diminuição da função hepática ou da liberação de substâncias intra-hepatocelulares para a circulação sistêmica. Essa hipótese é corroborada pela associação também encontrada entre AST e distúrbios da coagulação, já que quase todos os fatores de coagulação (com a exceção do fator de von Willebrand) são produzidos no fígado, sendo mesmo por vezes utilizados como marcadores da função hepática (SENZOLO; BURRA; CHOLONGITAS; BURROUGHS, 2006). No entanto, mais estudos, inclusive o estabelecimento de relação causal, são necessários antes que essas hipóteses possam ser confirmadas ou descartadas.

5. Convergências e divergências fenotípicas entre DG e DCGs

Nos trabalhos apresentados acima, foi utilizado conceito de fenótipo hepático para a determinação do conjunto de manifestações ligadas ao fígado em pacientes com DG e em pacientes com DCGs. Desse modo, obtém-se uma base comum para o entendimento do comprometimento e do processo patológico em vigor em ambos contextos, permitindo a sua comparação. Sabe-se atualmente que os lisossomos são reguladores da glicosilação devido a seu papel na reciclagem de glicanos (WINCHESTER, 2005) – a própria DG é, em última análise, um defeito da de-glicosilação, sendo a GCase a última enzima a remover os componentes glicídicos dos esfingolipídios (transformando GlcCer em glicose e ceramida). Essa visão é corroborada e expandida pelo achado, ainda não publicado, de que o soro e a urina de pacientes com DG apresentam um perfil de glicoproteínas truncadas similar ao de diversos DCG do tipo II (comunicação pessoal com a dra. Kimiyo Raymond, do Biochemical Genetics Laboratory da Mayo Clinic). Desse modo, a identificação das similitudes e diferenças entre o fenótipo hepático da DG e dos DCGs contribui para a compreensão dos processos patológicos subjacentes a ambos.

O primeiro ponto a ser analisado é a elevação dos marcadores de dano hepático: enquanto que na DG há aumento crônico e de baixo grau desses marcadores, incluindo hepatocelulares e biliares, o observado nos DCGs de um modo geral (e especialmente nos DCGs de tipo I) é o aumento extremo de marcadores hepatocelulares durante os primeiros anos de vida, com arrefecimento do dano hepático após e relativa preservação de valores normais dos marcadores biliares. Essa diferença reflete a natureza biológica distinta entre as duas entidades: enquanto que a DG é caracterizada por um acúmulo e um déficit de funções metabólicas progressivos que afetam tanto hepatócitos quanto vias biliares devido a sua natureza inespecífica (inflamação crônica), com possível contribuição de um dano direto conforme discutido na seção 2.5, os DCGs são caracterizados por uma falha metabólica evidente principalmente no início da vida, quando o processo de glicosilação de proteínas é mais intenso e ocorre principalmente nos hepatócitos, levando a um dano iniciado internamente (WIDE; ERIKSSON, 2019; ZHU; SUN; CHENG; CHEN *et al.*, 2014).

A esteatose parece à primeira vista ser mais evidente na DG que nos DCGs – no entanto, deve ser lembrado que as duas populações diferem quanto a sua composição demográfica, tendo os pacientes com DG média de idade mais avançada que os pacientes com DCGs, os quais tendem a ser pediátricos em sua maioria. Também deve-se levar em conta que a esteatose parece fazer parte principalmente do fenótipo estendido da DG, com pacientes sem tratamento tendo baixa prevalência desse fenômeno e da síndrome metabólica que contribui ao seu aparecimento: no artigo 1, apenas 7,6% dos pacientes com DG apresentaram esteatose previamente ao tratamento. Por fim, à US 4 pacientes com DCGs apresentaram esteatose, e à realização de BH, 3 pacientes com DCG e fibrose avançada apresentaram concomitância de esteatose, que foi macrovesicular, microvesicular, e mista respectivamente em um caso cada. Isso nos dá uma prevalência maior de esteatose em DCGs que em pacientes não-tratados com DG, levando à conclusão de que a esteatose é parte do fenótipo "primário" dos DCGs, mas do fenótipo estendido da DG.

Outro ponto de grande divergência entre o fenótipo hepático da DG e dos DCGs é a siderose: enquanto a deposição anômala de ferro parece ser uma das principais

características do fenótipo hepático da DG, sendo frequentemente conspícua à BH, esse processo foi ausente do fenótipo hepático dos DCGs, com pesquisa de ferro negativa em todas as 3 BHs de pacientes nos quais, devido ao fenótipo grave que apresentaram, seria esperado que essa manifestação fenotípica, se existente, fosse demonstrada.

F. Conclusões

- Objetivo específico 1: o fenótipo hepático clínico da DG inclui dano hepatocelular e biliar crônico com hemossiderose. Há evidência de que esse processo possa ser acompanhado de inflamação do parênquima hepático (hepatite). Uma grande parte dos pacientes apresenta evidências de fibrose hepática, sendo possíveis todos os graus de fibrose. Aproximadamente um terço dos pacientes com DG apresenta colelitíase. O fenótipo hepático estendido inclui esteatose hepática e possivelmente esteato-hepatite. O aumentado risco para o desenvolvimento de HC nos pacientes com DG reforça a recomendação proposta na literatura de que lesões hepáticas com características compatíveis com o diagnóstico de HC devam ser tratadas como HC, sem confirmação por biópsia, mesmo que o diagnóstico diferencial de um Gaucheroma seja considerado.

- Objetivo específico 2: o fenótipo hepático histológico quantitativo de pacientes com DG1 em tratamento inclui: aumento de características eucromáticas dos núcleos hepatocelulares e diminuição da ramificação dos canalículos biliares. O fenótipo hepático histológico descritivo de pacientes com DG1 inclui hemossiderose parenquimatosa e mesenquimal, fibrose perissinusoidal, fibrose portal com formação de pontes e progressão à cirrose, colestase canalicular, esteatose macrogoticular e esteato-hepatite. A presença local de células de Gaucher não parece ser necessária para o desenvolvimento das manifestações histológicas.

- Objetivo específico 3: não foram encontrados, entre os genes estudados, modificadores do fenótipo hepático. Um gene candidato a modificador do fenótipo geral da DG encontrado é o *CYBDR1*, especificamente a variante rs10455, com efeito protetor contra osteonecrose.

- Objetivo específico 4: em uma investigação ativa, aproximadamente metade dos pacientes com DG1 em tratamento têm fibrose hepática detectável ao uso de ETH,

sendo a maior parte dos pacientes portadores de fibrose hepática leve (F1). O escore clínico-laboratorial APRI apresentou maior acurácia entre os escores estudados, com sensibilidade adequada para o rastreio de fibrose significativa (F2 ou maior).

- Objetivo específico 5: o fenótipo hepático dos DCGs inclui: aumento específico de marcadores de dano hepatocelular, mas não de dano biliar, com tendência à diminuição em direção ao valor de normalidade após os 5 anos de idade em DCGs do grupo I, mas sem tendência observável em DCGs do grupo II; esteatose macrogoticular, microgoticular e mista; fibrose hepática de todos os graus, incluindo cirrose; depósito hepatocelular de glicogênio em pacientes com cirrose. Hemossiderose não é parte do fenótipo hepático dos DCGs estudados.

G. Perspectivas

Os estudos conduzidos durante essa tese respondem a diversas questões, mas suscitam outras ainda mais numerosas. Algumas perspectivas de pesquisa para os assuntos levantados são imediatas – isso é, já se encontram em andamento ou serão iniciadas dentro de pouco tempo – enquanto outras perspectivas são de longo prazo, e provavelmente darão origem a diversos outros projetos e linhas de pesquisa.

São perspectivas imediatas para a continuação da linha de pesquisa aqui explorada:

1. A expansão amostral do estudo detalhado no artigo 5, a fim de refinar os pontos de corte e os valores de sensibilidade e especificidade encontrados;
2. A finalização de um estudo, já em andamento, para o delineamento do fenótipo hepático de pacientes brasileiros com DCGs, a fim de identificar fatores específicos da nossa população e expandir os achados fenotípicos descritos no artigo 6;
3. A realização de novos estudos de histomorfometria de hepatócitos e canalículos biliares em doenças e condições diversas, a fim de expandir e refinar os usos dessa nova técnica analítica.

São perspectivas de longo prazo para a exploração de questões levantadas nesta tese:

1. A investigação experimental de transtornos canaliculares e hepatocelulares na DG, com uso de modelos experimentais (modelos animais, cultura de células), a fim de testar a hipótese do envolvimento do transportador ABCB1 na patogênese das manifestações canaliculares da DG;
2. A investigação sistemática de fibrose hepática em pacientes com DCGs e o estudo da acurácia de escores clínico-laboratoriais de fibrose hepática para essa população de pacientes;

3. A realização de estudos experimentais para confirmar o efeito modificador da variante rs10455 do gene *CYBDR1* sobre a ocorrência de osteonecrose na DG;

4. A busca por genes modificadores de fenótipo hepático e de fenótipo geral em pacientes com DCGs;

5. Uso de técnicas histomorfométricas para a quantificação de fenótipo hepático histológico em pacientes com DCGs.

6. Expansão da técnica histomorfométrica de biópsias hepáticas para estudo de outras doenças e novos parâmetros.

7. A expansão do estudo do fenótipo hepático para doenças relacionadas à DG, como a deficiência de esfingomielinase ácida, a doença de Niemann-Pick tipo C, ou a doença de Krabbe.

8. A investigação do papel da inflamação no desenvolvimento do fenótipo hepático da DG, haja vista a presença de esteato-hepatite em pacientes biopsiados, com identificação de citocinas e rotas envolvidas nesse processo.

H. Referências

ADAM, M. P.; ARDINGER, H. H.; PAGON, R. A.; WALLACE, S. E. *et al.* GeneReviews. *In*, 1993.

ADAR, T.; ILAN, Y.; ELSTEIN, D.; ZIMRAN, A. Liver involvement in Gaucher disease - Review and clinical approach. **Blood Cells Mol Dis**, Oct 2016.

AERTS, J. M. F. G.; KUO, C. L.; LELIEVELD, L. T.; BOER, D. E. C. *et al.* Glycosphingolipids and lysosomal storage disorders as illustrated by gaucher disease. **Curr Opin Chem Biol**, 53, p. 204-215, Dec 2019.

AFLAKI, E.; BORGER, D. K.; MOAVEN, N.; STUBBLEFIELD, B. K. *et al.* A New Glucocerebrosidase Chaperone Reduces α-Synuclein and Glycolipid Levels in iPSC-Derived Dopaminergic Neurons from Patients with Gaucher Disease and Parkinsonism. **J Neurosci**, 36, n. 28, p. 7441-7452, Jul 2016.

AFLAKI, E.; MOAVEN, N.; BORGER, D. K.; LOPEZ, G. *et al.* Lysosomal storage and impaired autophagy lead to inflammasome activation in Gaucher macrophages. **Aging Cell**, 15, n. 1, p. 77-88, Feb 2016.

ALIOTO, A. G.; GOMEZ, R.; MOSES, J.; PATERNOSTRO, J. *et al.* Quality of life and psychological functioning of pediatric and young adult patients with Gaucher disease, type 1. **Am J Med Genet A**, 182, n. 5, p. 1130-1142, May 2020.

ALMEIDA, V. B.; MONTEIRO, M. M. Investigação de Enzimas Hepáticas Alteradas. *In*: FERRAZ, M. L. G.;SILVA, A. E. B., *et al* (Ed.). **Manual de Hepatologia para Clínicos e Residentes**. 1st edition ed.: Atheneu, 2018. cap. 1, p. 1-8.

ALTASSAN, R.; PÉANNE, R.; JAEKEN, J.; BARONE, R. *et al.* International clinical guidelines for the management of phosphomannomutase 2-congenital disorders of glycosylation: Diagnosis, treatment and follow up. **J Inherit Metab Dis**, 42, n. 1, p. 5-28, 01 2019.

ANGULO, P.; HUI, J. M.; MARCHESINI, G.; BUGIANESI, E. *et al.* The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. **Hepatology**, 45, n. 4, p. 846-854, Apr 2007.

ATHYROS, V. G.; TZIOMALOS, K.; KATSIKI, N.; DOUMAS, M. *et al.* Cardiovascular risk across the histological spectrum and the clinical manifestations of non-alcoholic fatty liver disease: An update. **World J Gastroenterol**, 21, n. 22, p. 6820-6834, Jun 2015.

BARTON, N. W.; BRADY, R. O.; DAMBROSIA, J. M.; DI BISCEGLIE, A. M. *et al.* Replacement therapy for inherited enzyme deficiency--macrophage-targeted glucocerebrosidase for Gaucher's disease. **N Engl J Med**, 324, n. 21, p. 1464-1470, May 1991.

BATTS, K. P.; LUDWIG, J. Chronic hepatitis. An update on terminology and reporting. **Am J Surg Pathol**, 19, n. 12, p. 1409-1417, Dec 1995.

BEDOSSA, P. Pathology of non-alcoholic fatty liver disease. **Liver Int**, 37 Suppl 1, p. 85-89, 01 2017.

BEDOSSA, P. Diagnosis of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis: Why liver biopsy is essential. **Liver Int**, 38 Suppl 1, p. 64-66, 02 2018.

BEDOSSA, P.; PARADIS, V.; ZUCMAN-ROSSI, J. Cellular and Molecular Techniques. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th edition ed.: Elsevier, 2018. cap. 2, p. 88-110.

BEDOSSA, P.; POYNARD, T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. **Hepatology**, 24, n. 2, p. 289-293, Aug 1996.

BEN HAROSH-KATZ, M.; PATLAS, M.; HADAS-HALPERN, I.; ZIMRAN, A. *et al.* Increased prevalence of cholelithiasis in Gaucher disease: association with splenectomy but not with gilbert syndrome. **J Clin Gastroenterol**, 38, n. 7, p. 586-589, Aug 2004.

BLACKER, D.; WILCOX, M. A.; LAIRD, N. M.; RODES, L. *et al.* Alpha-2 macroglobulin is genetically associated with Alzheimer disease. **Nat Genet**, 19, n. 4, p. 357-360, Aug 1998.

BOHTE, A. E.; VAN DUSSEN, L.; AKKERMAN, E. M.; NEDERVEEN, A. J. *et al.* Liver fibrosis in type I Gaucher disease: magnetic resonance imaging, transient elastography and parameters of iron storage. **PLoS One**, 8, n. 3, p. e57507, 2013.

BORSOI VIANA, M. S.; TAKEI, K.; COLLARILE YAMAGUTI, D. C.; GUZ, B. *et al.* Use of AST platelet ratio index (APRI Score) as an alternative to liver biopsy for treatment indication in chronic hepatitis C. **Ann Hepatol**, 8, n. 1, p. 26-31, 2009 Jan-Mar 2009.

BOUAYADI, O.; LYAGOUBI, A.; AARAB, A.; LAMRABAT, S. *et al.* Gaucher Disease: An Underdiagnosed Pathology in the Eastern Moroccan Population. **EJIFCC**, 30, n. 1, p. 82-87, Mar 2019.

BOYD, A.; CAIN, O.; CHAUHAN, A.; WEBB, G. J. Medical liver biopsy: background, indications, procedure and histopathology. **Frontline Gastroenterol**, 11, n. 1, p. 40-47, Jan 2020.

CALÈS, P.; OBERTI, F.; MICHALAK, S.; HUBERT-FOUCHARD, I. *et al.* A novel panel of blood markers to assess the degree of liver fibrosis. **Hepatology**, 42, n. 6, p. 1373-1381, Dec 2005.

CHEDID, A. D.; CHEDID, M. F. Fígado, vias biliares e sistema porta. *In*: DE OLIVEIRA, V. F. C., MARCIO FERNANDES e GIRARDI, F. M. (Ed.). **Anatomia Clínica e Cirúrgica do Abdome**. First edition ed.: UFRGS, 2005. cap. 9, p. 225-262.

CHOI, R.; WOO, H. I.; CHOE, B. H.; PARK, S. *et al.* Application of whole exome sequencing to a rare inherited metabolic disease with neurological and gastrointestinal manifestations: a congenital disorder of glycosylation mimicking glycogen storage disease. **Clin Chim Acta**, 444, p. 50-53, Apr 2015.

CICHOŻ-LACH, H.; CELIŃSKI, K.; PROZOROW-KRÓL, B.; SWATEK, J. *et al.* The BARD score and the NAFLD fibrosis score in the assessment of advanced liver fibrosis in nonalcoholic fatty liver disease. **Med Sci Monit**, 18, n. 12, p. CR735-740, Dec 2012.

CONSORTIUM, U. UniProt: a worldwide hub of protein knowledge. **Nucleic Acids Res**, 47, n. D1, p. D506-D515, 01 2019.

CONSTANTINE, C. C.; ANDERSON, G. J.; VULPE, C. D.; MCLAREN, C. E. *et al.* A novel association between a SNP in CYBRD1 and serum ferritin levels in a cohort study of HFE hereditary haemochromatosis. **Br J Haematol**, 147, n. 1, p. 140-149, Oct 2009.

COX, T.; LACHMANN, R.; HOLLAK, C.; AERTS, J. *et al.* Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. **Lancet**, 355, n. 9214, p. 1481-1485, Apr 2000.

COX, T. M.; DRELICHMAN, G.; CRAVO, R.; BALWANI, M. *et al.* Eliglustat maintains long-term clinical stability in patients with Gaucher disease type 1 stabilized on enzyme therapy. **Blood**, 129, n. 17, p. 2375-2383, Apr 2017.

COX, T. M.; DRELICHMAN, G.; CRAVO, R.; BALWANI, M. *et al.* Eliglustat compared with imiglucerase in patients with Gaucher's disease type 1 stabilised on enzyme replacement therapy: a phase 3, randomised, open-label, non-inferiority trial. **Lancet**, 385, n. 9985, p. 2355-2362, Jun 2015.

CRAWFORD, J. M.; BIOULAC-SAGE, P.; HYTIROGLOU, P. Structure, Function, and Responses to Injury. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. Seventh edition ed.: Elsevier, 2018. cap. 1, p. 1-87.

DAMLE-VARTAK, A.; BEGHER-TIBBE, B.; GUNTHER, G.; GEISLER, F. *et al.* Pipe-3D: A Pipeline Based on Immunofluorescence, 3D Confocal Imaging, Reconstructions, and Morphometry for Biliary Network Analysis in Cholestasis. **Methods Mol Biol**, 1981, p. 25-53, 2019.

DAVIDSON, B. A.; HASSAN, S.; GARCIA, E. J.; TAYEBI, N. *et al.* Exploring genetic modifiers of Gaucher disease: The next horizon. **Hum Mutat**, 39, n. 12, p. 1739-1751, 12 2018.

DAWKINS, R. **The Selfish Gene**. 30th anniversary ed. Oxford University Press, 1976.

DAWKINS, R. Replicator selection and the extended phenotype. **Z Tierpsychol**, 47, n. 1, p. 61-76, May 1978.

DE FOST, M.; VOM DAHL, S.; WEVERLING, G. J.; BRILL, N. *et al.* Increased incidence of cancer in adult Gaucher disease in Western Europe. **Blood Cells Mol Dis**, 36, n. 1, p. 53-58, 2006 Jan-Feb 2006.

DE LOPE, C. R.; TREMOSINI, S.; FORNER, A.; REIG, M. *et al.* Management of HCC. **J Hepatol**, 56 Suppl 1, p. S75-87, 2012.

DEUGNIER, Y.; TURLIN, B. Pathology of hepatic iron overload. **World J Gastroenterol**, 13, n. 35, p. 4755-4760, Sep 2007.

DHITAL, R.; TIROSH, O. Fatty Liver Vulnerability to Hypoxic and Inflammatory Stress. *In*: TIROSH, O. (Ed.). **Liver Metabolism and Fatty Liver Disease**. 1st edition ed., 2015. cap. 2, p. 27-52.

DONEDA, D.; LOPES, A. L.; OLIVEIRA, A. R.; NETTO, C. B. *et al*. Gaucher disease type I: assessment of basal metabolic rate in patients from southern Brazil. **Blood Cells Mol Dis**, 46, n. 1, p. 42-46, Jan 2011.

DONEDA, D.; NETTO, C. B.; MOULIN, C. C.; SCHWARTZ, I. V. Effects of imiglucerase on the growth and metabolism of Gaucher disease type I patients: a systematic review. **Nutr Metab (Lond)**, 10, n. 1, p. 34, Apr 2013.

DONEDA, D.; VAIRO, F. P.; LOPES, A. L.; REISCHAK-OLIVEIRA, A. *et al.* Assessment of Basal Metabolic Rate and Nutritional Status in Patients with Gaucher Disease Type III. **JIMD Rep**, 14, p. 37-42, 2014.

DRAGICA, J. M.; KOSTANDINA, K. P. Applied 3-D anatomy of liver bile ducts in injection-corrosion casts. **Sanamed**, 8, n. 1, p. 10, 2013.

DRELICHMAN, G.; FERNÁNDEZ ESCOBAR, N.; BASACK, N.; AVERSA, L. *et al.* Skeletal involvement in Gaucher disease: An observational multicenter study of prognostic factors in the Argentine Gaucher disease patients. **Am J Hematol**, 91, n. 10, p. E448-453, Oct 2016.

EDMISON, J.; MCCULLOUGH, A. J. Pathogenesis of non-alcoholic steatohepatitis: human data. **Clin Liver Dis**, 11, n. 1, p. 75-104, ix, Feb 2007.

EL-SERAG, H. B.; MARRERO, J. A.; RUDOLPH, L.; REDDY, K. R. Diagnosis and treatment of hepatocellular carcinoma. **Gastroenterology**, 134, n. 6, p. 1752-1763, May 2008.

ELIAS, A. F.; JOHNSON, M. R.; BOITNOTT, J. K.; VALLE, D. Neonatal cholestasis as initial manifestation of type 2 Gaucher disease: a continuum in the spectrum of early onset Gaucher disease. **JIMD Rep**, 5, p. 95-98, 2012.

ELLEDER, M. Glucosylceramide transfer from lysosomes--the missing link in molecular pathology of glucosylceramidase deficiency: a hypothesis based on existing data. **J Inherit Metab Dis**, 29, n. 6, p. 707-715, Dec 2006.

ESSABAR, L.; MESKINI, T.; LAMALMI, L.; ETTAIR, S. *et al.* Gaucher's diseaase: report of 11 cases and review of the literature. **Pan-African Medical Journal**, 20, p. 8, 2015.

FERREIRA, C. R.; ALTASSAN, R.; MARQUES-DA-SILVA, D.; FRANCISCO, R. *et al.* Recognizable phenotypes in CDG. **J Inherit Metab Dis**, 41, n. 3, p. 541-553, 05 2018.

FERREIRA, C. S.; DA SILVA, L. R.; ARAÚJO, M. B.; TANNÚS, R. K. *et al.* Doença de Gaucher - uma desordem subdiagnosticada. **Revista Paulista de Pediatria**, 29, n. 1, p. 4, 2011.

FIGUEIREDO, F. D. A.; FIREMAN, M. A. D. A. PCDT - Protocolo de Diretrizes Clínicas e Terapêuticas para Doença de Gaucher. SAÚDE, M. D.**:** 1-25. p. 2017.

FLEMING, R. E.; MIGAS, M. C.; HOLDEN, C. C.; WAHEED, A. *et al.* Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. **Proc Natl Acad Sci U S A**, 97, n. 5, p. 2214-2219, Feb 2000.

FOSTER, J. R. Liver. *In*: SUTTIE, A. W. (Ed.). **Boorman's Pathology of the Rat**. 2nd edition ed.: Elsevier, 2018. cap. 9, p. 81-105.

FRANCISCO, R.; MARQUES-DA-SILVA, D.; BRASIL, S.; PASCOAL, C. *et al.* The challenge of CDG diagnosis. **Mol Genet Metab**, 126, n. 1, p. 1-5, 01 2019.

FUCUTA, P. D. S.; BILAR, J. M. Sobrecarga de Ferro e Hemocromatose Hereditária. *In*: FERRAZ, M. L. G. S., ANTONIO EDUARDO BENEDITO e SCHIAVON, J. L. N. (Ed.). **Manual de Hepatologia para Clínicos e Residentes**. 1st edition ed.: Atheneu, 2018. cap. 54, p. 433-444.

FUJIHARA, J.; YASUDA, T.; KIMURA-KATAOKA, K.; TAKESHITA, H. Association of SNPs in transferrin and transferrin receptor genes with blood iron levels in human. **Leg Med (Tokyo)**, 36, p. 17-20, Feb 2019.

GALARIS, D.; BARBOUTI, A.; PANTOPOULOS, K. Iron homeostasis and oxidative stress: An intimate relationship. **Biochim Biophys Acta Mol Cell Res**, 1866, n. 12, p. 118535, 12 2019.

GANDON, Y.; OLIVIÉ, D.; GUYADER, D.; AUBÉ, C. *et al.* Non-invasive assessment of hepatic iron stores by MRI. **Lancet**, 363, n. 9406, p. 357-362, Jan 2004.

GARY, S. E.; RYAN, E.; STEWARD, A. M.; SIDRANSKY, E. Recent advances in the diagnosis and management of Gaucher disease. **Expert Rev Endocrinol Metab**, 13, n. 2, p. 107-118, 03 2018.

GAWAD TANTAWY, A. A.; MONEAM ADLY, A. A.; MADKOUR, S. S.; SALAH EL-DIN, N. Y. Pulmonary manifestations in young Gaucher disease patients: Phenotype-genotype correlation and radiological findings. **Pediatr Pulmonol**, 55, n. 2, p. 441-448, 02 2020.

GIRARD, M.; POUJOIS, A.; FABRE, M.; LACAILLE, F. *et al.* CCDC115-CDG: A new rare and misleading inherited cause of liver disease. **Mol Genet Metab**, 124, n. 3, p. 228-235, 07 2018.

GREENBERGER, N. J.; PAUMGARTNER, G. Diseases of the Gallbladder and Bile Ducts. *In*: KASPER, D. L.;HAUSER, S. L., *et al* (Ed.). **Harrison's Principles of Internal Medicine**. 19th edition ed.: Mc-Graw Hill, 2015. v. 2, cap. 369, p. 2075-2085.

GUJJA, P.; ROSING, D. R.; TRIPODI, D. J.; SHIZUKUDA, Y. Iron overload cardiomyopathy: better understanding of an increasing disorder. **J Am Coll Cardiol**, 56, n. 13, p. 1001-1012, Sep 2010.

GUO, M. H.; PLUMMER, L.; CHAN, Y. M.; HIRSCHHORN, J. N. *et al.* Burden Testing of Rare Variants Identified through Exome Sequencing via Publicly Available Control Data. **Am J Hum Genet**, 103, n. 4, p. 522-534, 10 2018.

HADAS-HALPERN, I.; DEEB, M.; ABRAHAMOV, A.; ZIMRAN, A. *et al.* Gaucher disease: spectrum of sonographic findings in the liver. **J Ultrasound Med**, 29, n. 5, p. 727-733, May 2010.

HAGSTRÖM, H.; NASR, P.; EKSTEDT, M.; STÅL, P. *et al.* Accuracy of Noninvasive Scoring Systems in Assessing Risk of Death and Liver-Related Endpoints in Patients With

Nonalcoholic Fatty Liver Disease. **Clin Gastroenterol Hepatol**, 17, n. 6, p. 1148-1156.e1144, 05 2019.

HALL, A. R.; LE, H.; ARNOLD, C.; BRUNTON, J. *et al.* Aluminum Exposure from Parenteral Nutrition: Early Bile Canaliculus Changes of the Hepatocyte. **Nutrients**, 10, n. 6, Jun 2018.

HARRIS, Z. L. Ceruloplasmin. *In*: KERKAR, N. e ROBERTS, E. A. (Ed.). **Clinical and Translational Perspectives on Wilson Disease**. 1st edition ed.: Academic Press, 2019. cap. 9, p. 77-84.

HARRISON, S. A.; OLIVER, D.; ARNOLD, H. L.; GOGIA, S. *et al.* Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. **Gut**, 57, n. 10, p. 1441-1447, Oct 2008.

HEITNER, R.; ELSTEIN, D.; AERTS, J.; WEELY, S. *et al.* Low-dose N-butyldeoxynojirimycin (OGT 918) for type I Gaucher disease. **Blood Cells Mol Dis**, 28, n. 2, p. 127-133, 2002 Mar-Apr 2002.

HIGGINS, D.; OTERO, J.; JEFFERIS KIRK, C.; PAK, J. *et al.* Iron Laboratory Studies in Pediatric Patients With Heart Failure from Dilated Cardiomyopathy. **Am J Cardiol**, 120, n. 11, p. 2049-2055, Dec 2017.

HILL, S. C.; DAMASKA, B. M.; LING, A.; PATTERSON, K. *et al.* Gaucher disease: abdominal MR imaging findings in 46 patients. **Radiology**, 184, n. 2, p. 561-566, Aug 1992.

HORAI, Y.; KAKIMOTO, T.; TAKEMOTO, K.; TANAKA, M. Quantitative analysis of histopathological findings using image processing software. **J Toxicol Pathol**, 30, n. 4, p. 351-358, Oct 2017.

HUBERT, N.; HENTZE, M. W. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. **Proc Natl Acad Sci U S A**, 99, n. 19, p. 12345-12350, Sep 2002.

HUGHES, D.; MIKOSCH, P.; BELMATOUG, N.; CARUBBI, F. *et al.* Gaucher Disease in Bone: From Pathophysiology to Practice. **J Bone Miner Res**, 34, n. 6, p. 996-1013, Jun 2019.

IVANOVA, M.; LIMGALA, R. P.; CHANGSILA, E.; KAMATH, R. *et al.* Gaucheromas: When macrophages promote tumor formation and dissemination. **Blood Cells Mol Dis**, Oct 2016.

IYER, S.; SAM, F. S.; DIPRIMIO, N.; PRESTON, G. *et al.* Repurposing the aldose reductase inhibitor and diabetic neuropathy drug epalrestat for the congenital disorder of glycosylation PMM2-CDG. **Dis Model Mech**, 12, n. 11, 11 2019.

JAEKEN, J. Congenital disorders of glycosylation. **Handb Clin Neurol**, 113, p. 1737-1743, 2013.

JAEKEN, J.; MORAVA, E. Congenital Disorders of Glycosylation, Dolichol

and Glycosylphosphatidylinositol Metabolism. *In*: SAUDUBRAY, J.-M.;BAUMGARTNER, M. R., *et al* (Ed.). **Inborn Metabolic Diseases**. 6th edition ed.: Springer, 2016. cap. 41, p. 607-631.

JAGOE, R.; SOWTER, C.; SLAVIN, G. Shape and texture analysis of liver cell nuclei in hepatomas by computer aided microscopy. **J Clin Pathol**, 37, n. 7, p. 755-762, Jul 1984.

JAMES, S. P.; STROMEYER, F. W.; CHANG, C.; BARRANGER, J. A. LIver abnormalities in patients with Gaucher's disease. **Gastroenterology**, 80, n. 1, p. 126-133, Jan 1981.

JANSEN, J. C.; CIRAK, S.; VAN SCHERPENZEEL, M.; TIMAL, S. *et al.* CCDC115 Deficiency Causes a Disorder of Golgi Homeostasis with Abnormal Protein Glycosylation. **Am J Hum Genet**, 98, n. 2, p. 310-321, Feb 2016.

JOHANNSEN, W. The genotype conception of heredity. 1911. **Int J Epidemiol**, 43, n. 4, p. 989-1000, Aug 2014.

KANEL, G. C.; KORULA, J. Alcoholic and Non-Alcoholic Fatty Liver Diseases. *In*: KANEL, G. C. e KORULA, J. (Ed.). **Atlas of Liver Pathology**. 3rd edition ed.: Elsevier, 2011a. cap. 4, p. 71-92.

KANEL, G. C.; KORULA, J. Diseases of Hepatic Iron and Copper Metabolism. *In*: KANEL, G. C. e KORULA, J. (Ed.). **Atlas of Liver Pathology**. 3rd ed.: Elsevier, 2011b. cap. 9, p. 236-248.

KANEL, G. C.; KORULA, J. General Aspects of the Liver and Liver Diseases. *In*: KANEL, G. C. e KORULA, J. (Ed.). **Atlas of Liver Pathology**. 3rd edition ed.: Elsevier, 2011c. cap. 1, p. 3-15.

KANEL, G. C.; KORULA, J. Viral Hepatitis. *In*: KANEL, G. C. e KORULA, J. (Ed.). **Atlas of Liver Pathology**. 3rd edition ed.: Elsevier, 2011d. cap. 2, p. 16-51.

KANWAL, F.; SINGAL, A. G. Surveillance for Hepatocellular Carcinoma: Current Best Practice and Future Direction. **Gastroenterology**, 157, n. 1, p. 54-64, 07 2019.

KAYE, E. M. Therapeutic approaches to lysosomal storage diseases. **Curr Opin Pediatr**, 7, n. 6, p. 650-654, Dec 1995.

KHAN, A.; HANGARTNER, T.; WEINREB, N. J.; TAYLOR, J. S. *et al.* Risk factors for fractures and avascular osteonecrosis in type 1 Gaucher disease: a study from the International Collaborative Gaucher Group (ICGG) Gaucher Registry. **J Bone Miner Res**, 27, n. 8, p. 1839-1848, Aug 2012.

KLEIN, A. D.; FERREIRA, N. S.; BEN-DOR, S.; DUAN, J. *et al.* Identification of Modifier Genes in a Mouse Model of Gaucher Disease. **Cell Rep**, 16, n. 10, p. 2546-2553, 09 2016.

KLIMKOWSKA, M.; MACHACZKA, M.; PALMBLAD, J. Aberrant bone marrow vascularization patterns in untreated patients with Gaucher disease type 1. **Blood Cells Mol Dis**, 68, p. 54-59, 02 2018.

KOPPE, T.; DONEDA, D.; SIEBERT, M.; PASKULIN, L. *et al.* The prognostic value of the serum ferritin in a southern Brazilian cohort of patients with Gaucher disease. **Genet Mol Biol**, 39, n. 1, p. 30-34, Mar 2016.

KORULA, S.; OWENS, P.; CHARLTON, A.; BHATTACHARYA, K. Rare Case of Hepatic Gaucheroma in a Child on Enzyme Replacement Therapy. **JIMD Rep**, 32, p. 101-104, 2017.

KRISTIANSEN, M. N. B.; VEIDAL, S. S.; CHRISTOFFERSEN, C.; JELSING, J. *et al.* Molecular Characterization of Microvesicular and Macrovesicular Steatosis Shows Widespread Differences in Metabolic Pathways. **Lipids**, 54, n. 1, p. 109-115, 01 2019.

LACHMANN, R. H.; WIGHT, D. G.; LOMAS, D. J.; FISHER, N. C. *et al.* Massive hepatic fibrosis in Gaucher's disease: clinico-pathological and radiological features. **QJM**, 93, n. 4, p. 237-244, Apr 2000.

LANGEVELD, M.; DE FOST, M.; AERTS, J. M.; SAUERWEIN, H. P. *et al.* Overweight, insulin resistance and type II diabetes in type I Gaucher disease patients in relation to enzyme replacement therapy. **Blood Cells Mol Dis**, 40, n. 3, p. 428-432, 2008 May-Jun 2008.

LARIONOV, S.; DEDECK, O.; BIRKENMEIER, G.; THAL, D. R. Expression of alpha2-macroglobulin, neutrophil elastase, and interleukin-1alpha differs in early-stage and late-stage atherosclerotic lesions in the arteries of the circle of Willis. **Acta Neuropathol**, 113, n. 1, p. 33-43, Jan 2007.

LEE, P. L.; BEUTLER, E. Regulation of hepcidin and iron-overload disease. **Annu Rev Pathol**, 4, p. 489-515, 2009.

LEE, R. E. The fine structure of the cerebroside occurring in Gaucher's disease. **Proc Natl Acad Sci U S A**, 61, n. 2, p. 484-489, Oct 1968.

LEE, R. E.; BALCERZAK, S. P.; WESTERMAN, M. P. Gaucher's disease. A morphologic study and measurements of iron metabolism. **Am J Med**, 42, n. 6, p. 891-898, Jun 1967.

LEE, W. K.; KOLESNICK, R. N. Sphingolipid abnormalities in cancer multidrug resistance: Chicken or egg? **Cell Signal**, 38, p. 134-145, Oct 2017.

LEFEBER, D. J.; MORAVA, E.; JAEKEN, J. How to find and diagnose a CDG due to defective N-glycosylation. **J Inherit Metab Dis**, 34, n. 4, p. 849-852, Aug 2011.

LEFEBVRE, T.; REIHANI, N.; DAHER, R.; DE VILLEMEUR, T. B. *et al.* Involvement of hepcidin in iron metabolism dysregulation in Gaucher disease. **Haematologica**, 103, n. 4, p. 587-596, Apr 2018.

LI, J. F.; QU, F.; ZHENG, S. J.; REN, F. *et al.* Plasma sphingolipids: potential biomarkers for severe hepatic fibrosis in chronic hepatitis C. **Mol Med Rep**, 12, n. 1, p. 323-330, Jul 2015.

LI, S.; XIANG, C.; WEI, X.; SUN, X. *et al.* Early supplemental α2-macroglobulin attenuates cartilage and bone damage by inhibiting inflammation in collagen II-induced arthritis model. **Int J Rheum Dis**, 22, n. 4, p. 654-665, Apr 2019.

LINARI, S.; CASTAMAN, G. Hematological manifestations and complications of Gaucher disease. **Expert Rev Hematol**, 9, n. 1, p. 51-58, Jan 2016.

LIPIŃSKI, P.; SZYMAŃSKA-ROŻEK, P.; SOCHA, P.; TYLKI-SZYMAŃSKA, A. Controlled attenuation parameter and liver stiffness measurements using transient elastography by FibroScan in Gaucher disease. **Mol Genet Metab**, Nov 2019.

LOLLERT, A.; HOFFMANN, C.; LACHE, M.; KÖNIG, J. *et al.* Acoustic radiation force impulse point shear wave elastography of the liver and spleen in patients with Gaucher disease type 1: Correlations with clinical data and markers of disease severity. **Mol Genet Metab**, 130, n. 2, p. 140-148, Jun 2020.

LORBER, M. The occurrence of intracellular iron in Gaucher's disease. **Ann Intern Med**, 53, p. 293-305, Aug 1960.

LORBER, M.; NEMES, J. L. Identification of ferritin within Gaucher cells. An electron microscopic and immunofluorescent study. **Acta Haematol**, 37, n. 4, p. 189-197, 1967.

LUKAS, J.; COZMA, C.; YANG, F.; KRAMP, G. *et al.* Glucosylsphingosine Causes Hematological and Visceral Changes in Mice-Evidence for a Pathophysiological Role in Gaucher Disease. **Int J Mol Sci**, 18, n. 10, Oct 2017.

LUM, G.; GAMBINO, S. R. Serum gamma-glutamyl transpeptidase activity as an indicator of disease of liver, pancreas, or bone. **Clin Chem**, 18, n. 4, p. 358-362, Apr 1972.

MARQUES-DA-SILVA, D.; DOS REIS FERREIRA, V.; MONTICELLI, M.; JANEIRO, P. *et al.* Liver involvement in congenital disorders of glycosylation (CDG). A systematic review of the literature. **J Inherit Metab Dis**, 40, n. 2, p. 195-207, 03 2017.

MARTÍNEZ-MONSENY, A. F.; BOLASELL, M.; CALLEJÓN-PÓO, L.; CUADRAS, D. *et al.* AZATAX: Acetazolamide safety and efficacy in cerebellar syndrome in PMM2 congenital disorder of glycosylation (PMM2-CDG). **Ann Neurol**, 85, n. 5, p. 740-751, 05 2019.

MASHAV, N.; SHIBOLET, O. Clinical Aspects of Nonalcoholic Fatty Liver Disease. *In*: TIROSH, O. (Ed.). **Liver Metabolism and Fatty Liver Disease**. 1st edition ed., 2015. cap. 1, p. 3-26.

MCKIE, A. T.; BARROW, D.; LATUNDE-DADA, G. O.; ROLFS, A. *et al.* An iron-regulated ferric reductase associated with the absorption of dietary iron. **Science**, 291, n. 5509, p. 1755-1759, Mar 2001.

MEDRANO, C.; VEGA, A.; NAVARRETE, R.; ECAY, M. J. *et al.* Clinical and molecular diagnosis of non-phosphomannomutase 2 N-linked congenital disorders of glycosylation in Spain. **Clin Genet**, 95, n. 5, p. 615-626, 05 2019.

MEDRANO-ENGAY, B.; IRUN, P.; GERVAS-ARRUGA, J.; ANDRADE-CAMPOS, M. *et al.* Iron homeostasis and infIammatory biomarker analysis in patients with type 1 Gaucher disease. **Blood Cells Mol Dis**, 53, n. 4, p. 171-175, Dec 2014.

MEHTA, A.; BELMATOUG, N.; BEMBI, B.; DEEGAN, P. *et al.* Exploring the patient journey to diagnosis of Gaucher disease from the perspective of 212 patients with Gaucher disease and 16 Gaucher expert physicians. **Mol Genet Metab**, 122, n. 3, p. 122-129, 11 2017.

MEHTA, K. J.; FARNAUD, S. J.; SHARP, P. A. Iron and liver fibrosis: Mechanistic and clinical aspects. **World J Gastroenterol**, 25, n. 5, p. 521-538, Feb 2019.

MENTION, K.; LACAILLE, F.; VALAYANNOPOULOS, V.; ROMANO, S. *et al.* Development of liver disease despite mannose treatment in two patients with CDG-Ib. **Mol Genet Metab**, 93, n. 1, p. 40-43, Jan 2008.

MISTRY, P. K.; LIU, J.; SUN, L.; CHUANG, W. L. *et al.* Glucocerebrosidase 2 gene deletion rescues type 1 Gaucher disease. **Proc Natl Acad Sci U S A**, 111, n. 13, p. 4934-4939, Apr 2014.

MISTRY, P. K.; LOPEZ, G.; SCHIFFMANN, R.; BARTON, N. W. *et al.* Gaucher disease: Progress and ongoing challenges. **Mol Genet Metab**, 120, n. 1-2, p. 8-21, 2017 Jan - Feb 2017.

MORAVA, E. Galactose supplementation in phosphoglucomutase-1 deficiency; review and outlook for a novel treatable CDG. **Mol Genet Metab**, 112, n. 4, p. 275-279, Aug 2014.

NADEAU, J. H. Modifier genes in mice and humans. **Nat Rev Genet**, 2, n. 3, p. 165-174, Mar 2001.

NAITO, Y.; SAWADA, H.; OBOSHI, M.; IWASAKU, T. *et al.* Cardiac remodeling in response to chronic iron deficiency: role of the erythropoietin receptor. **J Hypertens**, 33, n. 6, p. 1267-1275, Jun 2015.

NASCIMBENI, F.; CASSINERIO, E.; DALLA SALDA, A.; MOTTA, I. *et al.* Prevalence and predictors of liver fibrosis evaluated by vibration controlled transient elastography in type 1 Gaucher disease. **Mol Genet Metab**, Aug 2018.

NASCIMBENI, F.; DALLA SALDA, A.; CARUBBI, F. Energy balance, glucose and lipid metabolism, cardiovascular risk and liver disease burden in adult patients with type 1 Gaucher disease. **Blood Cells Mol Dis**, Oct 2016.

NASCIMBENI, F.; DIONISI VICI, C.; VESPASIANI GENTILUCCI, U.; ANGELICO, F. *et al.* AISF update on the diagnosis and management of adult-onset lysosomal storage diseases with hepatic involvement. **Dig Liver Dis**, 52, n. 4, p. 359-367, Apr 2020.

NASLAVSKY, M. S.; YAMAMOTO, G. L.; DE ALMEIDA, T. F.; EZQUINA, S. A. M. *et al.* Exomic variants of an elderly cohort of Brazilians in the ABraOM database. **Hum Mutat**, 38, n. 7, p. 751-763, Jul 2017.

NGUYEN, Y.; STIRNEMANN, J.; BELMATOUG, N. [Gaucher disease: A review]. **Rev Med Interne**, 40, n. 5, p. 313-322, May 2019.

NGUYEN, Y.; STIRNEMANN, J.; LAUTREDOUX, F.; CADOR, B. *et al.* Immunoglobulin Abnormalities in Gaucher Disease: an Analysis of 278 Patients Included in the French Gaucher Disease Registry. **Int J Mol Sci**, 21, n. 4, Feb 2020.

OLIVEIRA, F. L.; ALEGRA, T.; DORNELLES, A.; KRUG, B. C. *et al.* Quality of life of brazilian patients with Gaucher disease and fabry disease. **JIMD Rep**, 7, p. 31-37, 2013.

PANDEY, M. K.; BURROW, T. A.; RANI, R.; MARTIN, L. J. *et al.* Complement drives glucosylceramide accumulation and tissue inflammation in Gaucher disease. **Nature**, 543, n. 7643, p. 108-112, 03 2017.

PANDEY, M. K.; GRABOWSKI, G. A.; KÖHL, J. An unexpected player in Gaucher disease: The multiple roles of complement in disease development. **Semin Immunol**, 37, p. 30-42, 06 2018.

PARISE, E.; BORGES, P. S. D. A. Doença Hepática Gordurosa Não Alcoólica. *In*: FERRAZ, M. L. G. S., ANTONIO EDUARDO BENEDITO e SCHIAVON, J. L. N. (Ed.). **Manual de Hepatologia para Clínicos e Residentes**. 1st edition ed.: Atheneu, 2018. cap. 56, p. 459-476.

PARTANEN, K.; PIKKARAINEN, P.; PASANEN, P.; ALHAVA, E. *et al.* Ultrasonography and computed tomography in diffuse liver disease with cholestasis. **Acta Radiol**, 31, n. 5, p. 477-482, Sep 1990.

PATLAS, M.; HADAS-HALPERN, I.; ABRAHAMOV, A.; ELSTEIN, D. *et al.* Spectrum of abdominal sonographic findings in 103 pediatric patients with Gaucher disease. **Eur Radiol**, 12, n. 2, p. 397-400, Feb 2002.

PATLAS, M.; HADAS-HALPERN, I.; REINUS, C.; ZIMRAN, A. *et al.* Multiple hypoechoic hepatic lesions in a patient with Gaucher disease. **J Ultrasound Med**, 21, n. 9, p. 1053-1055, Sep 2002.

PELUCCHI, S.; MARIANI, R.; CALZA, S.; FRACANZANI, A. L. *et al.* CYBRD1 as a modifier gene that modulates iron phenotype in HFE p.C282Y homozygous patients. **Haematologica**, 97, n. 12, p. 1818-1825, Dec 2012.

PENTCHEV, P. G.; GAL, A. E.; WONG, R.; MORRONE, S. *et al.* Biliary excretion of glycolipid in induced or inherited glucosylceramide lipidosis. **Biochim Biophys Acta**, 665, n. 3, p. 615-618, Sep 1981.

PEREL, Y.; BIOULAC-SAGE, P.; CHATEIL, J. F.; TRILLAUD, H. *et al*. Gaucher's disease and fatal hepatic fibrosis despite prolonged enzyme replacement therapy. **Pediatrics**, 109, n. 6, p. 1170-1173, Jun 2002.

PIETRANGELO, A.; TORBENSON, M. Disorders of Iron Overload. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th edition ed.: Elsevier, 2018. cap. 4, p. 275-307.

PIRES, M. M.; EMMERT, D. M.; CHMIELEWSKI, J. A.; HRYCYNA, C. A. ABCB1 and ABCG2: Deciphering the Role of Human Efflux Proteins in Cellular and Tissue Permeability. *In*: LINTON, K. J. e HOLLAND, B. (Ed.). **The ABC Transporters of Human Physiology and Disease**. 1st edition ed.: World Scientific, 2011. cap. 4, p. 85-126.

PORTINCASA, P.; MOSCHETTA, A.; PALASCIANO, G. Cholesterol gallstone disease. **Lancet**, 368, n. 9531, p. 230-239, Jul 2006.

PÉANNE, R.; DE LONLAY, P.; FOULQUIER, F.; KORNAK, U. *et al*. Congenital disorders of glycosylation (CDG): Quo vadis? **Eur J Med Genet**, 61, n. 11, p. 643-663, Nov 2018.

QUAGLIA, A.; ROBERTS, E. A.; TORBENSON, M. Developmental and Inherited Liver Disease. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th edition ed.: Elsevier, 2018. cap. 3, p. 111-274.

QUIGLEY, B. C.; ADSAY, V. Diseases of the Gallbladder. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th ed.: Elsevier, 2018. cap. 10, p. 594-635.

RAGGERS, R. J.; VAN HELVOORT, A.; EVERS, R.; VAN MEER, G. The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane. **J Cell Sci**, 112 (Pt 3), p. 415-422, Feb 1999.

RAGGERS, R. J.; VOGELS, I.; VAN MEER, G. Upregulation of the expression of endogenous Mdr1 P-glycoprotein enhances lipid translocation in MDCK cells transfected with human MRP2. **Histochem Cell Biol**, 117, n. 2, p. 181-185, Feb 2002.

REED, M. C.; SCHIFFER, C.; HEALES, S.; MEHTA, A. B. *et al.* Impact of sphingolipids on osteoblast and osteoclast activity in Gaucher disease. **Mol Genet Metab**, 124, n. 4, p. 278-286, 08 2018.

REGENBOOG, M.; BOHTE, A. E.; AKKERMAN, E. M.; STOKER, J. *et al.* Iron storage in liver, bone marrow and splenic Gaucheroma reflects residual disease in type 1 Gaucher disease patients on treatment. **Br J Haematol**, 179, n. 4, p. 635-647, Nov 2017.

REGENBOOG, M.; BOHTE, A. E.; SOMERS, I.; VAN DELDEN, O. M. *et al.* Imaging characteristics of focal splenic and hepatic lesions in type 1 Gaucher disease. **Blood Cells Mol Dis**, 60, p. 49-57, Sep 2016.

REGENBOOG, M.; VAN DUSSEN, L.; VERHEIJ, J.; WEINREB, N. J. *et al.* Hepatocellular carcinoma in Gaucher disease: an international case series. **J Inherit Metab Dis**, Feb 2018.

REGENBOOG, M.; VAN KUILENBURG, A. B.; VERHEIJ, J.; SWINKELS, D. W. *et al.* Hyperferritinemia and iron metabolism in Gaucher disease: Potential pathophysiological implications. **Blood Rev**, 30, n. 6, p. 431-437, Nov 2016.

REVEL-VILK, S.; SZER, J.; MEHTA, A.; ZIMRAN, A. How we manage Gaucher Disease in the era of choices. **Br J Haematol**, May 2018.

ROESER, H. P.; LEE, G. R.; NACHT, S.; CARTWRIGHT, G. E. The role of ceruloplasmin in iron metabolism. **J Clin Invest**, 49, n. 12, p. 2408-2417, Dec 1970.

ROGHI, A.; POGGIALI, E.; CASSINERIO, E.; PEDROTTI, P. *et al.* The role of cardiac magnetic resonance in assessing the cardiac involvement in Gaucher type 1 patients: morphological and functional evaluations. **J Cardiovasc Med (Hagerstown)**, 18, n. 4, p. 244-248, Apr 2017.

ROSENBAUM, H.; SIDRANSKY, E. Cholelithiasis in patients with Gaucher disease. **Blood Cells Mol Dis**, 28, n. 1, p. 21-27, 2002 Jan-Feb 2002.

ROZWADOWSKA, K.; RACZAK, G.; SIKORSKA, K.; FIJAŁKOWSKI, M. *et al.* Influence of hereditary hemochromatosis on left ventricular wall thickness: does iron overload exacerbate cardiac hypertrophy? **Folia Morphol (Warsz)**, Mar 2019.

RUCHLEMER, R.; MITTELMAN, M.; ZIMRAN, A. Gaucher disease, myelodysplastic syndrome and ICUS. **Blood Cells Mol Dis**, 80, p. 102373, 02 2020.

RYAN, E.; SEEHRA, G. K.; SIDRANSKY, E. Mutations, modifiers and epigenetics in Gaucher disease: Blurred boundaries between simple and complex disorders. **Mol Genet Metab**, 128, n. 1-2, p. 10-13, 2019 Sep - Oct 2019.

SANDRIN, L.; FOURQUET, B.; HASQUENOPH, J. M.; YON, S. *et al.* Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. **Ultrasound Med Biol**, 29, n. 12, p. 1705-1713, Dec 2003.

SASSO, M.; TENGHER-BARNA, I.; ZIOL, M.; MIETTE, V. *et al.* Novel controlled attenuation parameter for noninvasive assessment of steatosis using Fibroscan(®): validation in chronic hepatitis C. **J Viral Hepat**, 19, n. 4, p. 244-253, Apr 2012.

SAUDUBRAY, J.-M.; CAZORLA, A. G. Clinical Approach to Inborn Errors of Metabolism in Pediatrics. *In*: SAUDUBRAY, J.-M. B., MATTHIAS R e WALTER, J. (Ed.). **Inborn Metabolic Diseases**. 6th edition ed.: Springer, 2016. cap. 1, p. 3-70.

SCHIFF, M.; RODA, C.; MONIN, M. L.; ARION, A. *et al.* Clinical, laboratory and molecular findings and long-term follow-up data in 96 French patients with PMM2-CDG (phosphomannomutase 2-congenital disorder of glycosylation) and review of the literature. **J Med Genet**, 54, n. 12, p. 843-851, 12 2017.

SCHIFFMANN, R.; SEVIGNY, J.; ROLFS, A.; DAVIES, E. H. *et al.* The definition of neuronopathic Gaucher disease. **J Inherit Metab Dis**, Apr 2020.

SCHLOTTMANN, F.; VERA-AVILES, M.; LATUNDE-DADA, G. O. Duodenal cytochrome b (Cybrd1) ferric reductase functional studies in cells. **Metallomics**, 9, n. 10, p. 1389-1393, 10 2017.

SCHUELER, U. H.; KOLTER, T.; KANESKI, C. R.; BLUSZTAJN, J. K. *et al.* Toxicity of glucosylsphingosine (glucopsychosine) to cultured neuronal cells: a model system for assessing neuronal damage in Gaucher disease type 2 and 3. **Neurobiol Dis**, 14, n. 3, p. 595-601, Dec 2003.

SCHULZE, R. J.; SCHOTT, M. B.; CASEY, C. A.; TUMA, P. L. *et al.* The cell biology of the hepatocyte: A membrane trafficking machine. **J Cell Biol**, 218, n. 7, p. 2096-2112, 07 2019.

SENZOLO, M.; BURRA, P.; CHOLONGITAS, E.; BURROUGHS, A. K. New insights into the coagulopathy of liver disease and liver transplantation. **World J Gastroenterol**, 12, n. 48, p. 7725-7736, Dec 2006.

SERAI, S. D.; NAIDU, A. P.; ANDREW BURROW, T.; PRADA, C. E. *et al.* Correlating liver stiffness with disease severity scoring system (DS3) values in Gaucher disease type 1 (GD1) patients. **Mol Genet Metab**, 123, n. 3, p. 357-363, Mar 2018.

SHIHA, G. E.; EL-ETREBY, S.; BAHGAT, M.; HAMED, M. *et al.* Chronic Hepatitis C Patients with Obesity: Do we Need two Operators for Accurate Evaluation of Liver Stiffness? **Ann Hepatol**, 17, n. 5, p. 795-801, Aug 2018.

SIDRANSKY, E. Gaucher disease: complexity in a "simple" disorder. **Mol Genet Metab**, 83, n. 1-2, p. 6-15, 2004 Sep-Oct 2004.

SOLANICH, X.; CLAVER, E.; CARRERAS, F.; GIRALDO, P. *et al.* Myocardial infiltration in Gaucher's disease detected by cardiac MRI. **Int J Cardiol**, 155, n. 1, p. e5-6, Feb 2012.

SOTTRUP-JENSEN, L. Alpha-macroglobulins: structure, shape, and mechanism of proteinase complex formation. **J Biol Chem**, 264, n. 20, p. 11539-11542, Jul 1989.

SOUDEK, L.; SIDDIQUI, I.; GUERIN, A.; SONDHEIMER, N. *et al.* Liver transplantation for Gaucher disease presenting as neonatal cholestasis: Case report and literature review. **Pediatr Transplant**, p. e13718, Apr 2020.

STAROSTA, R. T.; PINTO E VAIRO, F.; DORNELLES, A. D.; CERSKI, C. T. S. *et al.* Hepatocellular carcinoma in Gaucher disease: Reinforcing the proposed guidelines. **Blood Cells Mol Dis**, 74, p. 34-36, Feb 2019.

STAROSTA, R. T.; VAIRO, F. P. E.; DORNELLES, A. D.; BASGALUPP, S. P. *et al.* Liver involvement in patients with Gaucher disease types I and III. **Mol Genet Metab Rep**, 22, p. 100564, Mar 2020.

StatPearls. *In*, 2020.

STEIN, P.; YU, H.; JAIN, D.; MISTRY, P. K. Hyperferritinemia and iron overload in type 1 Gaucher disease. **Am J Hematol**, 85, n. 7, p. 472-476, Jul 2010.

STIRNEMANN, J.; BELMATOUG, N.; CAMOU, F.; SERRATRICE, C. *et al.* A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. **Int J Mol Sci**, 18, n. 2, Feb 2017.

STONE, D. L.; GINNS, E. I.; KRASNEWICH, D.; SIDRANSKY, E. Life-threatening splenic hemorrhage in two patients with Gaucher disease. **Am J Hematol**, 64, n. 2, p. 140-142, Jun 2000.

SUKUMARAN, A.; CHANG, J.; HAN, M.; MINTRI, S. *et al.* Iron overload exacerbates age-associated cardiac hypertrophy in a mouse model of hemochromatosis. **Sci Rep**, 7, n. 1, p. 5756, 07 2017.

TADDEI, T. H.; DZIURA, J.; CHEN, S.; YANG, R. *et al.* High incidence of cholesterol gallstone disease in type 1 Gaucher disease: characterizing the biliary phenotype of type 1 Gaucher disease. **J Inherit Metab Dis**, 33, n. 3, p. 291-300, Jun 2010.

TAMARGO, R. J.; VELAYATI, A.; GOLDIN, E.; SIDRANSKY, E. The role of saposin C in Gaucher disease. **Mol Genet Metab**, 106, n. 3, p. 257-263, Jul 2012.

TAMÒ, R.; HOCHULI, M.; BEUSCHLEIN, F.; NOWAK, A. [Gaucher's disease - an overview about a sphingolipidosis]. **Ther Umsch**, 75, n. 4, p. 209-214, Nov 2018.

TAYLOR, M. E.; DRICKAMER, K. Carbohydrate Recognition in Cell Adhesion and Signalling. *In*: TAYLOR, M. E. e DRICKAMER, K. (Ed.). **Introduction to Glycobiology**. 3rd edition ed.: Oxford University Press, 2011a. cap. 9, p. 139-170.

TAYLOR, M. E.; DRICKAMER, K. Effects of Glycosylation on Protein Structure and Function. *In*: TAYLOR, M. E. e DRICKAMER, K. (Ed.). **Introduction to Glycobiology**. 3rd edition ed.: Oxford University Press, 2011b. cap. 8, p. 125-138.

TAYLOR, M. E.; DRICKAMER, K. Glycosylation and Disease. *In*: TAYLOR, M. E. e DRICKAMER, K. (Ed.). **Introduction to Glycobiology**. 3rd ed.: Oxford University Press, 2011c. cap. 13, p. 240-259.

TAYLOR, M. E.; DRICKAMER, K. N-linked Glycosylation. *In*: TAYLOR, M. E. e DRICKAMER, K. (Ed.). **Introduction to Glycobiology**. 3rd edition ed., 2011d. cap. 2, p. 17-32.

TAYLOR, T.; WHEATLEY, M. Jaundice in the emergency department: meeting the challenges of diagnosis and treatment. **Emerg Med Pract**, 20, n. 4, p. 1-24, Apr 2018.

TERK, M. R.; ESPLIN, J.; LEE, K.; MAGRE, G. *et al.* MR imaging of patients with type 1 Gaucher's disease: relationship between bone and visceral changes. **AJR Am J Roentgenol**, 165, n. 3, p. 599-604, Sep 1995.

THEISE, N. D. Liver and Gallbladder. *In*: KUMAR, V.;ABBAS, A. K., *et al* (Ed.). **Robbins and Cotran Pathologic Basis of Disease**. 9th edition ed.: Elsevier, 2015. cap. 18, p. 821-882

THEISE, N. D.; BODENHEIMER JR, H. C.; GUIDO, M. Viral Hepatitis. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th edition ed.: Elsevier, 2018. cap. 6, p. 372-415.

TINIAKOS, D. G.; ANSTEE, Q. M.; BURT, A. D. Fatty Liver Disease. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th ed.: Elsevier, 2018. cap. 5, p. 308-371.

TSOCHATZIS, E. A.; BOSCH, J.; BURROUGHS, A. K. Liver cirrhosis. **Lancet**, 383, n. 9930, p. 1749-1761, May 2014.

TURPIN-NOLAN, S. M.; BRÜNING, J. C. The role of ceramides in metabolic disorders: when size and localization matters. **Nat Rev Endocrinol**, 16, n. 4, p. 224-233, 04 2020.

TYLKI-SZYMAŃSKA, A.; GROENER, J. E.; KAMIŃSKI, M. L.; ŁUGOWSKA, A. *et al.* Gaucher disease due to saposin C deficiency, previously described as non-neuronopathic form--no positive effects after 2-years of miglustat therapy. **Mol Genet Metab**, 104, n. 4, p. 627-630, Dec 2011.

VALLET-PICHARD, A.; MALLET, V.; NALPAS, B.; VERKARRE, V. *et al.* FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. **Hepatology**, 46, n. 1, p. 32-36, Jul 2007.

VANIER, M. T.; CAILLAUD, C.; LEVADE, T. Disorders of Sphingolipid Synthesis, Sphingolipidoses, Niemann-Pick Disease Type C and Neuronal Ceroid Lipofuscinoses. *In*: SAUDUBRAY, J.-M. B., MATTHIAS R e WALTER, J. (Ed.). **Inborn Metabolic Diseases**. 6th edition ed.: Springer, 2016. cap. 38, p. 551-575.

VELAYATI, A.; DEPAOLO, J.; GUPTA, N.; CHOI, J. H. *et al.* A mutation in SCARB2 is a modifier in Gaucher disease. **Hum Mutat**, 32, n. 11, p. 1232-1238, Nov 2011.

VERHEIJEN, J.; TAHATA, S.; KOZICZ, T.; WITTERS, P. *et al.* Therapeutic approaches in Congenital Disorders of Glycosylation (CDG) involving N-linked glycosylation: an update. **Genet Med**, 22, n. 2, p. 268-279, Feb 2020.

VUJOSEVIC, S.; MEDENICA, S.; VUJICIC, V.; DAPCEVIC, M. *et al.* Gaucher disease in Montenegro - genotype/phenotype correlations: Five cases report. **World J Clin Cases**, 7, n. 12, p. 1475-1482, Jun 2019.

WAI, C. T.; GREENSON, J. K.; FONTANA, R. J.; KALBFLEISCH, J. D. *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. **Hepatology**, 38, n. 2, p. 518-526, Aug 2003.

WATSON, J. D.; CRICK, F. H. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. **Nature**, 171, n. 4356, p. 737-738, Apr 1953.

WEBB, M.; ZIMRAN, A.; DINUR, T.; SHIBOLET, O. *et al.* Are transient and shear wave elastography useful tools in Gaucher disease? **Blood Cells Mol Dis**, Dec 2016.

WEINREB, N. J. Encore! Oral therapy for type 1 Gaucher disease. **Blood**, 129, n. 17, p. 2337-2338, Apr 2017.

WEINREB, N. J.; CAPPELLINI, M. D.; COX, T. M.; GIANNINI, E. H. *et al.* A validated disease severity scoring system for adults with type 1 Gaucher disease. **Genet Med**, 12, n. 1, p. 44-51, Jan 2010.

WEISS, G.; GANZ, T.; GOODNOUGH, L. T. Anemia of inflammation. **Blood**, 133, n. 1, p. 40-50, 01 2019.

WHITTINGTON, R.; GOA, K. L. Alglucerase. A review of its therapeutic use in Gaucher's disease. **Drugs**, 44, n. 1, p. 72-93, Jul 1992.

WIDE, L.; ERIKSSON, K. Unique Pattern of N-Glycosylation, Sialylation, and Sulfonation on TSH Molecules in Serum of Children Up to 18 Months. **J Clin Endocrinol Metab**, 104, n. 10, p. 4651-4659, Oct 2019.

WINCHESTER, B. Lysosomal metabolism of glycoproteins. **Glycobiology**, 15, n. 6, p. 1R-15R, Jun 2005.

WINTERBOURN, C. C. Toxicity of iron and hydrogen peroxide: the Fenton reaction. **Toxicol Lett**, 82-83, p. 969-974, Dec 1995.

WITTERS, P.; HONZIK, T.; BAUCHART, E.; ALTASSAN, R. *et al.* Long-term follow-up in PMM2-CDG: are we ready to start treatment trials? **Genet Med**, 21, n. 5, p. 1181-1188, 05 2019.

WONG, S. Y.; GADOMSKI, T.; VAN SCHERPENZEEL, M.; HONZIK, T. *et al.* Oral D-galactose supplementation in PGM1-CDG. **Genet Med**, 19, n. 11, p. 1226-1235, 11 2017.

XU, R.; MISTRY, P.; MCKENNA, G.; EMRE, S. *et al.* Hepatocellular carcinoma in type 1 Gaucher disease: a case report with review of the literature. **Semin Liver Dis**, 25, n. 2, p. 226-229, 2005.

YILDIZ, Y.; HOFFMANN, P.; VOM DAHL, S.; BREIDEN, B. *et al.* Functional and genetic characterization of the non-lysosomal glucosylceramidase 2 as a modifier for Gaucher disease. **Orphanet J Rare Dis**, 8, p. 151, Sep 2013.

ZELBER-SAGI, S.; YESHUA, H.; SHLOMAI, A.; BLENDIS, L. *et al.* Sampling variability of transient elastography according to probe location. **Eur J Gastroenterol Hepatol**, 23, n. 6, p. 507-514, Jun 2011.

ZELESCO, M.; ABOTT, S.; O'HARA, S. Pitfalls and sources of variability in two dimensional shear wave elastography of the liver: An overview. **Sonography**, 5, n. 1, p. 9, 2017.

ZEN, Y.; HÜBSCHER, S. G.; NAKANUMA, Y. Bile Duct Diseases. *In*: BURT, A. D.;FERRELL, L. D., *et al* (Ed.). **MacSween's Pathology of the Liver**. 7th edition ed.: Elsevier, 2018. cap. 9, p. 515-593.

ZHANG, C. K.; STEIN, P. B.; LIU, J.; WANG, Z. *et al.* Genome-wide association study of N370S homozygous Gaucher disease reveals the candidacy of CLN8 gene as a genetic modifier contributing to extreme phenotypic variation. **Am J Hematol**, 87, n. 4, p. 377-383, Apr 2012.

ZHU, J.; SUN, Z.; CHENG, K.; CHEN, R. *et al.* Comprehensive mapping of protein N-glycosylation in human liver by combining hydrophilic interaction chromatography and hydrazide chemistry. **J Proteome Res**, 13, n. 3, p. 1713-1721, Mar 2014.

ZIMMERMANN, A.; POPP, R. A.; AL-KHZOUZ, C.; BUCERZAN, S. *et al.* Cholelithiasis in Patients with Gaucher Disease type 1: Risk Factors and the Role of ABCG5/ABCG8 Gene Variants. **J Gastrointestin Liver Dis**, 25, n. 4, p. 447-455, 12 2016.

ZIMRAN, A.; ALTARESCU, G.; PHILIPS, M.; ATTIAS, D. *et al.* Phase 1/2 and extension study of velaglucerase alfa replacement therapy in adults with type 1 Gaucher disease: 48-month experience. **Blood**, 115, n. 23, p. 4651-4656, Jun 2010.

ZIMRAN, A.; BRILL-ALMON, E.; CHERTKOFF, R.; PETAKOV, M. *et al.* Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. **Blood**, 118, n. 22, p. 5767-5773, Nov 2011.

ZIMRAN, A.; ELSTEIN, D.; LEVY-LAHAD, E.; ZEVIN, S. *et al.* Replacement therapy with imiglucerase for type 1 Gaucher's disease. **Lancet**, 345, n. 8963, p. 1479-1480, Jun 1995.

ZIMRAN, A.; KAY, A.; GELBART, T.; GARVER, P. *et al.* Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients. **Medicine (Baltimore)**, 71, n. 6, p. 337-353, Nov 1992.

ZMESKAL, O.; DZIK, P.; VESELY, M. Entropy of fractal systems. **Computers & Mathematics with Applications**, 66, n. 2, p. 12, 2013.

ČECHOVÁ, A.; ALTASSAN, R.; BORGEL, D.; BRUNEEL, A. *et al*. Consensus guideline for the diagnosis and management of mannose phosphate isomerase-congenital disorder of glycosylation. **J Inherit Metab Dis**, Apr 2020.

Anexo A: Carta de Aprovação do Projeto 2018-0639 pelo Comitê de Ética e Pesquisa do Hospital de Clínicas de Porto Alegre

UFRGS - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE DA UNIVERSIDADE FEDERAL

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Validação de escores clínico-laboratoriais de fibrose hepática para pacientes com doença de Gaucher através do uso da elastografia transitória hepática

Pesquisador: Ida Vanessa Doederlein Schwartz

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 96764818.3.0000.5327

Instituição Proponente: Hospital de Clínicas de Porto Alegre

Patrocinador Principal: Hospital de Clínicas de Porto Alegre

DADOS DO PARECER

Número do Parecer: 3.312.749

Apresentação do Projeto:

A doença de Gaucher (DG) é uma doença genética autossômica recessiva e uma das mais frequentes patologias de acúmulo lisossomal. Ela é caracterizada pelo acúmulo de lipídeos complexos, especialmente a glicosilceramida/glicocerebrosídeo, em macrófagos, que passam a ser chamados então de "células de Gaucher". A DG pode se apresentar na forma não-neuronopática (tipo I), para a qual as alternativas terapêuticas atuais oferecem bom prognóstico, ou nas formas neuronopáticas (tipos II e III), que possuem prognóstico reservado devido à ausência de resposta ao tratamento existente. O fenótipo de doença hepática na DG, tanto em suas formas neuronopáticas quanto na não-neuropática, é ainda, devido a sua heterogeneidade, bastante incompreendido, indo desde hepatomegalia até cirrose hepática. Desse modo, o manejo clínico hepatológico dos pacientes com DG é complexo e pouco preciso, sendo necessárias mais ferramentas validadas para essa doença a fim de melhorar a assistência aos pacientes que apresentam essa patologia. A biópsia hepática é o teste padrão na avaliação do comprometimento do fígado, mas é invasiva e apresenta riscos, o que tem tornado mais populares os métodos não invasivos. O FLI (Fatty Liver Index - índice de gordura hepática) pode ser usado na predição de esteatose. Existem diversas ferramentas de cálculo da probabilidade e da gravidade de fibrose hepática, como NFS (NAFLD fibrosis score - escore de fibrose da doença hepática gordurosa não

Endereço: Rua Ramiro Barcelos 2.350 sala 2229
Bairro: Santa Cecília **CEP:** 90.035-903
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3359-7640 **Fax:** (51)3359-7640 **E-mail:** cep@hcpa.edu.br

alcoólica), APRI (indicador da razão AST-plaquetas), BARD e FIB4 (escore de fibrose, versão 4). Essas ferramentas ("escalas") são amplamente utilizadas para balizar o manejo clínico de diversas doenças, como as hepatites virais e a doença hepática gordurosa não-alcoólica. No entanto, provavelmente por utilizarem em seus cálculos a concentração de plaquetas no sangue periférico, medida alterada tanto pela doença hepática quanto pela própria DG, nenhuma dessas escalas pode ser utilizada para pacientes com DG. A elastografia hepática (EH), através de FibroScan, ARFI, shear-wave ou ressonância magnética, é um método não-invasivo que estima a fibrose hepática e que tem sido usado com sucesso em várias doenças do fígado. No entanto, o uso de EH em pacientes com DG ainda é muito limitado e merece ser melhor investigado, tendo os estudos anteriores realizados no tema se limitado à mera análise descritiva ou à análise da relação entre depósito hepático de ferro, esplenectomia e fibrose hepática. Até hoje, nenhum estudo usou a EH em conjunto com escalas clínico-laboratoriais de fibrose e esteatose hepáticas. Nesse estudo é proposto o uso da EH concomitante à avaliação clínico-laboratorial de fibrose e esteatose hepáticas com o uso das escalas NAFLD-FS, APRI, FIB4, BARD e FLI em pacientes com DG, a fim de tanto caracterizar o fenótipo de doença hepática da coorte de pacientes com DG do Hospital de Clínicas de Porto Alegre, quanto para avaliar a acurácia do uso das respectivas escalas para a predição do fenótipo de doença hepática dos pacientes com DG.

Objetivo da Pesquisa:

Objetivo Geral

1. Caracterizar o grau e a prevalência de fibrose hepática dos pacientes da coorte de Doença de Gaucher do Centro de Referência em Gaucher do Hospital de Clínicas de Porto Alegre baseado em técnica não invasiva.

2. Avaliar a acurácia de escalas clínico-laboratoriais para predição de fibrose hepática, já utilizadas em outras patologias, na doença de Gaucher.

Objetivos específicos

1. Caracterizar o fenótipo de doença hepática dos pacientes com doença de Gaucher da coorte do Hospital de Clínicas de Porto Alegre com o uso da elastografia transitória hepática.

2. Avaliar a correlação entre os resultados obtidos com a elastografia transitória hepática e os provenientes dos escores NAFLD-FS, APRI, FIB4, FLI e BARD nos pacientes com doença de Gaucher.

3. Determinar valores de sensibilidade e especificidade dos escores para fibrose hepática na doença de Gaucher, com formulação de curvas ROC, usando como padrão a elastografia transitória hepática.

4. Correlacionar valores obtidos com a elastografia transitória hepática e dados clínicos,

laboratoriais, genéticos e anatomopatológicos dos pacientes.

Avaliação dos Riscos e Benefícios:

Os riscos dessa pesquisa são baixos, advindo principalmente de: coleta de sangue venoso periférico, que pode causar dor local leve temporária, equimoses de pequena monta, ou hemorragia controlável em pacientes com condições de saúde predisponentes, como hemofilia; aplicação de questionário de rastreamento para alcoolismo, que pode levar a algum desconforto psíquico; elastografia hepática transitória, que, apesar de não apresentar nenhum risco biológico, químico ou físico, pode levar a algum desconforto leve local transitório durante a sua realização. Os benefícios dessa pesquisa são: maior compreensão do comprometimento hepático dos pacientes com doença de Gaucher; possibilidade de uso futuro de escores pouco invasivos para predição de fibrose hepática nos pacientes com doença de Gaucher, o que levará à menor necessidade de realização de biópsias hepáticas, que são procedimentos invasivos e de risco não-negligenciável.

Comentários e Considerações sobre a Pesquisa:

Emenda 1 com a seguinte justificativa: Solicitamos a modificação do local de realização do exame de elastografia transitória para o consultório privado do professor doutor Mário Reis Álvares-da-Silva.

O exame de elastografia transitória é realizado com equipamento especializado de alto custo e pouca portabilidade. No Hospital de Clínicas de Porto Alegre (HCPA), local em que originalmente fora planejada a sua realização neste projeto, o exame não é atualmente realizado para atividades de pesquisa, sendo, portanto, inacessível para a realização deste estudo.

O professor doutor Mário Reis, participante do estudo desde sua concepção, possui consultório particular de prática médica especializada; neste consultório, localizado a aproximadamente 10 minutos de carro do HCPA, o professor tem acesso a aparelho de elastografia transitória já por si adquirido, que disponibiliza para o uso no projeto de pesquisa sem custos associados.

O transporte dos sujeitos de pesquisa será realizado do HCPA ao consultório e de volta, junto com pesquisador, em táxi ou carro de aplicativo (Uber, Cabify), sem custos para os sujeitos de pesquisa. Portanto, se justifica a inclusão dos pesquisadores do serviço de Radiologia e Diagnóstico por imagem no presente projeto.

O projeto é relevante pois pretende avaliar a acurácia de escalas clínico-laboratoriais não-

invasivas através da comparação com a Elastografia hepática. Além da contribuição na complementação da caracterização do fenótipo de doença em estudo e talvez fornecer alguma ferramenta clínica segura que permita o melhor manejo da doença.

Considerações sobre os Termos de apresentação obrigatória:

Apresentados.

Recomendações:

Embora os pesquisadores informem na carta de emenda que "Portanto, se justifica a inclusão dos pesquisadores do serviço de Radiologia e Diagnóstico por imagem no presente projeto", não localizamos nenhuma inclusão ou exclusão. Permanecem os mesmo pesquisadores do projeto inicial no registro do projeto na Plataforma Brasil.

Conclusões ou Pendências e Lista de Inadequações:

Não apresenta pendências.

Considerações Finais a critério do CEP:

Emenda 1 aprovada.

Projeto e TCLEs de 20/04/2019 aprovados.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1332939_E1.pdf	20/04/2019 00:01:22		Aceito
Outros	carta_emenda_fibroscan.doc	20/04/2019 00:01:00	RODRIGO TZOVENOS STAROSTA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_fibroscan_gaucher_emenda.doc	14/04/2019 20:58:46	RODRIGO TZOVENOS STAROSTA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_fibroscan_controles_emenda.docx	14/04/2019 20:58:08	RODRIGO TZOVENOS STAROSTA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Fibroscan_Gaucher_emenda.docx	14/04/2019 20:57:59	RODRIGO TZOVENOS STAROSTA	Aceito
Outros	emenda_PB_Ida.pdf	14/04/2019 20:57:37	RODRIGO TZOVENOS STAROSTA	Aceito

Folha de Rosto	Untitled_08172018_114738.pdf	17/08/2018 23:49:58	RODRIGO TZOVENOS STAROSTA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 08 de Maio de 2019

Assinado por:
Marcia Mocellin Raymundo
(Coordenador(a))

Endereço: Rua Ramiro Barcelos 2.350 sala 2229
Bairro: Santa Cecília **CEP:** 90.035-903
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3359-7640 **Fax:** (51)3359-7640 **E-mail:** cep@hcpa.edu.br

Anexo B: Carta de Aprovação do Projeto 2018-0654 pelo Comitê de Ética e Pesquisa do Hospital de Clínicas de Porto Alegre

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise digital histomorfométrica e imuno-histoquímica de imagem em Doença de Gaucher.

Pesquisador: CARLOS THADEU SCHMIDT CERSKI

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 2

CAAE: 95286518.1.0000.5327

Instituição Proponente: Hospitalde Clínicas de Porto Alegre

Patrocinador Principal: Hospital de Clínicas de Porto Alegre

DADOS DO PARECER

Número do Parecer: 2.919.083

Apresentação do Projeto:

A doença de Gaucher é uma patologia lisossômica autossômica recessiva caracterizada pela baixa atividade da enzima glicocerebrosidase levando ao acúmulo do lipídeo complexo glicosilceramida nos lisossomos de diversos tipos celulares, principalmente macrófagos. As manifestações da doença de Gaucher são diversas, incluindo hepatomegalia, esplenomegalia, anemia, plaquetopenia, deformidades e dores ósseas, necroses ósseas avasculares, doença pulmonar restritiva e, em poucos casos (chamados de tipos neuronopáticos), envolvimento neurológico. Com o advento de formas eficazes de tratamento para a doença de Gaucher, especialmente a terapia de reposição enzimática, o tempo de seguimento dos pacientes com essa patologia aumentou, e diversas manifestações menores puderam ser observadas: no fígado, notou-se que há aumento de esteatose, fibrose, depósito de ferro, sintomas biliares e hepatocarcinoma. No entanto, essas manifestações crônicas ainda não são totalmente elucidadas, sendo necessários mais estudos para a compreensão e posterior elaboração de planos terapêuticos. Objetivos: caracterizar o perfil histomorfométrico de amostras de tecido hepático de pacientes com doença de Gaucher, com foco no padrão de depósito de ferro, na arquitetura da cromatina nuclear, e na arquitetura dos canalículos biliares, comparando com controles normais, esteatóticos e cirróticos. Metodologia: estudo transversal com avaliação histomorfométrica prospectiva e coleta retrospectiva de

Endereço: Rua Ramiro Barcelos 2.350 sala 2229
Bairro: Santa Cecília **CEP:** 90.035-903
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3359-7640 **Fax:** (51)3359-7640 **E-mail:** cep@hcpa.edu.br

informações clínicas, laboratoriais e genéticas de prontuário e bancos de dados do Serviço de Genética Médica do HCPA. A execução desse projeto ocorrerá após aprovação no Comitê de Ética em Pesquisa do HCPA. As análises histomorfométricas serão realizadas em amostras de tecido hepático de biópsias hepáticas que tenham sido realizadas na assistência dos pacientes. Será usada estatística analítica e descritiva com intervalo de confiança de 95% e significância menor de 5%.

Objetivo da Pesquisa:

Objetivo Primário: Caracterizar o a arquitetura dos canalículos biliares, o depósito hepatocitário de ferro, e a heterogeneidade nuclear hepatocitária, macrofágica e de outros tipos celulares em biópsias hepáticas ou de outros tecidos realizadas por motivos assistenciais em pacientes com DG acompanhados no ambulatório de Genética Médica do Hospital de Clínicas de Porto Alegre.

Objetivo Secundário:

• Descrever os parâmetros histomorfométricos (heterogeneidade de cromatina, arquitetura canalicular, depósito de ferro) de biópsias de pacientes com DG;

• Buscar associação entre os parâmetros histomorfométricos e as características clínicas, laboratoriais ou genotípicas dos pacientes;

•Comparar os parâmetros histomorfométricos dos pacientes com DG com os parâmetros de controles do arquivo do Serviço de Patologia Cirúrgica do HCPA, a fim de descrever alterações que possam ser próprias da DG;

• Aperfeiçoar as técnicas de histomorfometria utilizadas.

Avaliação dos Riscos e Benefícios:

Segundo os pesquisadores,

Os riscos desse estudo são os inerentes à análise de informações sigilosas contidas em prontuários e em arquivos, isto é, a quebra incidental de sigilo de tais informações - esse risco, no entanto, será minimizado pelo cuidado zeloso que os participantes da pesquisa terão para com essas informações. Sendo um estudo que utilizará amostras de tecido já coletadas e estocadas em arquivo, não há nenhum risco conhecido à sáude dos pacientes. Como cada lâmina utiliza uma quantidade de material da ordem de milésimos de cada amostra, e são necessárias somente três lâminas por amostra, o risco de esgotamento do material é praticamente nulo.

Os benefícios esperados com o projeto são: - melhor caracterização e compreensão da patologia e patogênese do acometimento hepático na doença de Gaucher; - aperfeiçoamento de técnicas de baixo custo de análise de imagens e histomorfometria, especialmente as incipientes quantificação de ferro e análise de canalículos biliares; - possibilidade de, com base nos achados desse projeto, serem elaboradas novas linhas de pesquisa direcionadas a opções terapêuticas de alvo preciso para o acometimento hepático na doença de Gaucher.

Comentários e Considerações sobre a Pesquisa:
Projeto de doutorado acadêmico com objetivo de realizar análises histomorfométricas e de imunohistoquimica de imagem em biopsias já realizadas em pacientes com Doença de Gaucher. A amostra é de conveniência e está prevista a inclusão de 7 pacientes.

Considerações sobre os Termos de apresentação obrigatória:
Apresenta TCLE, TCUD e TCUMBIA.

Recomendações:
Nada a recomendar.

Conclusões ou Pendências e Lista de Inadequações:
As pendências emitidas para o projeto no parecer 2.840.222 foram adequadamente respondidas pelos pesquisadores, conforme carta de respostas adicionada em 11/09/2018. Não apresenta novas pendências.

Considerações Finais a critério do CEP:
Lembramos que a presente aprovação (versão projeto 11/09/2018 TCLE 11/09/2018 e demais documentos que atendem às solicitações do CEP) refere-se apenas aos aspectos éticos e metodológicos do projeto.

Os pesquisadores devem atentar ao cumprimento dos seguintes itens:
a) Este projeto está aprovado para inclusão de 32 participantes no Centro HCPA, de acordo com as informações do projeto. Qualquer alteração deste número deverá ser comunicada ao CEP e ao Serviço de Gestão em Pesquisa para autorizações e atualizações cabíveis.
b) O projeto deverá ser cadastrado no sistema AGHUse Pesquisa para fins de avaliação logística e financeira e somente poderá ser iniciado após aprovação final do Grupo de Pesquisa e Pós-

Graduação.

c) Qualquer alteração nestes documentos deverá ser encaminhada para avaliação do CEP. Informamos que obrigatoriamente a versão do TCLE a ser utilizada deverá corresponder na íntegra à versão vigente aprovada.

d) Deverão ser encaminhados ao CEP relatórios semestrais e um relatório final do projeto.

e) A comunicação de eventos adversos classificados como sérios e inesperados, ocorridos com pacientes incluídos no centro HCPA, assim como os desvios de protocolo quando envolver diretamente estes pacientes, deverá ser realizada através do Sistema GEO (Gestão Estratégica Operacional) disponível na intranet do HCPA.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1190964.pdf	11/09/2018 12:09:16		Aceito
Outros	carta_ao_cep_hmm_gaucher_1.docx	11/09/2018 12:08:38	RODRIGO TZOVENOS STAROSTA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_histomorfometria_gaucher_v2.doc	11/09/2018 12:08:15	RODRIGO TZOVENOS STAROSTA	Aceito
Projeto Detalhado / Brochura Investigador	histometria_gaucher_v2.docx	11/09/2018 12:08:02	RODRIGO TZOVENOS STAROSTA	Aceito
Folha de Rosto	folha_de_rosto_def.pdf	06/08/2018 18:47:20	RODRIGO TZOVENOS STAROSTA	Aceito
Outros	termo_util_dados_hmm.pdf	31/07/2018 17:59:32	RODRIGO TZOVENOS STAROSTA	Aceito
Declaração de Pesquisadores	delegacao_funcoes_gaucher_hmm.pdf	31/07/2018 17:58:22	RODRIGO TZOVENOS STAROSTA	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	termo_material_biologico_gaucher_hmm.pdf	31/07/2018 17:58:08	RODRIGO TZOVENOS STAROSTA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 26 de Setembro de 2018

Assinado por:
José Roberto Goldim
(Coordenador(a))

Endereço: Rua Ramiro Barcelos 2.350 sala 2229
Bairro: Santa Cecília **CEP:** 90.035-903
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3359-7640 **Fax:** (51)3359-7640 **E-mail:** cep@hcpa.edu.br

Apêndice A: Termo de Consentimento Livre e Esclarecido para Pacientes Participantes do Projeto de Pesquisa 2018-0639

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nº do projeto GPPG ou CAAE: 98764818.3.0000.5327

Título do Projeto: Validação de escores clínico-laboratoriais de fibrose hepática para pacientes com doença de Gaucher através do uso da elastografia transitória hepática.

Você está sendo convidado a participar de uma pesquisa cujo objetivo é analisar se podemos usar exames de sangue para saber aproximadamente como está o fígado de pacientes com doença de Gaucher sem que necessitemos fazer exames invasivos. Esta pesquisa está sendo realizada pelo Serviço de Genética Médica em conjunto com o Serviço de Gastroenterologia do Hospital de Clínicas de Porto Alegre (HCPA).

A doença de Gaucher é uma condição de origem genética (herdada dos pais) em que algumas células, conhecidas como macrófagos, acumulam substâncias que normalmente seriam destruídas pelo organismo, causando aumento de órgãos como o baço e o fígado, anemia, risco de sangramentos por diminuição das plaquetas na corrente sanguínea, alterações ósseas (enfraquecimento dos ossos, fraturas, deformações). Algumas pesquisas mostram que o fígado, além de estar aumentado de volume, pode ser atacado sutilmente pelas células anormais, causando, após muitos anos de ataques, manifestações como esteato-hepatite (inflamação do fígado com acúmulo de gordura) e fibrose (endurecimento do fígado com diminuição da sua função normal). O tratamento para a doença de Gaucher oferece bons resultados para a prevenção da maioria, senão todas, as manifestações.

A elastografia transitória, também conhecida pelo nome comercial de FibroScan, é um exame parecido externamente com uma ecografia, mas que envia ondas mecânicas ao invés de ondas sonoras. Ele é utilizado para determinar o grau de endurecimento do fígado, que corresponde ao grau de fibrose.

Se você aceitar participar da pesquisa, os procedimentos envolvidos em sua participação são os seguintes: coleta de exames de sangue, realização de elastografia transitória (que é um exame indolor e sem riscos conhecidos à saúde, assemelhado a uma ultrassonografia/ecografia), aplicação de questionário, e consulta a dados de prontuário.

Os possíveis riscos ou desconfortos decorrentes da participação na pesquisa são decorrentes da coleta de sangue, que, apesar de ser um procedimento de rotina, possui uma taxa (ainda que bastante baixa) de complicações como hemorragia ("sangramento") e formação de equimoses ("hematomas"). Desconfortos associados à pesquisa são os decorrentes do tempo de realização da elastografia transitória (aproximadamente 10 minutos), de aplicação do questionário (aproximadamente 5 minutos), e dor leve e temporária devida à coleta de sangue.

Os possíveis benefícios decorrentes da participação na pesquisa são a possibilidade de saber com um grau de confiança maior que o atual se um paciente com doença de Gaucher está tendo lesão do tipo "fibrose" em seu fígado sem que sejam necessários exames invasivos, como a biópsia hepática,

ou caros e demorados, como a ressonância magnética. Uma das possíveis manifestações da doença de Gaucher é a fibrose hepática, e saber se um paciente a está desenvolvendo é importante para ajustar a dose do tratamento e/ou tomar medidas preventivas. Os benefícios dessa pesquisa também incluem o aumento do conhecimento sobre a doença de Gaucher, podendo futuramente beneficiar de maneira direta ou indireta outros pacientes com a mesma condição.

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

A elastografia transitória será realizada no consultório privado do professor dr. Mário Reis Álvares-da-Silva, localizado próximo ao HCPA (Rua Mostardeiro, 780). O transporte até lá será provido pela equipe de pesquisa, sem custos ao participante.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos, porém, poderá ser ressarcido por despesas decorrentes de sua participação (ex.: despesas de transporte e alimentação), cujos custos serão absorvidos pelo orçamento da pesquisa.

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

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

Caso você tenha dúvidas, poderá entrar em contato com o pesquisador responsável Rodrigo Tzovenos Staroste, pelo telefone 51 98172 4749, ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 33597640, ou no 2º andar do HCPA, sala 2227, de segunda à sexta, das 8h às 17h.

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

Nome do participante da pesquisa	Nome do pesquisador que aplicou o Termo
_____	_____
Assinatura	Assinatura
	Local e Data:

Apêndice B: Termo de Consentimento Livre e Esclarecido para Indivíduos Controle Participantes do Projeto de Pesquisa 2018-0639

Projeto GPPG ou CAAE 98784818.3.0000.5327

Título do Projeto: Validação de escores clínico-laboratoriais de fibrose hepática para pacientes com doença de Gaucher através do uso da elastografia transitória hepática.

Você está sendo convidado a participar de uma pesquisa cujo objetivo é analisar se podemos usar exames de sangue para saber aproximadamente como está o fígado de pacientes com doença de Gaucher sem que necessitemos fazer exames invasivos. Você está sendo convidado como um grupo de comparação, chamado de controle, pois não possui a doença de Gaucher. Esta pesquisa está sendo realizada pelo Serviço de Genética Médica em conjunto com o Serviço de Gastroenterologia do Hospital de Clínicas de Porto Alegre (HCPA).

A doença de Gaucher é uma condição de origem genética (herdada dos pais) em que algumas células, conhecidas como macrófagos, acumulam substâncias que normalmente seriam destruídas pelo organismo, causando aumento de órgãos como o baço e o fígado, anemia, risco de sangramentos por diminuição das plaquetas na corrente sanguínea, alterações ósseas (enfraquecimento dos ossos, fraturas, deformações). Algumas pesquisas mostram que o fígado, além de estar aumentado de volume, pode ser atacado sutilmente pelas células anormais, causando, após muitos anos de ataques, manifestações como esteato-hepatite (inflamação do fígado com acúmulo de gordura) e fibrose (endurecimento do fígado com diminuição da sua função normal). O tratamento para a doença de Gaucher oferece bons resultados para a prevenção da maioria, senão todas, as manifestações.

A elastografia transitória, também conhecida pelo nome comercial de FibroScan, é um exame parecido externamente com uma ecografia, mas que envia ondas mecânicas ao invés de ondas sonoras. Ele é utilizado para determinar o grau de endurecimento do fígado, que corresponde ao grau de fibrose.

Se você aceitar participar da pesquisa, os procedimentos envolvidos em sua participação são os seguintes: coleta de exames de sangue, realização de elastografia transitória (que é um exame indolor e sem riscos conhecidos à saúde, assemelhado a uma ultrassonografia/ecografia), aplicação de questionário, e consulta a dados de prontuário.

Os possíveis riscos ou desconfortos decorrentes da participação na pesquisa são decorrentes da coleta de sangue, que, apesar de ser um procedimento de rotina, possui uma taxa (ainda que bastante baixa) de complicações como hemorragia ("sangramento") e formação de equimoses ("hematomas"). Desconfortos associados à pesquisa são os decorrentes do tempo de realização da elastografia transitória (aproximadamente 10 minutos), de aplicação do questionário (aproximadamente 5 minutos), e dor leve e temporária devida à coleta de sangue.

Os possíveis benefícios decorrentes da participação na pesquisa são a possibilidade de ajudar a saber com um grau de confiança maior que o atual se um paciente com doença de Gaucher está tendo lesão do tipo "fibrose" em seu fígado sem que sejam necessários exames invasivos, como a biópsia hepática, ou caros e demorados, como a ressonância magnética. Uma das possíveis manifestações da

doença de Gaucher é a fibrose hepática, e saber se um paciente a está desenvolvendo é importante para ajustar a dose do tratamento e/ou tomar medidas preventivas. Para que possamos estabelecer os valores normais da população e compará-los com os pacientes com doença de Gaucher, necessitamos de "controles", que são indivíduos que não tenham a doença sendo estudada – o seu papel na pesquisa, portanto, será servir de comparação para os pacientes com doença de Gaucher.

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

A elastografia transitória será realizada no consultório privado do professor dr. Mário Reis Álvares-da-Silva, localizado próximo ao HCPA (Rua Mostardeiro, 780). O transporte até lá será provido pela equipe de pesquisa, sem custos ao participante.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos, porém, poderá ser ressarcido por despesas decorrentes de sua participação (ex.: despesas de transporte e alimentação), cujos custos serão absorvidos pelo orçamento da pesquisa.

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

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

Caso você tenha dúvidas, poderá entrar em contato com o pesquisador responsável Rodrigo Tzovenos Starosta, pelo telefone 51 98172 4749, ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 33597640, ou no 2º andar do HCPA, sala 2227, de segunda à sexta, das 8h às 17h.

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

_____	_____
Nome do participante da pesquisa	Nome do pesquisador que aplicou o Termo
_____	_____
Assinatura	Assinatura

Local e Data:

_____

Apêndice C: Produção científica não-relacionada à tese

Autopsy Case Reports

Article / Autopsy Case Report

Misdiagnosis of *Streptococcus gallolyticus* endocarditis

Rodrigo Tzovenos Starosta[a], Raquel Rivero[b], Francine Hehn de Oliveira[b], Eron Lopes[b], Marcelle Reesink Cerski[b]

Starosta RT, Rivero R, Oliveira FH, Lopes E, Cerski MR. Misdiagnosis of *Streptococcus gallolyticus* endocarditis. Autopsy Case Rep [Internet]. 2016;6(3):29-33. http://dx.doi.org/10.4322/acr.2016.042

ABSTRACT

Death certificate inaccuracy is of major concern both in the public health domain and in individual health care, since it may yield untruthful data on the incidence, prevalence, and lethality of medical entities, and may hamper prophylactic measures among those who share, with the deceased, the common genetic, environmental, or behavioral risk factors. An effective way to settle this haziness relies on the increase of autopsy performance, increasing manifold the exactitude as well as facing surprising diagnoses. In this report, the authors present the case of a middle-aged woman who sought medical care because of back pain accompanied by weight loss. She died suddenly and unexpectedly in the Emergency Room. In this case, due to the unusual clinical presentation and the patient's unexpected death, the *causa mortis* would not have been elucidated if the autopsy had not been undertaken.

Keywords
Endocarditis, Subacute Bacterial; Streptococcus; Leriche Syndrome

CASE REPORT

A 53-year-old Caucasian woman came to the hospital with a 1 month history of lower back pain radiating to the right lower limb, hampering her normal gait. In the same period, she referred weight loss (10 kg) accompanied by permanent tiredness and weakness. In the week before the admission, petechiae in the lower limbs overspread to the trunk and a reported fever (39°C) ensued. A laboratory work-up was undertaken before her admission, which showed high C-reactive protein and erythrocyte sedimentation rate; and high determinations of serum alkaline phosphatase, bilirubin, and gamma-glutamyl transferase. The platelet count was 37,000 per mm^3, and leukocytosis of 14,800 per mm^3 without a shift to the left. The lumbar spine magnetic resonance imaging (MRI) showed vertebral disc degeneration with a hernia at L5-S1 space. The Doppler ultrasonography of the lower limbs did not reveal any signs of venous flow disturbance.

The physical examination at admission revealed blood pressure of 90/46 mmHg; a respiratory rate of 17 breaths per minute; a heart rate of 96 beats per minute, blood oxygen saturation of 98%; and an axillary temperature of 35.7°C. Jaundice and palpable purpura at the lower limbs were evident, but pulmonary and heart auscultation were unremarkable. The abdominal examination showed hepatomegaly, with the liver edge palpable up to 2 cm below the rib cage. No signs of edema or ischemia at the extremities were present.

[a] Faculty of Medicine - Universidade Federal do Rio Grande do Sul, Porto Alegre/ RS – Brazil.
[b] Service of Surgical Pathology - Hospital de Clinicas de Porto Alegre - Universidade Federal do Rio Grande do Sul, Porto Alegre/RS – Brazil.

During the observation period, the patient started complaining of pain in the left leg. She was re-examined, and, in the face of an unchanged physical examination, codeine was prescribed. During the following hours, the patient's condition evolved and she began to complain of uneasiness, anxiety, and shortness of breath. Her heart rate rose to 120 beats per minute, and on auscultation a pericardial friction bruit was detected. The electrocardiogram showed right bundle block, supraventricular tachycardia, and deviation of the electrical axis to the right. Serum troponin I was of 2.01 ng/mL (reference value: <0.16 ng/mL). Soon after, she presented cardiac arrest in asystole. Advanced cardiac life support maneuvers were unsuccessful and the patient died. She was then referred to the pathology service for an autopsy.

AUTOPSY FINDINGS

At autopsy, the patient was icteric and had several coalescing elevated petechiae bilaterally at the lower limbs, characterizing a palpable purpura. At the opening of the abdominal cavity, abscesses were found in the left adrenal gland, the liver parenchyma, the spleen, and the cortex of the left kidney, and hepatomegaly was confirmed. In the right internal iliac artery, there was an embolization resulting in subtotal occlusion of the lumen, which was likely to be the cause of the patient's leg pain. No thrombus or embolus was found at the lower limbs or the iliac veins. On microscopic examination, the abscesses of the adrenal, liver, spleen, and kidney were of septic origin, since bacterial clumps of Gram-positive cocci were found. The same findings were also present at the iliac artery embolus and in the glomerular capillaries—all of which yielded the diagnosis of systemic septic embolism.

In the thoracic cavity several bilateral pulmonary subpleural hemorrhagic infarctions, a focus of acute bronchopneumonia, and edema were depicted. Mitral and aortic valves had vegetations (Figure 1A), and a thrombus of 6 cm in length extended from the right ventricle until the second-generation bifurcation of the right pulmonary artery (Figure 1B). The pulmonary thromboembolism was considered as the immediate *causa mortis*. The right atrium had a large-sized thrombus adhered to the free wall.

Histology of the pulmonary embolus showed Zahn lines (alternating between erythrocyte and platelet-fibrin aggregates deposition) (Figure 2), confirming the diagnosis of an embolus, and ruling out the suspicion of a postmortem clot.

The lung parenchyma showed alveoli filled with plasma and several foci of hemosiderin-laden macrophages, which, along with the sinusoidal dilation and the hepatomegaly, were consistent with the diagnosis of cardiac insufficiency. The heart failure was interpreted to be much more a consequence of the valvular destruction than of the acute pulmonary embolism. There was a focus of bronchopneumonia in the right lung lower lobe, with a mixed inflammatory

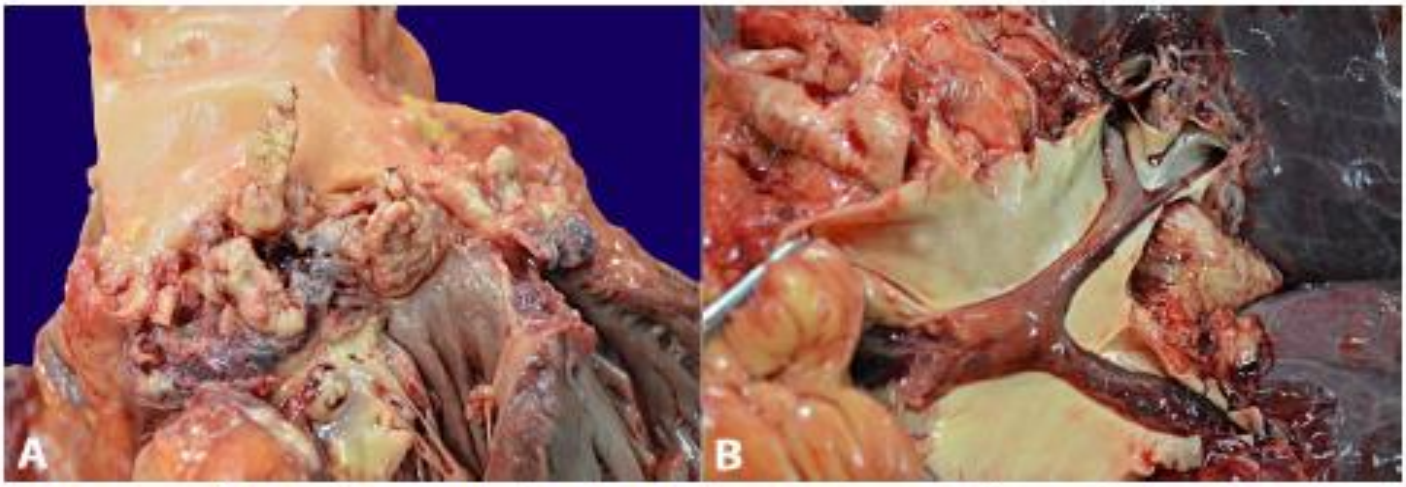


Figure 1. Gross findings of: **A** - Mitral valve with protruding vegetations; **B** - The opened pulmonary artery with saddle embolus.

infiltrate (both mononuclear and polymorphonuclear cells), engorged micro vessels, a low amount of alveolar and interstitial hemorrhage, and eosinophilic transudation material (Figure 3A). Gram stains were made but the results were inconclusive. Several micro emboli were found at the pulmonary microvasculature (Figure 3B), indicating an "embolic shower" and explaining the subpleural infarctions.

In the cardiac muscle, we observed stretched, pale pink myocardial fibers with the infiltration of a few polymorphonuclear inflammatory cells, which were consistent with a subacute ischemic myocardial injury. The vegetations of the affected valves were sent for microbiological analysis, which revealed the growth of *Streptococcus gallolyticus* in both, confirming the septic origin of the emboli (pathogen identification by Vitek-2 compact). A thorough search was undertaken for microscopic signs that could point towards the diagnosis of the chronic rheumatic valvular disease, but there were none. Previous history for rheumatic fever evaluation was unavailable.

At the cut surface of the brain, several reddish spots scattered on the white matter were found to be microemboli at histology (Figure 4). The pineal gland was enlarged, measuring about 1.2 cm at its longest axis and weighed less than 1 g. Four distinct nodular gray matter heterotopias were spotted in the cerebral white matter of the temporal and parietal lobes.

In the colonic lumen, there was a polypoid mass measuring 1.0 x 0.6 cm, which was diagnosed as a hyperplastic polyp at histology (Figure 5).

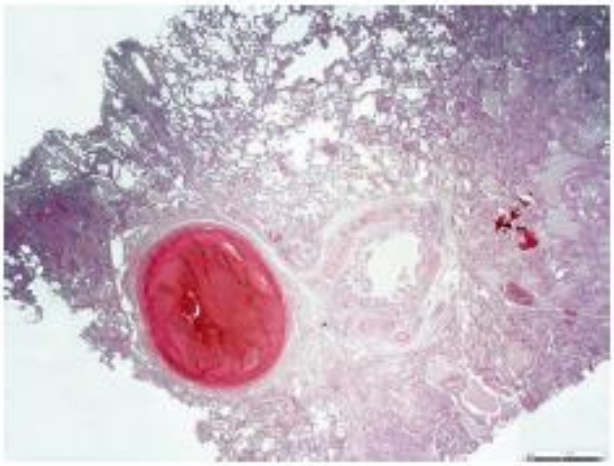


Figure 2. Photomicrography of an intralobar pulmonary artery with the end of the saddle embolus.

DISCUSSION

The microorganism *S. gallolyticus*, previously known as a subspecies of *Streptococcus bovis* biotype I,[1] is a common cause of infectious endocarditis in individuals with colonic neoplasia. In a recent systematic review and meta-analysis,[2] 64% of the patients with *S. bovis* endocarditis simultaneously presented gastrointestinal disease, although only 6% of all infectious endocarditides are caused by this pathogen,[3] which is associated with a low mortality rate when compared with other bacteria.[4] Although *S. gallolyticus* endocarditis and septicemia are classically associated with colonic neoplasia or dysplasia, there is

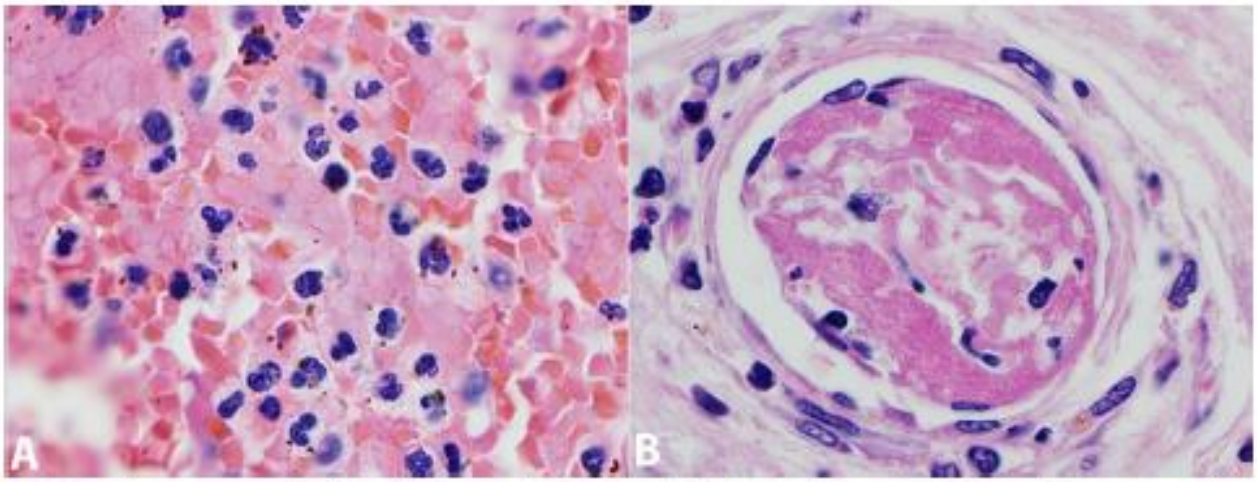


Figure 3. Photomicrography of the lung. **A** - Pulmonary alveoli filled with inflammatory cells and hyaline transudate; **B** - Peripheral pulmonary micro vessel with embolus.

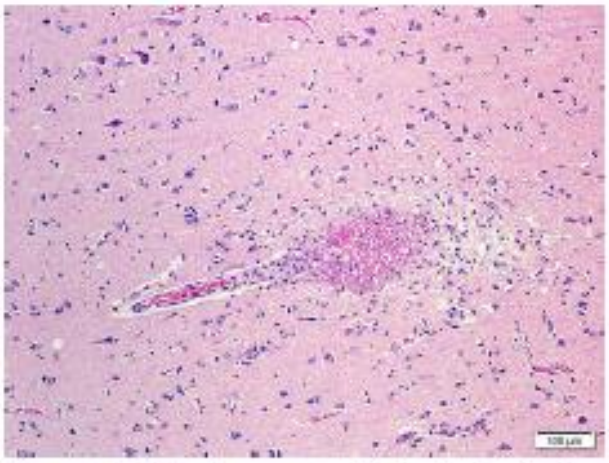


Figure 4. Photomicrography of the brain showing capillary vessel destroyed by micro thrombus. The pineal gland showed numerous full-formed cysts filled with amorphous, acellular, eosinophilic material of unknown origin, which is consistent with the diagnosis of simple pineal cysts. The central nervous system (CNS) microemboli, in contrast with those in the renal glomeruli, were of fibrinous origin and showed no bacteria or inflammatory cells.

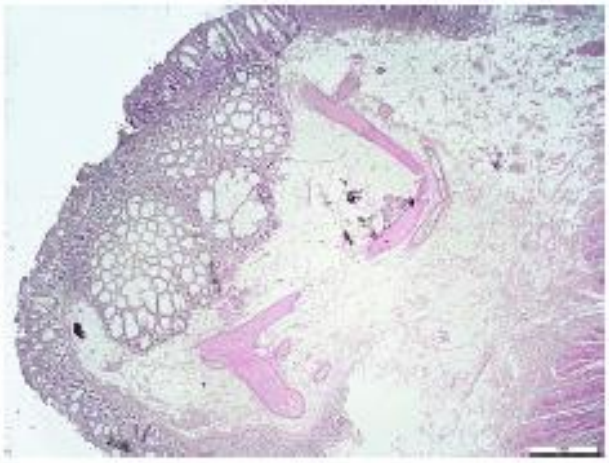


Figure 5. Photomicrography of the hyperplastic colonic polyp.

no evidence in the literature to support the hypothesis that hyperplastic polyps are also associated with it; therefore, in our case, we regarded the sole hyperplastic polyp to be an incidental finding. Several other findings in this autopsy could be attributed to the septicemia, including jaundice, bronchopneumonia, disseminated intravascular coagulation (especially in the brain and the lung), petechiae, bone marrow hypercellularity, and splanchnic abscesses. The bronchopneumonia was assumed also to be due to *S. gallolyticus*, but unfortunately we could not substantiate it even with the aid of the Gram stain and the culture.

The lack of fever at the time of the emergency consultation may likely account for the misdiagnosis; however, the patient presented fever in her medical history. The low back pain (her main complaint) was likely attributed to degenerative causes, such as osteoarthritis and osteoporosis, considering the epidemiological data. However, in the setting of the autopsy findings, her back pain could have been due to infectious discitis, vertebral osteomyelitis—both complications of infectious endocarditis—or distention of the renal capsule. At gross postmortem inspection there were no signs of discitis, and at microscopy of the vertebral bone marrow there were no signs of osteomyelitis. The vertebral hernia spotted on the MRI was very small and did not interfere with any adjacent structure. Hence, the distention of the renal capsule of the left kidney by the presence of the renal abscess remains the probable cause of her back pain.

Embolic events occur in nearly 27% of patients with infectious endocarditis; however, this rate is smaller in *S. bovis* endocarditis.[4] The main site of embolism involves the CNS, followed by the spleen, the kidney, the lungs, the peripheral arteries, the mesentery, and the eye.[5] Our patient had several septic emboli to the kidneys' microvasculature and one semi-occlusive septic embolus into the iliac artery, besides the less obvious septic microemboli in the pulmonary and cerebral microvasculature. Pulmonary embolism is a potentially lethal condition characterized by the impaction of an embolus in some part of the pulmonary arterial tree, which is called "saddle embolism" or Leriche's syndrome when the it impacts at the pulmonary artery bifurcation. Sometimes, the diagnosis of a pulmonary embolism is not straightforward, and estimates are that between 100,000 and 120,000 deaths occur each year in the United States because of this missed diagnosis.[6]

It is remarkable, in this case, that the clinical features and the final event were not enough to furnish a precise diagnosis. If the autopsy had not been performed, the case would have remained unclarified and certainly an inaccurate death certificate would have been reported. Death certificate information is a useful tool for epidemiologic and public health studies. The reliability of such certificates may be put under

suspicion when they are not endorsed by postmortem examination, especially in the case of unexpected death.[7] The *S. gallolyticus* infection and the pulmonary thromboembolism would not have been diagnosed without the autopsy. This information is important both to the family regarding the eventual colon cancer screening procedures,[8] and to the public health statistics, allowing more precise data of mortality by this infectious agent.

CONCLUSION

Although infectious endocarditis is a relatively common disorder, this case was atypical because no colonic neoplasia or dysplasia was associated with the infection by *S. bovis/gallolyticus*. Moreover, the massive pulmonary thromboembolism is also not classically associated with *S. gallolyticus* endocarditis and sepsis. Also remarkable is the fact that the patient presented to the Emergency Room with vague complaints, which highlights the importance of being aware of signs of more severe, acute conditions in patients who seem otherwise stable in every scenario.

REFERENCES

1. Gómez-Garcés JL, Gil Y, Burillo A, Wilhelmi I, Palomo M. Cuadros clínicos asociados a bacteriemia causada por las nuevas especies incluidas en el antiguo grupo Streptococcus bovis. Enferm Infecc Microbiol Clin. 2012;30(4):175-9. http://dx.doi.org/10.1016/j.eimc.2011.09.015. PMid:22377494.
2. Boleij A, van Gelder MM, Swinkels DW, Tjalsma H. Clinical Importance of Streptococcus gallolyticus infection among colorectal cancer patients: systematic review and meta-analysis. Clin Infect Dis. 2011;53(9):870-8. http://dx.doi.org/10.1093/cid/cir609. PMid:21960713.
3. Alozie A, Köller K, Pose L, et al. Streptococcus bovis infectious endocarditis and occult gastrointestinal neoplasia: experience with 25 consecutive patients treated surgically. Gut Pathol. 2015;7(1):27-31. http://dx.doi.org/10.1186/s13099-015-0074-0. PMid:26473016.
4. Kupferwasser I, Darius H, Müller AM, et al. Clinical and morphological characteristics in Streptococcus bovis endocarditis: a comparison with other causative microorganisms in 177 cases. Heart. 1998;80(3):276-80. http://dx.doi.org/10.1136/hrt.80.3.276. PMid:9875088.
5. Thuny F, Di Salvo G, Belliard O, et al. Risk of embolism and death in infective endocarditis: prognostic value of echocardiography: a prospective multicenter study. Circulation. 2005;112(1):69-75. http://dx.doi.org/10.1161/CIRCULATIONAHA.104.493155. PMid:15983252.
6. Calder KK, Herbert M, Henderson SO. The mortality of untreated pulmonary embolism in emergency department patients. Ann Emerg Med. 2005;45(3):302-10. http://dx.doi.org/10.1016/j.annemergmed.2004.10.001. PMid:15726055.
7. Mieno MN, Tanaka N, Arai T, et al. Accuracy of death certificates and assesment of factors for misclassification for underlying cause of death. J Epidemiol. 2016;26(4):191-8. http://dx.doi.org/10.2188/jea.JE20150010. PMid:26639750.
8. Boleij A, Muytjens CM, Bukhari SI, et al. Novel clues on the specific association of Streptococcus gallolyticus subsp gallolyticus with colorectal cancer. J Infect Dis. 2011;203(8):1101-9. http://dx.doi.org/10.1093/infdis/jiq169. PMid:21451000.

Conflict of interest: None

Submitted on: May 29th, 2016
Accepted on: July 17th, 2016

Correspondence
Rodrigo Tzovenos Starosta
Service of Pathology - Faculty of Medicine - Federal University do Rio Grande do Sul (UFRGS)
Rua Ramiro Barcellos, 2350 – Porto Alegre/RS – Brazil
CEP: 90035-003
Phone: +55 (51) 8172-4749
rodrigo.starosta@ufrgs.br

The specific impact of uremic toxins upon cognitive domains: a review

O impacto específico de toxinas urêmicas em domínios cognitivos: uma revisão

Authors

Álvaro de Oliveira Franco[1]
Rodrigo Tzovenos Starosta[1,2]
Matheus Roriz-Cruz[3,4]

[1] Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Porto Alegre, RS, Brasil.
[2] Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Genética e Biologia Molecular, Porto Alegre, RS, Brasil.
[3] Universidade Federal do Rio Grande do Sul, Departamento de Medicina Interna, Porto Alegre, RS, Brasil.
[4] Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brasil.

Abstract

One of the mechanisms proposed for chronic kidney disease (CKD)-related cognitive impairment is the accumulation of uremic toxins due to the deterioration of the renal clearance function. Cognition can be categorized into five major domains according to its information processing functions: memory, attention, language, visual-spatial, and executive. We performed a review using the terms 'uric acid', 'indoxyl sulfate', '*p*-cresyl sulfate', 'homocysteine', 'interleukins' and 'parathyroid hormone'. These are the compounds that were found to be strongly associated with cognitive impairment in CKD in the literature. The 26 selected articles point towards an association between higher levels of uric acid, homocysteine, and interleukin 6 with lower cognitive performance in executive, attentional, and memory domains. We also reviewed the hemodialysis effects on cognition. Hemodialysis seems to contribute to an amelioration of CKD-related encephalopathic dysfunction, although this improvement occurs more in some cognitive domains than in others.

Keywords: Uremia; Cognitive Dysfunction; Knowledge; Renal Insufficiency, Chronic; Renal Dialysis; Toxins, Biological; Memory; Executive Function; Attention.

Resumo

Um dos mecanismos propostos para explicar o comprometimento cognitivo relacionado à doença renal crônica (DRC) é o acúmulo de toxinas urêmicas devido à deterioração da função de depuração renal. A cognição pode ser categorizada em cinco domínios principais de acordo com suas funções de processamento de informações: memória, atenção, linguagem, visual-espacial e executiva. Realizamos uma revisão usando os termos "ácido úrico", "indoxil sulfato", "*p*-cresil sulfato", "homocisteína", "interleucinas" e "paratormônio". Estes são os compostos que se mostraram fortemente associados ao comprometimento cognitivo na DRC na literatura. Os 26 artigos selecionados apontam para uma associação entre níveis mais elevados de ácido úrico, homocisteína e interleucina-6 com menor desempenho cognitivo nos domínios executivo, atenção e de memória. Também revisamos os efeitos da hemodiálise na cognição. A hemodiálise parece contribuir para uma melhoria da disfunção encefalopática relacionada à DRC, embora essa melhora ocorra mais em alguns domínios cognitivos do que em outros.

Palavras-chave: Uremia; Disfunção Cognitiva; Conhecimento; Insuficiência Renal Crônica; Diálise Renal; Toxinas Biológicas; Memória; Função Executiva; Atenção.

Introduction

Many potentially toxic compounds build up in patients with chronic kidney disease (CKD); the biologically active ones are called uremic toxins (UTs)[1,1]. UTs comprise around 150 compounds that may cause many deleterious effects, such as systemic inflammation, cardiac failure, anemia, immune dysfunction, anorexia[1], neurological damage, and cognitive impairment.

CKD patients have a higher risk of developing cognitive impairment (CI) related to CKD (CKD-CI) even in the earlier stages[1-7], which affects their daily life and work capacity, and causes increased

Submitted on: 01/17/2018.
Approved on: 05/14/2018.

Correspondence to:
Álvaro de Oliveira Franco.
E-mail: alvaro.franco@ufrgs.br

DOI: 10.1590/2175-8239-JBN-2018-0033

periods of hospitalization[1]. Most importantly, CKD-CI is an independent predictor of mortality in patients submitted to dialysis and is associated with an almost three times greater mortality risk in 7 years[9]. More than 70% of hemodialysis (HD) patients older or equal to 55 years have moderate-to-severe CKD-CI[10].

CKD-CI's hypothetical mechanism can be divided into neurodegenerative[1,11-13] and cerebro-vascular[14-16] components. The former accounts for UTs' direct neurotoxicity, resulting in the alteration of the brain's redox environment, along with the promotion of central nervous system excitotoxicity through the activation of the glutamatergic pathways and the inhibition of the GABAergic ones[17]. The latter states that UTs[14] along with systemic hemodynamic impairment related to CKD[11] also cause a direct effect on the cerebral endothelium, resulting in oxidative stress, chronic inflammation, hypercoagulability[1,11,14], and disruption of the blood-brain barrier and cellular water transport[10,11]. This corroborates the fact that CKD patients have a higher incidence of cerebral microbleeds[11,17], silent brain infarcts, and white matter lesions (leukariosis)[18], even when adjusted for common risk factors (e.g.: hypertension and diabetes mellitus)[14,11]. Nevertheless, the impact of specific UTs upon cognition and the exact mechanisms by which they occur are still not completely understood, despite the increasing necessity for a systematic characterization that could improve the identification and management of CKD-CI[16].

Therefore, we reviewed literature data regarding the mechanisms by which homocysteine and uremic toxins with a higher impact on the emergence of CKD-CI[16] - uric acid, indoxyl sulphate, *p*-cresyl sulphate, interleukins 1-β and 6, and parathyroid hormone - can produce deleterious effects on cognition (data shown in Table 1). We conducted an analysis on the possible relationship between the main uremic toxins on one side, and the basic cognitive domains on the other (data shown in Table 2), which reveal a consistent cognitive deterioration pattern associated with CKD-CI. Finally, we also reviewed the influence of HD upon each different cognitive domain among CKD patients, identifying which domains benefit the most from this treatment.

Uremic toxins and cognitive domains

The categorization of cognition into discrete cognitive domains is a reductionist approach used in neurocognitive study and practice. This method allows researchers and clinicians to decompose the high-order feature called 'cognition' into less complex information processing units in order to identify patterns of impairment that can be associated with a certain disease, process, or toxin. Hence, the description of major cognitive domains - memory, executive functions, attention, language, and visual-spatial function - affected by a particular toxin can be used to establish a specific cognitive impairment pattern and identify its target areas. This can be achieved by the association between its serum levels and standardized specific neuropsychological tests.

Uric acid

High uric acid levels in the blood are associated with poorer attention, visual-processing speed, and cognitive flexibility in adolescent survivors of childhood acute lymphoblastic leukemia, but not in adult ones[17]. Increased uric acid in baseline levels was also associated with poorer working memory in a cohort study with cognitively healthy community-dwelling older women[11], as well as with white matter atrophy, poorer information-processing speed, decreased executive functionality[19] and cerebral ischemic burden[78]. It is also associated with faster cognitive decline in visual memory and visuo-construction skill in the baseline levels, although increased serum uric acid over-time was associated with a potential benefit for the attention domain and the processing speed among older men[71]. The authors of this study determined that this paradoxical situation might be attributed to uric acid antioxidant (primarily in plasma) and oxidant (primarily intracellular) function in neurons[71]. Aiming to resolve this possibility, Schretlen *et al.* performed a study that showed an association between uric acid and poor verbal and working memories, even after controlling for confounding factors[77].

Indoxyl sulphate and *P*-Cresyl sulphate

High serum indoxyl sulphate levels are associated with a poorer executive function in the early stages of CKD, despite the lack of a significant association between *p*-cresyl sulphate and cognitive impairment[74].

Homocysteine

Increased homocysteine (HCy) levels in the blood are related to greater cognitive and motor impairment, especially regarding frontal-executive

Table 1 Current mechanistic data on some of the most meaningful uremic toxins, as stated by Watanabe and colleagues[26] with the addition of HCy

Uric acid	Antioxidant and pro-oxidant effects, white matter atrophy, and cerebral ischemic burden. Uric acid is a major alarmin that induces pro-inflammatory cytokine expression and secretion, as well as inflammation; the underlying mechanism for these functions is the activation of the nuclear factor-κ B by toll-like receptor 4. This response was activated more in neurons than in glial cells when rat hippocampi were studied. The promotion of gliosis has also been observed. Uric acid is also associated with atherosclerosis, endothelial and cardiovascular disease burden, microvascular renal disease, glomerular hypertension, glomerulosclerosis, and renal interstitial fibrosis. 29 30 32 64 65 66
Indoxyl sulphate and *p*-cresyl sulphate	Direct neurotoxicity of indoxyl sulphate is suggested, but not proven. Indoxyl sulphate possibly causes a disruption of the brain efflux transport systems. Some of the transporters found in brain capillary endothelium are the same secretory transport molecules found in the basolateral membrane of proximal tubular cells; for instance, the organic anion transporter 3 (OAT3). Indoxyl sulphate was found to accumulate in uremic patients' brains. Indoxyl sulphate also causes nephrotoxic renal fibrosis through the accumulation in renal tubular cells, production of free radicals, inflammation, endothelial cell dysfunction, endothelial and proximal tubular cell senescence, atherosclerosis, and the disruption of rhythmicity regulation of clock genes (*rPer2*). 26 67 68
Homocysteine (HCy)	HCy increases oxidative stress, DNA damage, induction of apoptosis, production of homocysteic acid, excitotoxicity (mediated by NMDA glutamate receptor activation), white matter hyperintensities, cerebrovascular disease, and brain atrophy. Hyperhomocysteinemia is linked to cerebral microvascular rarefaction and dysfunction of the methylation of DNA, proteins, and phospholipids due to the inhibition of methyltransferase. This can lead to abnormal epigenetic regulation. Superoxide and hydrogen peroxide are formed by the oxidation of homocysteine, whose increased levels could cause a reduction in glutathione peroxidase activity and antioxidant potentials. Hyperhomocysteinemia also seems to cause alterations in the monoamine neurotransmitter system through mechanisms involving the inhibition of methyltransferase reactions and changes in the cellular redox state. Involving these same mechanisms, hyperhomocysteinemia also promotes the reduction of brain-derived neurotrophic factor (BDNF) levels in cerebrospinal fluid. BDNF is a protein related to cell maintenance, plasticity, growth and death. Hyperhomocysteinaemia also causes: endothelial dysfunction, prothrombogenic activity and cardiovascular disease. 13 18 19 26 69 70
Interleukin 1-β and interleukin 6	These interleukins cause brain inflammation, particularly through microglial cells and astrocytes; DNA damage; oxidative stress; the up-regulation of glutamate resulting in excitotoxicity; and brain and systemic aging-related changes. 26 51
Parathyroid hormone (PTH)	PTH promotes mineral bone disorder, metastatic calcification, increased brain circulating and neuronal cytosol calcium levels causing changing in brain function, the induction of apoptosis due to calcium overloading, reduced regional cerebral blood flow, and somatic, behavioural and motor abnormalities. 26 59

function, attention[72], verbal memory, fine motor speed[76], processing speed, episodic memory[77], visual, spatial and constructional ability, and processing speed[71]. High HCy levels impact negatively on task performance that assesses executive functioning[73,48] and executive-language functioning[41]. The impact of HCy on memory is controversial: while some studies have shown none, others have reported poorer memory, motor speed, dexterity, and visuo-spatial function with higher HCy levels[41]. Executive functions and verbal expression[47], attention, and visuoperception and construction[44] are also impaired by HCy. In another study, HCy was found to be significantly inversely correlated with attention, and delayed but not immediate memory recall[47]. When assessing memory, the mixed results found might suggest a more specific subcomponent impairment of this core cognitive domain. There is a significant positive correlation between HCy and interleukin (IL)-6 levels[47].

Table 2 Cognitive domains affected by each of the searched UTs. The references in the table correspond to the 26 selected articles

Cognitive domains	Uric acid	Indoxyl sulphate	Homocysteine	Interleukin 1-β	Interleukin 6	PTH
Executive	X	X	X		X	X
	27	34	35		50	58
	28		37		52	
	29		38		53	
	33		39		54*	
			40			
			41			
			42			
			43			
			Dubious:			
			44			
Attention	X		X		X	
	27		35		52	
	28		44		54	
			45			
Memory	X		X	X	X	
	31		37	46	54**	
	35		42	47	55	
			43	48	Dubious:	
			44		52***	
			45			
			Dubious:			
			35			
			36			
Language			X			
			41			
			43			
			Dubious:			
			35			
			42			
Visual-spatial	X		X			NA
	27		38			
	31		42			
			44			

Continuation table 2.

Motor	Dubious: 28	NA	X 42 Dubious: 36 43	NA	NA

X - Significant negative associations - $p<0.05$.

Dubious - Association lost significance after adjusting for other factors.

NA - Not assessed.

PTH - Parathyroid hormone.

* Association with processing speed in cross-sectional, but not in prospective analysis.

** Association only in prospective, but not in cross-sectional analysis.

*** Association with auditory recognition memory (before correcting for demographic characteristics) but not with general memory.

Interleukin 1-β

There is some evidence relating IL-1β to aging processes in the hippocampus, cognitive impairment in multiple domains, and to Alzheimer disease[46]. IL-1β also impairs spatial learning and memory in animal model studies[47,41]: higher IL-1β levels hamper the consolidation processes of contextual fear conditioning and have a particular effect in the rat dorsal hippocampus[47], which could be explained by the cytokine's interference with hippocampal long-term potentiation[48].

Interleukin 6

Higher IL-6 levels are associated with poorer executive function[18], aging processes, and degeneration of GABAergic interneurons, which are essential for normal information processing, encoding, and retrieval in the hippocampus and the cortex[11]. IL-6 levels also correlate inversely with performance in tests assessing auditory recognition memory, attention/working memory, and executive function, but surprisingly not with general memory[11]. This cytokine is also associated with poorer executive function in African Americans, but not in European Americans[18], with worse executive, attentional, and memory function, independent of cardiovascular disease and risk factors[14], and with low performance in memory tests[11].

Parathyroid hormone

Parathyroid hormone (PTH) crosses the blood-brain barrier[16] and has a wide receptor distribution in the central nervous system[17], which probably explains why alterations in calcium metabolism and, consequently, in the serum ionized calcium level (the main regulator of PTH), also impact brain function. A study has found significant negative associations between serum PTH levels and working memory capacity, and the speed of information processing[11]. A 2015 systematic review concluded that, despite mixed results, there is the suggestion of a link between PTH high serum levels and increased odds of poor cognition or dementia, although the evidence available offers weak support[19]. In this way, studies that conclusively differentiate the effects of PTH from those of the metabolites it modulates (e.g., calcium) are still necessary.

Hemodialysis and the cognitive domains

Evidence about the effectiveness of dialysis in reducing CKD-CI is extremely relevant when considering it as a treatment at the first signals of CI. Although dialysis causes great morbidity and is a nuisance to the patient, an untreated CKD-CI for a long period will lower the patient's quality of life and may result in increased periods of hospitalization and a higher risk of mortality[16]. This is a very relevant topic because of the high prevalence of CKD-CI[1], mainly among those older than 55 years[49].

The association of CKD with the impairment of target cognitive domains is now being elucidated. People treated with HD have significantly lower cognitive test scores than the general population in all the domains evaluated (orientation and attention, memory, language, construction and motor function, conceptualization and reasoning, executive function, and global cognition) except for one (perception), as concluded by a systematic review and meta-analysis of 42 randomized controlled trials, and both cohort and cross-sectional studies. Tests assessing orientation and attention, memory, and executive function scored the poorest in HD patients compared to the

general population. However, compared to chronic kidney disease patients not undergoing dialysis, limited evidence suggests that HD patients may perform better in memory and attention[40].

A cohort of Dutch CKD patients performed the worst in questions that demanded memory and attention, and presented a low verbal fluency. They were born before 1979 and had started chronic renal replacement therapy at age 0-14 years between 1972 and 1992. However, this study[41] made use of the Wechsler Adult Intelligence Scale; therefore, the cognitive domains could not be evaluated by individual tests. The authors concluded that the long duration of dialysis would enhance CI, a condition that could not be reversed even after renal transplantation, and that end-stage renal disease in childhood is associated with CKD-CI and impaired educational attainment levels in adulthood.

Starosta and colleagues[42], in a pilot study, matched CKD patients' cognition before HD with measurements taken after the beginning of the sessions. The results suggest that HD is an effective treatment for CKD-CI, even though cognition is not fully recovered to the level of a non-CKD patient (i.e. CKD-CI persisted, but was less severe). Cognitive domains were not assessed individually in this study. In another study, Schneider and colleagues[43] concluded that a single dialysis session (with testing performed 1 hour before and 19 hours after dialysis) improved the results in memory, attention, and executive functions. Despite the improvement, the performance of dialytic patients in post-dialysis assessments was significantly smaller than that of non-CKD patients, which highlights the lingering aspect of CKD-CI.

Interestingly, executive functions, memory, and attention are the cognitive domains most affected by the UTs that we researched in our review. This convergent pattern of cognitive domain impairments underlines the role and importance of these toxins in the genesis of CKD-CI. The fact that HD treatment ameliorates memory and attention, in comparison with non-dialyzed CKD patients, indicates that these removed UTs may affect some brain areas differently than others. We suggest that it would be beneficial for further studies to collect biochemical data about the uremic state of each patient and that cognition be assessed by domain-specific tests. In this way, specific factors may be matched more precisely with their impact on cognition, which would provide a better understanding of the mechanism of each toxic molecule and the cause of the impairment in each cognitive domain.

All these findings, together with the continual description of alterations in discrete domains, provide a finer resolution of the pattern of cognitive impairment found in uremic patients. Ultimately, these efforts have clinical significance as uremic encephalopathy needs to be distinguished from neurodegenerative diseases, delirium, cerebro-vascular diseases, and non-related psychiatric disorders, especially in a population of CKD patients, who exhibit multiple comorbidities many times.

Strengths and limitations

The main strength of our review is the extensive and comprehensive search on the topic of interest, which allowed us to gather a significant amount of data regarding the interaction between uremic toxins and specific cognitive domains. To the best of our knowledge, no study has reviewed such a relationship to such depth. We have also provided a strong body of evidence linking at least three UTs to three specific cognitive domains.

There are various limitations with this review: (1) the articles' biases were not systematically revised or graded after being selected for the review. Studies presenting patients with comorbidities (which make it difficult to attribute an isolated correlation between a UT and CKD) or with cognitive domain measurements made via telephone, were excluded in the selection process; (2) only articles in English were included; (3) only the PubMed database was searched; and (4) the data synthesis was not based directly on the tests used, but rather on their interpretations.

Conclusions

Higher levels of uric acid, HCy and IL-6 are significantly associated with lower cognitive performance in executive, attentional, and memory domains. These same three cognitive domains are the most impaired in patients under HD treatment; conversely, among the cognitive domains, they present the greatest performance improvement after HD treatment, according to our literature review. This suggests a protective effect derived from the removal of uremic toxins, and highlights the important role of these three compounds in the onset of CKD-CI. In fact, when studying uremic encephalopathy, it is important to keep in mind that different uremic toxins may have different effects upon different parts of the brain, which reflects the alterations in distinct cognitive domains.

This is important because it broadens the possibility of future symptomatic treatment based on the specific features a patient might present; it also helps to shed light on the biochemical background underlying each function. We hope that proper understanding of the pathophysiology of uremic encephalopathy will improve the diagnosis of cognitive impairment (which will become more clearly recognizable with the use of tests) and allow for appropriate treatment and care.

As occurred in the 20th century with cancer - a single entity that was found to be composed of a myriad of different mechanisms and types - cognitive dysfunction is bound to, in the present century, suffer the same process of deep understanding and enlightenment, since each of its facets is indeed composed of unique, distinct, underlying neural substrate. Furthermore, it is possible that each dysfunction may need a differential, mechanism-based approach in order to be tackled. With this review, we aimed to present a panorama of the intricate relationship between renal failure's uremic syndrome and the loss of full cognitive function - as both are issues to be solved per se - and to provide a window to the even most mysterious and intriguing ways of the brain.

References

1. Vanholder R, Glorieux G, De Smet R, Lameire N; European Uremic Toxin Work Group. New insights in uremic toxins. Kidney Int Suppl 2003;(84):S6-10.
2. Vanholder R, Van Laecke S, Glorieux G. What is new in uremic toxicity? Pediatr Nephrol 2008;23:1211-21.
3. Madan P, Kalra OP, Agarwal S, Tandon OP. Cognitive impairment in chronic kidney disease. Nephrol Dial Transplant 2007;22:440-4.
4. Buchman AS, Tanne D, Boyle PA, Shah RC, Leurgans SE, Bennett DA. Kidney function is associated with the rate of cognitive decline in the elderly. Neurology 2009;73:920-7.
5. Etgen T, Sander D, Chonchol M, Briesenick C, Poppert H, Förstl H, et al. Chronic kidney disease is associated with incident cognitive impairment in the elderly: the INVADE study. Nephrol Dial Transplant 2009;24:3144-50.
6. McQuillan R, Jassal SV. Neuropsychiatric complications of chronic kidney disease. Nat Rev Nephrol 2010;6:471-9.
7. Elias MF, Elias PK, Seliger SL, Narsipur SS, Dore GA, Robbins MA. Chronic kidney disease, creatinine and cognitive functioning. Nephrol Dial Transplant 2009;24:2446-52.
8. Bugnicourt JM, Godefroy O, Chillon JM, Choukroun G, Massy ZA. Cognitive disorders and dementia in CKD: the neglected kidney-brain axis. J Am Soc Nephrol 2013;24:353-63.
9. Griva K, Stygall J, Hankins M, Davenport A, Harrison M, Newman SP. Cognitive impairment and 7-year mortality in dialysis patients. Am J Kidney Dis 2010;56:693-703.
10. Murray AM. Cognitive impairment in the aging dialysis and chronic kidney disease populations: an occult burden. Adv Chronic Kidney Dis 2008;15:123-32.
11. Lu R, Kiernan MC, Murray A, Rosner MH, Ronco C. Kidney-brain crosstalk in the acute and chronic setting. Nat Rev Nephrol 2015;11:707-19.
12. Bernstein HG, Bogerts B, Keilhoff G. The many faces of nitric oxide in schizophrenia. A review. Schizophr Res 2005;78:69-86.
13. Lipton SA, Kim WK, Choi YB, Kumar S, D'Emilia DM, Rayudu PV, et al. Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 1997;94:5923-8.
14. Stinghen AE, Pecoits-Filho R. Vascular damage in kidney disease: beyond hypertension. Int J Hypertens 2011;2011:272637.
15. Silvestrini M, Pasqualetti P, Baruffaldi R, Bartolini M, Handouk Y, Matteis M, et al. Cerebrovascular reactivity and cognitive decline in patients with Alzheimer disease. Stroke 2006;37:1010-5.
16. O'Brien JT, Erkinjuntti T, Reisberg B, Roman G, Sawada T, Pantoni L, et al. Vascular cognitive impairment. Lancet Neurol 2003;2:89-98.
17. De Deyn PP, D'Hooge R, Van Bogaert PP, Marescau B. Endogenous guanidino compounds as uremic neurotoxins. Kidney Int Suppl 2001;78:S77-85.
18. Fassbender K, Mielke O, Bertsch T, Nafe B, Fröschen S, Hennerici M. Homocysteine in cerebral macroangiography and microangiopathy. Lancet 1999;353:1586-7.
19. Wright CB, Paik MC, Brown TR, Stabler SP, Allen RH, Sacco RL, et al. Total homocysteine is associated with white matter hyperintensity volume: the Northern Manhattan Study. Stroke 2005;36:1207-11.
20. Brouns R, De Deyn PP. Neurological complications in renal failure: a review. Clin Neurol Neurosurg 2004;107:1-16.
21. Benga O, Huber VJ. Brain water channel proteins in health and disease. Mol Aspects Med 2012;33:562-78.
22. Shima H, Ishimura E, Naganuma T, Yamazaki T, Kobayashi I, Shidara K, et al. Cerebral microbleeds in predialysis patients with chronic kidney disease. Nephrol Dial Transplant 2010;25:1554-9.
23. Yokoyama S, Hirano H, Uomizu K, Kajiya Y, Tajitsu K, Kusumoto K. High incidence of microbleeds in hemodialysis patients detected by T2*-weighted gradient-echo magnetic resonance imaging. Neurol Med Chir (Tokyo) 2005;45:556-60.
24. Weiner DE, Bartolomei K, Scott T, Price LL, Griffith JL, Rosenberg I, et al. Albuminuria, cognitive functioning, and white matter hyperintensities in homebound elders. Am J Kidney Dis 2009;53:438-47.
25. Wada M, Nagasawa H, Ikeki C, Takahashi Y, Sato H, Arawaka S, et al. Cerebral small vessel disease and chronic kidney disease (CKD): results of a cross-sectional study in community-based Japanese elderly. J Neurol Sci 2008;272:36-42.
26. Watanabe K, Watanabe T, Nakayama M. Cerebro-renal interactions: impact of uremic toxins on cognitive function. Neurotoxicology 2014;44:184-93.
27. Cheung YT, Edelmann MN, Mulrooney DA, Green DM, Chemaitilly W, Joha N, et al. Uric Acid and Neurocognitive Function in Survivors of Childhood Acute Lymphoblastic Leukemia Treated with Chemotherapy Only. Cancer Epidemiol Biomarkers Prev 2016;25:1159-67.
28. Vannorsdall TD, Kneider AM, Carlson MC, Schretlen DJ. Higher baseline serum uric acid is associated with poorer cognition but not rates of cognitive decline in women. Exp Gerontol 2014;60:136-9.
29. Verhaaren BF, Vernooij MW, Dehghan A, Vrooman HA, de Boer R, Hofman A, et al. The relation of uric acid to brain atrophy and cognition: the Rotterdam Scan Study. Neuroepidemiology 2013;41:29-34.
30. Schretlen DJ, Inscore AB, Vannorsdall TD, Kraut M, Pearlson GD, Gordon B, et al. Serum uric acid and brain ischemia in normal elderly adults. Neurology 2007;69:1418-23.
31. Beydoun MA, Canas JA, Dore GA, Beydoun HA, Rostant OS, Fanelli-Kuczmarski MT, et al. Serum Uric Acid and Its Association with Longitudinal Cognitive Change Among Urban Adults. J Alzheimers Dis 2016;52:1415-30.

32. Santin YY, Johnson RJ. Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids 2008;27:608-19.
33. Schretlen DJ, Inscore AB, Jinnah HA, Rao V, Gordon B, Pearlson GD. Serum uric acid and cognitive function in community-dwelling older adults. Neuropsychology 2007;21:136-40.
34. Yeh YC, Huang MF, Liang SS, Hwang SJ, Tsai JC, Liu TL, et al. Indoxyl sulfate, not p-cresyl sulfate, is associated with cognitive impairment in early-stage chronic kidney disease. Neurotoxicology 2016;53:148-52.
35. Sachdev PS, Valenzuela MJ, Brodaty H, Wang XL, Looi J, Lorentz L, et al. Homocysteine as a risk factor for cognitive impairment in stroke patients. Dement Geriatr Cogn Disord 2005;15:155-62.
36. Sachdev P, Parslow R, Salonikas C, Lux O, Wen W, Kumar R, et al. Homocysteine and the brain in midadult life: evidence for an increased risk of leukoaraiosis in men. Arch Neurol 2004;61:1369-76.
37. Narayan SK, Saxby BK, Firbank MJ, O'Brien JT, Harrington F, McKeith IG, et al. Plasma homocysteine and cognitive decline in older hypertensive subjects. Int Psychogeriatr 2011;23:1607-15.
38. Feng L, Ng TP, Chuah L, Niti M, Kua EH. Homocysteine, folate, and vitamin B-12 and cognitive performance in older Chinese adults: findings from the Singapore Longitudinal Ageing Study. Am J Clin Nutr 2006;84:1506-12.
39. Polito L, Poloni TE, Vaccaro R, Abbondanza S, Mangieri M, Davin A, et al. High homocysteine and epistasis between MTHFR and APOE: association with cognitive performance in the elderly. Exp Gerontol 2016;76:9-16.
40. Yeh YC, Huang MF, Hwang SJ, Tsai JC, Liu TL, Hsiao SM, et al. Association of homocysteine level and vascular burden and cognitive function in middle-aged and older adults with chronic kidney disease. Int J Geriatr Psychiatry 2016;31:713-20.
41. West RK, Beeri MS, Schmeidler J, Mitchell DB, Carlisle KR, Angelo G, et al. Homocysteine and cognitive function in very elderly nondemented subjects. Am J Geriatr Psychiatry 2011;19:673-7.
42. Schafer JH, Glass TA, Bolla KI, Mintz M, Jedlicka AE, Schwartz BS. Homocysteine and cognitive function in a population-based study of older adults. J Am Geriatr Soc 2005;53:381-8.
43. Hooshmand B, Solomon A, Kivipelto M, Kåreholt I, Rusanen M, Hänninen T, Leiviskä J, et al. Associations between serum homocysteine, holotranscobalamin, folate and cognition in the elderly: a longitudinal study. J Intern Med 2012;271:104-12.
44. van Raamt AF, Kalmijn S, Mali WP, van Zandvoort MJ, van der Graaf Y; SMART Study Group. Homocysteine level and cognitive function in patients with arterial disease: the second manifestations of ARTerial Disease Study. J Am Geriatr Soc 2006;54:575-9.
45. Agarwal A, Ilango K, Singh PK, Karmakar D, Singh GP, Kumari R, et al. Age-dependent levels of plasma homocysteine and cognitive performance. Behav Brain Res 2015;283:179-84.
46. Foclesan OV, Diaz BS, Talib LL, Mendonça VA, Ojopi EB, Gattaz WF, et al. Increased serum IL-1beta level in Alzheimer's disease and mild cognitive impairment. Dement Geriatr Cogn Disord 2009;28:507-12.
47. Palin K, Bluthé RM, Verrier D, Tridon V, Dantzer R, Lestage J. Interleukin-1beta mediates the memory impairment associated with a delayed type hypersensitivity response to bacillus Calmette-Guérin in the rat hippocampus. Brain Behav Immun 2004;18:227-30.
48. Cibelli M, Fidalgo AR, Terrando N, Ma D, Monaco C, Feldmann M, et al. Role of interleukin-1beta in postoperative cognitive dysfunction. Ann Neurol 2010;68:360-8.
49. Vereker E, Campbell V, Roche E, McEntee E, Lynch MA. Lipopolysaccharide inhibits long-term potentiation in the rat dentate gyrus by activating caspase-1. J Biol Chem 2000;275:26252-8.
50. Meyers CA, Albitar M, Estey E. Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome. Cancer 2005;104:788-93.
51. Dugan LL, Ali SS, Shekhtman G, Roberts AJ, Lucero J, Quick KL, et al. IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase. PLoS One 2009;4:e5518.
52. Marsland AL, Petersen KL, Sathanoori R, Muldoon MF, Neumann SA, Ryan C, et al. Interleukin-6 covaries inversely with cognitive performance among middle-aged community volunteers. Psychosom Med 2006;68:895-903.
53. Windham BG, Simpson BN, Lirette S, Bridges J, Bielak L, Peyser PA, et al. Associations between inflammation and cognitive function in African Americans and European Americans. J Am Geriatr Soc 2014;62:2303-10.
54. Mooijaart SP, Sattar N, Trompet S, Lucke J, Stott DJ, Ford I, et al.; PROSPER Study Group. Circulating interleukin-6 concentration and cognitive decline in old age: the PROSPER study. J Intern Med 2013;274:77-85.
55. Grassi-Oliveira R, Bauer ME, Pezzi JC, Teixeira AL, Brietzke E. Interleukin-6 and verbal memory in recurrent major depressive disorder. Neuro Endocrinol Lett 2011;32:540-4.
56. Johora C, Hetta J, Niklasson F, Rastad J, Wide L, Agren H, et al. Cerebrospinal fluid calcium, parathyroid hormone, and monoamine and purine metabolites and the blood-brain barrier function in primary hyperparathyroidism. Psychoneuroendocrinology 1991;16:211-22.
57. Weaver DR, Deeds JD, Lee K, Segre GV. Localization of parathyroid hormone-related peptide (PTHrP) and PTH/PTHrP receptor mRNAs in rat brain. Brain Res Mol Brain Res 1995;28:296-310.
58. Jorde R, Waterloo K, Saleh F, Haug E, Svartberg J. Neuropsychological function in relation to serum parathyroid hormone and serum 25-hydroxyvitamin D levels. The Tromsø study. J Neurol 2006;253:464-70.
59. Louridas I, Thompson-Coon J, Dickens CM, Soni M, Kuźma E, Kos K, et al. Parathyroid hormone, cognitive function and dementia: a systematic review. PLoS One 2015;10:e0127574.
60. O'Lone E, Connors M, Masson P, Wu S, Kelly PJ, Gillespie D, et al. Cognition in People With End-Stage Kidney Disease Treated With Hemodialysis: A Systematic Review and Meta-analysis. Am J Kidney Dis 2016;67:925-35.
61. Groothoff JW, Grootenhuis M, Dommerholt A, Gruppen MP, Offringa M, Heymans HS. Impaired cognition and schooling in adults with end stage renal disease since childhood. Arch Dis Child 2002;87:380-5.
62. Stavosta RT, Vidor MV, Rizzi L, Marques F, Rocis-Cruz M. Haemodialysis improves uraemic patients' cognition: a pilot study. Alzheimer Dement Cogn Neurol 2016;1:1-3.
63. Schneider SM, Malecki AK, Müller K, Schönfeld R, Girardt M, Mohr P, et al. Effect of a single dialysis session on cognitive function in CKD5D patients: a prospective clinical study. Nephrol Dial Transplant 2015;30:1551-9.
64. Feig DI, Kang DH, Johnson RJ. Uric acid and cardiovascular risk. N Engl J Med 2008;359:1811-21.
65. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007;81:1-5.
66. Shao N, Lu W, Gao F, Li D, Hu J, Li Y, et al. Uric Acid Induces Cognitive Dysfunction through Hippocampal Inflammation in Rodents and Humans. J Neurosci 2016;36:10990-1005.
67. Iwata K, Watanabe H, Morisaki T, Matsuzaki T, Ohmura T, Hamada A, et al. Involvement of indoxyl sulfate in renal and central nervous system toxicities during cisplatin-induced acute renal failure. Pharm Res 2007;24:662-71.

68. Sirich TL, Meyer TW, Gondouin B, Brunet P, Niwa T. Protein-bound molecules: a large family with a bad character. Semin Nephrol 2014;34:106-17.
69. Sachdev PS. Homocysteine and brain atrophy. Prog Neuropsychopharmacol Biol Psychiatry 2005;29:1152-61.
70. Gao L, Zeng XN, Guo HM, Wu XM, Chen HJ, Di RK, et al. Cognitive and neurochemical alterations in hyperhomocysteinaemic rat. Neurol Sci 2012;33:39-45.

Molecular Genetics and Metabolism Reports 21 (2019) 100544

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

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

Case Report

Rare *GBA1* genotype associated with severe bone disease in Gaucher disease type 1

Livia d'Avila Paskulin[a,b,*], Rodrigo Tzovenos Starosta[a], Vitória Schütt Zizemer[c], Suélen Basgalupp[d], Débora Bertholdo[e], Filippo Pinto e Vairo[f,g], Marina Siebert[h,i], Kristiane Michelin-Tirelli[b], Ida Vanessa Doederlein Schwartz[a,b,c,i]

[a] Post-Graduation Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Post-Graduation Program in Medical Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[d] Hospital Moinhos de Vento, Porto Alegre, RS, Brazil
[e] DASA Clinic, Curitiba, PR, Brazil
[f] Center of Individualized Medicine, Mayo Clinic, Rochester, MN, USA
[g] Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA
[h] Molecular and Protein Analysis Unit, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[i] BRAIN Laboratory, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

ARTICLE INFO

Keywords:
Gaucher disease
GBA1
Bone disease
Genotype
Phenotype

ABSTRACT

Introduction: Gaucher disease (GD) type 1 is a lysosomal disease characterised by hepatosplenomegaly, anemia, thrombocytopenia, bone changes, and bone marrow infiltration. The disease is caused by biallelic pathogenic variants in *GBA1* which codes for glucocerebrosidase, an enzyme involved in the catabolic pathway of complex lipids.

Aims: To report on the case of two sisters with GD type 1 who bear a genotype never reported in the literature.

Case report: Patient 1 is a 47-year-old female diagnosed at 42 years of age with chronic lumbar pain, mild splenomegaly, slightly reduced platelets and normal hemoglobin values, severe Bone Marrow Burden (BMB) score, high chitotriosidase activity, and low glucocerebrosidase. Patient 2 is a 50-year-old female, sister of patient 1, who was diagnosed after familial screening. At 45 years of age, she had osteonecrosis of the left femur and a total hysterectomy because of uncontrollable bleeding. At first evaluation, she had bone pain with a high BMB score, mild splenomegaly, normal hemoglobin, normal platelets count, elevated chitotriosidase activity, and low glucocerebrosidase activity. Both patients were found to be compound heterozygotes for the p.Glu388Lys and the p.Ser405Asn variants in *GBA1*.

Conclusions: This is the first family with GD and this combination of variants which causes a phenotype remarkable for severe bone disease with no or mild hematological manifestations.

1. Introduction

Gaucher disease (GD) is one of the most common lysosomal disorders with an overall frequency of one in 40,000 newborns worldwide [1]; it is caused by deficient activity of *GBA1*-coded lysosomal glucocerebrosidase (acid beta-glucosidase), which leads to a building up of glucocerebroside in macrophages thereby known as "Gaucher cells". Accumulation inside the macrophages causes malfunction and shifts their activation profile [2], ultimately leading to systemic inflammatory response [3] and symptoms such as hepatosplenomegaly, thrombocytopenia, osteonecrosis, and, in some patients, neurological deficits. GD is categorised into three types according to neurological compromising: GD type 1 is characterised by no overt neurological symptoms; GD type 2 (acute neuronopathic), by an acute and fatal neurological compromise at early age; and GD type 3 (chronic neuronopathic), by neurological compromise with onset at late childhood or at adulthood. The first specific treatment for GD was enzyme replacement therapy (ERT), with biweekly infusions of imiglucerase, velaglucerase alfa, or taliglucerase alfa. ERT offers a significant improvement for all clinical parameters, except neurological impairment. Substrate reduction therapy (SRT) with miglustat, a daily oral drug, also showed some improvement, but not as great as ERT. Eliglustat is also a SRT, and has been

* Corresponding author at: Serviço de Genética Médica – Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, Brazil.
E-mail address: livia.paskulin@gmail.com.br (L. d'Avila Paskulin).

https://doi.org/10.1016/j.ymgmr.2019.100544
Received 8 September 2019; Received in revised form 11 November 2019; Accepted 12 November 2019
Available online 22 November 2019

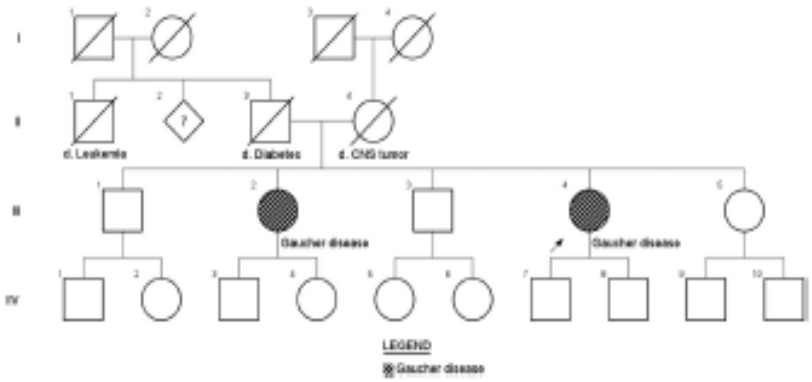


Fig. 1. Family history. Patient 1 is the III4 and patient 2 is the III2. The individuals III1, III3 and III5 were biochemically screened negative (normal glucocerebrosidase activity).

used as a first line therapy for GD [4,5]. GD presents a broad range of phenotypes that are partially explained by the different *GBA1* genotypes; therefore, we herein report on two sisters with a novel genotype associated with severe bone disease and mild or no hematological phenotype.

2. Case reports

Patient 1 is a 47-year-old female diagnosed with GD type 1 at the age of 42 years. She was born to a non-consanguineous couple and has 4 siblings of whom 3 were healthy and screened negative for GD, and one sister was symptomatic, described below as patient 2, see Fig. 1. There is no history of Parkinsonism or other neurologic symptoms in the family.

She was referred to the GD Reference Center at the Hospital de Clínicas de Porto Alegre (HCPA), Brazil, because of hyperferritinaemia (ferritin = 588 ng/mL) resistant to phlebotomy treatment; chronic lumbar pain (Visual Analogue Scale = 8); and recurrent epistaxis. Laboratory exams at admission showed hemoglobin of 13 g/dL, leukocyte count at 2320 cells/mm^3, platelets at 143,000/mm^3, and chitotriosidase activity at 9609 nmol/h/mL (NRV = 8.8–132). Abdominal ultrasonography revealed normal liver and spleen volumes. She had normal bone metabolism markers (parathyroid hormone, alkaline phosphatase, calcium, phosphate, and vitamin D), bone mineral density (BMD) with normal Z scores, however the bone marrow burden score (BMB) was 14/16 [Fig. 2] [6]. Glucocerebrosidase activity was 5 nmol/h/mg prot in leukocytes (NRV = 10–45 nmol/h/mg prot) and 132 nmol/h/mg prot in fibroblasts (NRV = 257–688 nmol/h/mg prot) confirming the diagnosis of GD type 1. At diagnosis, the Disease Severity Scoring System [7] (DSS) was 3.6/19 (scoring only in bone subscore) and the Severity Score Index [8] (SSI) was 5/49. The patient started treatment with miglustat 300 mg/day and followed a low-carbohydrate diet. Soon after, due to diarrhea and unintended 6 kg weight loss (10% of total body weight), the patient was found to also have lactose intolerance (lactase non-persistence CC-genotype) and strongyloidiasis, and received treatment with lactose-free diet and albendazol. Due to persistence of gastrointestinal symptoms and slight clinical improvement (see Table 1), miglustat was switched after one year to taliglucerase alfa 30UI/kg/biweekly; since the patient presented an allergic reaction after 2 months of infusions to taliglucerase, it was switched to imiglucerase 30UI/kg/biweekly (see Table 1) - which regimen has been kept uneventfully, with improvement of the symptoms (Table 1).

Patient 2 is a 50-year-old female diagnosed with GD type 1 when she was 45 years old. Four years before the first appointment with Medical Genetics, the patient underwent a prosthetic replacement of the left femoral-acetabular joint for osteonecrosis, and, one year after, underwent total hysterectomy for uncontrollable bleeding during uterine polyp removal surgery. Laboratory tests at admission to our Center showed hemoglobin at 11.5 g/dL, leukocyte count at 8710 cells/mm^3, platelets at 195,000/mm^3, ferritin of 880 ng/mL, and chitotriosidase activity of 2970 nmol/h/mL (NRV = 8.8–132). Further investigation revealed mild hepatosplenomegaly and hepatic steatosis by abdominal ultrasonography. She had normal bone metabolism markers, BMD with normal T scores and BMB of 14/16 [Fig. 3]. Glucocerebrosidase activity of 2.8 nmol/h/mg protein in leukocytes (NRV = 10-45 nmol/h/mg protein) and 60 nmol/h/mg protein in fibroblasts (NRV = 257-688 nmol/h/mg protein) confirmed the diagnosis of GD. The severity scores were DSS = 2/19 (scoring only in bone subscore) and SSI = 1/49. Because of needle phobia, she started on treatment with miglustat 300 mg/day together with a low-carbohydrate diet.

After 3 years of treatment with miglustat with unsatisfactory response (Table 1), treatment was switched to taliglucerase alfa 15UI/kg/biweekly. After 2 years of treatment with ERT, the hematological parameters and chitotriosidase activity improved, however ferritin remained high and bone marrow infiltration remained severe.

Upon genotyping with next-generation sequencing (NGS) both patients 1 and 2 were discovered to be compound heterozygotes for c.1162G > A (p.Glu388Lys) (E349K) and c.1214G > A (p.Ser405Asn) (S366N) *GBA1* pathogenic variants. Both patients tested negative for the chitotriosidase gene (CHIT1) null variant. Patients are also heterozygote for the *HFE1* pathogenic variant c.187C > G (p.His63Asp).

3. Discussion

3.1. Genotype

The patients described herein were compound heterozygotes for two uncommon *GBA1* pathogenic variants, E349K and S366N. The former has been previously described by Grabowski and colleagues in 2006 [9]; however, no clinical phenotype description nor if it was in homozygosis or compound heterozygosis with a different variant was provided. The latter, was described in compound heterozygosis with R48W (p.Arg87Trp) by Demina and Beutler in 1998 [10] in an African-American female GD type II patient whose sister had anemia, mild thrombocytopenia, mild neutropenia, and moderate hepatosplenomegaly – however, no more details on the patient's phenotype are provided. The E349K residue is on a coil motif at the eighth exon, in a region of neutral hydropathy. This variant is predicted to cause a reduction of 88% of the normal enzyme activity [11]. The S366N variant lays on an alpha-helix at the 3' end of the eighth exon, in a region of neutral hydropathy, and impairs a phosphorylation site. Of note, the combination of these variants in our patients caused enzyme activity higher than expected for classical GD patients.

Both variants are considered pathogenic when applying the ACMG [12] classification criteria: they are absent from gnomAD (PM2), were

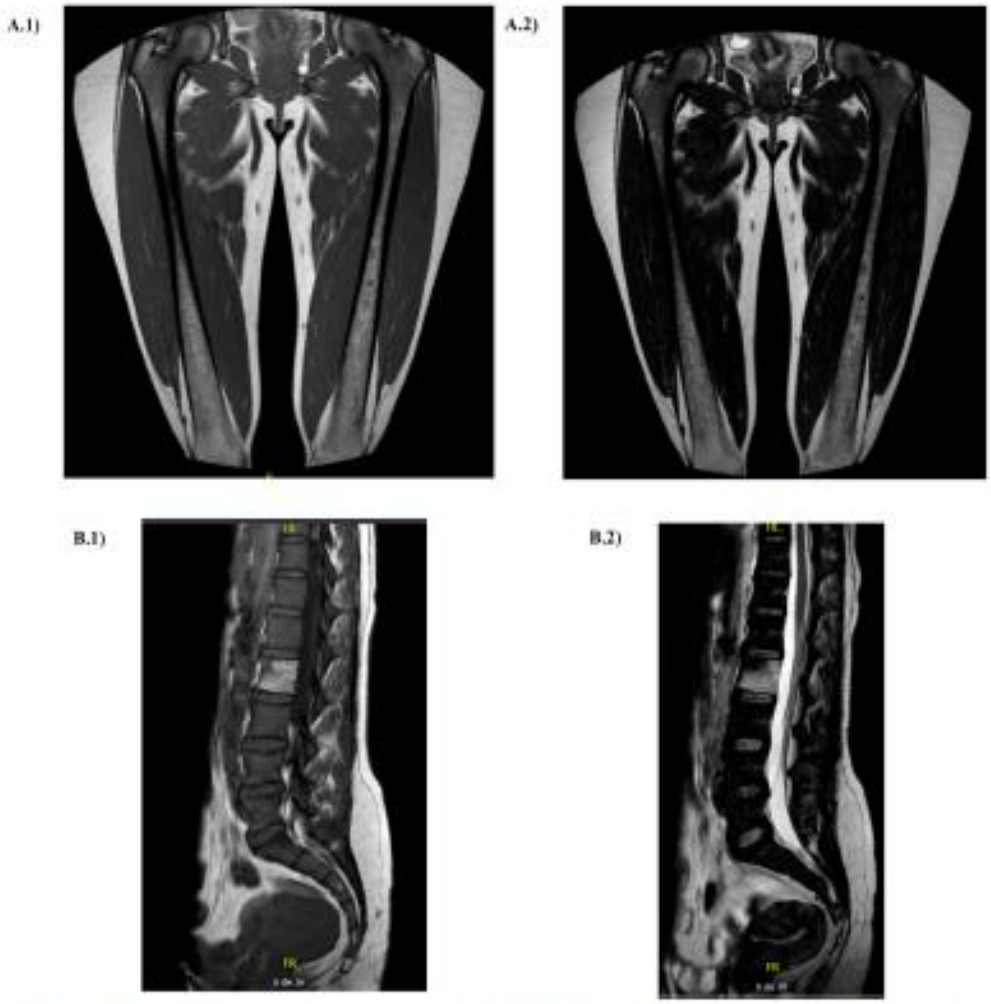


Fig. 2. MRI images of patient 1 at baseline. A.1) Coronal T1-weighted image of femurs. A.2) Coronal T2-weighted image of femurs. B.1) Sagittal T1-weighted image of lumbar spine. B.2) Sagittal T2-weighted image of lumbar spine. Femur total score = 6 (T1 = 2, T2 = 2, Sites = 2); Lumbar spine total score = 8 (T1 = 3, T2 = 2, Pattern = 3).

previously detected in trans with a pathogenic variant (PM3), multiple in silico algorithms (such as DANN, FATHMM-MKL, SIFT, LRT, MutationTaster) predict both variants to be deleterious (PP3), patient's phenotype and family history are highly specific for GD (PP4), UniProt classifies this variant as 'disease' (PP5), and the variant segregates with the phenotype in a gene definitively known to cause the disease (PP1).

4. Phenotype

Much is being studied about secondary modifier genes in Mendelian disorders, including GD [13–15]; however, still little is known about how strong is the genotype-phenotype association in GD. In the presented case, both patients harboured the same variants in *GBA1*, and although quite similar overall, there were some differences between the two sisters' phenotypes: while patient 2's bone phenotype may be considered somewhat more severe, patient 1's chitotriosidase – a biomarker for GD activity – was more than three times higher at admission than patient 2's. Liver and metabolic profiles, on the other side, were quite similar. This perhaps may be explained by the action of an unidentified modifier gene harboured by only one of the patients, or it may be due to environmental factors.

Osteonecrosis is a common manifestation of GD, with up to one third of GD patients experiencing it [16,17]. The most common site affected is the femoral head [18], as was the case of patient 2. In a study published by the International Collaborative Gaucher Group (ICGG) searching for risk factors for osteonecrosis [16], the only identified ones were anemia and splenectomy. Being their genotype for *GBA1* the same and neither having been submitted to splenectomy, we cannot but wonder whether patient 2 being anemic at admission was related to her having had osteonecrosis, and her sister, which was not anemic at admission, having it not.

Another common hallmark of GD is bone marrow infiltration, which can be best assessed through the method of Dixon quantitative chemical shift (Dixon's QCSI) [19], but unfortunately this method is not available worldwide. Because of that, different other semiquantitative methods are worldwide used to measure the bone marrow infiltration [6,20]. The method that correlates the best with the Dixon's QCSI method, and evaluates both the axial and the peripheral skeleton is the MRI-based BMB score published by Maas et al., which relies on signal intensity as a measure of fat substitution for Gaucher cells in the bone marrow of femurs and lumbar spine [6]. Bone manifestations of GD are secondary to Gaucher cells infiltration in the bone marrow, together with possible phenotype modifiers genes [21]. What constitutes severe bone disease in GD is open to debate. Although both sisters presented with a normal BMD and no fractures, and only one had hip necrosis, both had a severe BMB score, which may imply a more severe bone phenotype caused by

Table 1
Hematological parameters, chitotrioxidase activity, bone marrow burden score and DS3 subscores during treatment periods of both patients.

	Patient 1	Patient 2
***GBA1* genotype**	E349K/S366N	E349K/S366N
Glucocerebrosidase activity		
Leucocytes (NRV: 10–45 nmol/h/mg protein)	5	2.8
Fibroblast (NRV 257–668 nmol/h/mg protein)	132	60
Baseline		
Age at diagnosis (years)	42	45
Bone Marrow Burden score	13	14
Hb (g/dL)	14.6	11.6
Platelets (/μL)	143,000	192,000
Ferritin (ng/mL)	588	880
Chitotriosidase activity (nmol/h/mL)	15,581	3432
DS3 subscores		
Bone	3.6	2
Visceral	0	0
Hematological	0	0
After 1 year of treatment		
Drug	Miglustat 300 mg/day	Miglustat 300 mg/day
Time in months of the current treatment	12	12
Bone Marrow Burden score	13	14
Hemoglobin (g/dL)	14	11.7
Platelets (/μL)	172,000	207,000
Ferritin (ng/mL)	536	1160
Chitotriosidase activity (nmol/h/mL)	8591	1667
DS3 subscores		
Bone	3.6	2
Visceral	0	0
Hematological	0	0
After 2 years of treatment		
Drug	Imiglucerase 30 U/kg/inf	Miglustat 300 mg/day
Time in months of the current treatment	2	24
Bone Marrow Burden score	NA	14
Hemoglobin (g/dL)	14.5	11.8
Platelets (/μL)	224,000	184,000
Ferritin (ng/mL)	937	1117
Chitotriosidase activity (nmol/h/mL)	5821	1984
DS3 subscores		
Bone	3.6	2
Visceral	0	0
Hematological	0	0
After 3 years of treatment		
Drug	Imiglucerase 30 U/kg/inf	Taliglucerase 15U/kg/inf
Time in months of the current treatment	14	1
Bone Marrow Burden score	3	14
Hemoglobin (g/dL)	14.5	12.4
Platelets (/μL)	259,000	202,000
Ferritin (ng/mL)	608	1025
Chitotriosidase activity (nmol/h/mL)	1472	1689
DS3 subscores		
Bone	1.6	2
Visceral	0	0
Hematological	0	0
After 4 years of treatment		
Drug	Imiglucerase 30 U/kg/inf	Taliglucerase 15U/kg/inf
Time in months of the current treatment	36	13
Bone Marrow Burden score	NA	NA
Hemoglobin (g/dL)	15.3	13.2
Platelets (/μL)	287,000	234,000
Ferritin (ng/mL)	689	1053
Chitotriosidase activity (nmol/h/mL)	881	1133
DS3 subscores		
Bone	1.25	0
Visceral	0	0
Hematological	0	0

NA = Not Available.

the unusual combination of the E349K/S366N *GBA1* variants. Besides that, as the DS3 subscores show, for both patients the compromise of bone is more severe than the compromise of visceral and hematological systems. Also, the pattern of decrease of the BMB score during treatment shows that patient 1, who was being treated with ERT for 24 months, presented a fast response with a significantly drop in the total score when compared to patient 2, who was beign treated with SRT for 24 months and only 1 month with ERT. This is in accordance with previous studies that have shown that BMB tend to decrease during the first years of ERT, but this cannot be observed with SRT, and, also, the response is not known to reflect disease severity [22,23].

At admission, neither patient was profoundly thrombocytopenic nor anemic. Nor did the patients present overt hepatosplenomegaly, although patient 2 had mild hepatosplenomegaly and mild hepatic steatosis. Overall, the patients could be described as having predominantly severe bone disease and few, mild visceral and hematological manifestations. Whether this is due to environmental factors or indeed to the patients' rare genotype is still unclear, and more reports of patients with the same *GBA1* genotype are needed before a conclusion may be confidently drawn. Response to substrate reduction therapy with miglustat was not satisfactory for both sisters, whereas response to enzyme replacement therapy was satisfactory regarding hematological and visceral parameters; both patients reached their goals following the Brazilian Guideline [24] and the European Working Group on Gaucher Disease in 2018 [25].

Mehta *et al* published in 2019 [26] the presenting signs and patient co-variables in Gaucher disease, and highlighted that physicians can fail to recognise the early stages of GD, which can lead to significant diagnostic delays and sometimes irreversible but avoidable morbidities. When it comes to a classic GD phenotype with massive splenomegaly, bone pain and cytopenias, diagnosis is more intuitive. On the other hand, if the patient has mild symptoms, or, as in our patients' cases only bone disease, the diagnosis becomes trickier and less intuitive, requiring greater expertise to be defined.

5. Conclusions

This is the first GD family with the E349K/S366N *GBA1* genotype which is associated with severe bone disease and mild visceral and hematological manifestations. More genotype-phenotype studies are needed to fully establish a causational relationship between this rare genotype and the patients' unique phenotype.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the staff from Hospital de Clínicas, particularly

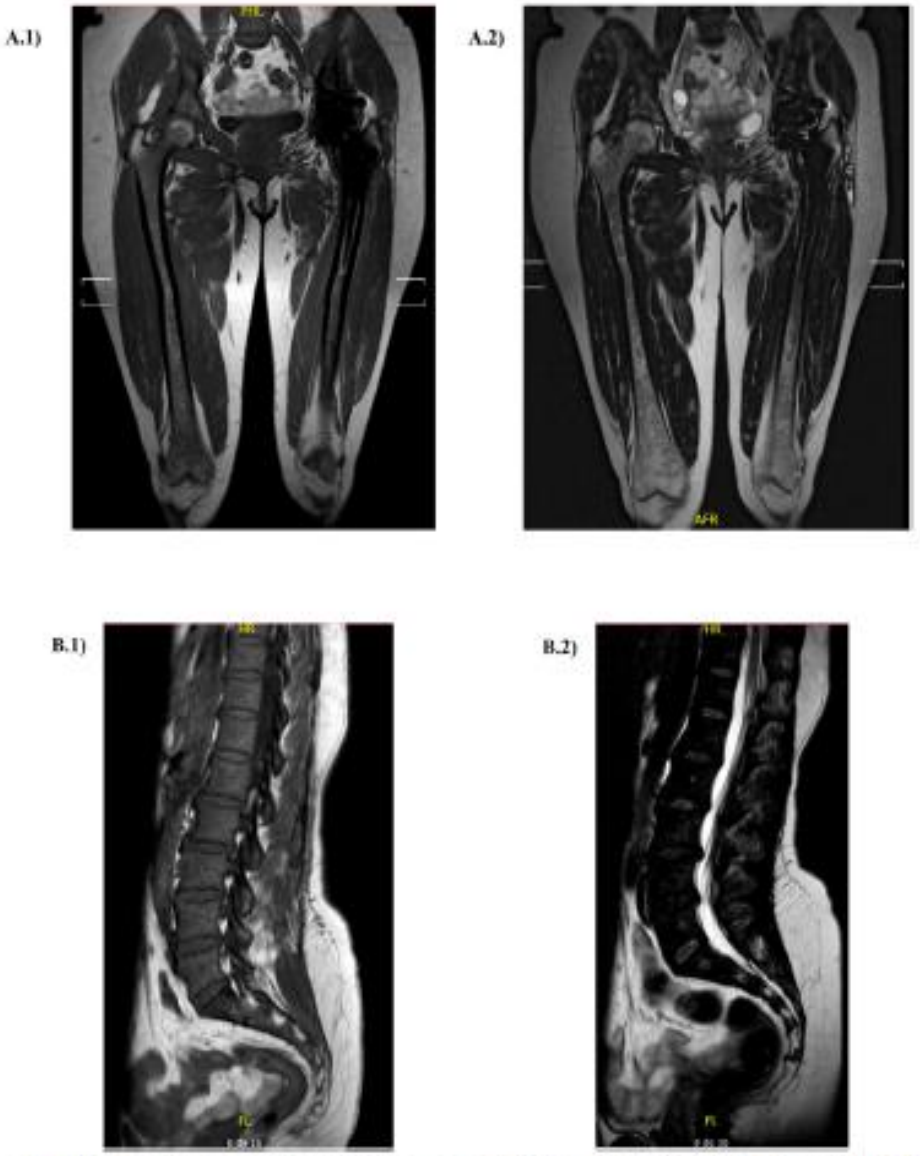


Fig. 3. MRI images of patient 2 at baseline. A.1) Coronal T1-weighted image of femurs. A.2) Coronal T2-weighted image of femurs. B.1) Sagittal T1-weighted image of lumbar spine. B.2) Sagittal T2-weighted image of lumbar spine. Femur total score = 7 (T1 = 2, T2 = 2, Sites = 3); Lumbar spine total score = 7 (T1 = 2, T2 = 2, Pattern = 3).

from the Medical Genetics Service, for their help, and Dr. Jorge Luiz dos Santos from the Gastroenterology and Hepatology Experimental, for the molecular analysis of Lactase Phlorizin Hydrolase. Financial support for this study was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundo de Incentivo à Pesquisa e Eventos (FIPE) of the HCPA.

References

[1] G.A. Grabowski, Phenotype, diagnosis, and treatment of Gaucher's disease, Lancet 372 (2008) 1263–1271.
[2] L.A. Boven, et al., Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages, Am. J. Clin. Pathol. 122 (2004) 359–369.
[3] T. Koppe, et al., The prognostic value of the serum ferritin in a southern Brazilian cohort of patients with Gaucher disease, Genet. Mol. Biol. 39 (2016) 30–34.
[4] S. Revel-Vilk, J. Szer, A. Mehta, A. Zimran, How we manage Gaucher disease in the era of choices, Br. J. Haematol. 182 (2018) 467–480.
[5] R.M. Poole, Eliglustat: First global approval, Drugs 74 (2014) 1829–1836.
[6] M. Maas, et al., Quantification of bone involvement in Gaucher disease: MR imaging bone marrow burden score as an alternative to Dixon quantitative chemical shift MR imaging—initial experience, Radiology 229 (2003) 554–561.
[7] N.J. Weinreb, et al., A validated disease severity scoring system for adults with type 1 Gaucher disease, Genet. Med. 12 (2010) 44–51.
[8] A. Zimran, et al., Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients, Med. 71 (1992) 337–353.
[9] B. Liou, et al., Analyses of variant acid β-glucosidases: effects of Gaucher disease mutations, J. Biol. Chem. 281 (2006) 4242–4253.
[10] D. A, E. Beutler, Six new Gaucher disease mutations, Acta Haematol. 99 (1998) 80–82.
[11] H.M. Berman, et al., The protein data bank, Nucleic Acids Res. 58 (2000) 235–242.
[12] S. Richards, et al., Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology 2015, Genet. Med. 17 (2015) 405–424.
[13] A.D. Klein, et al., Identification of modifier genes in a mouse model of Gaucher disease, Cell Rep. 16 (2016) 2546–2553.
[14] A. Greenwood, D. Eustace, A. Zimran, G. Abrahamov, Effect of vitamin D receptor (VDR) genotypes on the risk for osteoporosis in type 1 Gaucher disease, Clin. Rheumatol. 29 (2010) 1037–1041.
[15] F. Varki, et al., KIR genes and HLA class I ligands in Gaucher disease, Gene 516 (2013) 53–57.

[16] A. Khan, T. Hangartner, N.J. Weinreb, J.S. Taylor, P.K. Mistry, Risk factors for fractures and avascular osteonecrosis in type 1 Gaucher disease: A study from the international collaborative Gaucher group (ICGG) Gaucher registry, J. Bone Miner. Res. 27 (2012) 1839–1848.
[17] J. Cherrow, et al., The Gaucher registry: demographics and disease characteristics of 1698 patients with Gaucher disease, Arch. Intern. Med. 160 (2000) 2835–2843.
[18] M. Itzchaki, et al., Orthopedic considerations in Gaucher disease since the advent of enzyme replacement therapy, Acta Orthop. Scand. 75 (2004) 641–653.
[19] T.W. Dixon, Simple proton spectroscopic imaging, Radiology 153 (1984) 189–194.
[20] S. vom Dahl, et al., Evidence-based recommendations for monitoring bone disease and the response to enzyme replacement therapy in Gaucher patients, Curr. Med. Res. Opin. 22 (2006) 1045–1064.
[21] P. Mikosch, D. Hughes, An overview on bone manifestations in Gaucher disease, Wien. Med. Wochenschr. 160 (2010) 609–624.
[22] B. Fedida, et al., Bone marrow involvement in Gaucher disease at MRI: what long-term evolution can we expect under enzyme replacement therapy? Eur. Radiol. 25 (2015) 2969–2975.
[23] G.M. Pastores, N. Barnett, E. Kolodny, An open-label, noncomparative study of Miglustat in type 1 Gaucher disease: efficacy and tolerability over 24 months of treatment, Clin. Ther. 27 (2005) 1215–1227.
[24] P.D. Picon, M.I.P. Gadelha, R.F. Alexandre, Doença de Gaucher, Protoc. Clínicos E Diretrizes Ter. Secr. Atenção À Saúde - Brasília Ministério Da Saúde 2 (2014) 159–194.
[25] M. Biegstraaten, et al., Management goals for type 1 Gaucher disease: an expert consensus document from the European working group on Gaucher disease, Blood Cells Mol. Dis. 68 (2018) 203–208.
[26] A. Mehta, et al., Presenting signs and patient co-variables in Gaucher disease: outcome of the Gaucher Earlier Diagnosis Consensus (GED-C) Delphi initiative, Intern. Med. J. 49 (2019) 578–591.

Leite et al. Surgical and Experimental Pathology (2020) 3:4
https://doi.org/10.1186/s42047-020-0055-6

Surgical and Experimental Pathology

RESEARCH Open Access

Corrected integrated density: a novel method for liver elastic fibers quantification in chronic hepatitis C

Carine Leite[1*], Rodrigo Tzovenos Starosta[2], Eduardo Neubarth Trindade[3], Manoel Roberto Maciel Trindade[1,3], Mário Reis Álvares-da-Silva[1,4] and Carlos Thadeu Schmidt Cerski[1,5]

Abstract

Background: Elastic fibers deposition is triggered during liver fibrosis and is related to worse clinical prognosis in chronic hepatitis C patients. This study aimed to verify if a new method for elastic fiber quantification can be used to discriminate between different degrees of fibrosis in liver biopsies of patients with hepatitis C.

Methods: Individuals presenting with different degrees of fibrosis in liver biopsy were included. Slides of liver samples were stained with orcein with and without prior oxidation. Morphometric analysis was proceeded, and quantification accomplished by corrected integrated density.

Results: Twenty-seven patients, mean age 52 years-old, 59% women, were included. Elastic fibers density was higher in advanced fibrosis patients and there was a positive correlation with Metavir score (*Spearman* $r = 0.609$, $p < 0.001$), as well as with the noninvasive scores Fib-4 (*Pearson* $r = 0.46$, $p = 0.029$) and APRI ($r = 0.52$, $p = 0.01$).

Conclusion: Morphometric analysis by corrected integrated density demonstrates that elastic fibers abundance is higher in advanced stage of fibrosis in patients with hepatitis C.

Keywords: Liver fibrosis, Chronic hepatitis C, Elastic fibers

Background

Chronic hepatitis C affects 71 million people worldwide, and is a leading cause of cirrhosis and liver transplantation in most countries (Collaborators 2017). The grade of hepatic fibrosis is an important prognostic factor (Manns et al. 2017). To this purpose noninvasive methods have been developed, such as transient elastography, magnetic resonance elastography, and scores based on laboratory tests (Paranaguá-Vezozzo et al. 2017). Histological analysis of liver biopsy is the gold standard and allows for better staging, through the evaluation of fibrosis by the Metavir score (Bedossa and Poynard 1996).

During the development of liver fibrosis, new elastic fibers are deposited in the extracellular matrix, conferring tissue resistance. Elastin is an amorphous protein highly resistant to degradation; it is believed to be the most stable component among the extracellular matrix. It is degraded slowly and late in the reversion of fibrosis, and may lead to irreversibility of the process (Chen et al. 2019).

Elastic tissue is physiologically present in several human tissues, especially where greater elasticity is needed. In the healthy liver it is observed in small amounts, mainly in the wall of arterioles and in portal tracts (Kanta 2016). In these locations it exerts a structural function, providing stability to the bile ducts and vessels under conditions of high ductal pressure (Mederacke et al. 2013).

Elastic fibers are composed of two proteins, elastin and fibrillin, which are separated from each other instead of constituting bundles as is the case of collagen (Kielty et al. 2002). Fibrillin forms microfibrils, the mold to elastic fibers; elastin forms the fibers' core (Houghton et al. 2006). Chronic hepatitis C causes a cellular inflammatory response

*Correspondence: carlindoleite@hotmail.com
[1]Post-Graduate Program on Gastroenterology and Hepatology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
Full list of author information is available at the end of the article

as a result of oxidative stress and immune response to hepatocytes expressing viral epitopes (Neumann-Haefelin and Thimme 2013). This response generates release of cytokines and growth factors that activate stellate cells in myofibroblasts, which synthesize extracellular matrix (Geerts 2001, Franceschini et al. 2012). In this matrix small amounts of elastic fibers are found (Burt et al. 1990). Therefore, fibrogenesis results in deposition of elastin (Baiocchini et al. 2016). The immature (or oxytalan) fibers are present only as microfibrils in physiological conditions; however, during late elastogenesis, they generate mature elastic fibers. On histopathology, orcein is one of the selective methods for staining these fibers. When oxidation by peracetic acid or permanganate is accomplished, oxytalan fibers are visible (Nakayama et al. 2008, Kanta 2016).

An association of hepatic elastin with poor prognosis in patients with chronic hepatitis C has been found by some authors (Kendall et al. 2018). It is not well established if quantification of the fibers is directly related to fibrosis stage (Yasui et al. 2019).

We hypothesize that the hepatic inflammatory damage caused by the hepatitis C virus might lead to the deposition of elastin in the extracellular matrix. In this study, we aim at assessing whether a new method for elastic fiber quantification can be used to discriminate between different degrees of fibrosis in liver biopsies of patients with hepatitis C, thus confirming the quantitative relationship between elastin and fibrosis.

Methods

Patients

This was a retrospective study. Inclusion criteria were: chronic hepatitis C confirmed with positive hepatitis C virus PCR and anti-HCV test lasting for more than 6 months; ultrasound-guided liver biopsy performed between January 2010 and December 2012 at the Hospital de Clínicas de Porto Alegre (HCPA). Individuals with another liver disease or current alcohol abuse were excluded, as well as cases with samples measuring less than 0.7 cm in the longer axis (which are considered nondiagnostic). A total of 33 cases were included.

This study was approved by Ethics Committee of the HCPA number 3.227.637. Informed consent was waived due to its retrospective design, but authors signed a data usage responsibility term. The study was performed according to the guidelines of the Declaration of Helsinki, 1975.

Study variables

Demographic data, clinical history and biochemical tests were obtained from electronic records. Non-invasive fibrosis scores were calculated as follows: FIB-4 (Fibrosis-4) = age (years) x AST (IU/L) / platelets (10^9/L) x √ ALT (IU/L); APRI (AST to platelets Ratio Index) = AST (UI/L) / ALT upper limit of normal × 100 / platelets (10^9/L) (Trang et al. 2008).

Histopathological procedures

The paraffin-embedded liver samples were sectioned into 4 μm-thick sections, deparaffinized and stained according to the standard procedures of the Department of Surgical Pathology of the HCPA: one slide was stained with hematoxylin-eosin, the other with Sirius Red staining, for Metavir score. They were reviewed by a second expert pathologist. The blocks were reprocessed for staining with orcein to verify mature elastic fibers, and a second slide was submitted to oxidation by potassium permanganate to highlight oxytalan fibers. The elastic fibers distribution (restricted or not to portal tracts) was verified. Those slices were submitted to morphometry.

Morphometric analysis

The slides were analyzed with Olympus BX41 microscope in a magnification of 200X. Following microscope calibration, each fragment was photographed in its entire length with the Olympus DP73 microscope-attached camera and the CellSens® software. Five hundred images were generated and saved in TIFF format to allow for better resolution. The images were converted on ImageJ 1.8.0_112 to grayscale 8-bit images (Schneider et al. 2012) and analyzed morphometrically as follows: to find the optical density of the elastic fibers, the gray value threshold was determined manually for each image to distinguish the particles (that is, the areas with gray values within the threshold range) from the background. The micrometer-to-pixel scale was calculated from the scale generated by CellSens® when each image was obtained.

Area and mean gray value (MGV) of each particle were obtained with ImageJ. The corrected density in absorbance-micrometer^2 per particle was obtained by the formula (255-MGV)*area (Hartig 2013). Artifacts and white areas were manually removed using the "wand" tool with a tolerance of 10. The sum of the corrected densities of all particles (corrected integrated density, CID; also known as corrected absorbance) corresponds to the quantification of elastic fibers in that region of the fragment, and the sum of CIDs of all the photographs corresponds to the sum of all densities in that individual slide. The division of the summed CIDs by the number of photographs informs the mean CID of elastic fibers per photograph, which was used in our analysis. The same procedures were repeated by a second blinded observer.

Statistical analysis

To detect difference in the presence of elastic fibers outside portal tracts, a minimum of six early and advanced

fibrosis patients were needed (Nakayama et al. 2008). Categorical variables were shown as number and proportion in percentage, and continuous variables were presented as mean and standard deviation. To compare between groups, quantitative variables presented symmetric distributions and Student t-test was performed. To assess correlation between two continuous or ordinal variables, Pearson or Spearman's rank-test were performed. A 95% interval confidence calculated by Bland-Altman was used to verify concordance between observers. Statistical significance was defined as $p < 0.05$.

Results

A total of 33 patients were included, 5 were excluded due to insufficient sample (scarce material after reprocessing), and 1 due to the presence of concomitant hepatocellular carcinoma, comprising 27 individuals with ultrasound-guided needle biopsy. The mean age was 52 years, with a predominance of women. Arterial hypertension was the most frequent comorbidity (Table 1).

Elastin was verified in orcein-stained slides as described in methods (example in Fig. 1). Elastic fibers outside portal tracts were detected in only one of 15 patients F0–2 and in 8 of 12 advanced fibrosis ($p = 0.12$).

Table 1 Baseline sample characteristics

	Total 27
Age (years)	52.3 (11.15)
Female (%)	16 (59%)
White (%)	25 (92.59)
Hypertension (%)	7 (26%)
Former alcohol abuse (%)	8 (29.6%)
Genotype	
1	12
2	2
3	11
4	0
Coinfection 1 + 3	1
FIB-4	2.58 (1.77)
APRI	1.3 (0.9)
Platelets (10^3/mm^3)	191.11 (66.97)
INR*	1 (0.07)
Total bilirubin (mg/dL)	0.74 (0.22)
Albumin (mg/dL)	4.23 (0.26)
Fibrosis Grade - Metavir	
F0	4
F1	5
F2	6
F3	5
F4	7

* *INR* International normalized ratio, *FIB-4* Fibrosis-4, *APRI* AST to platelets Ratio Index, percentage or standard deviation between parenthesis

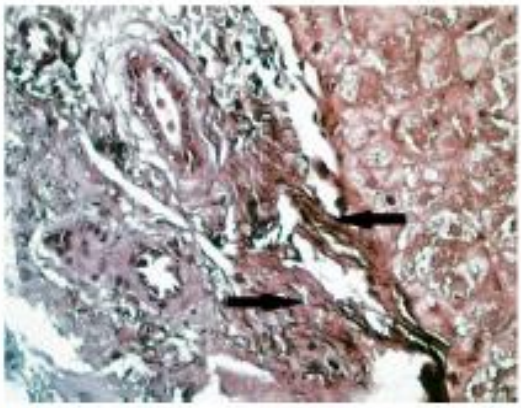


Fig. 1 Representative case of F4 fibrosis, stained with orcein, 400x magnification. Elastic fibers are thin structures (black arrows), surrounded by collagen (thicker and lighter color)

The mean elastic fibers density was significantly higher in the group with advanced fibrosis (Metavir F3-F4) compared to other individuals (Fig. 2). A higher mean was also observed in this group when analyzing oxytalan fibers, $p = 0.027$ (Fig. 3).

We also graded the quantification according to terciles. None of the F0-F2 patients was in the highest tercile, while 50% of the F3-F4 patients were in this group ($p = 0.003$). Descriptive analysis was undertaken. The group of patients with elastic fibers outside portal tracts had a higher fibers' density than the other individuals ($p = 0.015$).

The mean of difference in quantification between observers was -414.72×10^3 and was not significant (IC 95% -342.8 to 259.86, $p = 0.3$). The Metavir fibrosis stage correlated linearly with the elastic fibers' density (Fig. 4). There was no significant correlation between oxytalan fibers and degree of fibrosis (Spearman correlation coefficient $r = 0.38$ $p = 0.063$). Activity score and genotype did not correlate to elastic fibers density.

We also analyzed noninvasive markers of fibrosis. The Fib-4 score showed a positive significant correlation with the elastic fibers' quantification (Pearson correlation coefficient $r = 0.46$, $p = 0.029$), as well as APRI ($r = 0.52$, $p = 0.01$). There was a correlation in the limit of significance between Fib-4 and oxytalan fibers ($r = 0.453$, $p = 0.052$), but it was not observed for APRI ($r = 0.366$, $p = 0.12$).

All included cirrhotic individuals were classified clinically as Child-Pugh A. Only four among those had cirrhosis with complications (three cases of medium or large esophageal varices and one hepatocellular carcinoma). The mean elastic fibers density of this subgroup compared to others with advanced fibrosis were higher, but

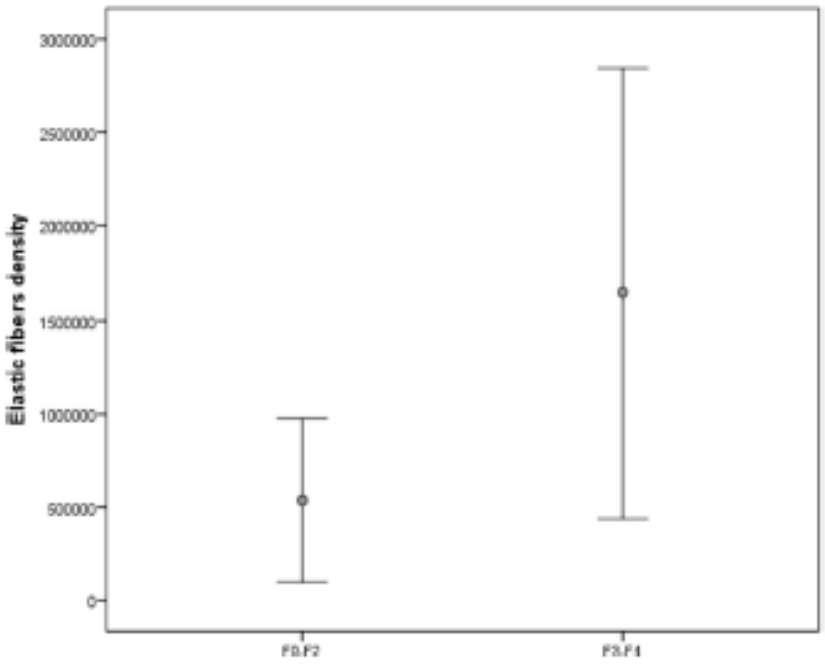


Fig. 2 Elastic fibers density in groups of early and advanced fibrosis. F0-F2: 536.72 × 10^3 absorbance-micrometer^2 (438.83 × 10^3) X F3-F4: 1641.04 × 10^3 absorbance-micrometer^2 (1202.75 × 10^3), $p = 0.001$

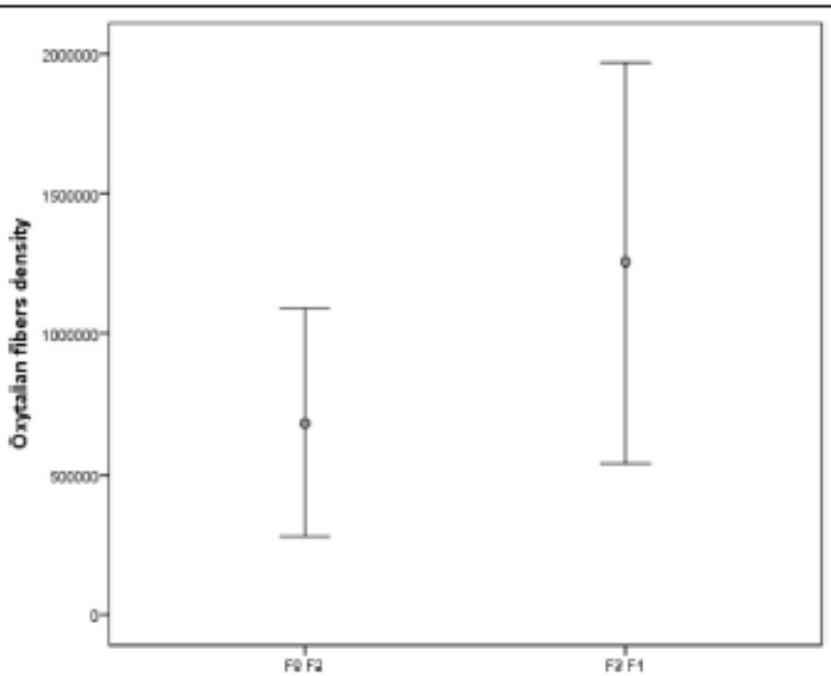


Fig. 3 Oxytalan fibers density in groups of early and advanced fibrosis. F0-F2: 683.87 × 10^3 absorbance-micrometer^2 (404.35) X F3-F4: 1254.4 × 10^3 absorbance-micrometer^2 (714.08 × 10^3), $p = 0.027$

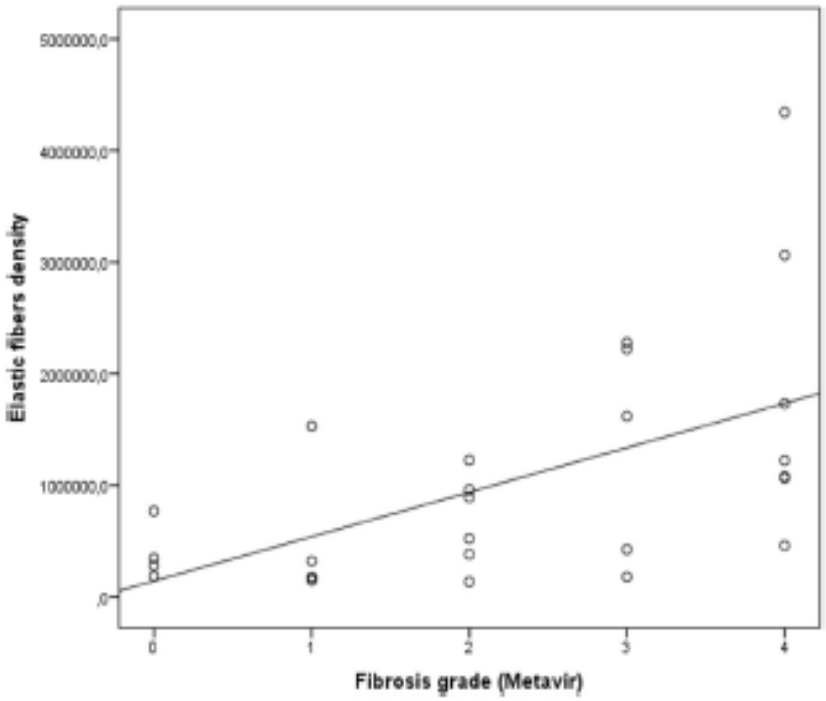


Fig. 4 Correlation of stage fibrosis and elastic fibers density, Spearman test, $r = 0.609$, $p < 0.001$

not significant: 2214.96×10^3 absorbance-micrometer^2 (standard deviation = 1845.73) versus 1449.74×10^3 absorbance-micrometer^2 (standard deviation = 986.26), $p = 0{,}1$.

Discussion

This study revealed a positive correlation between the grade of hepatic fibrosis and density of elastic fibers, especially those with amorphous material (elastin). We found that noninvasive scores may also presume the degree of deposition of hepatic elastin. Fibrosis scores such as Metavir are ordinal or semi-quantitative. Morphometric analysis allows a more reliable evaluation of the deposition of extracellular matrix structures in fibrogenic processes, since it is quantitative (Goodman et al. 2007). Other advantages over conventional visual assessment are the objectivity, reproducibility, and the ability to detect changes not apparent to the naked eye (Hamilton and Allen 1995). In hepatitis C patients who are non-responders to treatment, morphometric analysis demonstrated relevant worsening in fibrosis (80% increase of collagen content) in 4 years (Goodman et al. 2009). Only by direct measurement of fibrosis this change can become apparent, because histological stages are neither a continuous function nor a sensitive measure.

Hepatic elastic fibers have been first evaluated in non-alcoholic fat liver disease, with a higher amount found in the zone three of patients with advanced fibrosis (Nakayama et al. 2008). These fibers have emerged as new focus of research due to its possible relationship with worse prognosis in chronic hepatitis C and advanced fibrosis (Houghton et al. 2006, Kendall et al. 2018). Contrary to what was previously assumed, elastic fibers have been found in greater quantity in liver biopsies with F3 fibrosis in relation to F2, but there was no evidence of a linear relation with a fibrosis score by Metavir (Yasui et al. 2019). In these work, authors have quantified elastic fibers by measuring the percentage in which they occur in the total area of the fragment (Abe et al. 2013). In our work, on the other hand, we measure the absorbance by corrected integrated density. This measure is more reliable and accurate because it verifies not only the affected area, but also the elastic fiber concentration or density, which may explain the difference of our findings. As in previous published literature, we found a positive correlation between non-invasive fibrosis scores and the fibers quantification (Masugi et al. 2018, Yasui et al. 2019).

The reported association of elastin with poor prognosis (Kendall et al. 2018) might be explained by different stages of fibrosis in cirrhotic patients. The quantification of elastin allows more information than just the grade in Metavir, which does not reflect the heterogeneity of patients in the subgroup F4.

Our study has as limitation the non-evaluation by the other quantification method, the percentage of affected area, to compare the findings. This technique would make this study more expensive, mostly because of the

need of a scanner. However, we believe that the rigorous methodology makes our findings reliable. Also, the descriptive analysis correlated to our quantification. Another limitation was the small sample, which did not allow us to adequately measure statistical significance to evaluate the prognosis of the individuals. However, this was not a primary goal; we plan to conduct these analyses in another study with an increased sample. In addition, due to the cross-sectional design, this study does not provide information on more specific aspects of fibrosis triggers and elastic fibers synthesis.

Conclusion

The elastic fibers are in higher concentration in advanced stages of fibrogenesis. Our quantification method adds relevant information to liver pathology study in chronic hepatitis C. Although labor-intensive, this method has the advantage of being more precise than simple histopathological evaluation or less complex methods of quantification, mainly because it concatenates two dimensions of elastin deposition – the area occupied by elastic fibers and the density of these fibers in such areas – thus being a good fit for more complex experiments and for precision medicine. This method is also inexpensive and potentially applicable to other substances than elastin.

Due to its relationship with worse prognosis and possibly irreversibility of fibrosis, we believe that elastin may play an important role in understanding the pathogenesis and the predictability of prognosis in viral cirrhosis.

Abbreviations

ALT: Alanine aminotransferase; APRI: AST to platelets ratio index; AST: Aspartate aminotransferase; CID: Corrected integrated density; FIB-4: Fibrosis-4; HCPA: Hospital de Clínicas de Porto Alegre; INR: International normalized ratio; MGV: Mean gray value; PCR: Polymerase chain reaction

Acknowledgements

Not applicable.

Informed consent

The authors signed a declaration for Ethics Committee and are committed to keep patients' data confidential. Formal informed consent was waived by the HCPA Ethics Committee.

Authors' contribution

CL: design of study, participated in the acquisition of clinical data, analysis and interpretation of data, participated in writing manuscript. RTS: participated in digital analysis conceptualization and execution, and writing manuscript. ENT, CTSC, MRAS, MRMT: responsible for the conception and design of the study, besides writing manuscript. All authors read and approved the final manuscript.

Funding

The author(s) received financial support for the research by FIPE of Hospital de Clínicas.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was approved by Ethics Committee of the HCPA number 3.227.637.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Post-Graduate Program on Gastroenterology and Hepatology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. [2]Post-Graduate Program on Genetics and Molecular Biology, UFRGS, Porto Alegre, RS, Brazil. [3]Department of Surgery, Hospital de Clínicas de Porto Alegre (HCPA), UFRGS, Porto Alegre, RS, Brazil. [4]Department of Gastroenterology, HCPA, UFRGS, Porto Alegre, RS, Brazil. [5]Department of Surgical Pathology, HCPA, UFRGS, Porto Alegre, RS, Brazil.

Received: 9 August 2019 Accepted: 13 January 2020
Published online: 15 February 2020

References

Abe T, Hashiguchi A, Yamazaki K, Ebinuma H, Saito H, Kumada H, Izumi N, Maeki N, Sakamoto M (2013) Quantification of collagen and elastic fibers using whole-slide images of liver biopsy specimens. Pathol Int 63(6):305–310

Balocchini A, Montaldo C, Coniglaro A, Grimaldi A, Comeni V, Mura F, Ciccosanti F, Rotiroti N, Brenna A, Montalbano M, D'Offizi G, Capobianchi MR, Alessandro R, Piacentini M, Schininà ME, Maras B, Del Nonno F, Tripodi M, Mancone C (2016) Extracellular matrix molecular remodeling in human liver fibrosis evolution. PLoS One 11(3):e0151736

Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. the METAVIR cooperative study group. Hepatology 24(2):289–293

Burt AD, Griffiths MR, Schuppan D, Voss B, MacSween RN (1990) Ultrastructural localization of extracellular matrix proteins in liver biopsies using ultracryomicrotomy and immuno-gold labelling. Histopathology 16(1):53–58

Chen W, Yan X, Xu A, Sun Y, Wang B, Huang T, Wang H, Cong M, Wang P, Yang A, Jia J, You H (2019) Dynamics of elastin in liver fibrosis: accumulates late during progression and degrades slowly in regression. J Cell Physiol 234(12): 22613–22622

Collaborators: POH (2017) Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. Lancet Gastroenterol Hepatol 2(3):161–176

Francoschini D, Del Porto P, Piconese S, Trella E, Accapezzato D, Paroli M, Morrone S, Piccolella E, Spada E, Mele A, Sidney J, Sette A, Barnaba V (2012) Polyfunctional type-1, -2, and -17 CD8$^+$ T cell responses to apoptotic self-antigens correlate with the chronic evolution of hepatitis C virus infection. PLoS Pathog 8(6):e1002759

Geerts A (2001) History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis 21(3):311–335

Goodman ZD, Becker RL, Pockros PJ, Afdhal NH (2007) Progression of fibrosis in advanced chronic hepatitis C: evaluation by morphometric image analysis. Hepatology 45(4):886–894

Goodman ZD, Stoddard AM, Bonkovsky HL, Fontana RJ, Ghany MG, Morgan TR, Wright EC, Brunt EM, Kleiner DE, Shiffman ML, Everson GT, Lindsay KL, Dienstag JL, Morishima C, H.-C. T. Group (2009) Fibrosis progression in chronic hepatitis C: morphometric image analysis in the HALT-C trial. Hepatology 50(6):1738–1749

Hamilton P, Allen D (1995) Morphometry in histopathology. J Pathol 175:369–379

Hartig, S. M. (2013). Basic image analysis and manipulation in ImageJ. Curr Protoc Mol Biol Chapter 14: Unit14.15

Houghton AM, Quintero PA, Perkins DL, Kobayashi DK, Kelley DG, Marconcini LA, Mecham RP, Senior RM, Shapiro SD (2006) Elastin fragments drive disease progression in a murine model of emphysema. J Clin Invest 116(3):753–759

Kanta J (2016) Elastin in the liver. Front Physiol 7:491

Kendall TJ, Dolman GE, Duff CM, Palsh EC, Zaitoun A, Irving W, Fallowfield JA, Guha IN (2018) Hepatic elastin content is predictive of adverse outcome in advanced fibrotic liver disease. Histopathology 73(1):90–100

Kielty CM, Sherratt MJ, Shuttleworth CA (2002) Elastic fibres. J Cell Sci 115(Pt 14): 2817–2828

Manns MP, Buti M, Gane E, Pawlotsky JM, Razavi H, Terrault N, Younossi Z (2017) Hepatitis C virus infection. Nat Rev Dis Primers 3:17006

Masugi Y, Abe T, Tsujikawa H, Effendi K, Hashiguchi A, Abe M, Imai Y, Hino K, Higo S, Kawanaka M, Yamada G, Kage M, Korenaga M, Hiasa Y, Mizokami M, Sakamoto M (2018) Quantitative assessment of liver fibrosis reveals a

nonlinear association with fibrosis stage in nonalcoholic fatty liver disease. Hepatol Commun 2(1):58–68
Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, Pradere JP, Schwabe RF (2013) Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat Commun 4:2823–2843
Nakayama H, Itoh H, Kunita S, Kuroda N, Hiroi M, Matsuura H, Yasui W, Enzan H (2008) Presence of perivenular elastic fibers in nonalcoholic steatohepatitis fibrosis stage III. Histol Histopathol 23(4):407–409
Neumann-Haefelin C, Thimme R (2013) Adaptive immune responses in hepatitis C virus infection. Curr Top Microbiol Immunol 369:243–262
Paranaguá-Vezozzo DC, Andrade A, Mazo DF, Nunes V, Guedes AL, Ragazzo TG, Moutinho R, Nacif LS, Ono SK, Alves VA, Carrilho FJ (2017) Concordance of non-invasive mechanical and serum tests for liver fibrosis evaluation in chronic hepatitis C. World J Hepatol 9(8):436–442
Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675
Trang T, Petersen JR, Snyder N (2008) Non-invasive markers of hepatic fibrosis in patients co-infected with HCV and HIV: comparison of the APRI and FIB-4 indices. Clin Chim Acta 397(1–2):51–54
Yasui Y, Abe T, Kurosaki M, Matsunaga K, Higuchi M, Tamaki N, Watakabe K, Okada M, Wang W, Shimizu T, Takaura K, Masugi Y, Nakanishi H, Tsuchiya K, Takahashi Y, Itakura J, Sakurai U, Hashiguchi A, Sakamoto M, Izumi N (2019) Non-invasive liver fibrosis assessment correlates with collagen and elastic fiber quantity in patients with hepatitis C virus infection. Hepatol Res 49(1):33–41

Publisher's Note

European Journal of Medical Genetics 63 (2020) 103941

Contents lists available at ScienceDirect

European Journal of Medical Genetics

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

Bosch-Boonstra-Schaaf optic atrophy syndrome (BBSOAS) initially diagnosed as ALG6-CDG: Functional evidence for benignity of the *ALG6* c.391T > C (p.Tyr131His) variant and further expanding the BBSOAS phenotype

Rodrigo Tzovenos Starosta[a,b,*], Jessica Tarnowski[b,c], Filippo Pinto e Vairo[b,c], Kimiyo Raymond[c], Graeme Preston[b], Eva Morava[b,c]

[a] Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA
[c] Center for Individualized Medicine, Mayo Clinic, Rochester, MN, USA

ARTICLE INFO

Keywords:
Seizure
Visual loss
Autism spectrum disease
CDG
Transient ataxia
Protein-losing enteropathy

ABSTRACT

Bosch-Boonstra-Schaaf optic atrophy syndrome (BBSOAS) is a recently described autosomal dominant syndrome of developmental delay, cortical vision loss with optic nerve atrophy, epilepsy, and autism spectrum disorder. Due to its many overlapping features with congenital disorders of glycosylation (CDG), the differential diagnosis between these disorders may be difficult and relies on molecular genetic testing. We report on a 31-years-old female initially diagnosed with ALG6-CDG based on glycosylation abnormalities on transferrin isoelectrofocusing and targeted genetic testing, and later diagnosed with BBSOAS by whole-exome sequencing (WES). Functional studies on cultured fibroblasts including Western blotting and RT-qPCR, as well as mass spectrometry of glycosylated transferrin and MALDI-TOF glycan analysis in serum, demonstrated normal glycosylation in this patient. In this report, we extend the phenotype of BBSOAS with ataxia and protein-losing enteropathy. This case is illustrative of the utility of whole exome sequencing in the diagnostic odyssey, and the potential pitfalls of relying on focused genetic testing results for diagnosis of conditions with complex overlapping phenotypes.

1. Introduction

Bosch-Boonstra-Schaaf optic atrophy syndrome (BBSOAS) is an autosomal dominant disorder caused by pathogenic variants in the *NR2F1* gene, which codes for the nuclear receptor subfamily 2 group F member 1, also known as chicken ovalbumin upstream promoter-transcription factor I (*COUP-TFI*). *COUP-TFI* is a member of the steroid-thyroid hormone superfamily, and though its function has not been elucidated, it is known to participate in retinoic-acid associated cell differentiation during embryogenesis (Pickens et al., 2013; Harrison-Uy et al., 2013). BBSOAS is characterized by developmental delay, cortical visual impairment with optic atrophy, oromotor dysfunction, seizures, and behavioral abnormalities consistent with autism spectrum disorder (Bosch et al., 2014; Chen et al., 2016; Kaiwar et al., 2017; Park et al., 2019). ALG6-CDG (previously called CDG-Ic) is an autosomal recessive disorder caused by biallelic pathogenic variants in the *ALG6* gene, which codes for alpha-1,3-glucosyltransferase (Imbach et al., 1999; Hanefeld et al., 2000). It is a defect of N-glycosylation (Chang et al., 2018), characterized by disrupted glycan assembly in the endoplasmic reticulum leading to decreased protein glycosylation – reflected in a type I pattern on serum transferrin isoelectrofocusing (IEF) (Chang et al., 2018). The main manifestations of ALG6-CDG are developmental delay, hypotonia, seizures, ataxia, failure to thrive, hyperinsulinemic hypoglycemia, protein losing enteropathy, hepatopathy, autism spectrum disorder, and visual impairment (Morava et al., 2016; Haeuptle and Hennet, 2009).

Phenotypic overlap is a common phenomenon in genetic conditions, leading to long diagnostic odysseys and misdiagnosis of rare disorders. In the recent years, with the widespread use of whole-exome sequencing (WES) for diagnosis in the clinical practice, previously unsolved cases have been resolved, with a high proportion of unexpected diagnoses leading to changes in clinical management (Tan et al., 2017; Niguidula et al., 2018). In this article, we report on a case in which a diagnosis made based on phenotype-directed metabolic and gene panel

* Corresponding author. Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA.
E-mail address: rodrigo.starosta@ufrgs.br (R.T. Starosta).

https://doi.org/10.1016/j.ejmg.2020.103941
Received 17 February 2020; Received in revised form 29 April 2020; Accepted 2 May 2020
Available online 11 May 2020
1769-7212/

testing was later changed following WES.

2. Clinical features

A 31-year-old Caucasian female presented at infancy with hypotonia, developmental delay, hyperinsulinism without hypoglycemia, and history of protein-losing enteropathy confirmed by laboratory testing of stool samples. She was born at full term by repeat cesarean section after an uneventful pregnancy. Birth weight was approximately 2900 g. At birth, poor latching and sucking and generalized hypotonia were noted. During the first three years of life, she presented to the emergency room on more than 30 documented occasions with febrile episodes and dehydration, occasionally with myoclonus and generalized seizures concomitantly. She displayed marked global developmental delay, with independent walking at age 3 due to ataxia and hypotonia, speaking in sentences at age 4, and toilet training at age 7. Although the ataxia and the hypotonia improved gradually during childhood, visual acuity steadily declined, and she was considered legally blind by age 10. There was subjective improvement in her symptoms with low-fat and low-protein diet with carnitine supplementation. Serum transferrin isoelectrofocusing was suggestive of a congenital disorder of glycosylation (type I pattern), and targeted genetic testing by direct sequencing of *PMM2*, *MPI*, and *ALG6* identified a homozygous missense NM_013339.3:c.391T > C; p.(Tyr131His); rs35383149) variant in *ALG6*.

During adolescence she developed aggressive behavior, depression, and hallucinations. Puberty was mildly delayed (thelarche at 13 years, menarche at 15 years), as described elsewhere (Miller et al., 2011). She underwent multiple dental procedures due to dental abscesses causing facial cellulitis, developing unilateral deep venous thrombosis after general anesthesia; and endometrial ablation for control of menorrhagia, with recurrence of regular menses afterward. Complete blood counts showed intermittent mild anemia. At the age of 25, she developed episodes of abdominal pain, and an abdominal ultrasound showed multiple gallstones without gallbladder wall thickening or pericystic fluid, with resolution of symptoms after cholecystectomy. On histopathological examination of the gallbladder, > 75 mixed stones and cholesterolosis of the gallbladder wall were identified. Brain MRI at age 29 showed atrophic optic nerves, chiasm, and tracts; atrophic splenium of the corpus callosum; and a complex pituitary cyst. Coagulation, platelet aggregation, and fibrinolysis studies, including prothrombin time, activated partial thromboplastin time, factor IX activity, factor XI activity, and antithrombin-III activity, were normal. Liver function tests were normal except during acute cholelithiasis episodes, with normalization after cholecystectomy. Transthoracic echocardiogram and bone mineral density scan were normal.

On physical exam, bilateral enophthalmos was noted with mild malar hypoplasia, bilateral inverted nipples, bilateral fifth finger clinodactyly, bilateral pes planus, generalized hypotonia with mildly decreased strength in all four extremities, and brisk deep tendon reflexes. No other abnormalities were noted on the physical exam.

At age 30, affinity chromatography-mass spectrometry carbohydrate-deficient transferrin and apolipoprotein-CIII ("Apo-CIII") study was normal with transferrin mono-oligo/di-oligo ratio of 0.03 (reference ≤ 0.06), a-oligo/di-oligo ratio of 0.002 (reference ≤ 0.011), tri-sialo/di-oligo ratio of 0.04 (reference ≤ 0.05), Apo–CIII–1/Apo–CIII–2 ratio of 1.73 (reference ≤ 2.91), and Apo–CIII–0/Apo–CIII–2 ratio of 0.22 (reference ≤ 0.48). A follow-up genetic testing through Invitae Genetics Laboratory (San Francisco, CA, USA) was negative for pathogenic, possibly pathogenic, or unknown significance variants in the *ALG6* gene as the p.(Tyr131His) has been reclassified as a benign variant after the 2015 American College of Medical Genetics and Genomics variant interpretation consensus (Richards et al., 2015) and the 2017 Invitae Sherloc algorithm (Nykamp et al., 2017), as well as the recent finding of this variant in homozygous state in a cohort of healthy centenarians (Freudenberg-Hua et al., 2014) and of its high frequency in a healthy Croatian population (Goreta et al., 2012). Given the evolving clinical features of the patient and the reclassification of her previous "disease-causing" variant as benign, WES was performed and a heterozygous *de novo* pathogenic variant NM_005654.5(NR2F1):c.319A > G; p.(Lys107Glu) was found.

3. Methods

3.1. Cell culture

Patient fibroblasts had previously been isolated from a skin biopsy performed at age 9 years and frozen in DMSO on liquid nitrogen. Patient and commercially-obtained control fibroblasts (Coriell Institute, Camden, N.J, USA) were cultured in Minimum Essential Media (MEM; Gibco Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) at 37 °C, 5% CO2 and 5% O2.

3.2. Western blot

Cells were harvested at 90% confluence by scraping with 5 mL ice-cold phosphate-buffered saline (PBS) (for ICAM-1) or by trypsinization with 0.05% Trypsin-EDTA (Gibco). Cells were solubilized in ice-cold RIPA Buffer (Sigma, St Louis, MO, USA) and centrifuged for 10 min at 10,000 rpm. Protein concentration was determined using the Pierce BCA Kit (ThermoFisher, Waltham, MA, USA). Lysates were incubated for 30 min at 70 °C in 1X NuPAGE™ Sample Buffer (Novex, Carlsbad, CA, USA) containing 1X NuPAGE™ Sample Reducing Agent (Invitrogen, Carlsbad, CA, USA). SDS-PAGE was performed in 10% Novex™ Bis-Tris gels using NuPAGE™ MOPS SDS running buffer (Novex) at 200V for 60 min. Proteins were transferred to 0.45μm nitrocellulose membranes (Bio-Rad laboratories, Germany) in NuPAGE™ Transfer Buffer (Novex) at 35V for 180 min on ice. Membranes were blocked in SEA Block blocking buffer (Thermo Scientific, Rockford, IL, USA). Membranes were incubated in primary antibody (ICAM-1 mouse monoclonal antibody, Santa Cruz Biotechnology, 1:500; LAMP2 mouse monoclonal antibody, H4B4 clone, Invitrogen, 1:1000; ACTB rabbit monoclonal antibody, ABclonal, 1:10,000) diluted in SEA Block overnight at 4 °C, then washed 6 × 10 min at room temperature (RT) in PBS with 0.1% Tween-20 (Fisher Bioreagents, Fair Lawn, NJ, USA). Membranes were incubated in secondary antibody (donkey anti-mouse cross-adsorbed secondary antibody, DyLight 800 conjugate; donkey anti-rabbit cross-adsorbed secondary antibody, DyLight 680 conjugate; Invitrogen) diluted 1:500 in SEA Block for 1 h at 4 °C, then washed 6 × 10 min at RT with 0.1% Tween-20 in PBS, followed by a final wash with PBS. Blots were visualized using an Odyssey Fc imager (Li-Cor Biosciences, Lincoln, NE, USA).

3.3. Real-time quantitative polymerase chain reaction

RNA was isolated from fibroblasts using the RNeasy Plus Mini-Kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) using the following primers (Integrated DNA Technologies, Coralville, IA, USA): *ALG6* forward 5′-GGACTAACAGTACGATGGACAGTG-3′, *ALG6* reverse 5′-GAAGCTCAGAGACACTGGCA-3′; *ICAM-1* forward 5′-GGCCTCAGTC AGTGTGA-3′, *ICAM-1* reverse 5′-AACCCCATTCAGCGTCA-3′; and the housekeeping genes 18S (forward 5′-GTAACCCGTTGAACCCCATT-3′, reverse 5′-CCATCCAATCGGTAGTAGCG-3′), *GAPDH* (forward 5′-GCCA AAAGGGTCATCATCTC-3′, reverse 5′-GGCCATCCACAGTCTTCT-3′), and *ACTB* (forward 5′-CATGTACGTTGCTATCCAGGC-3′, reverse 5′-CTCCTTAATGTCACGCACGAT-3′) in a reverse-transcriptase polymerase chain reaction (RT-PCR) with the following cycles: 65 °C for 5 min, 25 °C for 10 min, 50 °C for 50 min, 85 °C for 5 min. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in 10uL reactions containing 1X SYBR Green master mix (Applied Biosystems, Warrington, UK), 1 μL each forward and reverse primers,

and 1 μl. cDNA template, on the LightCycler 480 II (Roche Molecular Systems, Rotkreuz, Switzerland) using the following conditions: pre-incubation at 95 °C for 5 min; 45 cycles of amplification at 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s; melting curve at 95 °C for 5 s and 65 °C for 1 min; and cooling at 40 °C for 30 s.

3.4 Glycomics by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis in plasma.

MALDI-TOF was performed as previously described (Balakrishnan et al., 2019). Briefly, serum was diluted with water in a 96-well plate and heated briefly in a thermal cycler. The serum solution is incubated with PNGase in sodium phosphate buffer to selectively release protein-bound N-glycans. The released N-glycans are captured and purified on graphitized carbon cartridges. Solvent is removed under a heated flow on nitrogen and the N-glycan residue is reconstituted with DMSO, water, and iodomethane. This solution is then applied to a 96-well plate containing sodium hydroxide beads. The slurry is sonicated at RT to permethylate the N-glycans. Following quenching with acid the reaction solution is purified by solid supported liquid extraction (SLE). Extracts are then further purified using reverse phase solid phase extraction (SPE). Elution solvent is evaporated under heated nitrogen, the residue reconstituted, and then spotted onto a MALDI plate. MALDI matrix is spotted directly onto the sample and allowed to dry at ambient conditions. The MALDI spots are then recrystallized in ethanol, yielding a homogenous MALDI sample. The samples are analyzed using the MALDI TOF-TOF operated in MS reflector mode across the range of m/z 900 to 4000 with a laser pulse rate of 400 Hz, collecting 2000 shots per spectrum.

4. Results

4.1. Bioinformatic analysis

The *NR2F1* gene is intolerant to missense and loss-of-function variants based on constraint metrics in gnomAD (http://gnomad.broadinstitute.org) (Karczewski et al., 2019). The variant is not reported in general population databases such as gnomAD, nor in the clinical databases ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) (Landrum et al., 2014) and Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/). *In silico* prediction tools M-CAP (Jagadeesh et al., 2016) and REVEL (Ioannidis et al., 2016) deemed the variant deleterious. The amino acid position is highly conserved among paralogs and orthologues and lies within the DNA-binding domain, a region scored intolerant to variations by subRVIS (Gussow et al., 2016). This variant was reported as pathogenic by the clinical laboratory following the ACMG 2015 guidelines (Richards et al., 2015).

4.2. Western Blot and RT-qPCR

Western Blot (WB) showed a profoundly decreased abundance of mature ICAM-1 protein relative to control fibroblasts (Fig. 1A). However, no differences in LAMP2 abundance or migration were detected compared to control fibroblasts. RT-qPCR for *ICAM-1* showed a decrease in mRNA abundance that mirrored the decreased ICAM-1 found by WB (Fig. 1B), suggesting that the decreased ICAM1 expression is due to decreased gene expression, instead of decreased protein translation/maturation. A similar phenomenon has been observed in other CDG (Radenkovic et al., 2019). No differences in *LAMP2* or *ALG6* gene expression were detected compared to controls.

4.3. MALDI-TOF mass spectrometry

In the serum sample, N-glycan, transferrin, and apolipoprotein CIII isoform analyses by MALDI-TOF mass spectrometry were essentially normal.

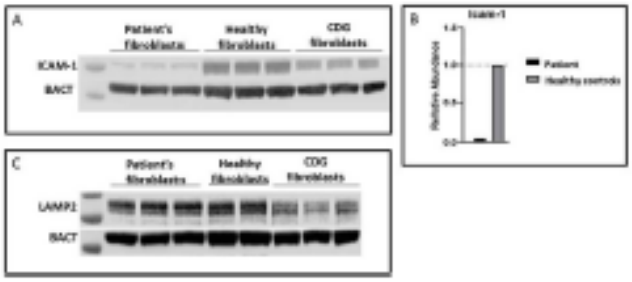


Fig. 1. A – Western blotting of ICAM-1 using beta-actin (ACTB) as a load control (protein load: 20 μg), showing a 62% lower abundance of mature ICAM-1 in the patient's cultured fibroblasts compared to control. This N-glycosylated screening marker shows decreased expression in several CDG types, however decreased ICAM1 protein expression can also be secondary to decreased *ICAM1* gene expression in some glycosylation defects. B – Results from RT-qPCR showing a reduced transcript abundance of *ICAM1* in the patient's fibroblasts after normalization to three housekeeping transcripts (*RNA18S*, *GAPDH*, and *ACTB*) in the patient fibroblasts compared to healthy control fibroblasts. This nonspecific finding has been shown in glycosylation defects, and suggest that decreased gene expression is the underlying cause of ICAM1 protein expression. C – Western blotting of LAMP2 using ACTB as a load control (protein load: 20 μg), showing no significant difference in glycosylated LAMP2 protein abundance between patient and control. This finding does not support a glycosylation defect.

5. Discussion

Here we describe an individual with BBSOAS initially diagnosed with ALG6-CDG. The clinical presentation and biochemical abnormalities (abnormal CDG screening by capillary electrophoresis, and type 1 TIEF pattern in serum) were in line with the suspected diagnosis of ALG6-CDG. The detection of homozygous variants, which were previously suggested to affect glycosylation and lead to mild dysfunction in a cell model system (Miller et al., 2011) further supported the diagnosis. Normalization of the transferrin glycosylation in adults with CDG is common and did not suggest a misdiagnosis *per se*. However, major behavioral changes at the adult age lead to a reevaluation of the primary diagnosis.

This complex phenotype, while common in young individuals with CDG, includes several unique features including protein losing enteropathy, which is undescribed in BBSOAS, and ataxia, which has been previously described in only one patient with BBSOAS (Al-Kateb et al., 2013). Although it is possible that some of these features could be explained by other concomitant disorders, we believe that the careful evaluation of whole-exome sequencing data in this patient makes a second diagnosis less likely, and that these manifestations further extend the BBSOAS phenotype.

In this reported case, a number of features common to both disorders can be observed, including febrile seizures (Hino-Fukuyo et al., 2017; Al-Owain et al., 2010), visual loss and optic atrophy (Bosch et al., 2014; Al-Kateb et al., 2013; Kahook et al., 2006), hypotonia, failure to thrive, developmental delay and intellectual disability (Bosch et al., 2014; Al-Kateb et al., 2013; Morava et al., 2016; Park et al., 2019). Notably, one of the most commonly performed screening tests for CDG, serum transferrin IEF, can offer false positives under certain conditions (Francisco et al., 2019; Lefeber et al., 2011). We believe that some of the reasons for the initial misdiagnosis were unspecific phenotypes, phenotypic overlap between both syndromes, the limited availability of genetic testing when the patient was first evaluated, and the lack of easily accessible population databases to check for variant frequency.

In disorders without a highly compelling clinical picture, targeted genetic testing can become a potential pitfall in a diagnostic odyssey. Although evidence-based criteria for variant curation have decreased the false-positive rate of molecular genetic diagnosis (Mirshahi et al., 2019), the finding of a variant that could explain the clinical findings

when analyzing a limited number of genes still does not rule out a more fitting variant present in non-analyzed genes.

The c.391T > C *ALG6* variant has previously been reported in patients diagnosed with ALG6-CDG (Westphal et al. 2000, 2003), suggesting that additional patients may have been misdiagnosed based on the presence of this variant. Questions regarding the true pathogenicity of this variant have already been raised based both on clinical pictures that did not precisely match the expected ALG6-CDG phenotype and its high population frequency (Westphal et al., 2003; Goreta et al., 2012). However until now no functional evaluation had confirmed the benignity of the variant. Based on Western blot, RT-qPCR, mass spectrometry of glycosylated transferrin and MALDI-TOF we proved that the NM_013339.3:c.391T > C; p.(Tyr131His) variant in *ALG6* does not alter the expression of the protein or induce any definite glycosylation abnormality. Even though ICAM-1 expression is decreased in several CDG types, and decreased ICAM-1 gene expression has been observed in CDG (Radenkovic et al., 2019), we concluded that without any additional glycosylation changes in plasma or cultured fibroblasts in this patient, the lower expression of ICAM-1 is a non-specific finding. Together with the populational evidence available, we conclude that there is compelling evidence for its classification as benign. These findings raise the concern for reinitiating the diagnostic efforts for other patients previously diagnosed with ALG6-CDG based on this variant.

We believe that the patient's phenotype can be fully explained by the novel *de novo* pathogenic variant found in the *NR2F1* gene and that protein-losing enteropathy and transient ataxia should be proposed as additional phenotypes associated with BBSOAS.

CRediT authorship contribution statement

Rodrigo Tzovenos Starosta: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Jessica Tarnowski:** Conceptualization, Data curation, Writing - review & editing. **Filippo Pinto e Vairo:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Kimiyo Raymond:** Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **Graeme Preston:** Conceptualization, Formal analysis, Methodology, Resources, Software, Writing - review & editing. **Eva Morava:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Acknowledgements

This work was supported by the Programa Institucional de Internacionalização of the Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (PRINT/CAPES, Brazil) and funded by the grant titled Frontiers in Congenital Disorders of Glycosylation (1U54NS115198-01) from the National Institute of Neurological Diseases and Stroke (NINDS) and the National Center for Advancing Translational Sciences (NCATS), and the Rare Disorders Clinical Research Network (RDCRN), at the National Institute of Health.

References

Al-Kateb, H., Shimony, J.S., Vineyard, M., Manwaring, L., Kulkarni, S., Shinawi, M., 2013. NR2F1 haploinsufficiency is associated with optic atrophy, dysmorphism and global developmental delay. Am. J. Med. Genet. 161A (2), 377–381. https://doi.org/10.1002/ajmg.a.35650.

Al-Owain, M., Mohamed, S., Kaya, N., Zayed, A., Matthijs, G., Jaeken, J., 2010. A novel mutation and first report of dilated cardiomyopathy in ALG6-CDG (CDG-Ic): a case report. Orphanet J. Rare Dis. 5, 7. https://doi.org/10.1186/1750-1172-5-7.

Balakrishnan, B., Verheijen, J., Lupo, A., Raymond, K., Tuysuz, C., Yang, Y., Carter, K.J., Whitehead, K.J., Kozicz, T., Morava, E., Lai, K., 2019. A novel phosphoglucomutase-deficient mouse model reveals aberrant glycosylation and early embryonic lethality. J. Inherit. Metab. Dis. 42 (5), 998–1007. https://doi.org/10.1002/jimd.12110.

Basch, D.G., Bronstra, F.N., Gonzaga-Jauregui, C., Xia, M., de Ligt, J., Shangjuni, S., Wozniewski, W., Muzny, D.M., Yntema, H.G., Pfundt, R., Vissers, L.E., Spruijt, L., Blokland, E.A., Chen, C.A., Lewis, R.A., Tsai, S.Y., Gibbs, R.A., Tsai, M.J., Lupski, J.R., Zoghbi, H.Y., Cremers, F.P., de Vries, B.B., Schaaf, C.P., Baylor-Hopkins Center for Mendelian Genomics, 2014. NR2F1 mutations cause optic atrophy with intellectual disability. Am. J. Hum. Genet. 94 (2), 303–309. https://doi.org/10.1016/j.ajhg.2014.01.002.

Chang, I.J., He, M., Lam, C.T., 2018. Congenital disorders of glycosylation. Ann. Transl. Med. 6 (24), 477. https://doi.org/10.21037/atm.2018.10.45.

Chen, C.A., Basch, D.G., Cho, M.T., Rosenfeld, J.A., Shinawi, M., Lewis, R.A., Mann, J., Jayakar, P., Payne, K., Walsh, L., Moss, T., Schreiber, A., Schoonveld, C., Monaghan, K.G., Elmslie, F., Douglas, G., Bonnstra, F.N., Millan, F., Cremers, F.P., McKnight, D., Richard, G., Jaunela, J., Kendall, F., Ramsey, K., Anyane-Yeboa, K., Malkin, R., Chung, W.K., Niyazov, D., Pascual, J.M., Walkiewicz, M., Velurhamy, V., Li, C., Hisama, F.M., de Vries, B.B., Schaaf, C., 2016. The expanding clinical phenotype of Basch-Bonstra-Schaaf optic atrophy syndrome: 20 new cases and possible genotype-phenotype correlations. Genet. Med. 18 (11), 1143–1150. https://doi.org/10.1038/gim.2016.18.

Francisco, R., Marques-da-Silva, D., Brasil, S., Pascoal, C., Dos Reis Ferreira, V., Morava, E., Jaeken, J., 2019. The challenge of CDG diagnosis. Mol. Genet. Metabol. 126 (1), 1–8. https://doi.org/10.1016/j.ymgme.2018.11.003.

Freudenberg-Hua, Y., Freudenberg, J., Vacic, V., Abhyankar, A., Emde, A.K., Ben-Avraham, D., Barzilai, N., Cochwald, D., Christen, E., Koppel, J., Greenwald, R., Darnell, R.B., Gerner, S., Atzmon, G., Davies, P., 2014. Disease variants in genomes of 44 centenarians. Mol Genet Genomic Med 2 (5), 438–450. https://doi.org/10.1002/mgg3.86.

Goreta, S.S., Dabelic, S., Pavlinic, D., Lauc, G., Dumic, J., 2012. Frequency determination of α-1,3 glucosyltransferase p.Y131H and p.F304S polymorphisms in the Croatian population revealed five novel single nucleotide polymorphisms in the hALG6 gene. Genet. Test. Mol. Biomarkers 16 (1), 50–53. https://doi.org/10.1089/gtmb.2011.0093.

Gussow, A.B., Petrovski, S., Wang, Q., Allen, A.S., Goldstein, D.B., 2016. The intolerance to functional genetic variation of protein domains predicts the localization of pathogenic mutations within genes. Genome Biol. 17, 9. https://doi.org/10.1186/s13059-016-0869-4.

Haeuptle, M.A., Hennet, T., 2009. Congenital disorders of glycosylation: an update on defects affecting the biosynthesis of dolichol-linked oligosaccharides. Hum. Mutat. 30 (12), 1628–1641. https://doi.org/10.1002/humu.21126.

Hansefeld, F., Körner, C., Holzbach-Eberle, U., von Figura, K., 2000. Congenital disorder of glycosylation-Ic: case report and genetic defect. Neuropediatrics 31 (2), 60–62. https://doi.org/10.1055/s-2000-7486.

Harrison-Uy, S.J., Siegenthaler, J.A., Faedo, A., Rubenstein, J.L., Pleasure, S.J., 2013. Coup TFI interacts with retinoic acid signaling during cortical development. PloS One 8 (3), e58219. https://doi.org/10.1371/journal.pone.0058219.

Hino-Fukuyo, N., Kikuchi, A., Yokoyama, H., Inuma, K., Hirose, M., Haginoya, K., Niihori, T., Nakayama, K., Aoki, Y., Kure, S., 2017. Long-term outcome of a 26-year-old woman with West syndrome and an nuclear receptor subfamily 2 group F member 1 gene (NR2F1) mutation. Seizure 50, 144–146. https://doi.org/10.1016/j.seizure.2017.06.018.

Imbach, T., Burda, P., Kuhnert, P., Wevers, R.A., Aebi, M., Berger, E.G., Hennet, T., 1999. A mutation in the human ortholog of the Saccharomyces cerevisiae ALG6 gene causes carbohydrate-deficient glycoprotein syndrome type-Ic. Proc. Natl. Acad. Sci. U. S. A. 96 (12), 6982–6987. https://doi.org/10.1073/pnas.96.12.6982.

Ioannidis, N.M., Rothstein, J.H., Pejaver, V., Middha, S., McDonnell, S.K., Baheti, S., Musolf, A., Li, Q., Holzinger, E., Karyadi, D., Cannon-Albright, L.A., Teerlink, C.C., Stanford, J.L., Isaacs, W.B., Xu, J., Cooney, K.A., Lange, E.M., Schleutker, J., Carpten, J.D., Powell, I.J., Cussenot, O., Cancel-Tassin, G., Giles, G.G., MacInnis, R.J., Maier, C., Hsieh, C.L., Wiklund, F., Catalona, W.J., Foulkes, W.D., Mandal, D., Eeles, R.A., Kote-Jarai, Z., Bustamante, C.D., Schaid, D.J., Hastie, T., Ostrander, E.A., Bailey-Wilson, J.E., Radivojac, P., Thibodeau, S.N., Whittemore, A.S., Sieh, W., 2016. REVEL: an ensemble method for predicting the pathogenicity of rare missense variants. Am. J. Hum. Genet. 99 (4), 877–885. https://doi.org/10.1016/j.ajhg.2016.08.016.

Jagadeesh, K.A., Wenger, A.M., Berger, M.J., Guturu, H., Stenson, P.D., Cooper, D.N., Bernstein, J.A., Bejerano, G., 2016. M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. Nat. Genet. 48 (12), 1581–1586. https://doi.org/10.1038/ng.3703.

Kahook, M.Y., Manahan, N., Bateman, J.B., Thomas, J.A., 2006. Glycosylation type Ic disorder: idiopathic intracranial hypertension and retinal degeneration. Br. J. Ophthalmol. 90 (1), 115–116. https://doi.org/10.1136/bjo.2005.080648.

Kaiwar, C., Zimmermann, M.T., Ferber, M.J., Niu, Z., Urrutia, R.A., Klee, E.W., Babovic-Vuksanovic, D., 2017. Novel Cold Spring Herb Mol Case Stud 3 (6). https://doi.org/10.1101/mcs.a002162.

Karczewski, Konrad J., Francioli, Laurent C., Grace, Tiao, Beryl, B Cummings, Alföldi, Jessica, Wang, Qingbo, Collins, Ryan L., 2019. Variation across 141,456 Human Exomes and Genomes Reveals the Spectrum of Loss-Of-Function Intolerance across Human Protein-Coding Genes. (bioRxiv).

Landrum, M.J., Lee, J.M., Riley, G.R., Jang, W., Rubinstein, W.S., Church, D.M., Maglott, D.R., 2014. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res. 42 (Database issue), D980–D985. https://doi.org/10.1093/nar/gkt1113.

Lefeber, D.J., Morava, E., Jaeken, J., 2011. How to find and diagnose a CDG due to defective N-glycosylation. J. Inherit. Metab. Dis. 34 (4), 849–852. https://doi.org/10.1007/s10545-011-9370-0.

Miller, B.S., Pravos, H.H., Hoffmann, G.F., Sarafoglou, K., 2011. Pubertal development in ALG6 deficiency (congenital disorder of glycosylation type Ic). Mol. Genet. Metabol. 103 (1), 101–103. https://doi.org/10.1016/j.ymgme.2011.01.016.

Minhahi, U.L., Luo, J.Z., Mantcham, K., Warfield, A.H., Minhahi, T., Murray, M.F., Carey, D.J., 2019. Trajectory of exonic variant discovery in a large clinical population:

implications for variant curation. Genet. Med. 21 (6), 1417–1424. https://doi.org/10.1038/s41436-018-0353-5.

Morava, E., Tiemes, V., Thiel, C., Seta, N., de Lonlay, P., deKlerk, H., Mulder, M., Rubio-Gozalbo, E., Visser, G., van Hasselt, P., Horowitz, D.D.G., de Sousa, C.F.M., Schwartz, I.V.D., Groen, A., Al-Owain, M., Thiel, G., Sigauty, S., Chabrol, B., van Spronsen, F.J., Steinert, M., Romini, E., Wurm, D., Bevot, A., Ayadi, A., Huijben, K., Derksen, M., Witters, P., Jaeken, J., Matthijs, G., Lefeber, D.J., Wevers, R.A., 2016. ALG6-CDG: a recognizable phenotype with epilepsy, proximal muscle weakness, ataxia and behavioral and limb anomalies. J. Inherit. Metab. Dis. 39 (5), 713–723. https://doi.org/10.1007/s10545-016-9945-x.

Niguidula, N., Alamillo, C., Shahmirzadi Mowlavi, L., Powis, Z., Cohen, J.S., Farwell Hagman, K.D., 2018. Clinical whole-exome sequencing results impact medical management. Mol Genet Genomic Med 6 (6), 1068–1078. https://doi.org/10.1002/mgg3.484.

Nykamp, K., Anderson, M., Powers, M., Garcia, J., Herrera, B., Ho, Y.Y., Kobayashi, Y., Patil, N., Thusberg, J., Westbrook, M., Topper, S., Invitae Clinical Genomics Group, 2017. Sherloc: a comprehensive refinement of the ACMG-AMP variant classification criteria. Genet. Med. 19 (10), 1105–1117. https://doi.org/10.1038/gim.2017.37.

Park, S.E., Lee, J.S., Lee, S.T., Kim, H.Y., Han, S.H., Han, J., 2019. Targeted panel sequencing identifies a novel. Ophthalmic Genet. 40 (4), 359–361. https://doi.org/10.1080/13816810.2019.1650074.

Pickett, B.S., Teets, B.W., Sopranno, K.J., Sopranno, D.R., 2013. Role of COUP-TFI during retinoic acid-induced differentiation of P19 cells to endodermal cells. J. Cell. Physiol. 228 (4), 791–800. https://doi.org/10.1002/jcp.24228.

Radenkovic, S., Bird, M.J., Emmerzaal, T.L., Wong, S.Y., Felgueira, C., Stiers, K.M., Sabbagh, L., Himmelreich, N., Poschet, G., Windmolders, P., Verhelst, J., Witters, P., Altassan, R., Honzik, T., Eminoglu, T.F., Jaeken, J., Edmondson, A.C., Bertoncini, J., Konicz, T., Thiel, C., Vermeersch, P., Cassiman, D., Beamer, L., Morava, E., Ghesquière, B., 2019. The metabolic map into the pathomechanism and treatment of PGM1-CDG. Am. J. Hum. Genet. 104 (5), 835–846. https://doi.org/10.1016/j.ajhg.2019.03.003.

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., Rehm, H.L., ACMG Laboratory Quality Assurance Committee, 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and Genomics and the association for molecular pathology. Genet. Med. 17 (5), 405–424. https://doi.org/10.1038/gim.2015.30.

Tan, T.Y., Dillon, O.J., Stark, Z., Schofield, D., Alam, K., Shrestha, R., Chong, B., Phelan, D., Brett, G.R., Creed, E., Jarmolowicz, A., Yap, P., Walsh, M., Downie, L., Amor, D.J., Savarirayan, R., McGillivray, G., Young, A., Peters, H., Robertson, S.J., Robinson, A.J., Mastrocola, L., Sadedin, S., Bell, K., Oshlack, A., Georgeson, P., Thorne, N., Gaff, C., White, S.M., 2017. Diagnostic impact and cost-effectiveness of whole-exome sequencing for ambulant children with suspected monogenic conditions. JAMA Pediatr 171 (9), 855–862. https://doi.org/10.1001/jamapediatrics.2017.1755.

Westphal, V., Murch, S., Kim, S., Sekhsaria, G., Winchester, B., Day, R., Preece, H.H., 2000. Reduced heparan sulfate accumulation in enterocytes contributes to protein-losing enteropathy in a congenital disorder of glycosylation. Am. J. Pathol. 157 (6), 1917–1925. https://doi.org/10.1016/S0002-9440(10)64830-4.

Westphal, V., Xiao, M., Revak, P.Y., Preece, H.H., 2003. Identification of a frequent variant in ALG6, the cause of congenital disorder of glycosylation-ic. Hum. Mutat. 22 (5), 420–421. https://doi.org/10.1002/humu.9195.