Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Genética e Biologia Molecular

Glicogenoses hepáticas: estudo do uso de diferentes amidos e caracterização do perfil de parâmetros do metabolismo do ferro

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Orientadora: Profa. Dra. Ida Vanessa Doederlein Schwartz

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

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APRESENTAÇÃO

Conforme formato requerido pelo Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, esta tese está dividida em: *Introdução; Justificativa; Objetivos; Capítulos* (substituirão as seções de Material e Métodos e Resultados descrevendo os resultados obtidos no período sob forma de artigos científicos publicados e artigos em elaboração); *Discussão; Conclusões; Perspectivas; Referências Bibliográficas e Apêndices.* Este trabalho foi desenvolvido no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre e na Universidade de Groningen/Países Baixos. O estudo foi financiado pelo Fundo de Incentivo à Pesquisa e Eventos (FIPE) do Hospital de Clínicas de Porto Alegre, verbas de editais Nº 31/2013 – Doenças Metabólicas e Endócrinas – Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e PPSUS/2013 – Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), e *unrestricted grants* da empresa Vitaflo. A aluna recebeu bolsa de estudos concedida pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e bolsa de doutorado-sanduíche concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Todos os experimentos com seres humanos apresentados neste trabalho estão incluídos em projeto de pesquisa aprovado em seus aspectos éticos e metodológicos pelo Comitê de Ética em Pesquisa do Grupo de Pesquisa e Pós-Graduação do Hospital de Clínicas de Porto Alegre sob número 12-0429.

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SUMÁRIO

ABREVIATURAS E SIGLAS

AMC – Amido de milho cru

ALT – Alanina aminotransferase

AR – Autossômica recessiva

AST – Aspartato aminotraferase

IBD – *Inflammatory bowel disease*

BMI – *Body mass index*

BMP6 – *Bone morphogenetic protein* 6

BR – *Brazil*

CPK – Creatinofosfoquinase

CNGDF – *Continuous nocturnal gastric drip feeding*

GSD – Glicogenoses

GSD – *Glycogen Storage Diseases*

GTI – *Glycemic TNO index method*

G6Pase – Glicose-6-fosfatase

G6PT – Transportador de glicose-6-fosfato

HAMP - *Hepcidin antimicrobial peptide*

HCPA – Hospital de Clínicas de Porto Alegre

HFE – Proteína da hemocromatose

HJV – Hemojuvelina

IL-6 – Interleucina – 6

IL-6 - *Interleukin – 6*

IEM – *Inborn errors of metabolism*

JAK – *Janus Kinase*

LX – Ligada ao X

MT2 – Matriptase-2

NL – *the Netherlands*

RAG- *Rapidly available glucose*

RS – *Resistant starch*

SAG – *Slowly available glucose*

SGM-HCPA - Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre

STAT3 - tradutor de sinal e ativador de transcrição 3

TfR2 – Receptor de transferrina 2

TIM-1 – *TNO in vitro model of the gastrointestinal tract - 1*

TG – *Total glucose*

UCCS – *Uncooked cornstarch*

UFRGS – Universidade Federal do Rio Grande do Sul

UK – *United Kingdom*

USA – *United States of America*

WHO - *World Health Organization*

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RESUMO

Introdução: As Glicogenoses (GSD) hepáticas são doenças genéticas relativamente frequentes. Entre as suas manifestações clínicas, a que mais se destaca é a ocorrência de hipoglicemias de repetição. O tratamento com amido de milho cru é o mais largamente utilizado atualmente. Contudo, tem sido observado na prática clínica que os pacientes apresentam resposta metabólica diferente ao uso de diferentes amidos de milho. Além disso, complicações a longo prazo vêm sendo descritas nos pacientes com GSD hepáticas, especialmente na GSD I, considerada a mais grave, entre elas anemia ferropriva e de doença crônica.

Objetivos: 1) Analisar a digestão de amidos de milho de diferentes marcas e provenientes de diferentes países, e de possíveis substitutos aos mesmos, por meio do modelo gastrointestinal, *in vitro,* TIM-1 *(TNO in vitro model of the gastrointestinal tract - 1);* 2) Determinar as frações de amido e a razão de amilose/amilopectina de amidos de milho de diferentes marcas e provenientes de diferentes países, e possíveis substitutos aos mesmos; 3) Caracterizar o perfil de hepcidina, de IL-6 e de outros parâmetros do metabolismo do ferro (ferritina, ferro e saturação de transferrina), em plasma de pacientes com GSD hepáticas, e avaliar a sua associação com a presença de anemia, de forma a contribuir para a melhor compreensão da fisiopatogenia dessa complicação nessas condições.

Metodologia: Para as análises com amidos, foram estudados as seguintes marcas: Argo® e Great Value® provenientes dos Estados Unidos da América; Maizena Duryea® e Yoki® provenientes do Brasil; Maizena Duryea® proveniente dos Países Baixos; Glycosade®, um amido modificado; e polvilho doce, amido brasileiro extraído da mandioca. As frações de amido (amido rapidamente disponível – RAG; amido lentamente disponível – SAG; e amido resistente – RS) foram analisadas por meio do método GTI (*Glycemic TNO Index*). A razão de amilose/amilopectina foi determinada por meio de ensaio com kit comercial. A digestão dos amidos foi determinada por meio do sistema TIM-1 (TNO *in vitro* model of the gastrointestinal tract - 1), um modelo gastrointestinal dinâmico, controlado por computador, que simula os processos sucessivos do estômago e do intestino curto. Para estudo de hepcidina e IL-6 nos pacientes com GSD

hepáticas, foi realizado um estudo transversal com amostragem por conveniência. Foram determinadas as concentrações de hepcidina e de IL-6, por meio de kits comerciais ELISA, e de parâmetros do metabolismo do ferro (Hb, ferro, ferritina, transferrina e saturação de transferrina), em 32 pacientes com GSD hepática em tratamento com amido de milho (GSD Ia= 18, GSD Ib= 7, GSD III= 3, GSD IXa= 3; GSD IXb= 1; sexo feminino= 17; mediana de idade= 9,5 anos). Dados adicionais de tratamento, clínicos e bioquímicos, foram obtidos por meio de revisão de prontuário dos pacientes. Para fins de comparação, foram determinadas concentrações de hepcidina em 20 controles, não aparentados aos pacientes (sexo feminino= 10, mediana de idade= 12 anos). Adicionalmente foram determinados níveis de hepcidina e IL-6 em 8 indivíduos heterozigotos para GSD hepáticas (mediana de idade= 33,5 anos; mães de pacientes= 6). Para análise dos dados, foram utilizados métodos não paramétricos.

Resultados: Nos experimentos com os amidos, todos os amidos de milho estudados apresentaram valores semelhantes de frações de amido, o Glycosade® apresentou valor superior de RAG e inferior de RS, enquanto o polvilho doce apresentou valor inferior de RAG e superior de RS, comparado aos demais amidos. O estudo da razão de amilose/amilopectina apresentou valores semelhantes para os amidos de milho, o Glycosade® apresentou valor superior de amilopectina, conforme descrito pelo fabricante, e o polvilho apresentou valor ligeiramente superior de amilopectina, comparado aos amidos de milho, e apresentou estabilidade nos diferentes lotes estudados. No estudo de digestão, utilizando a TIM-1, a quantidade final digerida foi entre 84 e 86% para os amidos de milho e para o Glycosade®, e foi de 75,5% para o polvilho doce. Aos 180 minutos do experimento, um ponto de tempo importante para pacientes com GSD, a quantidade digerida dos amidos era correspondente a 67,9-71,5% para os amidos de milho e o Glycosade®, enquanto para o polvilho doce esse valor era de 55,5%. No experimento realizado com uma mistura de amido de milho (Maizena Duryea® brasileira) e polvilho doce, a quantidade final digerida foi de 78,4%, enquanto o valor no momento de 180 minutos foi de 61,7%. No estudo com pacientes com GSD hepática, nove pacientes (GSD Ia= 3; GSD Ib= 6) apresentavam anemia (leve= 4; moderada=5). Cinco pacientes apresentavam

adenoma hepático. A hepcidina correlacionou-se positivamente com os níveis de ferritina (r = 0,375; p= 0,034). Já a IL-6 correlacionou-se com níveis de Hb (r= -0,572; p= 0,001), ferro (r= -0,538; p= 0,001), transferrina (r = -0,550; p= 0,001) e saturação de transferrina (r= -0,425; p= 0,015). Não foi encontrada correlação entre os níveis de hepcidina e IL-6 (p= 0,057) e dessas variáveis em relação a outros parâmetros bioquímicos e nutricionais estudados. Os pacientes com GSD Ib apresentaram níveis mais elevados de IL-6. Não foi encontrada relação entre a presença de adenomas e anemia, e pacientes com doença inflamatória intestinal apresentaram valores superiores de hepcidina. Pacientes apresentaram níveis de IL-6 superiores aos indivíduos heterozigotos (p= 0,003). Além disso, os níveis de hepcidina diferiram entre os grupos estudados (p= 0,006), estando aumentados em pacientes (mediana= 58,6 ng/mL; IQR= 41,7-71,9) e em indivíduos heterozigotos (mediana= 60,7 ng/mL, IQR= 51,7-68,3) quando comparados aos controles (mediana= 42,8 ng/mL, IQR= 30,9-50,5) (p= 0,005 e 0,006, respectivamente).

Conclusões: Os amidos de milho e o amido comercial estudados apresentaram pequenas diferenças na liberação de glicose nos experimentos realizados no sistema TIM-1. Esses achados sugerem que diferenças nas respostas glicêmicas em pacientes com GSD hepáticas, podem ser relacionadas à variações inter- e intraindivíduos e/ou ainda relacionadas à adesão ao tratamento. Cabe ressaltar que as pequenas diferenças encontradas na digestão *in vitro* podem ter o seu efeito amplificado *in vivo*, quando variáveis como a resposta hormonal e o fenótipo da doença estão presentes. O polvilho doce apresentou uma liberação de glicose mais lenta e parece ser um produto interessante para ser melhor estudado como alternativa de tratamento, com intuito de prolongar a normoglicemia, para pacientes com GSD hepáticas. Em relação a anemia, a mesma apresentou-se como um achado frequente nas GSD hepáticas, sendo mais frequente na GSD Ib, tipo que apresenta os níveis mais elevados de IL-6. Nossos achados sugerem que a inflamação está relacionada à ocorrência de anemia nas GSD hepáticas, principalmente na GSD Ib.

ABSTRACT

Background: The hepatic glycogen storage diseases (GSD) are relatively common genetic disorders. Among their clinical manifestations, the most remarkable is recurrent hypoglycemia. Uncooked cornstarch (UCCS) administration is the most widely used treatment today. However, it has been observed in clinical practice that patients exhibit different metabolic responses to different cornstarches. Furthermore, long-term complications – including iron deficiency anemia and the anemia of chronic disease – have been described in patients with hepatic GSD, especially those with type I, which is considered the most severe form.

Objectives: 1) To analyze the digestion of different brands of cornstarch from different countries, and of possible substitutes for these substances, using the TIM-1 (TNO in vitro model of the gastrointestinal tract - 1); 2) To determine the starch fractions and amylose/amylopectin ratios of cornstarches from different brands and countries and of possible substitutes for these substances; 3) To characterize levels of hepcidin, IL-6, and other markers of iron metabolism (ferritin, iron, transferrin saturation) in plasma of patients with hepatic GSD and evaluate the association of these parameters with the presence of anemia, so as to contribute to a better understanding of the pathogenesis of this complication in GSD.

Methodology: The following brands and types of straches were studied: Argo® and Great Value®, from the United States of America; Maizena Duryea® and Yoki®, from Brazil; Maizena Duryea®, from the Netherlands; Glycosade®, a modified starch; sweet polvilho, a cassava-derived starch, from Brazil. Starch fractions (rapidly available glucose, RAG; slowly available glucose, SAG; and resistant starch, RS) were analyzed by the glycemic TNO index (GTI) method. The amylose/amylopectin ratio was determined using a commercial kit. Starch digestion was determined in the TIM-1 system, a dynamic, computer-controlled in vitro model of the gastrointestinal tract that simulates successive digestive processes in the stomach and small intestine. To study hepcidin and IL-6 profiles

in patients with hepatic GSD, a cross-sectional study with convenience sampling was conducted. Plasma hepcidin and IL-6 levels (using commercial enzyme-linked immunoassay kits) and iron metabolism parameters (hemoglobin, iron, ferritin, transferrin, and transferrin saturation) were measured in 32 patients with hepatic GSD on cornstarch treatment (GSD Ia =18; GSD Ib= 7; GSD III= 3; GSD IXa= 3; GSD IXb= 1; 17 females; median age, 9.5 years). Additional data, about treatment, clinical and biochemical variables, were obtained from patients' medical records. For comparison purposes, hepcidin levels were quantitated in 20 unrelated healthy controls (10 females; median age= 12 years). Additionally, hepcidin and IL-6 levels were evaluated in eight subjects heterozygous for hepatic GSD (median age= 33.5 years; 6 were mothers of patients with GSD). Nonparametric methods were used for data analysis.

Results: All analyzed cornstarches displayed similar values for all starch fractions. Glycosade® had higher RAG and lower RS values, while sweet polvilho had lower RAG and higher RS values, as compared with the other starches. Analysis of amylose/amylopectin ratio revealed similar values for the tested UCCS. Glycosade® had a higher amylopectin content, as described by the manufacturer, whereas sweet polvilho had a slightly higher amylopectin content as compared with cornstarches and was stable across different studied batches. In the digestion study using the TIM-1 system, the final digested amount ranged from 84–86% for the UCCS and Glycosade®, but was 75.5% for sweet polvilho. At the 180-min point of the experiment, an important time point for GSD patients, the digested amount was 67.9–71.5% for UCCS and Glycosade®, and 55.5% for sweet polvilho. In an experiment conducted with a mixture of cornstarch (Brazilian Maizena Duryea®) and sweet polvilho, a final digested amount of 78.4% was found, while the value at 180 min was 61.7%. On analysis of GSD patients, nine (GSD Ia= 3; GSD Ib= 6) were anemic (mild= 4; moderate= 5). Five patients had hepatic adenomas. Hepcidin correlated positively with ferritin levels ($r= 0.375$; $p= 0.034$), while IL-6 correlated with hemoglobin ($r= -0.572$; $p= 0.001$), iron ($r= -0.538$; $p= 0.001$), transferrin ($r= -0.550$; $p= 0.001$), and transferrin saturation ($r= -0.425$; $p= 0.015$). There was no correlation between hepcidin and IL-6 levels ($p= 0.057$), or between these variables and the other biochemical and nutritional parameters

of interest. Patients with GSD Ib had the highest IL-6 levels. There was no association between presence of hepatic adenoma and anemia. Patients with inflammatory bowel disease exhibited higher hepcidin levels. Patients exhibited IL-6 levels higher than those of heterozygotes (p=0.003). Furthermore, hepcidin levels were significantly different across the tested groups (p= 0.006), being elevated in patients (median= 58.6 ng/mL, IQR 41.7–71.9) and in heterozygous individuals (median= 60.7 ng/mL, IQR 51.7–68.3) as compared with controls (median= 42.8 ng/mL, IQR 30.9–50.5) (p= 0.005 and 0.006, respectively)

Conclusions: In experiments performed in the TIM-1 system, the cornstarches and commercial modified starch analyzed in this study displayed only small differences in glucose release. These findings suggest that the differences in glucose response reported in GSD patients may be related to inter- and intraindividual variations and/or to treatment compliance. It is important to take into account that the small difference in glucose release found among the starches could be clinically important in vivo when taking variables such as hormonal responses and disease phenotype into account. Sweet polvilho was associated with slower glucose release and seems to be an interesting product that warrants more in-depth study as an alternative treatment, aiming to extend normoglycemia in patients with hepatic GSD. Anemia is a common finding in hepatic GSD, and was most common in GSD Ib, the type of GSD associated with the highest IL-6 levels. These findings suggest that inflammation is associated with development of anemia in hepatic GSD, particularly in GSD Ib.

1. INTRODUÇÃO

1.1. Glicogenoses hepáticas

As Glicogenoses (GSD) consistem em condições patológicas de etiologia genética que resultam em alterações no metabolismo do glicogênio (Wolfsdorf e Weinstein, 2003). Essas condições podem ser classificadas em diferentes tipos, nomeadas de acordo com o defeito enzimático específico e os órgãos afetados. Cada um dos diferentes tipos caracterizados atualmente é causado por diferentes genes, diferentes enzimas e tipos variados de herança que acabam acarretando variações nas manifestações clínicas e mesmo na forma de tratar as doenças. Novos subtipos ainda estão sendo descritos, devido às diferentes características clínicas, bioquímicas e genéticas (Laforêt *et al*, 2012).

A concentração de glicose no sangue é provavelmente uma das mais bem reguladas em nosso organismo. Uma das principais razões para a regulação rigorosa dos níveis sanguíneos de glicose é que o cérebro depende de um suprimento contínuo dessa substância, ainda que possa se adaptar a níveis mais baixos ou mesmo usar corpos cetônicos a partir da degradação de gorduras. Essa adaptação é essencial nos períodos de jejum (Shils *et al*, 2003). A glicogenólise hepática (quebra do glicogênio para formar glicose), na ausência de uma fonte de alimentação exógena para prover glicose, é um mecanismo que recompõe as taxas de glicose de uma forma rápida (Campbell, 2000). O fígado representa uma função importante na regulação do estoque de glicose e de armazenamento de glicose (Nordlie e Foster, 1999).

Durante o período prandial imediato, a produção de glicose endógena é cessada e a glicose exógena pode ser metabolizada em piruvato ou armazenada na forma de glicogênio no fígado e nos músculos. A glicose é armazenada no fígado e nos músculos como polímeros ramificados de glicogênio. As enzimas que controlam o metabolismo do glicogênio hepático são reguladas por uma série complexa de fosforilação e desfosforilação e por mecanismos alostéricos e sob influência hormonal (Shils *et al*, 2003).

A frequência estimada global das GSD é de 1/20.000 a 1/25.000 nascidos vivos (Sanjurjo, 2010), o que as caracteriza como doenças raras. As formas mais comuns são os tipos I, II, III e, entre os tipos com envolvimento hepático, os mais comuns são as do tipo I, III e IX. Não são conhecidos dados acerca da frequência das GSD no Brasil, e sua incidência pode estar subestimada pela falta de acesso aos métodos diagnósticos adequados e à suspeita clínica. No entanto, sugere-se que os tipos mais frequentes em nosso país são os tipos I e III (dados obtidos de levantamento dos pacientes do Hospital de Clínicas de Porto Alegre e da Universidade de Campinas).

Para o tratamento das GSD hepáticas, a estratégia mais utilizada é a administração frequente de amido de milho cru, visando a manutenção da normoglicemia e a prevenção de distúrbios metabólicos secundários. Restrições e suplementações dietéticas são realizadas de acordo com os diferentes tipos de GSD. O seguimento do tratamento é um desafio tanto para pacientes como seus familiares/cuidadores, exigindo cuidados rigorosos e disciplina. Além disso, não há um consenso sobre a dieta, havendo internacionalmente diferentes recomendações de tratamento.

Na figura 1 é apresentado o metabolismo do glicogênio e a classificação das GSD hepáticas e na tabela 1 são apresentadas as características das mesmas (Wolfsdorf e Weinstein, 2003; Beauchamp *et al*, 2007; Kishnani *et al*, 2010; Hicks *et al*, 2011; Dagli *et al*, 2012; Bali *et al*, 2013; Chen *et al*, 2014; Kishnani *et al*, 2014). As GSD I, III e IX, por serem os tipos mais prevalentes, serão detalhadas a seguir.

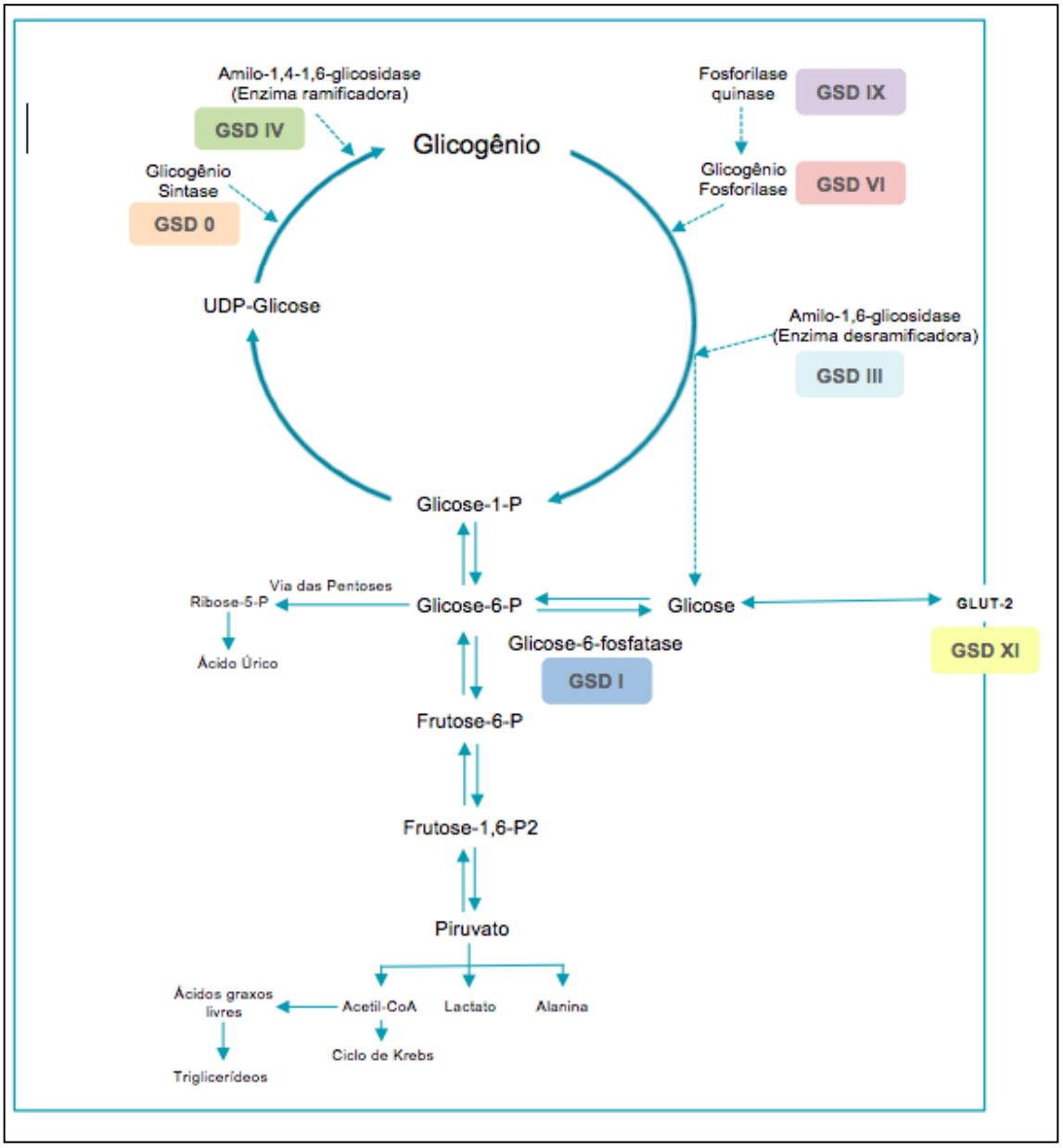

Figura 1 - Metabolismo do Glicogênio e Classificação das Glicogenoses hepáticas
Fonte: Criada com dados provenientes de Wolfsdorf e Weinstein, 2003; Beauchamp *et al*, 2007; Hicks *et al*, 2011; Dagli *et al*, 2012; Bali *et al*, 2013; Chen *et al*, 2014

Tabela 1. Classificação das glicogenoses hepáticas e resumo de suas características

Tipo (MIM)	Enzima envolvida	Gene	Herança	Clínica/Bioquímica	Tratamento
0,forma hepática (240600)	Glicogênio sintase (hepática)	*GYS2*	AR	Hipoglicemia cetótica ao jejum, hiperglicemia, hiperlactacidemia e hiperlipidemia pós-prandial. Ausência de hepatomegalia.	Dieta hiperproteica e com carboidratos complexos de baixo índice glicêmico; uso noturno de amido de milho cru
Ia (232200)	Glicose-6-fosfatase	*G6PC*	AR	Hipoglicemia, hepatomegalia, retardo do crescimento, acidose lática, hiperuricemia e hiperlipidemia	Uso de amido de milho cru; restrição de galactose, frutose, lactose e sacarose
Ib (232220)	Transportador de Glicose-6-fosfato	*SLC37A4*	AR	Idem Ia, acompanhando de neutropenia (infecções recorrentes, doença inflamatória intestinal)	Uso de amido de milho cru; restrição de galactose, frutose, lactose e sacarose. Uso de estimulador de colônia (filgastrima)
IIIa e IIIb (232400)	Enzima desramificadora de glicogênio	*AGL*	AR	Hepatomegalia, hipoglicemia cetótica; retardo de crescimento, hiperlipidemia, elevação da AST e ALT, CPK. Fraqueza muscular e cardiomiopatia ocorrem no subtipo IIIa	Uso de amido de milho cru; dieta hiperproteica; restrição de sacarose.
IV (232500)	Enzima ramificadora de glicogênio	*GBE1*	AR	Hepatomegalia, retardo do crescimento e cirrose	Transplante de fígado nos casos graves
VI (232700)	Glicogênio fosforilase hepática	*PYGL*	AR	Hepatomegalia, retardo do crescimento, hipoglicemia, hiperlipidemia e hipercetose leves	Se sintomático, aumento de carboidrato e alimentação frequente e dieta hiperproteica.
IXa1 e IXa2 (306000)	Fosforilase quinase (subunidade alfa)	*PHKA2*	LX	Hepatomegalia, hipoglicemia cetótica ao jejum, retardo do crescimento, elevação de AST/ALT e hiperlipidemia leves	Uso de amido de milho cru; dieta hiperproteica; evitar grandes quantidade de sacarose
IXb (261750)	Fosforilase quinase (subunidade beta)	*PHKB*	AR	Semelhante IXa	Uso de amido de milho cru; dieta rica em proteínas; evitar grandes quantidade de sacarose
IXc (613027)	Fosforilase quinase (subunidade gama)	*PHKG2*	AR	Semelhante IXa e cirrose hepática	Uso de amido de milho cru; dieta rica em proteínas; evitar grandes quantidade de sacarose
XI (227810)	Transportador de glicose-2	*GLUT2*	AR	Hipoglicemia, déficit de crescimento, raquitismo e abdome protuberante devido ao aumento do tamanho do fígado e rins	Restrição da ingestão de galactose, suplementação de água, amido de milho, eletrólitos e vitamina D

AR=autossômica recessiva; LX= ligada ao X

Fonte: **(**Wolfsdorf e Weinstein, 2003; Beauchamp *et al*, 2007; Kishnani *et al*, 2010; Hicks *et al*, 2011; Dagli *et al*, 2012; Bali *et al*, 2013; Chen *et al*, 2014; Kishnani *et al*, 2014)

1.1.1. Glicogenose tipo I

A GSD Ia, também denominada de doença de Von Gierke (MIM: 232200), possui um padrão de herança autossômico recessivo, e é caracterizada pela deficiência da atividade de glicose-6-fosfatase (G6Pase) (Koeberl *et al*, 2007). Essa enzima localiza-se no retículo endoplasmático sendo expressa principalmente no fígado, rins e intestino (Grinshpun *et al*, 2010; Froissart *et al*, 2011). O gene *G6Pase* localiza-se no cromossomo 17q21.31 (Brody *et al*, 1995). A GSD Ib (MIM:232220) ocorre por uma alteração no transportador de glicose-6-fosfato (G6PT). Em humanos, o gene *SLC37A4* foi mapeado no cromossomo 11q23.3 (Annabi *et al*, 1998) . A GSD Ib é menos comum do que defeitos na G6Pase e representa cerca de 20% de todos os casos de GSD I. A GSD I tem incidência estimada de 1:100.000 (Bali *et al*, 2013) e não há dados epidemiológicos sobre a frequência em nosso meio.

A G6Pase é um complexo funcional constituído por uma unidade catalítica com o centro ativo no lúmen do retículo endoplasmático e por transportadores que permitem a entrada do substrato, glicose-6fosfato, e a saída dos produtos da reação, glicose e fosfato (Santos-Antunes e Fontes, 2009). A G6Pase, juntamente com o G6PT, formam um complexo responsável pela produção de glicose catalisando os pontos finais da glicólise e da gliconeogênese, sendo que ambas estão alteradas em pacientes com GSD I (Bali *et al*, 2013).

A maioria dos pacientes com GSD I pode ser diagnosticada por meio de uma combinação de achados clínicos, testes bioquímicos e genéticos (Laforêt *et al*, 2012). Além disso, pode ser realizado o estudo do sistema G6Pase que requer uma amostra hepática de fígado não congelado. A medida da atividade de G6Pase é realizada em microssomas intactos e em microssomas rompidos. A GSD Ia é caracterizada pela deficiência de atividade de G6Pase em microssomas hepáticos intactos e rompidos, já na GSD Ib a atividade de G6Pase é deficiente em microssomas intactos e (sub)normal em microssomas rompidos (Laforêt *et al*, 2012). Na maioria dos pacientes com GSD Ia, a atividade residual da G6Pase é inferior a 10% do normal (Bali *et al*, 2013). O diagnóstico da GSD Ia pode ser

também estabelecido por meio da análise do gene *G6PC*. A pesquisa de mutações em *G6PT* deve ser realizada se os pacientes sofrem de neutropenia e/ou infecções recorrentes (achados comuns em GSD Ib), bem como todas as outras características clínicas e bioquímicas já descritas para pacientes com GSD Ia (Laforêt *et al*, 2012).

Pela produção endógena de glicose estar alterada ou ausente e pelas rotas alternativas que a glicose-6-fostato segue, ocorrem alterações metabólicas significativas nesses pacientes, como: hipoglicemia, aumento de lactato, aumento de ácido úrico, hipertrigliceridemia e hiperlipidemia, especialmente após um curto período de permanência em jejum (Bali *et al*, 2013).

Os sintomas podem estar presentes ao nascimento ou nas primeiras semanas de vida. O mais comum é o aparecimento, aos 3 - 4 meses de vida, de hepatomegalia ou convulsão associada à hipoglicemia. Alterações frequentemente descritas incluem face de boneca, obesidade troncular, abdômen globoso pela hepatomegalia, resultante do acúmulo de glicogênio e esteatose hepática, hipotonia e baixa estatura. Também são descritos anemia, manifestações intestinais, como diarreia intermitente (Sanjurjo, 2010), enteropatia relacionada à GSD (Rake *et al*, 2002) e dano da mucosa intestinal (Bhattacharya, 2011). A GSD I também manifesta-se com doença renal progressiva (Moses, 2002), alterações hepáticas teciduais tais como: hepatócitos edemaciados, esteatose, hiperglicogenação nuclear (Talente *et al*, 1994), e adenomas hepáticos em 22%-75% dos pacientes adultos. Os adenomas hepáticos podem ser solitários ou múltiplos e como complicação podem apresentar hemorragia ou malignização (risco de 10%). A regressão dos adenomas com a instituição da dieta adequada é descrita (Lee, 2002).

O retardo do crescimento e a baixa estatura são achados praticamente universais (Smit, 1993). Nos pacientes com tratamento inadequado, sabe-se que a acidose metabólica crônica pode alterar a atividade do hormônio de crescimento. Evidências mostram que um bom controle metabólico desses pacientes pode favorecer o crescimento (Moses, 2002). Alguns pacientes apresentam osteoporose, a qual, por sua vez, pode estar relacionada à nutrição

inadequada, à falta de vitaminas, aos efeitos do ácido láctico e ao hipogonadismo (Cabrera-Abreu *et al*, 2004).

Na GSD Ib é descrita ainda neutropenia crônica e déficit de função de neutrófilos e monócitos. A neutropenia resulta em infecções bacterianas recorrentes além de úlceras orais e na mucosa intestinal (Bali *et al*, 2013). A doença inflamatória intestinal semelhante à Doença de Crohn é um achado frequente na GSD Ib, sendo usualmente a maior causa de morbidade nesses pacientes (Weinstein *et al*, 2008).

O tratamento da GSD I é basicamente dietético e tem como objetivo proporcionar uma fonte contínua de glicose, manter normoglicemia (glicose > 4mmol/L [70 mg / dL]) e prevenir distúrbios metabólicos secundários (Laforêt *et al*, 2012). As estratégias atuais de tratamento incluem dieta contínua noturna por sonda nasogástrica e/ou administração frequente e a intervalos regulares de amido de milho cru (AMC) (Rake *et al*, 2002).

O uso de sonda nasogastrica foi introduzida em 1974, possibilitando a manutenção da normoglicemia (Burr *et al*, 1974; Greene *et al*, 1976). Em 1984, foi descrita a utilização de AMC, na dose de 1,75 a 2,5g/kg de peso a cada 4 a 6 horas, como uma boa alternativa de tratamento para a GSD I (Chen *et al*, 1984). Por ser um polissacarídeo, o AMC apresenta uma degradação lenta e mantém assim a normoglicemia plasmática. A dose de AMC para os pacientes com GSD I varia de acordo com a idade e o controle metabólico do paciente, com intervalos de 3-5 horas durante o dia e 4-6 horas no período da noite (Wolfsdorf e Weinstein, 2003).

A distribuição calórica da dieta dos pacientes deve ser 60 a 65% de carboidratos, sendo destes, 30 a 45% sob a forma de amido de milho cru, 20 a 25% de lipídeos e 10 a 15% de proteínas. A sacarose, frutose e lactose devem ser rigidamente restritas, pois transformam-se em glicose e são absorvidas rapidamente e transformada em glicogênio, aumentando progressivamente o glicogênio hepático, gerando uma extensa lista de alimentos que não são permitidos na dieta desses pacientes (Rake *et al*, 2002; Laforêt *et al*, 2012; Kishnani *et al*, 2014).

A glicemia deve ser monitorada diariamente, possibilitando a realização de ajustes no uso do AMC, tanto para dose quanto para horários, além de ajustes na prescrição da dieta (Kishnani *et al*, 2014).

As suplementações de vitaminas e minerais devem ser realizadas de acordo com o tipo de dieta oferecida e a idade, baseadas no recordatório alimentar e de acordo com as recomendações da Organização Mundial de Saúde. Esses pacientes têm várias restrições dietéticas como de frutas e produtos lácteos, que resultam em limitada oferta, por exemplo, de cálcio e vitamina D (Kishnani *et al*, 2009). Em estudo americano, observou-se que de 26 pacientes com diagnóstico de GSD I, dezesseis (61,5%) apresentavam níveis subótimos de 25-hidroxivitamina D (<30 ng/ml), mesmo recebendo oferta de vitamina D e cálcio conforme recomendação da Organização Mundial da Saúde. Foram sugeridas como causadoras de tais achados, a natureza restritiva da dieta, as alterações metabólicas e a má absorção intestinal (Banugaria *et al*, 2010). As restrições dietéticas que focam na manutenção da normoglicemia podem resultar em deficiências nutricionais, como a ingestão de ferro e vitaminas (Kishnani *et al*, 2014).

O tratamento farmacológico da GSD I objetiva principalmente os controles da hiperuricemia, da acidose persistente e da perda urinária de proteína. Acrescenta-se ainda o uso de estimulador de colônia de neutrófilos (filgastrima) para aqueles com GSD Ib (Rake *et al*, 2002).

O transplante de fígado é uma opção terapêutica apenas para os pacientes que apresentam complicações graves como descompensação metabólica frequente, atraso grave no desenvolvimento e surgimento de neoplasias (Morioka *et al*, 2005).

1.1.2. Glicogenose tipo III

A GSD III (MIM: 232400), também conhecida como Doença de Cori ou Doença de Forbe, é uma doença autossômica recessiva que ocorre quando há

deficiência da enzima desramificadora (amilo-1,6-glicosidase) (MIM: 610860). A liberação de glicose do glicogênio requer a ação tanto da glicogênio fosforilase quanto da enzima desramificadora (Endo *et al*, 2006; Ozen, 2007). Quando há ausência ou deficiência da enzima desramificadora, a glicogenólise é interrompida nos pontos de ramificação mais externos. Como consequência, ocorre o acúmulo anormal de glicogênio nos órgãos afetados (fígado, coração, musculatura esquelética, leucócitos no tipo IIIa, e fígado no tipo IIIb). O gene *AGL* localiza-se no cromossomo 1p21.2 (Bao *et al*, 1996).

Existem dois subtipos principais de GSD III: o tipo IIIa, o qual afeta tanto o fígado quanto os músculos, e compreende cerca de 85% dos casos, e o tipo IIIb que afeta somente o fígado e compreende cerca de 15% de todos os pacientes com GSD III (Ozen, 2007). Estes dois fenótipos clinicamente distintos são causados por mutações no mesmo gene (*AGL*) (Goldstein *et al*, 2010) e são explicados por diferenças na expressão da enzima deficiente em diferentes tecidos (Endo *et al*, 2006). É descrita uma incidência estimada de 1:100.000 para a doença (Dagli *et al*, 2012).

O diagnóstico inicia a partir da suspeita clínica (hepatomegalia, hipoglicemia, alterações de transaminases e creatinaquinase), pode ser confirmado por meio da medida anormal de glicogênio no fígado/músculo ou por meio da medida da atividade da amilo-1,6-glicosidase no fígado, músculo e nos eritrócitos. A medida da enzima no músculo é necessária para diferenciar os subtipos IIIa e IIIb, pois pacientes com GSD IIIa podem apresentar função muscular normal bem como níveis normais de creatina quinase na idade adulta e podem ser erroneamente diagnosticados com GSD IIIb (Goldstein *et al*, 2010). Contudo, devido a dificuldade técnicas, dificilmente essa investigação é realizada na prática. A análise de mutações é um método diagnóstico fácil e não invasivo e pode ser feita a partir de amostras de sangue, biópsia do fígado, músculo esquelético e cardíaco, eritrócitos e fibroblastos cultivados (Ozen, 2007; Hicks *et al*, 2011).

Os indivíduos com GSD III apresentam hepatomegalia, hipoglicemia, baixa estatura, miopatia esquelética e miocardiopatia. A maioria dos pacientes tem tipo

IIIa e apresentam envolvimento hepático e muscular, já os demais pacientes, com tipo IIIb, apresentam apenas envolvimento hepático (Kishnani *et al*, 2009).

A doença hepática ocorre progressivamente ao longo da vida com o desenvolvimento da fibrose do fígado e, em alguns casos, cirrose e carcinoma hepatocelular. Adenomas hepáticos têm sido observados em 25% dos pacientes com GSD III, contudo, a relação entre a formação de lesões e o controle metabólico ainda não é bem conhecida (Dagli *et al*, 2012). Alguns pacientes apresentam hipertrigliceridemia, mas ácido úrico e lactato costumam estar dentro da faixa considerada normal (Laforêt *et al*, 2012). A presença de cetose urinária e plasmática é um marcador bioquímico de mau controle metabólico.

Cardiomiopatia hipertrófica ocorre na maioria dos indivíduos com tipo IIIa, mas sua significância clínica continua incerta e a maioria dos pacientes são assintomáticos. A miopatia está ausente ou é mínima na infância e progride lentamente, tornando-se proeminente na terceira ou quarta década de vida. Osteoporose e osteopenia têm sido observados em GSD III assim como em outras doenças de depósito de glicogênio, provavelmente associadas à baixa ingestão de cálcio. Além disso, pode ser encontrada a doença do ovário policístico, mas a fertilidade não parece ser afetada. O crescimento desses pacientes pode ser comprometido pelo mau controle metabólico, contudo a retomada do crescimento pode ser observada com o estabelecimento de um bom controle metabólico, baseado em uma dieta adequada, indicada para GSD (Dagli *et al*, 2012).

Os sintomas podem ser considerados menos graves que na GSD I, sendo que a tolerância ao jejum sofre variação e a hipoglicemia em geral é menos grave (Dornelles *et al*, 2010).

O principal foco do tratamento é a oferta regular de glicose, a fim de manter seus níveis sanguíneos acima de 70 mg/dL (Laforêt *et al*, 2012). A terapia com AMC é prescrita com dose de acordo com a idade e o controle metabólico do paciente a cada 4-6 horas, sendo capaz de manter a normoglicemia, aumentar a velocidade de crescimento e diminuir a concentração das transaminases (Weinstein *et al*, 2008). Galactose e frutose podem ser utilizadas, contudo as suas

quantidades devem ser adaptadas a cada paciente, evitando o armazenamento excessivo de glicogênio (Laforêt *et al*, 2012).

A gliconeogênese está preservada nesses pacientes, sendo recomendada uma ingestão de 3g/kg/dia de proteína, visto que essa pode ser utilizada como uma fonte de energia eficaz. A alta ingestão proteica impede ainda a quebra de proteína muscular em momentos de jejum, preservando assim os músculos esqueléticos e cardíacos (Dagli *et al*, 2012). A dieta recomendada para esses pacientes compreende valores de 55-60% de carboidratos, 15-20% de proteína e 20-25% de lipídeos (Weinstein *et al*, 2008).

Devido à possibilidade de osteopenia e osteoporose, a suplementação de vitamina D e cálcio também é recomendada nessa condição (Dagli *et al*, 2012).

1.1.3. Glicogenose tipo IX

A GSD IX é causada pela deficiência da enzima fosforilase quinase e pode ser dividida em subtipos. A GSD IXa é o subtipo mais comum, sendo causada por mutações em *PHKA2,* com localização citogenética em Xp22.13 e tem padrão de herança ligada ao X. A GSD IXb é causada por alterações no gene *PHKB,* com localização citogenética em 16q12.1 e tem padrão de herança autossômica recessiva. A GSD IXc é causada por mutações em *PHKG2*, com localização citogenética em 16p11.2 e tem padrão de herança autossômica recessiva. É descrita uma incidência estimada de 1:100.000 para deficiência de *PHK* hepática (Goldstein *et al*, 2011; Laforêt *et al*, 2012).

O diagnóstico pode ser realizado em diferentes tecidos, mas sua sensibilidade é baixa pelas diferentes isoformas da enzima que esses apresentam. A análise de mutações em diferentes genes pode ser utilizada quando estiver disponível (Weinstein *et al*, 2008).

A apresentação clínica da deficiência de fosforilase quinase hepática inicia nos primeiros anos de vida, sendo característicos os sintomas de hepatomegalia

e retardo de crescimento, sendo esse mais pronunciado na infância, quando na vida adulta os pacientes podem alcançar estatura normal.

Podem ser encontrados também hipoglicemia hipercetótica, sendo essa geralmente leve, a puberdade pode ser atrasada e presença de fibrose hepática que pode progredir mais raramente para cirrose (Goldstein *et al*, 2011). São descritos ainda elevação de transaminases hepáticas, hipercolesterolemia e hipertrigliceridemia (Laforêt *et al*, 2012).

Apesar dessa ser tipicamente classificada como um tipo leve de GSD, alguns pacientes podem apresentar sintomas típicos de GSD I e III, com hipoglicemia, grave hepatomegalia e aumento de transaminases (Laforêt *et al*, 2012).

O tratamento dessa condição visa prevenir a hipoglicemia e a cetose. Preconiza-se dieta frequente e complementada com a utilização de AMC, com dose e frequência de acordo com a idade e a tolerância ao jejum e com os sintomas clínicos (Wolfsdorf e Weinstein, 2003). O percentual de consumo proteico varia entre 15 e 25%, visto que a via de gliconeogenêse está preservada, essa pode ser uma fonte de glicose (Goldstein *et al*, 2011). Portanto, recomenda-se de 3-3,5g/kg/dia de proteína.

1.2. Caracterização de amidos

Os humanos produzem uma série de enzimas que hidrolisam o amido. A saliva contém α-amilase, contudo o alimento permanece pouco tempo na boca e essa produz um pequeno efeito na digestão do amido, já o pâncreas libera α-amilase pancreática para o lúmen do duodeno produzindo glicose, oligossacarídeos e dextrinas, desempenhando um papel crucial na digestão do amido. A glicose pode ser absorvida no intestino delgado, contudo maltose e dextrinas necessitarão da ação de enzimas que são produzidas por células epiteliais que revestem as vilosidades intestinais (Tester *et al*, 2004).

O amido nativo é sintetizado como grânulos esféricos grosseiros em

diferentes tecidos de plantas, como pólen, folhas, caules, raízes, tubérculos, bulbos, rizomas, frutos e sementes. Os amidos comercialmente disponíveis são extraídos de forma pura a partir de uma variedade de fontes como arroz, batata e mandioca, e, predominantemente, do milho. O amido puro consiste predominantemente de uma mistura de amilose (moléculas relativamente pequenas e lineares) e amilopectina (moléculas maiores e fortemente ramificadas). As plantas sintetizam o amido como uma forma compacta semicristalina para diminuir o espaço de armazenamento e otimizar a concentração de energia (Tester *et al*, 2004). O valor da razão de amilose e amilopectina tem uma influência importante na taxa e na extensão que o amido será digerido (Bjorck *et al*, 1994).

O amido extraído do milho, quando na forma crua, comporta-se como um carboidrato lento, que libera glicose lentamente, através da atividade hidrolítica da amilase pancreática, suficiente para muitas horas. O cozimento do amido de milho altera suas características físico-químicas, passando a ser uma fonte de liberação rápida de glicose (Reis *et al*, 1999).

A mandioca é uma das mais tradicionais culturas agrícolas brasileiras, e é amplamente produzida no território nacional (Aplevicz e Demiate, 2007). Entre os principais produtos que são extraídos da mandioca está o polvilho que pode ser classificado em doce ou azedo de acordo com o teor de acidez (Brasil, 1978). O polvilho doce e o azedo são largamente utilizados na culinária brasileira, sendo considerados ingredientes básicos na produção de biscoitos e pão de queijo (Pereira *et al*, 2004).

1.2.1. Amidos e as Glicogenoses hepáticas

O tratamento das GSD hepáticas e que cursam com hipoglicemia é essencialmente dietético e baseia-se no fornecimento de carboidratos exógenos (via oral ou enteral), a fim de que sejam mantidos os valores normais de glicemia e que sejam corrigidos os defeitos metabólicos secundários (Laforêt *et al*, 2012).

A dieta contínua noturna foi introduzida em 1974, sendo demonstrado que a normoglicemia poderia ser mantida, que as alterações metabólicas secundárias poderiam ser corrigidas, e que haveria melhora da qualidade de vida dos pais e pacientes com GSD, devido à preservação do sono ininterrupto à noite, com o uso dessa estratégia (Burr *et al*, 1974; Greene *et al*, 1976). Em 1984, foi descrita a utilização de AMC, na dose de 1,75 a 2,5g/kg de peso a cada 4 a 6 horas, como uma boa alternativa de tratamento para a GSD I (Chen *et al*, 1984). Uma vez que o amido é um polissacarídeo constituído de amilose e amilopectina, a tolerância ao mesmo pode ser reduzida até os dois anos de idade devido à insuficiência da amilase pancreática característica dessa faixa etária (Hayde e Widhalm, 1990); o AMC, portanto, não se constitui em terapia inicial em lactentes com idade inferior a 6-8 meses de idade. O AMC não deve ser acrescentado à água morna ou quente ou a cítricos, como suco de limão, pois tal procedimento acelera a hidrólise do amido, consequentemente diminuindo sua ação de manter a normoglicemia (Reis *et al*, 1999).

Ambas as terapias, infusão contínua e uso de AMC, são capazes de prevenir a hipoglicemia e corrigir alterações metabólicas secundárias. Cada terapia apresenta vantagens e desvantagens, visto que diferem em certos aspectos entre si, como na idade de introdução, duração da normoglicemia, necessidade ou não de acordar no durante a noite, conteúdo calórico, índice glicêmico, risco de falha do equipamento com consequente risco metabólico, dificuldades para pais e/ou pacientes, invasividade e custos (Derks *et al*, 2013). Uma metanálise avaliou os efeitos das intervenções dietéticas sobre a manutenção da normoglicemia na GSD Ia. Neste estudo, foi realizada uma comparação entre o uso de AMC intermitente, infusão contínua com dextrose, amido de milho modificado e mistura de amido de milho com dextrose. A conclusão foi a de que, tanto a curto como a longo prazo, a administração intermitente, dia e noite, de AMC previne a hipoglicemia, inclusive a hipoglicemia noturna, de forma mais eficaz do que a infusão contínua com dextrose (Shah e O'Dell, 2013).

Apesar da utilização de AMC no tratamento das GSD ser bem sucedido, como descrito anteriormente, não existe o amido de milho que possa ser

considerado ideal e compreenda todos os requisitos de tratamento dos pacientes. Além da premissa de sustentar a normoglicemia durante toda a noite, o amido considerado o mais ideial na perspectiva do paciente deveria ser palatável, prático, com poucos efeitos colaterais e mantendo o apetite normal, além de evitar o ganho de peso excessivo (Bhattacharya *et al*, 2007). Sabe-se que os riscos envolvidos com um "supertratamento" em pacientes com GSD I incluem alta concentração de insulina e obesidade (Derks *et al*, 2013).

Considerando essas variáveis, algumas tentativas de desenvolvimento de um amido melhorado para tratamento das GSD tem sido realizadas. Bhattacharya *et al*, (2007), realizou um estudo randomizado, duplo cego, cruzado, no qual foram incluídos 21 pacientes com GSD I e III com idade entre 3 e 47 anos. O mesmo comparou a utilização de AMC em relação a um amido de milho cru fisicamente modificado (WMHM20). Foram realizadas análises da composição de ambos os amidos, onde o amido modificado (WMHM20) apresentava maior percentual de umidade, conteúdo de amilopectina e resistência, e valor semelhante de total de carboidrato. Os pacientes receberam 2g/kg de amido (máximo de 120g) misturado em água fria e foram coletadas amostras de sangue e ar expirado. O teste foi finalizado quando o paciente estava em jejum por 10h, glicose sanguínea <3mmol/L ou o paciente solicitou finalizar o mesmo. A glicose plasmática diminuiu mais lentamente e o lactato mais rapidamente quando utilizado WMHN20 em relação ao AMC, além disso, o pico de excreção de hidrogênio foi maior quando AMC foi ingerido, sendo que essa última medida é uma técnica para mensurar a malabsorção de amido. Esses dados sugerem uma maior duração de normoglicemia e melhor controle metabólico em curto prazo quando utilizado WMHN20, além disso, o estudo destaca a maior fermentação e o maior potencial malabortivo do AMC.

Correia *et al*, (2008) realizaram um estudo randomizado, duplo-cego e cruzado, comparando AMC clássico (Argo - ACH Food Companies Inc, Memphs, TN) e um amido desenvolvido, denominado Glycosade® (Vitaflo International Ltd, Liverpool, United Kingdom) em 12 pacientes com GSD I e idade entre 14 e 34 anos. Os pacientes receberam 100g de amido e os testes foram realizados em duas noites consecutivas. O amido experimental (Glycosade®) apresentava maior

percentual de umidade, conteúdo de amilopectina e resistência, e menor percentual de carboidrato total e menor índice glicêmico. Foram realizadas medidas de glicose e lactato até o momento em que a concentração de glicose alcançou valor de 60mg/dL ou após jejum de 10 horas. O amido experimental manteve valores maiores de 60mg/dL por um período estatisticamente mais longo que o amido clássico. Além disso, o amido clássico levou a um pico mais elevado de glicose e uma queda mais acelerada da mesma em relação ao novo amido.

Um novo estudo (Bhattacharya *et al*, 2015) avaliou a eficácia e a segurança de um novo amido chamado WMHMS (Vitaflo Ltd, Liverpool, UK), que segundo os autores é o WMHMS20, preparado em uma forma emulsificada. Esse foi um estudo randomizado, duplo-cego, onde o uso desse amido foi comparado ao uso de AMC tradicional. Foram recrutados 10 pacientes com GSD Ia e Ib, e os dados de 6 pacientes foram disponíveis para a fase de acompanhamento de 16 semanas com cada produto. Os dados da sobrecarga com os amidos estão disponíveis para 7 pacientes de forma pareada. Esse estudo conclui que o amido modificado apresentou uma diminuição significativa na liberação de insulina e reduziu o quantidade utilizada de amido em alguns dos pacientes estudados.

1.3. Metabolismo do ferro, hepcidina e interleucina-6

O ferro é uma substância considerada essencial para uma série de funções biológicas, tanto a nível sistêmico como a nível celular, atuando no transporte de oxigênio e atividade catalítica de uma série de enzimas (Heeney e Andrews, 2004).

Em relação à absorção de ferro, sob condições fisiológicas normais, o ferro entra no corpo humano por meio do intestino delgado. O ferro proveniente da dieta é mais tipicamente classificado como ferro heme e não heme. O ferro heme é encontrado de forma abundante em proteínas de origem animal, como na carne, como parte das hemeproteínas, hemoglobina e mioglobina (Fuqua *et al*, 2012). A maior parte do ferro não heme é fornecida por vegetais e cereais (Grotto, 2008).

A maior parte da necessidade diária de ferro é suprida pela recuperação de ferro dos eritrócitos, onde é realizada degradação de hemoglobina através da fagocitose dos eritrócitos senescentes por macrófagos do sistema reticuloendotelial. Este ferro reciclado é então disponibilizado para os precursores eritróides em desenvolvimento na medula óssea (Grotto, 2008).

Para circular no plasma, o ferro se liga à glicoproteína transferrina. A ligação à transferrina mantém o ferro na sua forma solúvel, sendo assim o veículo principal de entrega às células e limita a geração de radicais tóxicos (Hentze *et al*, 2010). A saturação de transferrina é um indicador do armazenamento de ferro no corpo, mas também reflete o balanço entre a liberação reticuloendotelial de ferro e a absorção na medula óssea (Gkouvatsos *et al*, 2012). Uma saturação de transferrina baixa indica deficiência de ferro e uma saturação alta é sinal de que está ocorrendo acúmulo de ferro. Quando a saturação excede 60%, o ferro não ligado à transferrina começa a se acumular na circulação e a danificar células parenquimatosas (Hentze *et al*, 2010).

A principal forma de armazenamento de ferro é através da ferritina (Shi *et al*, 2008). O armazenamento de ferro na ferritina é um processo reversível, o que possibilita sua liberação pela mesma em períodos de privação de ferro no organismo (Millonig *et al*, 2010). Células reticuloendoteliais (macrófagos) e hepatócitos são os dois principais locais de armazenamento de ferro em humanos, e os dois tipos celulares contribuem para o fornecimento de ferritina circulante (Gkouvatsos *et al*, 2012). Em condições fisiológicas normais, a ferritina plasmática se correlaciona com o ferro acumulado (Crook, 2012).

Transtornos na homeostase de ferro devido à deficiência ou ao acúmulo do mesmo são a causa de algumas das doenças humanas mais comuns. A chave para a homeostase de ferro está na regulação adequada dos níveis plasmáticos deste elemento (Hentze *et al*, 2010).

O metabolismo do ferro é balanceado por dois sistemas regulatórios, um que funciona de modo sistêmico e conta com o hormônio hepcidina e com a proteína transportadora de ferro ferroportina, e outro que controla predominantemente o metabolismo celular de ferro através de proteínas

regulatórias do ferro que se ligam aos elementos responsivos do ferro em mRNAs (Hentze *et al*, 2010).

Os hepatócitos desempenham um papel duplo no metabolismo sistêmico do ferro: eles são o principal sítio de armazenamento de ferro, e eles secretam a hepcidina, o principal hormônio regulatório da homeostase do ferro. A hepcidina, um hormônio peptídico composto com 25 aminoácidos é codificada pelo gene *Hepcidin antimicrobial peptide* (*HAMP*) (MIM606464) que exerce suas funções por meio do complexo hepcidina-ferroportina, o qual regula as concentrações de ferro intra e extracelular. Inicialmente a hepcidina possui 84 aminoácidos, a preprohepcidina. Após duas clivagens enzimáticas é liberada a forma com 25 aminoácidos que é a forma biologicamente ativa. As duas outras isoformas são secretadas e considerados produtos de degradação da mesma (Zhao *et al*, 2013; Kali *et al*, 2015).

A ferroportina é um receptor transmembrana que realiza a exportação celular de ferro. Quando os níveis de hepcidina aumentam, a mesma se liga à ferroportina, induz sua internalização e degradação, consequentemente ocorre a diminuição da liberação de ferro para o plasma, através da inibição da absorção de ferro intestinal pelos enterócitos e da inibição da mobilização dos estoques corporais do mesmo. Quando o nível de hepcidina diminui, a ferroportina fica exposta disponibilizando o ferro advindo da dieta e dos estoques dos macrófagos e hepatócitos, figura 2. Em condições normais, a hepcidina é regulada por *feedback*, onde sua transcrição é induzida por altos níveis de ferro e diminuída quando o nível do mesmo decresce (Ganz, 2003; Nemeth *et al*, 2004; Ganz, 2013; Zhao *et al*, 2013; Kali *et al*, 2015).

Os níveis de ferro regulam a expressão de hepcidina em um processo coordenado que envolve uma série de proteínas, entre eles, a hemojuvelina (HJV), BMP6 (*bone morphogenetic protein* 6), proteína da hemocromatose (HFE), receptor de transferrina 2 (TfR2), matriptase-2 (MT2), neogenina e receptores de BMP. Outra forma de regulação da expressão da hepcidina também pode ser realizada por meio de estimulo inflamatório, envolvendo citocinas. Um mecanismo de ação proposto envolve a rota de sinalização JAK-STAT3, onde a IL-6 se liga

ao seu receptor que ativa a JAK (*Janus Kinase*), levando a fosforilação e ativação da STAT3 (tradutor de sinal e ativador de transcrição 3), que induz então a produção de hepcidina (Zhao *et al*, 2013).

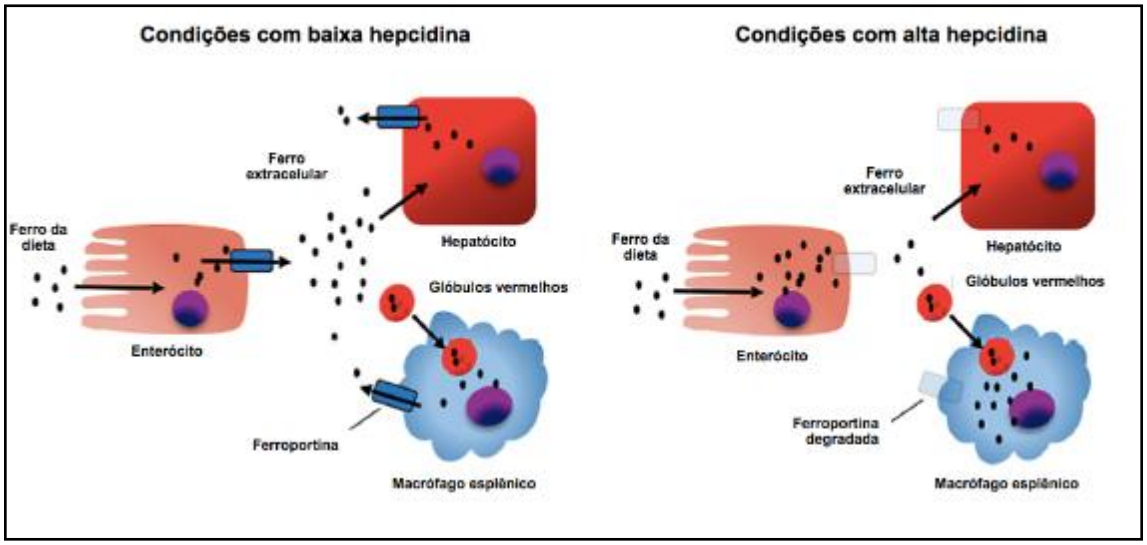

Figura 2. Atuação da hepcidina na homeostase do ferro.
Adaptado de Michels *et al*, (2015).

Alterações genéticas ou adquiridas que desregulam a produção de hepcidina levam à deficiência de ferro (altos níveis de hepcidina) ou ao acúmulo de ferro (baixos níveis de hepcidina) (Hentze *et al*, 2010). Alterações na regulação da hepcidina podem ser descritas em uma série de condições como na anemia de doença crônica, na anemia por deficiência de ferro refratária ao ferro (*iron refractory iron deficiency anemia*), na hemocromatose hereditária e na β-talassemia (Zhao *et al*, 2013).

Sugere-se que as citocinas inflamatórias, particularmente a IL-6, sejam desencadeantes para o aumento de hepcidina e esse mecanismo parece ser responsável pelo desenvolvimento da anemia por inflamação ou anemia por doença crônica (Nemeth e Ganz, 2009; Ganz, 2011). Estudo em ratos expostos à inflamação crônica demonstrou que os mesmos apresentaram maior concentração de hepcidina e desenvolveram anemia. Em contrapartida, os níveis de hepcidina não sofreram alteração naqueles ratos com deficiência de IL-6 e os mesmos não apresentaram anemia (Nicolas *et al*, 2001; Nicolas *et al*, 2002).

As citocinas são um grupo de proteínas solúveis, de baixo peso molecular, secretadas por linfócitos e outros tipos celulares do organismo, responsáveis por atuar na comunicação entre as diversas células do sistema imunológico em resposta a vários estímulos específicos, sendo sua secreção um evento breve (Kindt et al., 2007). As citocinas têm ainda uma ação importante sobre a regulação do crescimento de células, reparo tecidual, secreção de anticorpos e de outras citocinas, indução da resposta inflamatória e regulação da hematopoiese (Guimarães e Scroferneker, 2007). Elas possuem os atributos de pleiotropia, redundância, sinergismo, antagonismo e indução de cascata, permitindo regulação da atividade celular de uma forma coordenada e interativa (Kindt et al., 2007).

Uma forma de classificação das mesmas é em pró-inflamatórias e anti-inflamatórias, de acordo com a sua ação (Remick, 2003). A IL-6, juntamente com a IL-1 e o TNF, é uma das mais importantes citocinas pró-inflamatórias do sistema imune inato, tendo efeitos tanto locais como sistêmicos. É produzida mediante infecções e danos teciduais, sendo responsável por vários sinais clínicos encontrados em doenças infecciosas e inflamatórias. A IL-6 atua, entre outros, na produção hepática de outros mediadores inflamatórios, estimulando a síntese de proteínas da fase aguda, e a produção de neutrófilos na medula óssea e a diferenciação de linfócitos T auxiliares. A síntese da IL-6 é realizada por macrófagos, células endoteliais e linfócitos T e seu receptor atua na via de sinalização que ativa o fator de transcrição STAT3 (Abbas, 2011).

1.3.1. Anemia por deficiência de ferro e anemia por doença crônica

A anemia é definida, segundo a Organização Mundial da Saúde, como valores de hemoglobina no sangue abaixo do normal, a partir de valores de referência de acordo com idade e sexo (WHO, 2011).

A deficiência de ferro atinge uma grande parcela da população mundial. Entre as caudas da deficiência de ferro podem ser descritas, entre outras, a ingestão insuficiente de ferro, que pode resultar da falta de alimento, mal nutrição

ou restrições dietéticas, como a dieta vegetariana; causas genéticas como a anemia por deficiência de ferro refratária ao ferro; relacionada ao uso de medicamentos; e perda crônica de sangue, entre outras (Camaschella, 2015).

A anemia ferropriva é frequentemente assintomática e, dessa forma, muitas vezes não é diagnosticada. O balanço entre o ferro disponível e utilizado é negativo por um longo período, o que leva à diminuição dos estoques do mesmo. Sintomas inespecíficos dessa condição são cansaço, fadiga, dificuldade de concentração, entre outros. O tratamento mais amplamente utilizado é a suplementação de ferro. Na condição de deficiência de ferro a transcrição de hepcidina é inibida, sendo esse um mecanismo adaptativo para aumentar a absorção de ferro. Essa anemia se caracteriza por baixos estoques de ferro corporal, além da baixa hemoglobina (Camaschella, 2015).

A anemia por doença crônica, ou anemia da inflamação, tem como característica se apresentar como uma anemia leve ou moderada. Os valores de hemoglobina e ferro estão baixos, apesar dos estoques corporais estarem adequados. Ela geralmente ocorre por infecção, inflamação ou malignização. O diagnóstico é estabelecido quando ferro está baixo e ferritina normal ou alta. Contudo, cabe ressaltar, que a ferritina pode sofrer aumento pela inflamação e por dano tecidual, especialmente no fígado (Nemeth e Ganz, 2014). A suplementação não costuma ser benéfica nessa condição devido a diminuição de absorção pelos enterócitos (Stein, 2012).

A diferenciação da anemia por doença crônica da anemia ferropriva é em muitos casos considerada difícil, sendo que as duas podem ainda ser apresentadas concomitantemente (Nemeth e Ganz, 2014; Camaschella, 2015)

Uma série de estudos tem avaliado a relação entre hepcidina e IL-6 e a presença de anemia nas doenças crônicas, como na doença cardíaca, linfoma, mielola múltiplo e doença renal (Hohaus *et al*, 2010; Matsumoto *et al*, 2010; Eleftheriadis *et al*, 2014; Mei *et al*, 2014; Li *et al*, 2015; Lukaszyk *et al*, 2015).

A anemia também é a manifestação extraintestinal mais frequente na doença inflamatória intestinal, sendo sua causa, na maioria das vezes, associada à anemia ferropriva, devido à perda sanguínea e à alteração da absorção

intestinal, secundária à a inflamação tecidual, e a anemia pela inflamação crônica, secundária ao mecanismo citocinas (IL-6) e hepcidina (Kaitha *et al*, 2015).

1.3.2. Anemia, hepcidina e interleucina-6 nas Glicogenoses hepáticas

A anemia tem sido descrita como um achado frequente nas GSD hepáticas. Talente *et al*, (1994) descreveram a presença de anemia em 26 (81%) de 32 pacientes adultos americanos com GSD Ia e em 5 dos pacientes com GSD Ib.

Estudo europeu avaliou a concentração de hemoglobina em uma coorte de 231 pacientes com GSD Ia e 57 pacientes com GSD Ib e encontrou uma prevalência de anemia de aproximadamente 25% nos pacientes com o tipo Ia e 50% dos pacientes com tipo Ib pré púberes, 40% e 70% para tipo Ia e tipo Ib adolescentes, respectivamente e para 45% e 100% dos adultos com tipo Ia e tipo Ib, respectivamente (Rake *et al*, 2002).

Estudo mais recente (Wang *et al*, 2012), que avaliou 202 pacientes com GSD I, oriundos dos Estados Unidos da América, encontrou frequência de anemia de 41,7% para pacientes com tipo Ia e 71,8% para pacientes com tipo Ib. Segundo o mesmo estudo, a anemia ferropriva seria mais frequente em pacientes com GSD I na pré-adolescência, e anemia da doença crônica em pacientes adultos. A anemia severa associou-se com a presença de adenoma na GSD Ia e com a inflamação na GSD Ib.

As restrições dietéticas imposta no tratamento das GSD hepáticas focam na manutenção da normoglicemia, mas podem resultar em deficiências nutricionais, como a pobre ingestão de ferro, vitamina B12 e ácido fólico (Kishnani *et al*, 2014), além de ser descrito uma possível alteração na absorção do ferro relacionada à utilização crônica e contínua de amido de milho por pacientes com GSD hepática (Wang *et al*, 2012).

Acrescenta-se ainda que a presença de doença inflamatória intestinal, semelhante à Doença de Crohn, é um achado frequente em pacientes com GSD

Ib (Weinstein *et al*, 2008), e tem sido recentemente descrita em pacientes com GSD Ia (Lawrence *et al*, 2014; Lawrence *et al*, 2015). Estudo anterior avaliou indivíduos com Doença de Crohn ativa e inativa em relação aos níveis de IL- 6 e hepcidina. Os indivíduos com a doença ativa apresentaram níveis mais elevados de IL-6 plasmática e hepcidina urinária em relação aos com doença inativa, os pacientes com doença ativa apresentaram comprometimento na absorção de ferro oral (Semrin *et al*, 2006).

O papel das IL-6 nas GSD hepáticas tem sido pouco explorado. Um estudo de Marfaing-Koka *et al*, (2003) analisou valores plasmáticos de IL-6 em 27 pacientes com GSD Ia (média de idade= 15 anos, amplitude= 2 – 35 anos), quatorze pacientes com GSD tipos III e VI (média de idade= 16 anos, amplitude= 4 – 28 anos) e 30 adultos saudáveis como controles (média de idade= 28 anos, amplitude= 22 – 38 anos). Desses pacientes, 15 apresentavam adenomas (GSD Ia= 14; GSD III= 1). Nesse estudo não foi encontrada diferença de valores de IL-6 entre as diferentes GSD, e em relação ao controles saudáveis. Além disso, não foi encontrada diferença entre pacientes com e sem adenoma.

O envolvimento da hepcidina na patogênese da anemia nas GSD hepáticas foi levantado após a observação que cinco pacientes com tipo Ia apresentavam grandes adenomas hepáticos e sofriam de anemia severa não responsiva ao ferro. O estudo do tecido do adenoma de um dos pacientes demonstrou que o mesmo apresentava aumento na expressão do mRNA de hepcidina e esse aumento não era encontrado no tecido hepático não afetado pelo adenoma. Além disso, após a ressecção do adenoma ou o transplante hepático a anemia era resolvida. Não foi encontrado aumento da expressão de mRNA de IL-6 no tecido hepático ou do adenoma no paciente estudado (Weinstein *et al*, 2002).

Estudos tem sugerido que a anemia por doença crônica envolvendo a hepcidina seja a causa de grande parte das anemias na GSD I (Wang *et al*, 2012; Kishnani *et al*, 2014), contudo, não há dados disponíveis de dosagem de hepcidina e IL-6 plasmáticos e estudos de associação com anemia e variáveis clínicas e de tratamento em pacientes com GSD hepáticas.

2. JUSTIFICATIVA

O Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (SGM-HCPA) é um centro de tratamento e diagnóstico de GSD hepáticas, altamente reconhecido, sendo o único local do Brasil que realiza dosagem enzimática e análise molecular para o diagnóstico das mesmas. O acompanhamento dos pacientes nesse serviço é realizado por equipe multidisciplinar com ampla experiência no tratamento dessa condição.

As GSD hepáticas são doenças monogênicas relativamente frequentes, que costumam não comprometer as funções cognitivas dos pacientes, mas que estão associadas à alta morbidade na infância. Entre as suas manifestações clínicas incluem-se a ocorrência de hipoglicemias de repetição (e de suas consequências, tais como crises convulsivas) e a predisposição ao desenvolvimento de tumores hepáticos.

Uma das estratégias mais comumente utilizadas no tratamento das GSD hepáticas é a administração frequente de AMC. Apesar do tratamento utilizando AMC ser bem sucedido, não existe um amido de milho que possa ser considerado ideal. Algumas tentativas de desenvolvimento de amidos modificados têm sido feitas, contudo produtos médicos industrializados não são de fácil introdução, especialmente em países em desenvolvimento, e os pacientes com GSD acabam dependendo da utilização de amido de milho tradicional. Sabe-se da importância da variável “resposta individualizada do paciente” ao consumo de diferentes marcas de amido de milho cru, existindo alguma evidência, oriunda principalmente da experiência clínica, de que a resposta dos pacientes ao tratamento depende da marca de amido de milho que é administrado. Estudos sistemáticos, de boa qualidade, sobre a comparação da composição de vários produtos e de possíveis substitutos, são praticamente inexistentes na literatura.

Outro ponto a ser levantando têm sido os estudos de investigação do papel da hepcidina e da inflamação na patogênese da anemia nas doenças crônicas, caso das GSD hepáticas. A hepcidina é produzida principalmente pelo fígado e desempenha um papel central no metabolismo do ferro. É descrito ainda que a

inflamação pode induzir a síntese de hepcidina através de citocinas, especialmente a IL-6, ocasionando anemia por doença crônica. A existência de componente inflamatório nas GSD hepáticas, incluindo doença inflamatória intestinal semelhante a Doença de Crohn, é descrita principalmente na GSD Ib e mais recentemente na GSD Ia. Sabe-se ainda, que a anemia é uma manifestação comum da GSD I e uma das complicações descritas a longo prazo nessa condição. Pouco se sabe do papel da hepcidina e da IL-6 nessas condições, além de sua associação com metabolismo do ferro, parâmetros bioquímicos, clínicos e de tratamento.

Dessa forma, destaca-se a importância da realização de um estudo de caracterização e análise do processo de digestão de diferentes amidos de milho clássicos e de outros produtos que possam vir a ser utilizados no tratamento de pacientes com GSD hepáticas, a fim de buscar o produto com a composição mais adequada e que compreenda todos os requisitos de tratamento desses pacientes. Além disso, acredita-se que um estudo que avalie os níveis de hepcidina, IL-6 e parâmetros do metabolismo do ferro em pacientes com GSD hepáticas e que busque verificar o envolvimento dos mesmos nessa doença e na presença de anemia seja de extrema importância para o compreensão da mesma.

3. OBJETIVOS

3.1. Objetivos Gerais

1) Analisar a digestão de amidos de milho de diferentes marcas e provenientes de diferentes países, e de possíveis substitutos aos mesmos, por meio do modelo gastrointestinal, *in vitro,* TIM-1;

2) Determinar as frações de amido e a razão de amilose/amilopectina de amidos de milho de diferentes marcas e provenientes de diferentes países, e possíveis substitutos aos mesmos;

3) Caracterizar o perfil de hepcidina, de IL-6 e de outros parâmetros do metabolismo do ferro (ferritina, ferro e saturação de transferrina), em plasma de pacientes com Glicogenoses hepáticas, e avaliar sua associação com a presença de anemia, de forma a contribuir para a melhor compreensão da fisiopatogenia dessa complicação nessas condições.

3.2. Objetivos específicos

1) Comparar os achados de frações de amido, razão de amilose/amilopectina e a digestão *in vitro* entre amidos de milho estudados e em relação aos seus possíveis substitutos;

2) Identificar os amidos que seriam mais benéficos para o tratamento de pacientes com Glicogenoses hepáticas;

3) Verificar a frequência da presença de anemia em uma amostra brasileira de pacientes com Glicogenoses hepáticas;

4) Comparar os níveis de hepcidina entre pacientes com Glicogenoses hepáticas, heterozigotos e controles saudáveis;

5) Comparar os níveis de IL-6 entre pacientes com Glicogenoses hepáticas e

heterozigotos;

6) Caracterizar os diferentes tipos de Glicogenose hepática em relação aos níveis de hepcidina, de IL-6 e de anemia.

4. CAPÍTULOS

4.1. Capítulo 1 - *In vitro digestion of starches in a dynamic gastrointestinal model: an innovative study to optimize dietary management of patients with hepatic glycogen storage diseases*

Título do manuscrito: In vitro digestion of starches in a dynamic gastrointestinal model: an innovative study to optimize dietary management of patients with hepatic glycogen storage diseases.

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GLYCOGENOSES

In vitro digestion of starches in a dynamic gastrointestinal model: an innovative study to optimize dietary management of patients with hepatic glycogen storage diseases

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Abstract Uncooked cornstarch (UCCS) is a widely used treatment strategy for patients with hepatic glycogen storage disease (GSD). It has been observed that GSD-patients display different metabolic responses to different cornstarches. The objective was to characterize starch fractions and analyze the digestion of different starches in a dynamic gastrointestinal in vitro model. The following brands of UCCS were studied: Argo® and Great Value® from the United States of America; Brazilian Maizena Duryea® and Yoki® from Brazil; Dutch Maizena Duryea® from the Netherlands. Glycosade®, a modified starch, and sweet polvilho, a Brazilian starch extracted from cassava, were also studied. The starch fractions were analyzed by glycemic TNO index method and digestion analyses were determined by the TIM-1 system, a dynamic, computer-controlled, in vitro gastrointestinal model, which simulates the stomach and small intestine. The final digested amounts were between 84 and 86 % for the UCCS and Glycosade®, but was 75.5 % for sweet povilho. At 180 min of the experiment, an important time-point for GSD patients, the digested amount of the starches corresponded to 67.9–71.5 for the UCCS and Glycosade®, while it was 55.5 % for sweet povilho. In an experiment with a mixture of sweet polvilho and Brazilian Maizena Duryea®, a final digested amount of 78.4 % was found, while the value at 180 min was 61.7 %. Sweet polvilho seems to have a slower and extended

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release of glucose and looks like an interesting product to be further studied as it might lead to extended normoglycemia in GSD-patients.

Abbreviations

CNGDF	Continuous nocturnal gastric drip feeding
GTI method	Glycemic TNO index method
GSD	Glycogen storage disease
RAG	Rapidly available glucose
RS	Resistant starch
SAG	Slowly available glucose
TIM-1	TNO intestinal model-1
UCCS	Uncooked cornstarch

Introduction

Hepatic glycogen storage diseases (GSD) are rare inborn errors of glycogen metabolism associated with fasting hypoglycemia as endogenous glucose production is impaired. GSD type I (GSD I; OMIM /#232200) is caused by a deficiency of the glucose-6-phosphatase (G6Pase) complex (EC 3.1.3.9). GSD I is the most severe type of hepatic GSD, and both glycogenolysis and gluconeogenesis are defective. As a result, no endogenous glucose production can occur, and shunting of glucose-6-phosphase away from the enzymatic defect leads to hyperuricemia, hyperlactatemia, and hypertriglyceridemia. Clinically, GSD I patients present with seizures, lethargy, failure to thrive and hepatomegaly, and long-term complications include liver adenomas, progressive renal disease, and osteoporosis (Weinstein et al 2001; Moses 2002; Rake et al 2002; Di Rocco et al 2008; Bali et al 2010).

Dietary management is the mainstay treatment for GSD I patients. It aims to maintain sufficient exogenous carbohydrate intake to correct for the defective endogenous glucose production while avoiding over storage of carbohydrate in the liver (Laforêt et al 2012). In the 1970s, continuous nocturnal gastric drip feeding (CNGDF) was introduced as a treatment for GSD patients (Burr et al 1974; Greene et al 1976). In the 1980s, uncooked cornstarch (UCCS) was introduced as an alternative to extend the fasting period during the night (Chen et al 1984). We have previously discussed the advantages and disadvantages of both nocturnal treatments (Derks et al 2013). Recently, a modified starch was developed, and the first clinical studies report a longer maintenance of normoglycemia with the modified starch compared with UCCS (Bhattacharya et al 2007; Correia et al 2008). However, to date, modified cornstarches are not generally available worldwide. Therefore, many patients in developing countries depend on the classical cornstarch or their possible alternatives that are available locally.

Inter- and intra-individual variations complicate the in vivo studies of starches in GSD patients, and the duration of normoglycemia after using UCCS can vary between patients (Lee et al 1996; Weinstein and Wolfsdorf 2002). In clinical practice, we also observe different fasting responses in and between GSD patients depending on the brand of UCCS (unpublished data). The TNO in vitro model of the gastrointestinal tract-1 (TIM-1) is a dynamic, computer-controlled model of the upper gastrointestinal tract created to study digestibility in vitro. The model simulates the successive dynamic processes in the stomach and the small intestine. It has been validated with clinical data on carbohydrate digestion (Minekus et al 1995; Bellmann et al 2010; Lafond et al 2011), and has been used for screening to study digestibility of a number of different starches (Fassler et al 2006).

This study characterized the digestion of different starches under standardized conditions, using the dynamic in vitro gastrointestinal system TIM-1. We hypothesized that observed clinical variations may be explained by differences in starch digestibility due to differences between starches. In addition to testing the commonly used brands of starches, we included local Brazilian brands of starch derived from cassava to determine the potential for treatment of GSD I patients.

Materials and methods

Samples

Table 1 presents the starches included in this study. The products sour polvilho, sweet polvilho, tapioca, and cassava flour are extracted from cassava and are local products commonly used in Brazilian cuisine.

In vitro analysis of starch fractions

The starch fractions in the samples were analyzed using the glycemic TNO index method (GTI method), as described by Englyst et al, 1999, with small modifications. In brief, starch samples containing approximately 0.6 g carbohydrate were weighed (without pre-treatment), water was added to make the volume 5 mL, and they were incubated in pepsin-HCl for 30 min. The pH was then adjusted to pH 5.0, and the samples were incubated with pancreatin and a mixture of the microbial enzymes amyloglucosidase (Megazyme Co.,Wicklow, Ireland), invertase (Fisher Scientific Co, United Kingdom) and ß-galactosidase (Amano Enzyme Europe Ltd, United Kingdom) for 2 h. Glucose release was measured after 20 min (G20) and 120 min (G120) of incubation time. Glucose present at G20 was designated the "rapidly available glucose" fraction (RAG), while the amount produced between 20 and 120 min of incubation was designated the "slowly

Table 1 In vitro analysis of starch fractions in the samples by the GTI method

Sample[a]	Product	Origin	Starch fraction (%)			
			RAG	SAG	RS	TG
Argo®	Cornstarch	USA	19.9	32.2	38.9	95.3
Great value®	Cornstarch	USA	16.0	41.5	30.7	91.6
Brazilian Maizena Duryea®	Cornstarch	BR	19.4	42.3	28.0	92.8
Yoki®	Cornstarch	BR	17.7	44.4	30.8	96.3
Dutch Maizena Duryea®	Cornstarch	NL	23.0	38.9	29.3	94.5
Glycosade®	Modified starch	UK	32.3	43.1	11.0	87.6
Sour polvilho	Sour polvilho	BR	14.0	32.8	41.7	93.0
Sweet polvilho	Sweet polvilho	BR	12.7	30.5	43.8	91.9
Tapioca	Tapioca	BR	86.0	9.0	2.7	97.9
Cassava flour	Cassava flour	BR	78.8	2.9	0.1	81.8

[a] Cornstarches and the modified starch samples are called by the brand; Sour and Sweet Polvilho brand: Fritz e Frida®; Tapioca brand: Yoki®; and Cassava Flour brand: Brasileirinha®. BR, Brazil; NL, The Netherlands; RAG, rapidly available glucose; RS, resistant starch; SAG, slowly available glucose; TG, total glucose; UK, United Kingdom; USA, United States of America

available glucose" fraction (SAG). After 120 min, the samples were treated with sodium hydroxide to solubilize resistant starch (RS). This RS was converted quantitatively into glucose and total glucose was measured (TG). The resistant starch fraction was calculated by subtracting G120 from TG and multiplying the result by 0.9. Glucose was measured using a commercial kit (Megazyme Co.,Wicklow, Ireland).

Determination of the digestibility of test-compounds in the TNO dynamic in vitro system of the stomach and small intestine (TIM-1)

The dynamic, computer-controlled TIM-1 system was previous described by Minekus et al (1995). The system comprises four serial compartments simulating stomach, duodenum, jejunum, and ileum. Parameters such as body temperature (37 °C), food transit, peristalsis, concentrations of gastric acid, intestinal secretion fluids, and uptake of digestion products closely simulate the human digestion process. The flexible walls contract periodically mimicking the peristaltic movements of the gastrointestinal tract. Secretions of gastric juice, bile, and pancreatin are introduced via pumps connected to the compartments. The pH is regulated by secretion of hydrochloric acid in the stomach and sodium bicarbonate in the intestinal compartments. Hollow fiare dialyzers, composed of semi-permeable membranes that simulate absorption are connected to jejunal and ileal compartments (Hatanaka et al 2012) (Fig. 1).

Digestibility of the samples was studied for 6 h in duplicate experiments in TIM-1. In the fed state, 50 grams of starch, dissolved in water and with the addition of electrolyte solution, salivary amylase and citrate buffer (total volume 295 mL), were introduced in the gastric compartment. For the first 3 h, samples were taken every 15 min from the dialysate in the jejunum and ileum (Fig. 1o) and from the ileal efflux (indigestible fraction; Fig. 1h). Thereafter, samples were taken from the dialysates and ileal effluents every 30 min for another 3 h. In those 6 h, approximately 90–95 % of the 'meal' had passed the ileocecal valve into the colon or in this case to the collected ileal effluents. Undigested material and residues remaining in the model after 6 h were taken and analyzed as well, to be able to make a complete mass-balance.

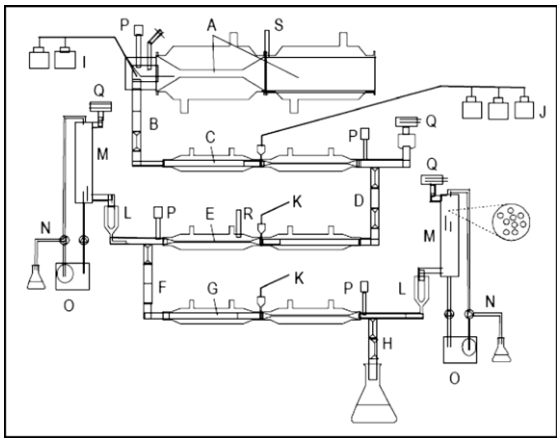

Fig. 1 Schematic of the TNO in vitro model of the stomach and small intestine (TIM-1). **a** = stomach compartment; **b** = pyloric sphincter; **c** = duodenum compartment; **d** = peristaltic valve; **e** = jejunum compartment; **f** = peristaltic valve; **g** = ileum compartment; **h** = ileo-caecal sphincter; **i** = stomach secretion; **j** = duodenum secretion; **k** = jejunum/ileum secretion; **l** = pre-filter; **m** = semi-permeable membrane; **n** = water absorption; **o** = collected dialysate; **p** = pH electrodes; **q** = level sensors; **r** = temperature sensor; **s** = pressure sensor. Reproduced from Hatanaka et al (2012) with permission

The glucose in samples from TIM-1 experiments was measured after acid hydrolysis using a commercial kit (Roche, Almere, The Netherlands) on a Cobas Mira plus autoanalyzer (Roche) by Bio-aNAlytiX (Mook, The Netherlands).

Calculation of digestibility

The digestibility of the products was expressed as a percentage of the amount of carbohydrate ingested (% of intake) to correct for small differences in the starch content of the various products. For this, the amounts of glucose present in the individual jejunal and ileal dialysates were summed and divided by the total glucose recovered in all fractions from the system, including the ileal effluents (indigestible fraction) and residues, after correction for recovery. These values are provided as individual time points and the cumulative values.

Statistical analysis

A two-tailed paired Student's *t test* was used to compare the results of the cumulative data of each starch against all of the other starches. The level of significance was set at 5 % ($p<0.05$).

Results

Data from the in vitro analysis of starch fractions in the samples are presented in Table 1. Based on these data, the following samples were selected to be studied in TIM-1: Argo®, Great Value®, Brazilian Maizena Duryea®, Yoki®, Dutch Maizena Duryea®, Glycosade® and sweet polvilho. The criterion for this was primarily a low RAG.

Table 2 presents the combined jejunum and ileum absorption according to collection time points, the total absorption, the ileal efflux, and the residue that remained in the model after the end of the experiment for the starches analyzed in the TIM-1 experiments. The cumulative jejunum and ileum absorption according to collection time points is presented in Fig. 2a. Table 3 represents the indices of significance (p-value) for two-tailed pairwise comparison of cumulative digestibility, between different substrates at time points 180 and 360 min.

Based on the results with the individual starches, an experiment was performed with a mixture of products in order to search for a product with a slower glucose release compared to UCCS and with the smallest possible amount of indigestible material (RS). A curve of cumulative combined jejunum and ileum absorption was estimated for a mixture of 60 % of Sweet Polvilho (30 g) and 40 % of Brazilian Maizena Duryea® (20 g), in which the expected total absorption was 79.9 % of the intake. The results from the experiment with the mixture are also shown in Table 2 and Fig. 2a.

Figure 2b presents the cumulative combined jejunum and ileum absorption according to collection time points focusing on the period 150 min (2.5 h) and 300 min (5 h). We consider this an important time interval derived from the clinical practice with hepatic GSD patients, when patients are usually treated in 3–5 hourly intervals. The differences in cumulative digestibility up to time point 180 min (within this period which we consider important) was significant between Sweet povilho and Brazilian Maizena Duryea®, Yoki® and Glycosade®. At this time point the mixture was significantly different from Argo® and Great Value® (Table 3).

Discussion

This is the first standardized comparative in vitro study of medically applied starches with implications for the dietary treatment of GSD patients. It was hypothesized that clinically observed differences between brands and/or countries of origin would be explained by the differences in digestibility of the cornstarches. This study reports small differences in digestibility between different brands of UCCS under standardized in vitro conditions. In developing countries or in countries with a large difference in social status, modified cornstarches and commercial UCCS are not generally available or affordable, which necessitates searching for local alternatives. Interestingly, sweet polvilho, a Brazilian cassava derived starch, demonstrated a digestibility pattern superior to many of the traditionally used cornstarches.

Starch consists of a mixture of amylose (relatively smaller, linear molecules) and amylopectin (larger, heavily branched molecules). Commercially available starches are extracted in pure form from a variety of sources, but predominantly corn (Tester et al 2004). The starch fractions RAG, SAG, and RS are interesting parameters to characterize the starches and some studies have been conducted in healthy men in order to analyze the influence of the starch fractions distribution in foods on the glycemic response (Anderson et al 2010; Eelderink et al 2012a; Eelderink et al 2012b). In the present study, the cornstarches did not show large differences in either the starch fractions or the experiments in TIM-1. In particular, they all present a small proportion of RS, or in this case of undigested material, in the TIM-1 experiments. The same behavior is seen for sweet polvilho; it presents a lower quantity of RAG in the GTI and higher value of RS. The TIM-1 experiment confirmed the lower glucose release and the larger quantity of undigested material for this product, but with a smaller quantity of undigested material than expected from RS determination in the GTI method. Glycosade® presented a higher value of RAG (32.5 %), compared to cornstarches (mean: 19.5 %) but this difference is not seen in the TIM-1

Table 2 Combined jejunum and ileum absorption from starches per time point (see Fig. 1o), total absorption (∑15–360 min) and ileal efflux (see Fig. 1h), in the TIM-1 experiments[a]

Time (minutes)	Argo®		Great value®		Brazilian Maizena Duryea®		Yoki®		Dutch Maizena Duryea®		Glycosade®		Sweet polvilho		Mixture[b]	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
15	0.1	0.2	0.3	0.2	0.1	0.2	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.6	0.2	0.3
30	1.3	0.9	1.0	1.3	1.0	1.3	1.1	1.0	1.4	1.1	1.1	1.2	0.9	1.1	1.0	0.7
45	4.1	4.1	3.9	3.3	3.9	3.7	3.9	4.2	3.8	3.9	3.7	4.2	3.1	2.9	3.5	2.9
60	4.8	4.5	4.8	4.7	4.6	5.6	4.3	4.7	6.0	4.3	4.9	4.6	3.6	4.4	4.5	4.3
75	7.9	7.0	8.2	7.8	6.9	6.4	6.8	6.7	8.6	7.3	7.2	7.7	5.6	5.3	6.2	6.9
90	8.3	7.1	7.6	8.0	8.2	9.4	8.1	8.7	7.9	8.5	8.4	8.4	5.6	6.0	7.3	6.6
105	8.7	8.6	9.1	8.1	9.2	9.8	8.7	9.1	8.6	8.6	8.7	9.0	6.5	6.7	7.4	8.2
120	7.2	8.7	8.8	8.7	8.3	8.7	7.7	8.9	8.4	8.2	8.1	8.4	5.8	5.8	7.7	7.2
135	9.0	8.6	7.8	8.7	8.7	8.5	8.3	7.4	7.3	7.6	6.9	7.4	5.8	6.1	6.9	7.4
150	7.7	7.4	6.8	7.3	7.0	6.7	6.5	7.8	7.1	7.3	6.8	7.5	6.6	6.0	6.0	5.5
165	5.9	7.3	6.0	5.5	6.6	7.1	6.2	5.7	6.4	6.2	5.5	5.7	5.3	6.2	5.5	6.1
180	5.7	5.5	4.5	5.0	5.3	5.6	5.4	5.9	4.8	5.9	5.0	4.8	5.2	5.7	5.5	5.6
210	5.1	5.3	4.2	4.4	5.2	5.1	5.0	5.5	4.2	5.3	4.8	4.3	4.9	5.3	5.2	5.1
240	4.5	5.2	4.0	4.0	5.0	4.4	4.7	5.0	3.9	4.6	4.6	3.9	4.7	4.6	4.9	4.4
270	3.1	4.0	3.7	3.4	3.0	3.0	3.2	3.9	3.4	4.5	4.4	3.5	4.6	4.0	3.4	3.9
300	1.5	1.8	2.1	1.6	1.4	1.5	1.4	1.6	1.5	2.0	2.1	1.7	2.9	3.5	1.6	1.9
330	0.5	0.9	1.3	0.7	0.5	0.5	0.5	0.6	0.8	0.7	0.9	1.0	1.7	1.7	0.8	1.0
360	0.2	0.5	1.1	0.3	0.2	0.2	0.3	0.2	0.5	0.3	0.4	0.5	0.9	1.1	0.4	0.6
Total absorption	86.0	87.4	85.2	83.1	85.1	87.7	82.2	87.2	84.8	86.5	83.7	84.2	74.2	76.8	78.0	78.9
Ileal effux	12.7	12.1	12.7	15.4	14.6	11.1	16.3	11.5	12.3	11.5	14.5	14.6	24.1	22.8	20.8	19.7
Residue[c]	1.2	0.5	2.1	1.5	0.3	1.2	1.5	1.3	2.9	2.0	1.8	1.2	1.7	1.2	1.2	1.4

[a] Data from the individual experiments of each product

[b] Mixture: 60 % of Sweet Polvilho plus 40 % of Maizena Duryea®

[c] Residue that remained in the model after the end of the experiment

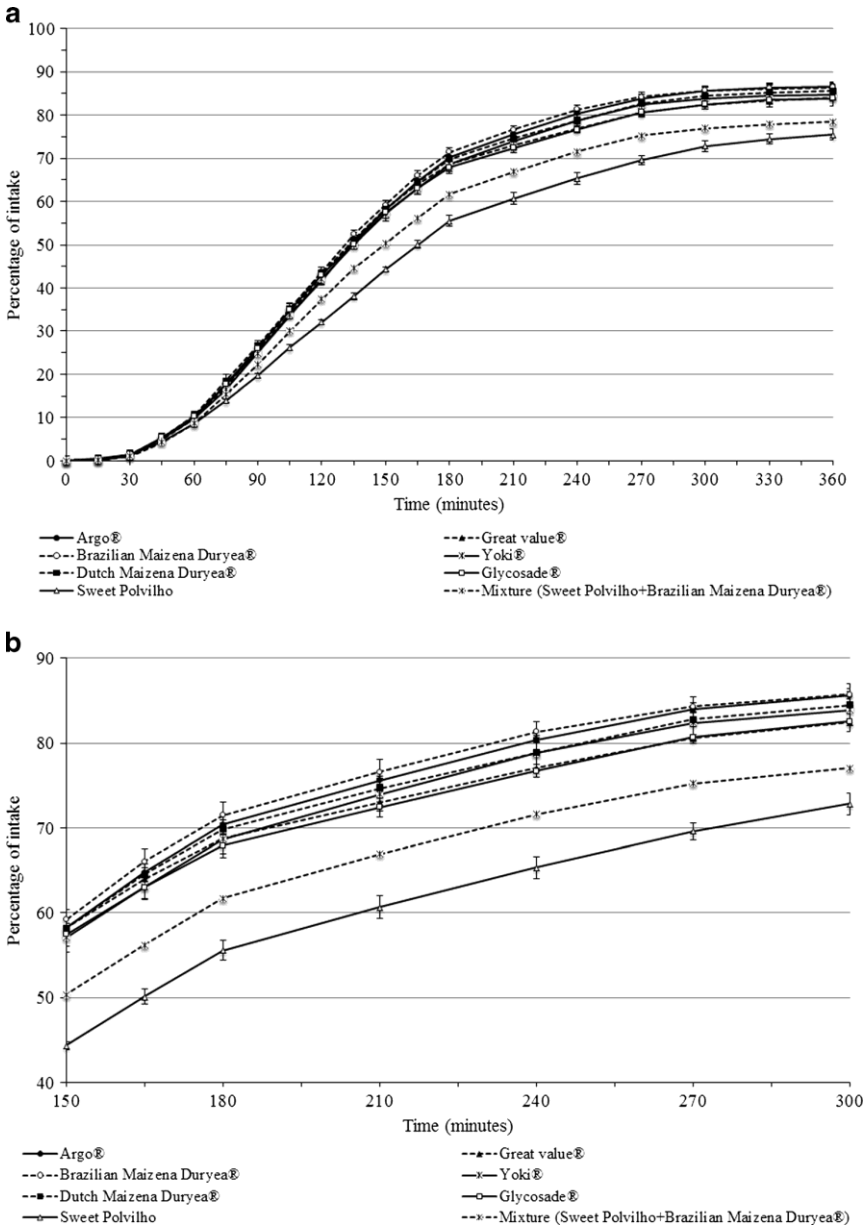

Fig. 2 **a** Cumulative combined jejunum and ileum absorption from starches per time point, in TIM-1 experiments. Data are the average of the values found in 2 experiments with each product with minimum/maximum error bars (range). **b** Cumulative combined jejunum and ileum absorption from starches per time point, in TIM-1 experiments, focusing on the time period of 150 to 300 min. Data are the average of the values found in two experiments with each product with minimum/maximum error bars (range)

results. A previous study (Anderson et al 2010) demonstrated that the rapidly digestible starch in food is correlated with a higher blood glucose concentration at 30 min, but there was a lower glucose concentration 120 min after ingestion. It is also known that the insulin response is related to the amylose/amylopectin ratio in meals (van Amelsvoort and Weststrate 1992) and one of the modifications described for Glycosade® is the higher quantity of amylopectin compared to traditional cornstarch (Correia et al 2008). In the TIM-1 system, hormonal influence is not taken into account and these two variables (RAG content and amylose/amylopectin ratio) can lead to a different hormonal response in some GSD patients and result in a difference in their plasma glucose concentrations.

To date, the perfect starch that meets all requirements for GSD patients does not exist. We included previously untested local Brazilian products extracted from cassava. They have the advantage of being widely available, and the relatively low

Table 3 Indices of significance (*p*-value) for two-tailed pairwise comparison of cumulative digestibility, between different substrates at time points 180 and 360 min

Samples	Argo®		Great value®		Brazilian Maizena Duryea®		Yoki®		Dutch Maizena Duryea®		Glycosade®		Sweet polvilho		Mixture	
	180 min	360 min	180 min	360 min	180 min	360 min	180 min	360 min	180 min	360 min	180 min	360 min	180 min	360 min	180 min	360 min
Argo®	–	–	0.187	0.394	0.692	0.719	0.6	0.464	0.111	0.09	0.433	0.11	0.075	0.035	0.05	0.022
Great value®	–	–	–	–	0.346	0.519	0.988	0.907	0.298	0.583	0.682	0.89	0.061	0.172	0.017	0.162
Brazilian Maizena Duryea®	–	–	–	–	–	–	0.029	0.387	0.602	0.337	0.034	0.26	0.016	0.002	0.096	0.07
Yoki®	–	–	–	–	–	–	–	–	0.708	0.661	0.251	0.794	0.026	0.079	0.145	0.203
Dutch Maizena Duryea®	–	–	–	–	–	–	–	–	–	–	0.517	0.223	0.082	0.03	0.06	0.038
Glycosade	–	–	–	–	–	–	–	–	–	–	–	–	0.011	0.082	0.131	0.022
Sweet polvilho	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.11	0.191
Mixture	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Paired Student's *t test*

Mixture: 60 % of Sweet Polvilho plus 40 % of Maizena Duryea®

Min, minutes

cost is advantageous for application in GSD patients. Sweet polvilho is more palatable than sour polvilho, and it displayed a slower glucose release compared to the other starches. After 3 h, 55.5 % of sweet polvilho was digested, while for the UCCS the values are approximately 70 % (Fig. 2b). Differences in total cumulative digestibility were significantly different between Sweet povilho and Argo®, Brazilian Maizena Duryea®, and Dutch Maizena Duryea® (Table 3). A possible disadvantage of sweet polvilho was the higher indigestible starch in the ileum effluent (23.1 %) compared to other starches (mean: 13.3 %). It is therefore likely that more undigested material will pass to the colon. In order to find a better product, the experiment with the mixture of sweet polvilho and the traditional UCCS was conducted. The mixture product presented a slower glucose release in relation to traditional UCCS used (Fig. 2a and b) and, a greater finally digested amount compared to sweet polvilho (78.4 % versus 75.5 %). A product with a slower glucose release without a large increase in the amount of undigested material can provide to patients a longer maintenance of plasma glucose, and may allow more uninterrupted sleep during the night. In vivo studies need to be conducted to confirm these findings and to determine the potential of sweet polvilho in the treatment of patients with hepatic GSD and other diseases, that are associated with fasting intolerance.

Several methodological factors of this study need to be addressed. TIM-1 has many advantages over traditional in vivo testing including standardized digestion and lack of intra-individual variability. TIM-1, however, does not take into account the gastrointestinal hormonal responses to carbohydrate ingestion, and this can be considered a limitation since the hormonal factors may be related to the difference in the glucose response in GSD patients observed in vivo. Secondly, the tolerability of starches is not only defined by jejunal and ileal factors, large intestine factors may also be involved, including the fermentation of carbohydrates and short-chain fatty acid production. A model that simulates the large intestine, called TIM-2, is available (Minekus et al 1999) and is an option to future studies to investigate these factors. Thirdly, the digestability in the TIM-1 does not necessarily correspond to tolerance in vivo.

Dietary compliance is of major importance for achievement of metabolic control and prevention of long-term complications in GSD patients. It is estimated that around 50 % of the subjects with chronic diseases do not completely follow the treatment recommendations received (WHO, 2003). Despite the major implications of the compliance to the dietary treatment in GSD, to the best of our knowledge, there are no formal studies addressing this issue.

In conclusion, the UCCS and the commercial modified starch analyzed in this study showed small differences in the glucose release in the TIM-1 system, which is a highly validated system that is used to predict in vivo outcome.

Therefore, we hypothesize that the reported difference in the glucose response in GSD patients may be related to inter- and intra-individual variations and/or the treatment compliance. Furthermore, it is important to take into account that the small difference in the glucose release found among the starches could be an important difference in patients who display severe fasting intolerance, taking the hormonal responses and the phenotype from the disease into account. These findings reinforce the importance of individualized treatment to hepatic GSD patients. In vivo studies using sweet polvilho will be helpful in the process to develop the perfect starch.

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Conflict of interest None.

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4.2. Capítulo 2 - *Determination of amylose/amylopectin ratio of starches.*

Título do manuscrito: Determination of amylose/amylopectin ratio of starches.

Autores: Tatiéle Nalin, Fernanda Sperb-Ludwig, Koen Venema, Terry G. J. Derks, Ida Vanessa D. Schwartz.

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LETTER TO THE EDITORS

Determination of amylose/amylopectin ratio of starches

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Dear editor,

Nalin et al 2014, in a paper recently published in the JIMD, tested in vitro digestion of seven different starches in a dynamic gastro-small intestine model (TIM-1), and did not find large differences between different brands of uncooked cornstarches (UCCS) and of a modified starch (Glycosade®) (Correia et al 2008). However, the authors found that sweet polvilho, and the mixture of sweet polvilho and UCCS, seem to have a slower and extended release of glucose, which looks promising as an option for the treatment of diseases associated with fasting intolerance, such as hepatic glycogen storage diseases. We would like to report herein the experiment we performed to determine the percentage of amylose and amylopectin in the same starch samples analyzed by Nalin et al 2014.

Starch consists of a mixture of amylose (linear chain) and amylopectin (branched chain) (Tester et al 2004). The amylose/amylopectin ratio has an important influence on the rate and extent of starch digestion (Björck et al 1994), which may, in turn, influence the treatment of patients with fasting intolerance.

The amylose/amylopectin ratio was measured using a commercial kit (Megazyme Co., Wicklow, Ireland), according to the manufacturer's recommendations. For a better characterization of the sweet polvilho, we also analyzed two different batches of this product and, in addition, two samples of the same batch but with different expiration dates (Table 1).

The different brands of UCCS did not differ regarding the amylose/amylopectin ratio. As expected, the Glycosade® presented the highest amylopectin content. The sweet polvilho was found to present a slightly higher value of amylopectin compared to the UCCS. Furthermore, little variation was found between different batches or within the same batch of sweet polvilho, demonstrating the stability of the composition of this product (Table 1).

Although the data presented herein supports some of the findings described by Nalin et al 2014, e.g., different brands of UCCS present small differences among themselves, they did not explain the slower and lower digestibility found for sweet polvilho in the TIM-1 model. This is not a surprising finding since many other factors, besides the amylose/amylopectin ratio, may be responsible for the differences in the glucose and insulin responses, such as the solubility of the starch. Additional studies using the TIM-2, which includes the large intestine

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Table 1 Amylose/amylopectin ratio in the samples

Samples[a]	Product	Origin	Percentage of amylose	Percentage of amylopectin
Argo®	Cornstarch	USA	28.7	71.3
GreatValue®	Cornstarch	USA	27.6	72.4
Brazilian Maizena Duryea®	Cornstarch	BR	27.8	72.2
Yoki®	Cornstarch	BR	27.4	72.6
Dutch Maizena Duryea®	Cornstarch	NL	26.6	73.4
Glycosade®	Modified starch	UK	8.0	92.0
Sweet polvilho*	Sweet polvilho	BR	23.8	76.2
Sweet polvilho**	Sweet polvilho	BR	22.2	77.8
Sweet polvilho***	Sweet polvilho	BR	21.9	78.1

[a] Cornstarches and the modified starch samples are called by the brand; Sweet Polvilho brand: Fritz e Frida®

BR, Brazil; NL, The Netherlands; UK, United Kingdom; USA, United States of America

*batch: 00113

**batch: 00114; expiration date: Jun 22, 2016

***batch: 00114; expiration date: Sep 22, 2016

model, or even clinical trials, should be performed to better characterize the effect of sweet polvilho in the treatment of fasting intolerance.

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Competing interest Tatiéle Nalin, Fernanda Sperb-Ludwig, Koen Venema, Terry G J Derks, and Ida V D Schwartz declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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4.3. Capítulo 3 - *Hepcidin levels in hepatic glycogen storage diseases: evidence of clinical manifestations in heterozygotes?*

Título do manuscrito: Hepcidin levels in hepatic glycogen storage diseases: evidence of clinical manifestations in heterozygotes?

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Hepcidin levels in hepatic glycogen storage diseases: evidence of clinical manifestations in heterozygotes?

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By definition, recessive inheritance implies absence of clinical manifestations in heterozygotes. In X-linked diseases, the occurrence of symptomatic heterozygotes is usually explained by non-random X chromosome inactivation (Pinto et al 2010). However, clinical manifestations have also been reported in individuals heterozygous for some autosomal recessive inborn errors of metabolism (IEM), such as CPT II deficiency (Endres 1997; Joshi et al 2012). Recognizing that individuals heterozygous for recessive diseases may develop clinical manifestations has fundamental implications for follow-up and genetic counseling of these individuals and their families.

Hepatic glycogen storage diseases (GSD) are autosomal recessive IEM (except for GSD IXa, which is an X-linked disorder) characterized by recurrent hypoglycemia, dyslipidemia, anemia and increased risk for hepatocellular carcinoma, among other manifestations (Wolfsdorf and Weinstein 2003). Anecdotal reports suggest that individuals heterozygous for these diseases may show subtle clinical manifestations typical of GSD, such as recurrent episodes of fasting hypoglycemia and abnormal lipid profile.

Hepcidin is a novel peptide hormone of hepatic origin encoded by the hepcidin antimicrobial peptide (*HAMP*) gene (Kali et al 2015). This peptide operates through the hepcidin–ferroportin complex, which regulates intracellular and extracellular iron concentrations. Elevated hepcidin levels decrease the amount of serum iron available for erythropoiesis by inhibiting iron absorption by enterocytes in the bowel and inhibiting mobilization of body iron stores. Inflammation may induce hepcidin synthesis mediated by cytokines, particularly IL-6, which leads to anemia (Kali et al 2015). The literature suggests that hepcidin is implicated in the anemia of chronic disease seen in GSD, particularly in patients with hepatic adenomas (Weinstein et al 2002).

The present study was based on the hypothesis that patients with hepatic GSD have higher levels of hepcidin. To test this hypothesis, hepcidin was measured in 32 patients with hepatic GSD seen at Hospital de Clínicas de Porto Alegre, Brazil (GSD Ia= 18, GSD Ib= 7, GSD III= 3, GSD IXa= 3, GSD IXb= 1; females= 17; median age= 9.5 years), and 20 unrelated healthy controls (females=

10; median age= 12 years). There was no difference between the ages of patients with GSD and controls (p= 0.224). Additionally, eight heterozygotes for hepatic GSD (median age= 33.5 years; 6 mothers of patients [GSD Ia =1, GSD Ib= 3, GSD III= 1, GSD IXb= 1] and 2 fathers of patients [GSD Ia= 1, GSD Ib= 1]) were also evaluated. All heterozygous individuals and controls denied the presence of any clinical manifestations at the time of study inclusion. Among patients, nine presented anemia and five presented hepatic adenoma at inclusion.

Plasma hepcidin levels were measured using a commercially available enzyme-linked immunoassay kit (Hepcidin-25 [human] ELISA Kit, Peninsula Laboratories International, Inc., USA). All analyses were performed in duplicate, averaged, and the median taken into account for analysis. No subject had a ≥30% difference in levels between test duplicates. The Kruskal–Wallis test followed by post-hoc analyses was used for comparisons, and the significance level was set at 5%.

Hepcidin levels were significantly different across the three tested groups (p= 0.006), being its levels elevated in patients (median= 58.6 ng/mL, IQR= 41.7–71.9) and in heterozygous individuals (median= 60.7 ng/mL, IQR= 51,7–68.3) as compared to controls (median= 42.8 ng/mL, IQR= 30.9–50.5) (p=0.005 and 0.006, respectively) (Figure 1). Therefore, despite the small sample size, our findings suggest that patients with hepatic GSD do present higher levels of hepcidin, and that biochemical phenotype of individuals heterozygous for hepatic GSD may be abnormal. We point out that, although the median age of controls and heterozygotes is not similar, reference data for hepcidin is available in the literature only for people above 18 years, and according to this data, men show constant and higher values than women until the age of 55, when values for women equal those for men (Galesloot et al 2011). If we use the values by Galesloot et al (2011) for comparison, all heterozygotes show high levels of hepcidin. The hypothesis raised is that the increase of hepcidin, found in heterozygous, may be mediated by inflammation and cytokines.

Future studies should explore the possibility that heterozygous individuals for hepatic GSD develop subtle clinical manifestations of GSD, including hepatocellular carcinoma.

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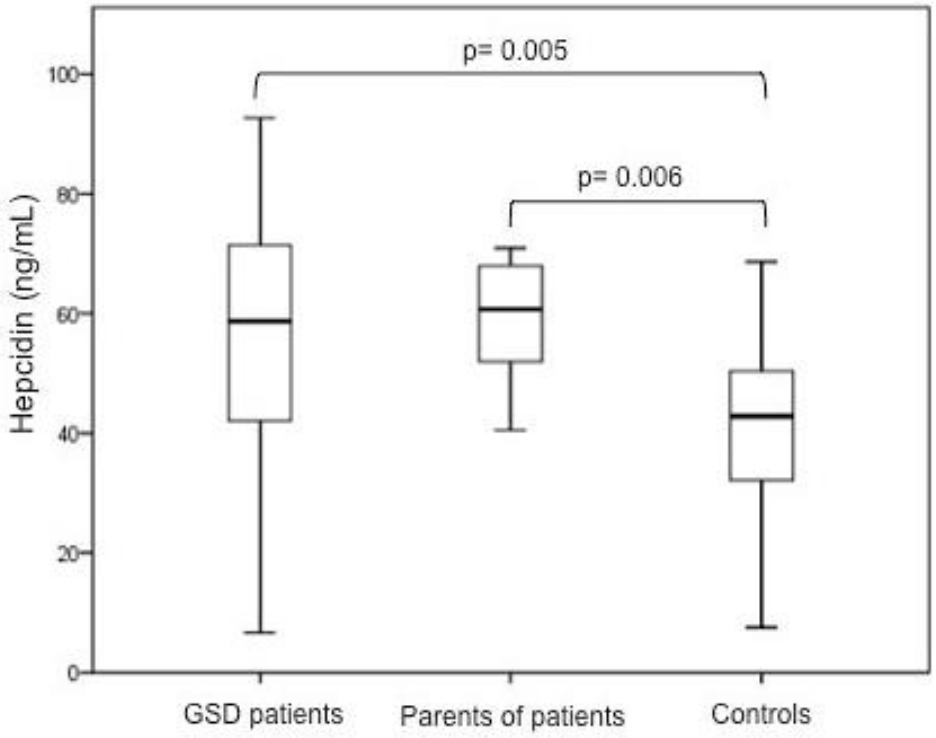

Figure 1: Comparison of hepcidin levels in patients with GSD (n=32), parents of patients (n=8) and controls (n=20).
GSD, glycogen storage disease.

4.4. Capítulo 4 - *Hepcidin, interleukin-6 levels and iron metabolism parameters in patients with hepatic glycogen storage diseases: a cross-sectional study*

Título do manuscrito: Hepcidin, interleukin-6 levels and iron metabolism parameters in patients with hepatic glycogen storage diseases: a cross-sectional study

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Hepcidin, interleukin-6 levels and iron metabolism parameters in patients with hepatic glycogen storage diseases: a cross-sectional study

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Abstract

Introduction: Hepatic glycogen storage diseases (GSD) are genetic diseases characterized by recurrent episodes of hypoglycemia. Anemia has been being recognized as a frequent complication of these disorders.

Objective: To evaluate hepcidin and IL-6 concentrations in patients with hepatic GSD and their association with anemia and other parameters of iron metabolism.

Methods: This was a cross-sectional study with a convenience sampling strategy. Levels of hepcidin, IL-6, and markers of iron metabolism (hemoglobin, iron, ferritin, transferrin, and transferrin saturation) were measured in 32 patients receiving uncooked cornstarch therapy for GSD (GSD Ia= 18; GSD Ib= 7; GSD III= 3; GSD IXa= 3; GSD IXb= 1; median age 9.5 years). IL-6 concentrations were compared to those of 8 individuals heterozygous for GSD. Additional data were obtained by means of a chart review. Nonparametric methods were used for data analysis.

Results: Nine patients (GSD Ia= 3/17; GSD Ib= 6/7) were anemic (mild= 4; moderate= 5). Five patients had hepatic adenomas. IL-6 levels were higher in patients than in heterozygotes (p= 0.003). Eight patients had hyperferritinemia, and one had elevated transferrin saturation as well. Hepcidin correlated positively with ferritin levels (r= 0.375; p= 0.034). IL-6 correlated with hemoglobin (r= -0.572; p= 0.001), iron (r= -0.538; p= 0.001), transferrin (r= -0.550; p= 0.001), and transferrin saturation (r= -0.425; p= 0.015). There was no correlation between hepcidin and IL-6 levels (p= 0.057). Patients with GSD Ib had the highest IL-6 levels. There was no association between presence of hepatic adenoma and anemia.

Conclusions: Anemia is a common finding in hepatic GSD, especially in GSD Ib, the type of GSD associated with the highest IL-6 levels. These findings suggest that inflammation is strongly associated with development of anemia in hepatic GSD, particularly in GSD Ib.

Keywords: Glycogen storage diseases, cytokines, anemia, iron metabolism, hepcidin

Introduction

The hepatic glycogen storage diseases (GSD) are inborn errors of metabolism characterized by abnormal endogenous glucose production and, consequently, fasting hypoglycemia. These conditions are classified into different types and receive different names depending on the underlying enzyme defect, and differ in terms of chemical and biochemical manifestations (Table 1). The most common management strategy is frequent administration of uncooked cornstarch, aiming at maintaining normal blood glucose levels and preventing secondary metabolic derangements. Additional dietary modifications may be made depending on the type of GSD [1-8].

Studies have investigated the role of hepcidin in the pathogenesis of anemia of chronic disease [9-11]. Hepcidin, a 25-amino acid peptide hormone encoded by the hepcidin antimicrobial peptide (*HAMP*) gene (MIM606464), is produced mainly in the liver and plays a key role in iron metabolism. This peptide operates through the hepcidin–ferroportin complex, which regulates intracellular and extracellular iron concentrations. Ferroportin is a transmembrane receptor that exports cellular iron. When levels of hepcidin increase, it binds to ferroportin and induces its internalization and degradation. Consequently, iron delivery into plasma is decreased through inhibition of iron absorption by enterocytes in the bowel and inhibition of mobilization of body iron stores. When levels of hepcidin decline, ferroportin is exposed, thus making both dietary iron and iron from macrophage and hepatocyte stores available. Under normal conditions, hepcidin is regulated by a feedback loop whereby increased iron concentrations induce its transcription and decreased iron levels reduce it [9, 12-15]. Hepcidin levels are decreased in several pathological conditions, such as hereditary hemochromatosis, a disease most commonly caused by pathogenic variations in the *HFE* gene and characterized by a buildup of iron in the body, particularly in the liver [9]. In iron deficiency anemia, decreased iron availability inhibits hepcidin expression, thus increasing absorption of iron from the diet and its release from macrophages [16]. In the liver adenoma tissue of two GSD Ia patient who were also anemic, it was found an increase of mRNA levels of hepcidin, and anemia

was found to resolve after adenoma resection and liver transplantation, respectively [17].

It has been reported that inflammation may induce hepcidin synthesis mediated by cytokines, particularly IL-6, thus leading to the anemia of chronic disease [18-20]. The involvement of an inflammatory component in hepatic GSD, including a Crohn's disease-like inflammatory bowel disorder, has been described, especially in GSD Ib [21]. This condition has recently been reported in GSD Ia as well [22, 23]. Furthermore, anemia is known to be a common manifestation of GSD I and one of its long-term complications [24]. It may be a result of iron deficiency or develop as anemia of chronic disease, particularly in cases of mild or moderate anemia.

This study sought to evaluate plasmatic hepcidin and IL-6 profiles in patients with hepatic GSD and their association with anemia and other clinical and laboratory parameters in these conditions.

Patients and Methods

The study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee, and was conducted in accordance with the Declaration of Helsinki. Study procedures were only begun after written informed consent had been obtained from all participants or their legal guardians.

Participant recruitment

Patients were eligible for inclusion in the study if they had a diagnosis of hepatic GSD, confirmed by measurement of enzyme activity and/or molecular analysis, and were receiving follow-up at the outpatient metabolic disorders clinic of the Medical Genetics Service, Hospital de Clínicas de Porto Alegre (ATDM-SGM/HCPA, Brazil). Recruitment took place from June 2014 to April 2015.

At the time of the study, 42 patients with GSD were seen at ATDM-SGM/HCPA. Of these, 32 (GSD Ia= 18; GSD Ib= 7; GSD III= 3; GSD IXa= 3; GSD IXb= 1; females= 17), from 30 unrelated families, were included. Two patients were aged <3 years and were not included due to the technical difficulty of blood

sampling. One patient refused to participate, and seven did not attend their visits during the recruitment period. The ATDM-SGM/HCPA follow-up protocol includes 3- to 6-monthly visits. Tests considered necessary for assessment of metabolic control (glucose, triglyceride levels, total cholesterol, lactate) are ordered at each visit, while other tests, including abdominal imaging, are performed once yearly.

For comparison of IL-6 levels, eight parents of patients, with a median age of 33.5 years (IQR= 27; 37.5), were also included in analyses (GSD Ia= 3; GSD Ib= 3; GSD III= 1; GSD IXb= 1; females= 6).

The exclusion criteria were that neither patients nor parents could present with comorbidities known to affect cytokine levels (e.g., autoimmune diseases). Known complications of GSD that are associated with elevated cytokine levels, such as inflammatory bowel disease, were not among the criteria for exclusion.

Sample collection

In the morning hours, blood samples from patients (for hepcidin, IL-6, iron, ferritin, and transferrin measurement) and parents (for IL-6 measurement) were collected into heparin sodium tubes. Blood samples were chilled immediately after collection and plasma separated by centrifugation (3000 rpm, 20 min, 4°C) within 30 minutes of collection. Plasma samples were than aliquoted and immediately frozen at -80°C until the time of testing.

Laboratory evaluation

Plasma hepcidin levels were measured using a commercially available enzyme-linked immunoassay kit (Hepcidin-25 [human] ELISA Kit, Peninsula Laboratories International, Inc., USA). Plasma IL-6 levels were also measured with a commercially available ELISA kit (IL-6 [Human] ELISA Kit, Invitrogen Corporation, USA). Both hepcidin and IL-6 tests were performed in duplicate and the average of the two measurements taken into account for analysis. No subject had a ≥30% difference in levels between test duplicates.

Iron, ferritin, and transferrin measurements were performed by the HCPA clinical laboratory, and transferrin saturation values were derived using the formula (iron/[transferrin × 1.28]) × 100 [25].

Anthropometric evaluation

Weight and height were measured on the day of blood draws and used to calculate the BMI, using the formula BMI = (weight [kg]/height [m]^2). Classification of nutritional status was based on BMI z scores calculated in the *Anthro plus* v.1.0.4 software environment. Individuals were classified as underweight, normal weight, overweight, or obese, as age-appropriate, using World Health Organization (WHO) criteria.

Chart review

Data on other clinical, biochemical, and treatment-related variables, including presence or absence of inflammatory bowel disease, were collected through a chart review. Complete blood count, AST, ALT, vitamin B12, lactate, glucose, triglyceride, and total cholesterol measurements were considered available if performed within 3 months before or after study inclusion; in all instances, the most recent value was used for analyses. Imaging (abdominal ultrasound or magnetic resonance imaging) was considered acceptable if performed no more than 1 year before or after. The median time elapsed in days from sample collection for AST/ALT measurement to sample collection for iron and hepcidin/IL-6 measurement was 2.5 days; 7 days for vitamin B12; 0.5 days to 1 day for cholesterol, triglycerides, glucose, and lactate; and 7.5 days for complete blood count. The median time elapsed in months between most recent imaging and sample collection for the present study was 4.4 months.

The diagnosis of anemia in patients was also based on WHO recommendations [26], using the following reference ranges of Hb levels (g/dL): a) children aged 6–59 months: >11.0 = normal; 10.0–10.9 = mild anemia; 7.0–9.9 = moderate anemia; <7 = severe anemia; b) children aged 5–11 years: >11.5 = normal; 11.0–11.4 = mild anemia; 8.0–10.9 = moderate anemia; <8 = severe anemia; c) children aged 12–14 years: >12.0 = normal; 11.0–11.9 = mild anemia; 8.0–10.9 = moderate anemia; <8 = severe anemia; d) females: >12.0 = normal; 11.0–11.9 = mild anemia; 8.0–10.9 = moderate anemia; <8 = severe anemia; e) males: >13.0 = normal; 11.0–12.9 = mild anemia; 8.0–10.9 = moderate anemia; <8 = severe anemia.

The following reference values were used for the other markers of interest: Mean corpuscular volume (MCV): a) children aged 2–12 years: 82–95 fL; b) females: 82–96 fL; b) males: 83–98 fL. Ferritin: a) females: 9–120 ng/mL; b) males: 18–370 ng/mL. Iron: a) females: 49–151 µg/dL; b) males: 53–167 µg/dL. Transferrin saturation. 20–50%. Transferrin: 200–360 mg/dL. Vitamin B12: 180–900 pg/mL.

Statistical analysis

The descriptive analysis included absolute and relative frequencies. Due to the small sample size, continuous variables were expressed as median and interquartile range (IQR). The Mann–Whitney *U* test was used for comparisons. The Kruskal–Wallis test followed by post-hoc analysis was used to compare variables across three groups. Correlations were analyzed using Spearman coefficients. The significance level was set at 5%. Statistical analysis was carried out in the *Statistical Package for the Social Sciences* (SPSS) version 22.0 software environment (SPSS Inc., Chicago, IL).

Results

The patients' clinical and laboratory characteristics are summarized in Table 2. Parental consanguinity was present in 7 of 30 families (23.33%).

At the time of enrollment, all patients were on uncooked cornstarch therapy, with a median dose of 325 g (IQR= 240 - 418.5; 1.33 g/kg/dose). Regarding medications, five patients were on ferrous sulphate (Table 3); five, all with GSD Ib, were taking granulocyte colony-stimulating factor; and 19 of 32 (GSD Ia= 11; GSD 1b= 5; GSD III= 2; GSD IXa=1) were taking a multivitamin.

Nine patients (30%) were anemic (Table 3). Four had mild anemia and five had moderate anemia. Five of 32 (15.6%) had inflammatory bowel disease (all with GSD Ib); five of 28 (17.8%) had hepatic nodules (all in the GSD Ia group). Finally, 22 of 32 (68.8%) were classified as having excess weight (GSD Ia= 14; GSD Ib= 5; GSD III= 2; GSD IX= 1): nine as overweight and 13 as obese. No patient had vitamin B12 deficiency.

Analysis of iron metabolism parameters

Eight patients (25%) had hyperferritinemia; of these, only one had elevated transferrin saturation levels, indicative of hereditary hemochromatosis. Five of these patients were anemic (Table 3).

Regarding IL-6, the median value measured in patients was 2.33 pg/mL (IQR= 1.55; 3.65), versus 1.26 pg/mL (IQR= 1.03; 1.85) in patients' parents (p= 0.003). Statistical significance remained even after removing from analysis all five patients who were on iron supplementation (data not shown).

Figure 1 illustrates hepcidin and IL-6 levels, stratified by GSD type. There were no statistically significant between-group differences in hepcidin levels (p= 0.069). IL-6 levels differed across GSD types (p= 0.022). Patients with GSD Ib had higher values than did those with GSD Ia and those with GSD III/IX.

Hepcidin correlated with ferritin levels only (r= 0.375; p= 0.034). IL-6 correlated with Hb (r= -0.572; p= 0.001), iron (r= -0.538; p= 0.001), transferrin (r=-0.550; p=0.001), and transferrin saturation (r= -0.425; p= 0.015). There was no correlation between hepcidin and IL-6 levels (r= 0.339; p= 0.057). Upon exclusion of the five patients who were on ferrous sulphate therapy, the correlation between IL-6 and transferrin saturation lost statistical significance (r= -0.340; p= 0.083), as did the correlation between hepcidin and ferritin (r= 0.267; p= 0.178). The other correlations remained statistically significant.

Correlation with clinical variables

No significant correlations were found between hepcidin, IL-6, or Hb values in relation to cornstarch intake, lactate, triglycerides, glucose, total cholesterol, weight, height, or BMI (data not shown). Overweight and obese patients did not differ regarding the variables of interest, nor did patients with adenoma and those without (data not shown). Analysis of patients with inflammatory bowel disease revealed a significant difference only in hepcidin values, which were higher in patients with inflammatory bowel disease than in those without this condition (p= 0.046). IL-6 values were also higher in patients with the condition, but the difference was not statistically significant (p= 0.092). Patients with anemia had higher IL-6 values than non-anemic ones (p= 0.003).

Discussion

To the best of our knowledge, this was the first study to investigate the association between plasma levels of hepcidin and IL-6 in patients with hepatic GSD. Our findings confirmed that anemia is especially prevalent in GSD Ib, the type of GSD most commonly associated with inflammatory bowel disease. Furthermore, the hepcidin and IL-6 concentrations found strengthen the hypothesis that the presence of anemia in patients with GSD is influenced by an underlying inflammatory state. It is important to point out that iron deficiency anemia also appears to be common in these conditions, which is unsurprising in view of its associations with inflammatory bowel disease; with potentially low iron intake due to a carbohydrate-rich diet [3]; and with possibly abnormal iron absorption as a result of cornstarch intake by patients [24].

Anemia is a common manifestation of inflammatory bowel disease, and its etiology is most often associated with iron deficiency and chronic inflammation. Less common causes of anemia in inflammatory bowel disease are vitamin B12 deficiency and folic acid deficiency [27]. In the present study, the majority of patients with GSD Ib had anemia associated with inflammatory bowel disease, whereas a smaller portion of patients with GSD Ia were deemed to have anemia. Patients with the other GSD types did not exhibit this condition. A study by Wang et al. [24], with a larger sample (n=202) of U.S. patients with GSD Ia and Ib, found rates of anemia different from those observed in our study, with a higher rate in GSD Ia (41.7% vs. 17.6%) and a lower rate in GSD Ib (71.8% vs 85.7%). According to the same study, iron deficiency anemia would be more common in preadolescent patients with GSD I, whereas anemia of chronic disease would be more common in adult patients. Due to the small size and relatively young age of our sample, we were unable to test for associations between type of anemia and age. However, it is clear that macrocytic anemia is not a common finding in patients with hepatic GSD; the differential diagnosis is restricted to the causes of microcytic and normocytic anemia in general, and to iron deficiency anemia and anemia of chronic disease in particular. Unfortunately, the ferritin levels may not reflect the iron status of these patients.

In the present study, patients with GSD Ib had significantly higher levels of IL-6 than did patients with GSD Ia and GSD III/IX. However, some patients with GSD Ia had IL-6 values similar to those of patients with GSD Ib. Elevated IL-6 levels are to be expected in chronic inflammation, and may be associated with the fact that inflammatory bowel disease is a frequent finding in GSD Ib. Furthermore, the presence of gastrointestinal symptoms and manifestations of inflammatory bowel disease has been recently described as a possible finding in GSD Ia [22], and markers of this condition may be present even in asymptomatic patients [23]. In addition, the IL-6 levels measured in patients with hepatic GSD were higher than those of their parents, suggesting a possible increase in this marker in this population. A prior study evaluated IL-6 levels by ELISA assay in 27 patients with GSD Ia (mean age=15 years, range= 2–35), fourteen patients with GSD III/VI (mean age= 16 years, range= 4–28), and 30 healthy adult controls (mean age= 28 years, range= 22–38). Hepatic adenoma was present for 15 patients (GSD Ia= 14; GSD III= 1). No difference was in IL-6 values, whether between the different GSD or in relation to healthy controls. Moreover, no differences were found between patients with and without adenoma [28]. It bears stressing that the study did not use sex- or age-matched controls; indeed, controls had a more advanced median age than patients, which is a bias since there is an age-associated increase in IL-6 levels, especially in late life [29]. As patients with GSD Ib had elevated values in relation to all other GSD types in the present study, the finding of no difference in IL-6 levels in this prior investigation may be also attributable to the fact that patients with GSD Ib were not included in the sample.

IL-6 was associated with Hb, iron, transferrin, and transferrin saturation. In addition, patients with anemia had higher IL-6 levels than those without anemia. These findings suggest that IL-6 as a marker is strongly associated with anemia of chronic disease in hepatic GSD. In a study by Miyamoto et al. [30], adult patients with congenital heart disease and a history of anemia exhibited higher IL-6 levels than patients without a history of anemia.

No positive correlation between hepcidin and IL-6 was found in the present study. Previous studies are ambiguous regarding this finding; some found a positive correlation between hepcidin and IL-6 [31-33], whereas others found no

significance for this correlation [34-36]. The lack of significance in the present study may be secondary to the small sample size. It is important to note that other cytokines may be involved in hepcidin regulation [12] and influence correlation between these variables.

Nalin et al. (manuscript in preparation), using the same sample of the present study and a group of healthy controls, found that hepcidin levels are probably elevated in GSD, as in other diseases associated with chronic anemia [11, 33, 34, 37]. In the present study, hepcidin levels did not differ significantly across different GSD types, although they were numerically higher in GSD Ib, followed by GSD Ia and GSD III/IX. This distribution of hepcidin values was expected, in view of the frequency of anemia in these GSD subtypes. Hepcidin levels did not differ significantly on comparison of patients with vs. without anemia. As expected, there was a positive correlation between levels of hepcidin and ferritin, markers that respond similarly to inflammation and to iron stores [38].

The possibility of involvement of hepcidin in the pathogenesis of anemia in hepatic GSD was raised after finding that five patients with GSD Ia had large hepatic adenomas and severe anemia nonresponsive to iron supplementation. Examination of adenoma tissue from two of these patients revealed increased hepcidin mRNA levels and anemia was found to resolve after adenoma resection or liver transplantation [17]. In our study, we found no association between presence of adenomas and levels of inflammatory markers. This finding may be attributable to the fact that imaging studies for detection of adenoma were not performed on the same day as blood draws. Regarding inflammatory bowel disease, we were able to detect an increase in levels of hepcidin, but not of IL-6, in patients with this condition. This was probably due to the small number of patients diagnosed with inflammatory bowel disease in the sample. In a study conducted by Semrin et al. [31], patients with active Crohn's disease had IL-6 and urinary hepcidin levels higher than those with quiescent disease. Although no between-group differences in hemoglobin or serum iron levels were found, oral iron absorption did differ.

Conclusions

Anemia is an especially common finding in GSD Ib. In patients with hepatic GSD, anemia is associated with levels of inflammatory markers, such as hepcidin and IL-6. A better understanding of the mechanisms involved in anemia in hepatic GSD may help the future introduction of new therapies and in preventing anemia in these diseases.

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Table 1. Classification of hepatic glycogen storage disorders and summary of their characteristics

Type (MIM)	Enzyme involved	Gene	Inheritance pattern	Clinical/Biochemical	Treatment
0, liver (240600)	Glycogen synthase (liver)	*GYS2*	AR	Fasting ketotic hypoglycemia; postprandial hyperglycemia, hyperlactatemia, and hyperlipidemia. No liver enlargement.	Protein-rich diet, low-glycemic index complex carbohydrates; bedtime uncooked cornstarch.
Ia (232200)	Glucose-6-phosphatase	*G6PC*	AR	Hypoglycemia, hepatomegaly, growth retardation, lactic acidosis, hyperuricemia, hyperlipidemia.	Uncooked cornstarch; galactose, fructose, lactose, and sucrose restriction.
Ib (232220)	Glucose-6-phosphate translocase	*SLC37A4*	AR	As in Ia, with neutropenia (recurrent infections, inflammatory bowel disease).	Uncooked cornstarch; galactose, fructose, lactose, and sucrose restriction. Granulocyte colony-stimulating factor (filgrastim).
IIIa and IIIb (232400)	Glycogen debranching enzyme	*AGL*	AR	Hepatomegaly, hyperketotic hypoglycemia; growth retardation, hyperlipidemia, elevated AST, ALT, CPK. Muscle weakness and cardiomyopathy occur in subtype IIIa.	Uncooked cornstarch; protein-rich diet; sucrose restriction.
IV (232500)	Glycogen branching enzyme	*GBE1*	AR	Hepatomegaly, growth retardation, cirrhosis.	Liver transplantation in severe cases.
VI (232700)	Glycogen phosphorylase (liver)	*PYGL*	AR	Hepatomegaly, growth retardation; mild hypoglycemia, hyperlipidemia, hyperketosis.	If symptomatic: increased carbohydrate intake, frequent feedings, protein-rich diet.
IXa1 and IXa2 (306000)	Phosphorylase kinase (subunit alpha)	*PHKA2*	XL	Hepatomegaly, fasting ketotic hypoglycemia, growth retardation, mild AST/ALT elevation and hyperlipidemia.	Uncooked cornstarch; protein-rich diet; avoidance of large amounts of sucrose.
IXb (261750)	Phosphorylase kinase (subunit beta)	*PHKB*	AR	As in IXa.	Uncooked cornstarch; protein-rich diet; avoidance of large amounts of sucrose.
IXc (613027)	Phosphorylase kinase (subunit gamma)	*PHKG2*	AR	As in IXa, plus cirrhosis.	Uncooked cornstarch; protein-rich diet; avoidance of large amounts of sucrose.
XI (227810)	Glucose transporter 2	*GLUT2*	AR	Hypoglycemia, failure to thrive, rickets, protuberant abdomen due to enlarged liver and kidneys.	Restricted galactose intake; uncooked cornstarch; supplementation of water, electrolytes, and vitamin D.

AR, autosomal recessive; XL, X-linked.

Source: Wolfsdorf and Weinstein 2003, Beauchamp et al. 2007, Kishnani et al. 2010, Hicks et al. 2011, Dagli et al. 2012, Bali et al. 2013, Chen et al. 2014, Kishnani et al. 2014 [1-8].

Table 2: Summary of patient characteristics.

Variable	Value (median, IQR)	n (32)
Age (years)	9.5 (8.0; 16.75)	32
Weight (kg)	40.8 (25.7; 62.57)	32
Height (cm)	138 (1.18; 1.54)	32
BMI (kg/m2)	22.6 (18.3; 27.0)	32
Hematocrit (%)	36.1 (34.0; 38.4) / 37.5 (35.6; 38.5)*	30/25*
Hemoglobin (g/dL)	12.3 (11.3; 13.4) / 12.6 (11.8; 13.6)*	30/25*
MCV (fL)	81.0 (77.9; 84.3) / 80.4 (77.8; 84.5)*	30/25*
Platelets (µL)	392.5 (318.8; 462.0)	28
Glucose (mg/dL)	87.0 (80.5; 95.5)	29
Triglycerides (mg/dL)	212.5 (93.8; 386.5)	26
Total cholesterol (mg/dL)	174.5 (150.8; 200.0)	26
Lactate (mmol/L)	2.0 (1.28; 2.73)	26
AST (U/L)	27.0 (23.0; 57)	26
ALT (U/L)	24.0 (16.0; 63.5)	26
Iron (µg/dL)	67.0 (44.3; 89.8) / 68.0 (52.0; 92.0)*	32/27*
Ferritin (ng/mL)	90.8 (55.6; 164.2) / 85.6 (55.1; 122.2)*	32/27*
Transferrin (mg/dL)	313.5 (272.5; 334.8) / 318.0 (282.0; 335)*	32/27*
Transferrin saturation (%)	15.6 (12.2; 23.2) / 17.5 (12.9; 25.3)*	32/27*
Vitamin B12 (pg/mL)	436.5 (335.5; 627.5) / 419.0 (330.0; 572.0)*	20/15*

* Excluding patients on oral iron supplementation.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index.

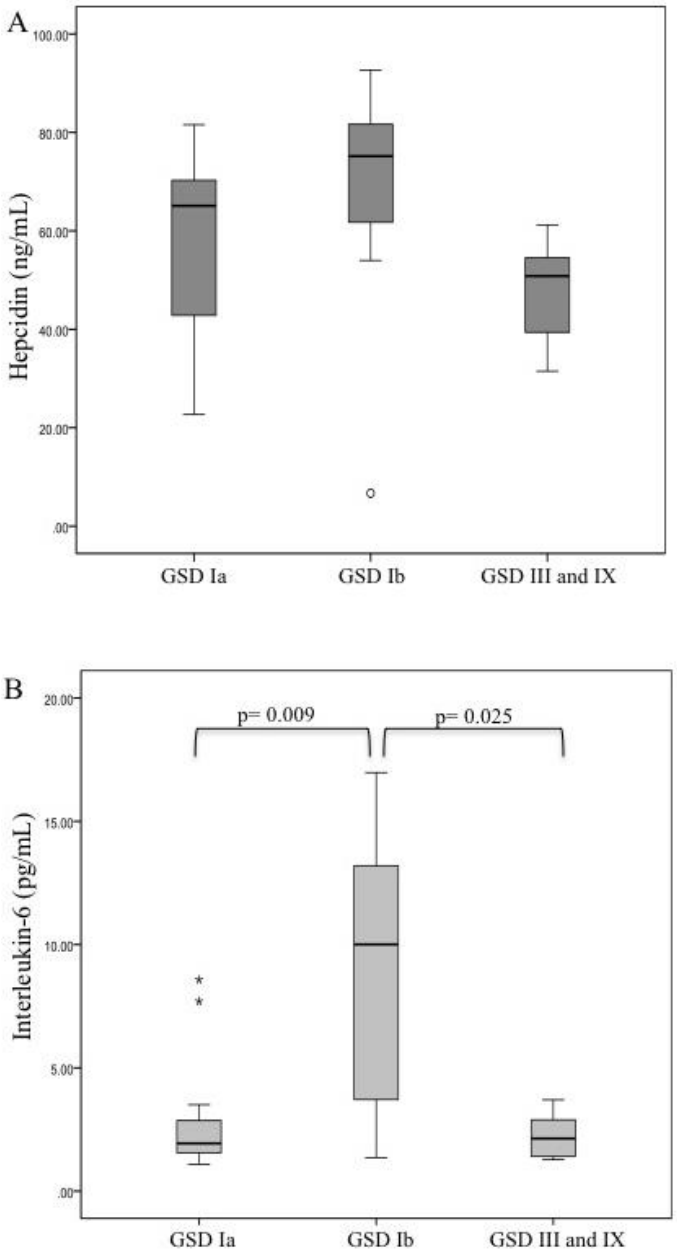

Figure 1: A. Comparison of hepcidin levels stratified by type of GSD; B. Comparison of interleukin-6 levels stratified by type of GSD. GSD Ia, n=18; Ib, n=7; III + IX, n=7.
GSD, glycogen storage disease.

Table 3: Characteristics of GSD patients with anemia (n=9/30)

Patient	Sex	Age (years)	Type of GSD	IBD	Liver adenoma	Overweight*	Multivitamin supplementation	Oral iron supplementation	Hb (g/dL)	MCV (fL)	Iron (µg/dL)	Ferritin (ng/mL)	Transferrin (mg/dL)	Transferrin sat. (%)	Vitamin B12 (pg/mL)	IL-6 (pg/mL)	Hepcidin (ng/mL)
1	F	17	Ia	No	Yes	No	No	Yes	9.1	92.8	93	199.7	598	12.1	441	1.7	70.27
2	F	8	Ia	No	No	Yes	No	Yes	10.5	75	21	27.2	290	5.7	269	7.7	65.2
3	M	10	Ia	No	No	Yes	No	No	11.3	77.9	66	241.8	355	14.5	432	1.82	30.51
4	F	32	Ib	Yes	NA	Yes	Yes[♯]	Yes (prophylactic)	7.8	82.6	29	166.7	182	12.4	650	15.43	77.24
5	M	3	Ib	No	No	Yes	No	No	10.8	75.3	17	44.1	238	5.6	572	16.97	53.97
6**	M	14	Ib	Yes	No	Yes	Yes[¶]	No	11.3	80	34	64.4	402	6.6	NA	3.52	6.69
7**	F	3	Ib	Yes	No	No	No	No	9.2	71	14	164.8	291	3.8	NA	10.97	92.67
8	F	13	Ib	Yes	No	Yes	Yes[♯]	Yes (prophylactic)	11.6	81	79	216	277	22.3	641	3.91	86.05
9	F	6	Ib	No	No	No	Yes[¶]	Yes	9.7	82.6	28	452.5	261	8.4	932	10.01	69.48

F, female; M, male; NA, not available;
GSD, glycogen storage disease; IBD, inflammatory bowel disease; IL-6: interleukin-6; Transferrin sat., transferrin saturation.
* Subjects were classified as overweight on the basis of body mass index, in accordance with World Health Organization recommendations.
**Iron had been prescribed, but the patient did not take it.
[♯]Vitamins A, B1, B2, B3, B5, B6, B9, B12, C, D, E, H, and K, calcium, chloride, copper, chromium, iron, phosphorus, iodine, magnesium, manganese, molybdenum, potassium, selenium, zinc.
[¶]Vitamins A, B1, B2, B6, B12, C, D3, E, nicotinamide, folic acid, panthenol.
Reference ranges:
MCV: a) children aged 2–12 years: 82–95 fL; b) females: 82–96 fL; b) males: 83–98 fL.
Ferritin: a) females: 9–120 ng/mL; b) males: 18–370 ng/mL.
Iron: a) females: 49–151 µg/dL; b) males: 53–167 µg/dL.
Transferrin saturation. 20–50%.
Transferrin: 200–360 mg/dL.
Vitamin B12: 180–900 pg/mL

5. DISCUSSÃO

As GSD hepáticas constituem-se em um grupo de doenças genéticas graves que se apresentam na infância e que se caracterizam mais frequentemente pela ocorrência de hipoglicemias de repetição e dislipidemia (Chen *et al*, 2014). Com o mais amplo acesso aos meios de diagnóstico e maior informação dos profissionais da saúde sobre essas condições, o número de pacientes diagnosticados com essa doença vem crescendo no Brasil.

Apesar do tratamento com amido de milho cru ser bem-sucedido em relação ao controle glicêmico, a duração da normoglicemia após o uso do mesmo pode variar entre pacientes (Weinstein e Wolfsdorf, 2002), e relatos da prática clínica sugerem respostas distintas dos pacientes ao uso de diferentes marcas de amido de milho. A mais recente diretriz americana de diagnóstico e tratamento para a GSD I apresenta que, segundo o relato de pacientes, a marca Argo® de amido de milho é a preferida entre eles pela palatabilidade e sustentabilidade. Os autores ainda indicam que outras marcas de amido de milho devem ser usadas com cautela e não recomendam a troca frequente entre as marcas (Kishnani *et al*, 2014).

Outro ponto de destaque é a necessidade do uso frequente do amido de milho, inclusive durante o período da noite, que influencia na qualidade de vida, tanto de pacientes, como de seus pais ou cuidadores. Têm havido um crescente interesse nos últimos anos no desenvolvimento de amidos modificados visando prolongar a normoglicemia e, consequentemente, melhorar a qualidade de vida desses pacientes (Bhattacharya *et al*, 2007; Correia *et al*, 2008; Bhattacharya *et al*, 2015). Um amido modificado com valor superior de amilopectina já está sendo comercializado, contudo, produtos médicos industrializados não são de fácil introdução, especialmente em países em desenvolvimento, entre outros motivos, pelo elevado custo. Com isso, os pacientes com GSD hepática acabam dependendo da utilização de amido de milho tradicional, frequentemente em grandes quantidades.

Nossos achados de análises das frações de amido, utilizando o modelo de digestão gastrointestinal *in vitro,* TIM-1, e a razão amiliose/amilopetina em amidos de milho de diferentes marcas e originários de diferentes países indicaram que os mesmos apresentam pequenas diferenças entre si em todos os parâmetros estudados. Esses achados sinalizam que a possível resposta distinta ao uso dos mesmos pode estar relacionada a variações individuais, como na resposta hormonal, que não foi simulada nos experimentos, ou ainda, relacionada à adesão ao tratamento, sabidamente uma grande dificuldade encontrada no tratamento das doenças crônicas. O amido modificado apresentou liberação de glicose semelhante aos amidos de milho com uma pequena diferença após 180 minutos de experimento. É importante salientar que as pequenas diferenças encontradas *in vitro* podem ter o seu efeito amplificado *in vivo*, na presença, por exemplo, de resposta hormonal, especialmente as diferenças após 180 minutos, visto que a ingestão de amido de milho cru se dá geralmente a cada 3-4 horas.

O polvilho doce, um produto derivado da mandioca largamente utilizado na culinária brasileira, foi incluído no estudo como um possível substituto ao amido de milho tradicional no tratamento das GSD hepáticas. Os resultados encontrados são promissores, visto sua diferença na liberação de glicose, possibilitando a manutenção de normoglicemia por um período mais longo, e a estabilidade apresentada quando analisados diferentes lotes e datas de validade do produto. Por apresentar um valor superior de amido resistente, a possibilidade de uma mistura com amido de milho, em proporções já estudadas na TIM-1, buscando a diminuição da quantidade de amido resistente e mantendo a velocidade de digestão inferior ao amido de milho, visa a busca de um produto que mais se adeque ao tratamento de pacientes com GSD hepáticas. Estudos *in vivo* são necessários para confirmar os achados *in vitro* em relação ao polvilho doce, e avaliar a eficácia e segurança da utilização do mesmo para tratamento dessas condições.

A possibilidade de tratamento com a oferta contínua de glicose na GSD hepática melhorou a expectativa de vida dos pacientes e, com isso, ampliou o número dos que desenvolvem complicações ditas como de longo prazo. Entre as complicações que vêm sendo descritas nesses pacientes estão a presença de

anemia, adenoma hepático (Kishnani *et al*, 2014) e doença inflamatória intestinal (Weinstein *et al*, 2008; Lawrence *et al*, 2014), sendo que muitas vezes a presença de adenomas hepáticos e de doença inflamatória intestinal está associada com a presença de anemia (Wang *et al*, 2012; Kaitha *et al*, 2015).

Estudos têm investigado o papel da hepcidina na patogênese da anemia nas doenças crônicas (Jankowska *et al*, 2013; Zhao *et al*, 2013; Mei *et al*, 2014), caso das GSD hepáticas. A hepcidina, um hormônio peptídico com 25 aminoácidos, é produzido principalmente pelo fígado e desempenha um papel central no metabolismo do ferro. Sugere-se que as citocinas inflamatórias, particularmente a IL-6, desencadeiem para o aumento de hepcidina e esse mecanismo parece ser responsável pelo desenvolvimento da anemia por inflamação ou anemia por doença crônica (Nemeth e Ganz, 2009; Ganz, 2011). A hepcidina também tem um importante papel no caso da anemia ferropriva, onde a diminuição da disponibilidade de ferro leva a inibição da expressão de hepcidina, aumentando a absorção de ferro da dieta e a liberação a partir macrófagos (Nicolas *et al*, 2002).

Os nossos dados confirmam os dados da literatura (Wang *et al*, 2012) que indicam que a anemia é especialmente frequente na GSD Ib, o tipo de GSD mais frequentemente associado à doença inflamatória intestinal, seguida da GSD Ia. Pacientes com GSD III e IX não apresentaram anemia em nosso estudo e não foram incluídos pacientes com esses tipos de GSD no estudo anterior.

Nossos achados sugerem, ainda, que pacientes com GSD hepática podem apresentar valores elevados de hepcidina, comparando os mesmos a controles saudáveis, assim como descrito em outros estudos que encontraram valores aumentados de hepcidina em pacientes com doenças crônicas, comparados a controles saudáveis (Hohaus *et al*, 2010; Eleftheriadis *et al*, 2014; Mei *et al*, 2014; Samouilidou *et al*, 2014). Além disso, apesar do pequeno tamanho amostral, os achados sugerem um possível aumento da hepcidina em indivíduos heterozigotos para GSD hepática, que deve ser mais profundamente estudada, na busca de uma possível alteração de fenótipo bioquímico e qual sua relevância clínica nessa população. Na literatura são descritos casos de manifestação clínica em

indivíduos heterozigotos para um erro inato do metabolismo, com padrão de herança autossômico recessivo, caso da deficiência de CPT II (Endres, 1997; Joshi *et al*, 2012).

As concentrações encontradas de hepcidina e IL-6 em pacientes com GSD hepáticas reforçam a hipótese de que a presença de anemia em pacientes com essa condição tem sua ocorrência influenciada pela inflamação subjacente a essa doença, especialmente nos pacientes com GSD Ib, que apresentam os maiores valores de IL-6 e hepcidina. Cabe ressaltar que a anemia ferropriva também pode ocorrer nessas doenças, haja vista a sua associação com doença inflamatória intestinal; as restrições dietéticas que focam na manutenção da normoglicemia, mas que podem resultar em deficiências nutricionais, como a ingestão de ferro e vitaminas (Kishnani *et al*, 2014) e a uma possível alteração na absorção do ferro relacionada à utilização de amido de milho pelos pacientes (Wang *et al*, 2012).

Os resultados encontrados em nossos estudos primeiramente reforçam a necessidade de realização de um tratamento individualizado para pacientes com GSD hepáticas. E, finalmente, destaca-se a importância dos achados do presente estudo no âmbito das GSD hepáticas, os quais, abrem caminhos e direcionam para estudos futuros, que cada vez mais são necessários, visto as lacunas de conhecimento existentes nessas condições e a importância do aperfeiçoamento contínuo nas escolhas de condutas para acompanhamento e tratamento dessa população.

6. CONCLUSÕES

As conclusões do presente trabalho serão apresentadas abaixo, de acordo com o objetivos específicos.

1) Comparar os achados de frações de amido, razão de amilose/amilopectina e a digestão in vitro entre amidos de milho estudados e em relação aos seus possíveis substitutos;

Por meio do nosso estudo foi possível demonstrar que os amidos de milho apresentam pequenas diferenças para todas as variáveis estudadas. O amido modificado apesar de apresentar valor superior de RAG e amilopectina em relação aos amidos de milho, no experimento que avaliou a digestão, o mesmo apresentou uma liberação de glicose semelhante aos demais amidos de milho. Esses achados sugerem que diferenças relatadas nas respostas glicêmicas em pacientes com GSD hepática, podem estar relacionadas às variações inter- e intraindivíduos e/ou ainda relacionadas à adesão ao tratamento.

O polvilho doce apresentou valor inferior de RAG e ligeiramente superior de amilopectina, e os achados da razão amilose/amilopectina foram semelhantes entre os diferentes lotes estudados do mesmo, demonstrando a estabilidade do produto. Nos experimentos de digestão realizados no sistema TIM-1 o polvilho doce apresentou uma liberação de glicose mais lenta, comparado aos demais amidos e um valor de material não digerido também superior. Os achados indicam que o mesmo pode ser digerido mais lentamente comparado aos demais amidos estudados e que misturas de polvilho e amido de milho podem ser uma alternativa para manter a liberação de glicose mais lenta e diminuir a quantidade de amido não digerido ao final.

2) Identificar os amidos que seriam mais benéficos para o tratamento de pacientes com Glicogenoses hepáticas;

Nossos achados indicam que em relação à digestão e liberação de glicose os amidos de milho e o amido modificado são semelhantes entre si, contudo, ressaltamos que pequenas diferenças encontradas na digestão *in vitro* podem ter o seu efeito amplificado *in vivo*, quando variáveis como a resposta hormonal e o fenótipo da doença estão presentes e devem ser observadas individualmente.

O uso do polvilho doce, de forma isolada ou associado a amido de milho, surge como uma potencial alternativa no tratamento de pacientes com GSD hepáticas e deve ser melhor estudado em estudos *in vivo*, na busca do amido que melhor preencha os critérios de tratamento das GSD hepáticas.

3) Verificar a frequência da presença de anemia em uma amostra brasileira de pacientes com Glicogenoses hepáticas;

Em nosso estudo a anemia foi um achado frequente (30%) na amostra de pacientes com GSD hepática estudada, indicando que pacientes brasileiros também apresentam anemia como uma complicação dessa condição, conforme descrito na literatura.

4) Comparar os níveis de hepcidina entre pacientes com Glicogenoses hepáticas, heterozigotos e controles saudáveis;

Os valores de hepcidina encontrados em pacientes com GSD hepática foram superiores àqueles encontrados em controles saudáveis, pareados por sexo e idade, indicando um aumento dessa substância em pacientes com GSD hepática. Além disso, indivíduos heterozigotos apresentaram valores semelhantes de hepcidina aos pacientes, o que pode indicar uma possível apresentação de fenótipo bioquímico alterado em indivíduos heterozigotos para essa doença, que deve ser melhor estudado.

5) Comparar os níveis de IL-6 entre pacientes com Glicogenoses hepáticas e heterozigotos;

Os valores de IL-6 foram superiores em pacientes com GSD hepática, comparados aos de indivíduos heterozigotos estudados, indicando um possível aumento desse marcador inflamatório em pacientes com essa condição, especialmente na GSD Ib.

6) Caracterizar os diferentes tipos de Glicogenose hepática em relação aos níveis de hepcidina, de IL-6 e de anemia.

A anemia foi mais frequente na GSD Ib, o tipo que mais frequentemente se associou à doença inflamatória intestinal e que apresentou valores superiores de IL-6, em relação aos demais grupos. Anemia também esteve presente em pacientes com GSD Ia e alguns pacientes apresentaram nível de IL-6 semelhante à pacientes com tipo Ib. Pacientes com GSD III e IX não apresentaram anemia, indicando que essa não parece ser uma complicação frequente nesses tipos de GSD.

Os valores de hepcidina, não diferiram estatisticamente entre os tipos de GSD, embora tenham sido numericamente superiores na GSD Ib, seguido das GSD Ia e GSD III/IX.

Nos pacientes estudados a IL-6 associou-se com os níveis de Hb, ferro, transferrina e saturação de transferrina. Já os valores de hepcidina apresentaram correlação positiva com os níveis de ferritina. Pacientes com anemia apresentaram valores superiores de IL-6 comparados aos sem anemia. Não foi encontrada, em nosso estudo, correlação estatisticamente significativa entre os níveis de hepcidina e IL-6. A falta de significância pode ser secundária ao relativo pequeno tamanho amostral. Cabe ressaltar que outras citocinas podem estar envolvidas na regulação da hepcidina e influenciar a correlação dessas variáveis.

Os nossos achados sugerem que inflamação está fortemente relacionada à ocorrência de anemia nas GSD hepáticas, principalmente na GSD Ib.

7. PERSPECTIVAS

A realização deste trabalho evidenciou a necessidade de realização de mais estudos na área das GSD hepáticas, visto que as lacunas existentes no conhecimento dessas condições são enormes, constituindo um vasto campo para a pesquisa. Estudos futuros são fundamentais para o estabelecimento de novas condutas para o tratamento e manejo dessas condições.

Com base nos achados do presente estudo, sugere-se realizar estudos com objetivo de:

- Avaliar a eficácia e a segurança da utilização do polvilho doce cru no tratamento de pacientes com GSD hepática, por meio da realização de um ensaio clínico randomizado e controlado, utilizando amido de milho e polvilho doce;

- Caracterizar o perfil inflamatório de pacientes com GSD hepática, por meio da determinação dos níveis de citocinas, e avaliar sua associação com achados clínicos, em especial a anemia, com os valores de hepcidina e com o tipo de GSD hepática;

- Investigar a possibilidade de indivíduos heterozigotos para a GSD hepática desenvolverem manifestações clínicas sutis para essa condição e avaliar sua relevância clínica.

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9. APÊNDICES

9.1. Apêndice 1 - Carta de aprovação do projeto no Comitê de Ética em Pesquisa

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

COMISSÃO CIENTÍFICA

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

Projeto: 120429
Data da Versão do Projeto:

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
TATIELE NALIN

Título: Estudo das Glicogenoses I e III: avaliação dos níveis de citocinas inflamatórias e de hepcidina e sua relação com variáveis clínicas, bioquímicas e nutricionais.

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

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

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

Porto Alegre, 10 de dezembro de 2012.

Profª Nadine Clausell
Coordenadora CEP/HCPA

9.2. Apêndice 2 - Termo de Consentimento Livre e Esclarecido - PACIENTES

Projeto: Estudo das Glicogenoses Hepáticas: avaliação dos níveis de citocinas inflamatórias e de hepcidina e sua relação com variáveis clínicas, bioquímicas e nutricionais.

Pesquisador responsável: Dra. Ida Vanessa D. Schwartz. Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos, 2350. Porto Alegre-RS. Tel: 51-3359 8011.

Nome do paciente:_____

A Glicogenose é uma doença hereditária causada por falta de uma enzima do fígado, dependendo do tipo de Glicogenose diferentes enzimas estão faltando. Quando não temos uma dessas enzimas e temos então Glicogenose, nas situações em que ficamos em jejum, não conseguimos manter o açúcar do sangue normal.

O açúcar baixo no sangue traz como sintomas: tremores, suor frio, cansaço fácil, sonolência e até convulsão (ataque). O tratamento é a alimentação frequente, com alimentos que mantêm os níveis de açúcar no sangue elevados por mais tempo, como é o caso do amido de milho (Maisena) cru.

Além do açúcar baixo no sangue, que pode com o tempo não ser percebido, o não cumprimento das orientações dietéticas pode levar a outros problemas, como aumento do fígado com ou sem tumores, pedra nos rins, perda de proteína pelo rim, atraso no crescimento (baixa estatura). Assim, o tratamento é essencial para prevenir ou impedir o avanço de tais complicações, além promover o crescimento e desenvolvimento adequados.

O envolvimento do sistema imunológico, que é responsável por defender nosso corpo, não tem sido muito estudado entre os pacientes com Glicogenose.

Uma das substâncias que podem ser avaliadas do sistema imunológico são as citocinas (moléculas que são produzidas por células específicas do sistema imunológico e emitem sinais localmente entre células e, assim, tem um efeito em outras células). As citocinas podem ser indutoras da inflamação (pró-inflamatórias) ou combaterem a inflamação (antiinflamatórias). Também não conhecemos o papel de uma substância chamada hepcidina, que está relacionada a presença de anemia nas pessoas. Essa substância pode estar alterada em pacientes com Glicogenose e essa alteração pode estar relacionada às citocinas descritas anteriormente.

Nesse estudo desejamos verificar o envolvimento do sistema imunológico nos pacientes com Glicogenose, como é o seu caso, através da dosagem de algumas citocinas e dosaremos também a expressão de hepcidina, para assim verificar se as citocinas podem influenciar a nível desse hormônio. Vamos também avaliar a relação dessas citocinas e hepcidina com informações suas, como seu tipo de Glicogenose, seu peso, altura e em relação à quantidade e número de vezes que consome amido de milho durante o dia.

Caso você decida participar desta pesquisa, o seu prontuário será consultado, a fim de que sejam obtidas informações clínicas, laboratoriais e nutricionais, como peso, estatura, uso do amido de milho, idade e resultados de exames já realizados. Durante uma de suas consultas ambulatoriais, será solicitado também que você/seu filho(a) realize a coleta de uma amostra de sangue para dosagem de citocinas e expressão de hepcidina.

Se você permitir, o material coletado que restar após a realização dos exames previstos neste estudo, serão armazenados por cinco anos e poderão ser utilizados, neste período, em estudos aprovados eticamente pelos órgãos ou comissões responsáveis. Em relação ao armazenamento e utilização de algum material (sangue) que tenha restado após a realização dos exames previstos neste estudo, você declara que autorizou:

( ) que este material poderá ser armazenado por cinco anos e poderá vir a ser utilizado em estudos futuros aprovados eticamente pelos órgãos ou comissões responsáveis, desde que você revise e assine o termo de

consentimento de tais estudos futuros. Após cinco anos, este material será obrigatoriamente descartado.

( ) que este material não poderá ser armazenado por cinco anos e não poderá vir a ser utilizado em estudos futuros aprovados eticamente pelos órgãos ou comissões responsáveis. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado, sendo obrigatoriamente descartado.

RISCOS E BENEFÍCIOS

Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). O desconforto e os riscos associados a estas avaliações serão minimizados pela realização da coleta por profissional treinado. Cabe salientar que esse estudo talvez não traga benefícios para você, mas pode contribuir para um melhor entendimento desta doença e a melhorar, futuramente, o acompanhamento e tratamento da mesma.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz (Fone: (51) 3359 8011), no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. Além disso, você pode entrar em contato com o Comitê de Ética em Pesquisa do HCPA, que aprovou esse projeto, através de telefone (51 – 3359 8304) ou localmente na rua Ramiro Barcelos, 2350, 2º andar na sala 2227 do Hospital de Clínicas de Porto Alegre. O Horário de funcionamento é de segunda-feira à sexta-feira, das 8 horas até as 17 horas.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

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

CONFIDENCIALIDADE DAS INFORMAÇÕES

As informações dessa pesquisa serão mantidas em sigilo, sendo apenas utilizadas de forma científica, e sem identificação do seu nome, em relatos especializados. Caso alguma informação derivada desse estudo for importante a você, todo esforço será realizado para informá-lo.

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

Data: ___/___/_____

Paciente: _____

Responsável
legal:_____

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

Data: ____/_____/_____

Pesquisador Nome:_____

Pesquisador Assinatura:_____

9.3. Apêndice 3 - Termo de Consentimento Livre e Esclarecido - CONTROLES

Projeto: Estudo das Glicogenoses Hepáticas: avaliação dos níveis de citocinas inflamatórias e de hepcidina e sua relação com variáveis clínicas, bioquímicas e nutricionais.

Pesquisador responsável: Dra. Ida Vanessa D. Schwartz. Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos, 2350. Porto Alegre-RS. Tel: 51-3359 8011.

Nome:_____

A Glicogenose é uma doença hereditária causada por falta de uma enzima do fígado, dependendo do tipo de Glicogenose diferentes enzimas estão faltando. Quando não temos uma dessas enzimas e temos então Glicogenose, nas situações em que ficamos em jejum, não conseguimos manter o açúcar do sangue normal.

O açúcar baixo no sangue traz como sintomas: tremores, suor frio, cansaço fácil, sonolência e até convulsão (ataque). O tratamento é a alimentação frequente, com alimentos que mantêm os níveis de açúcar no sangue elevados por mais tempo, como é o caso do amido de milho (Maisena) cru.

Além do açúcar baixo no sangue, que pode com o tempo não ser percebido, o não cumprimento das orientações dietéticas pode levar a outros problemas, como aumento do fígado com ou sem tumores, pedra nos rins, perda de proteína pelo rim, atraso no crescimento (baixa estatura). Assim, o tratamento é essencial para prevenir ou impedir o avanço de tais complicações, além promover o crescimento e desenvolvimento adequados.

O envolvimento do sistema imunológico, que é responsável por defender nosso corpo, não tem sido muito estudado entre os pacientes com Glicogenose.

Uma das substâncias que podem ser avaliadas do sistema imunológico são as citocinas (moléculas que são produzidas por células específicas do sistema imunológico e emitem sinais localmente entre células e, assim, tem um efeito em outras células). As citocinas podem ser indutoras da inflamação (pró-inflamatórias) ou combaterem a inflamação (antiinflamatórias). Também não conhecemos o papel de uma substância chamada hepcidina, que está relacionada a presença de anemia nas pessoas. Essa substância pode estar alterada em pacientes com Glicogenose e essa alteração pode estar relacionada às citocinas descritas anteriormente.

Nesse estudo desejamos verificar o envolvimento do sistema imunológico nos pacientes com Glicogenose através da dosagem de algumas citocinas e dosaremos também a expressão de hepcidina, para assim verificar se as citocinas podem influenciar a nível desse hormônio.

Os dados dos pacientes com Glicogenose serão comparados com o de pessoas de mesma idade e sexo sem a doença, como no seu caso. Esses são o que chamamos de controles do estudo.

Caso você decida participar desta pesquisa será solicitado que você/seu filho(a) realize a coleta de uma amostra de sangue para dosagem de citocinas e expressão de hepcidina

Se você permitir, o material coletado que restar após a realização dos exames previstos neste estudo, serão armazenados por cinco anos e poderão ser utilizados, neste período, em estudos aprovados eticamente pelos órgãos ou comissões responsáveis. Em relação ao armazenamento e utilização de algum material (sangue) que tenha restado após a realização dos exames previstos neste estudo, você declara que autorizou:

( ) que este material poderá ser armazenado por cinco anos e poderá vir a ser utilizado em estudos futuros aprovados eticamente pelos órgãos ou comissões responsáveis, desde que você revise e assine o termo de consentimento de tais estudos futuros. Após cinco anos, este material será obrigatoriamente descartado.

( ) que este material não poderá ser armazenado por cinco anos e não poderá vir a ser utilizado em estudos futuros aprovados eticamente pelos órgãos ou comissões responsáveis. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado, sendo obrigatoriamente descartado.

RISCOS E BENEFÍCIOS

Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). O desconforto e os riscos associados a estas avaliações serão minimizados pela realização da coleta por profissional treinado. Cabe salientar que esse estudo talvez não traga benefícios para você, mas pode contribuir para um melhor entendimento desta doença e a melhorar, futuramente, o acompanhamento e tratamento da mesma.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz (Fone: (51) 3359 8011), no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. Além disso, você pode entrar em contato com o Comitê de Ética em Pesquisa do HCPA, que aprovou esse projeto, através de telefone (51 – 3359 8304) ou localmente na rua Ramiro Barcelos, 2350, 2º andar na sala 2227 do Hospital de Clínicas de Porto Alegre. O Horário de funcionamento é de segunda-feira à sexta-feira, das 8 horas até as 17 horas.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

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

CONFIDENCIALIDADE DAS INFORMAÇÕES

As informações dessa pesquisa serão mantidas em sigilo, sendo apenas utilizadas de forma científica, e sem identificação do seu nome, em relatos

especializados. Caso alguma informação derivada desse estudo for importante a você, todo esforço será realizado para informá-lo.

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

Data: ___/___/_____

Participante: _____

Responsável
legal:_____

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

Data: ____/_____/_____

Pesquisador Nome:_____

Pesquisador Assinatura:_____

9.4 Apêndice 4 – Artigo publicado no JIMD Reports, 2013 - *Enzyme Replacement Therapy in a Patient with Gaucher Disease Type III: A Paradigmatic Case Showing Severe Adverse Reactions Started a Long Time After the Beginning of Treatment*

Artigo publicado no JIMD Reports, 2013

Título:

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

Autores:

Filippo Vairo; Cristina Netto; Alicia Dorneles; Suzana Mittelstadt; Matheus Wilke; Divair Doneda; Kristiane Michelin; Camila Blos Ribeiro; Amanda Quevedo; Tatiane Vieira; **Tatiele Nalin**; Sônia Lueska; Ida Vanessa D. Schwartz

JIMD Reports
DOI 10.1007/8904_2013_214

CASE REPORT

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

Filippo Vairo • Cristina Netto • Alicia Dorneles • Suzana Mittelstadt • Matheus Wilke • Divair Doneda • Kristiane Michelin • Camila Blos Ribeiro • Amanda Quevedo • Tatiane Vieira • Tatiele Nalin • Sônia Lueska • Ida Vanessa D. Schwartz

Received: 27 October 2012 / Revised: 18 January 2013 / Accepted: 23 January 2013

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

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

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

Communicated by: Robin Lachmann

Competing interests: None declared

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Introduction

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

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

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

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

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

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

Case Report

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

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

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

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

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

Discussion

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

Table 1 Follow-up of laboratory parameters and imaging findings

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

[a] Difficult to measure due to bone changes

[b] Shortly before first imiglucerase infusion

[c] On ultrasound

[d] On X-rays

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

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

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

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

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

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

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

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Contributors

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

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9.5 Apêndice 5 - Artigo publicado no Jornal de Pediatria, 2014 - *Glycogen storage disease type I: clinical and laboratory profile.*

Artigo publicado no Jornal de Pediatria, 2014

Título:

Glycogen storage disease type I: clinical and laboratory profile.

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ORIGINAL ARTICLE

Glycogen storage disease type I: clinical and laboratory profile☆,☆☆

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KEYWORDS
Inborn errors of metabolism;
Glycogen storage disease type I;
Clinical aspects;
Diagnoses;
Nutritional status

Abstract
Objectives: To characterize the clinical, laboratory, and anthropometric profile of a sample of Brazilian patients with glycogen storage disease type I managed at an outpatient referral clinic for inborn errors of metabolism.
Methods: This was a cross-sectional outpatient study based on a convenience sampling strategy. Data on diagnosis, management, anthropometric parameters, and follow-up were assessed.
Results: Twenty-one patients were included (median age 10 years, range 1–25 years), all using uncooked cornstarch therapy. Median age at diagnosis was 7 months (range, 1–132 months), and 19 patients underwent liver biopsy for diagnostic confirmation. Overweight, short stature, hepatomegaly, and liver nodules were present in 16 of 21, four of 21, nine of 14, and three of 14 patients, respectively. A correlation was found between height-for-age and BMI-for-age Z-scores ($r = 0.561$; $p = 0.008$).

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☆☆ Study conducted at the Universidade Federal do Rio Grande do Sul, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.
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Conclusions: Diagnosis of glycogen storage disease type I is delayed in Brazil. Most patients undergo liver biopsy for diagnostic confirmation, even though the combination of a characteristic clinical presentation and molecular methods can provide a definitive diagnosis in a less invasive manner. Obesity is a side effect of cornstarch therapy, and appears to be associated with growth in these patients.

PALAVRAS-CHAVE
Erros inatos do metabolismo;
Doença de depósito de glicogênio tipo I;
Aspectos clínicos;
Diagnósticos;
Estado nutricional

Doença de depósito de glicogênio tipo I: perfil clínico e laboratorial

Resumo
Objetivos: Caracterizar o perfil clínico, laboratorial e antropométrico de uma amostra de pacientes brasileiros com doença de depósito de glicogênio tipo I tratados em um ambulatório de referência para erros inatos do metabolismo.
Métodos: Este foi um estudo ambulatorial transversal com base em uma estratégia de amostragem de conveniência. Foram avaliados os dados com relação ao diagnóstico, tratamento, parâmetros antropométricos e acompanhamento.
Resultados: Foram incluídos 21 pacientes (idade média de 10 anos, faixa 1-25 anos de idade), e todos se encontravam em terapia de amido de milho cru. A idade média na época do diagnóstico foi de sete meses (faixa, 1-32 meses), e 19 pacientes foram submetidos a biópsia hepática para confirmação do diagnóstico. Sobrepeso, baixa estatura, hepatomegalia e nódulos hepáticos foram fatores presentes em 16 de 21, quatro de 21, nove de 14 e três de 14 pacientes, respectivamente. Foi encontrada uma correlação entre os escores z para peso para idade e IMC para idade ($r = 0{,}561$; $p = 0{,}008$).
Conclusões: O diagnóstico da doença de depósito de glicogênio tipo I tem sido tardio no Brasil. A maioria dos pacientes foi submetida a confirmação do diagnóstico, apesar de o quadro clínico característico e os métodos moleculares poderem fornecer um diagnóstico definitivo de forma menos invasiva. Obesidade é um efeito colateral da terapia com amido de milho e parece estar associada a crescimento nesses pacientes.

Introduction

Glycogen storage disease type I (GSDI, or von Gierke's disease) is caused by deficiency of glucose-6-phosphatase (G6Pase), an enzyme that catalyzes hydrolysis of glucose-6-phosphate (G6P) into glucose and inorganic phosphate (Pi), a key step in the maintenance of glucose homeostasis. Two major subtypes of GSDI are recognized: GSD type Ia (GSDIa), which is the result of a mutation affecting the catalytic subunit of G6Pase-alpha (or G6PC), and GSD type Ib (GSDIb), which is caused by a defect in G6P translocase (or G6PT).[1] GSDI is inherited in an autosomal recessive pattern, and its incidence is estimated at one in 100,000 live births, making it the most common of the hepatic GSDs.[2]

Patients with GSDIa present with hepatomegaly, a characteristic ''doll-like'' face, short stature, and chronic fatigue. Laboratory findings suggestive of GSDIa include hypoglycemia after a four to six hour fast, lactic acidosis, hypertriglyceridemia, and hyperuricemia. Functional tests for differential diagnosis of hypoglycemia show absence of glycemic response to glucagon injection and aggravation of hyperlactacidemia,[3] whereas histopathological analysis of hepatic biopsy specimens shows glycogen buildup in the liver. In GSDIb, the clinical presentation is quite similar to that of GSDIa, but may be accompanied by neutropenia with recurrent infections (particularly of the gastrointestinal tract) and an increased incidence of inflammatory bowel disease.[4] Although the gold-standard methods for diagnosis of GSDIa are the measurement of G6PC or G6PT activity in liver tissue and/or detection of pathogenic mutations in the genes that code for G6PC and G6PT, specific therapy can be initiated based solely on the clinical and histopathological findings.[3] Access to the DNA/enzyme tests is limited since they are only provided by a select few national and international centers, usually within the framework of research projects.

Management of GSDI is essentially dietary,[3] and consists of frequent meals – preferably containing slow-release carbohydrates such as uncooked cornstarch – at regular intervals, and restriction of fructose, sucrose, and lactose intake. In infants, the recommended management strategy includes frequent meals and continuous nocturnal infusion of glucose at a rate of 6-8 mg/kg/min through a nasogastric or gastrostomy tube. Treatment efficacy is measured by monitoring growth and biochemical parameters, as well as by abdominal ultrasound for assessment of liver volume and presence of nodules. Proper dietary management decreases the risk of long-term complications, which include short stature, osteoporosis or bone mineral loss, kidney disease with hypertension, proteinuria, renal calculi,

nephrocalcinosis, hepatocellular adenomas (with potential for malignant transformation), pancreatitis secondary to hypertriglyceridemia, and potentially life-threatening hypoglycemia.[5,6]

The objective of this study was to assess the clinical and laboratory profile of a sample of Brazilian patients with GSDI recruited from an outpatient referral center for inborn errors of metabolism. The main research hypothesis was that diagnosis of GSDI is delayed in Brazil, both due to a lack of access to diagnostic methods and due to poor awareness of the condition by healthcare providers, thus hindering early access to specific treatment and genetic counseling.

Methods

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA, Brazil). All subjects signed an informed consent prior to study participation.

This was an outpatient-based case series with cross-sectional analysis of the variables of interest. A convenience sampling strategy was used. The study was conducted between March of 2011 and January of 2013. The criterion for inclusion was a diagnosis of GSDI established using at least two of the following methods (the diagnosis was independently confirmed by the authors in all patients): a) clinical diagnosis, defined by over 12 months of specialist care (led by hepatologist or medical geneticist) and clinical manifestations consistent with GSDI (hypoglycemia with hyperlactatemia, hypertriglyceridemia, hyperuricemia, hepatomegaly, and/or growth failure and short stature, and normal levels of creatine phosphokinase [CPK]) at the time of diagnosis or at the time of study inclusion; b) positive family history consistent with autosomal recessive inheritance, as long as GSDI had been confirmed by enzymatic methods or DNA analysis in the affected relative(s); c) histopathological diagnosis, defined as the presence of histological changes in liver tissue consistent with GSD, such as hyperglycogenated nuclei, mild fibrosis, and fatty changes with lipid vacuoles;[7] d) enzymatic diagnosis, defined by negligible activity (< 10%) of G6Pase in fresh or frozen liver tissue samples; or e) molecular diagnosis, defined by the presence of pathogenic mutations in the *G6PC* gene (for patients with GSDIa) or in the *SLC37A*[3] gene (for those with GSDIb) as detected by molecular methods. The distinction between GSDIa and GSDIb was mostly based on clinical findings (absence or presence of neutropenia, respectively), as molecular diagnostics were unavailable to the majority of patients.

Patients were invited to take part in the study after routine visits. Those who agreed to participate were all assessed by the same researcher and underwent a targeted history, physical examination, and anthropometric assessment. The latest laboratory values (blood glucose, lactate, cholesterol, triglycerides, uric acid) and imaging findings available for each patient were obtained by means of a chart review. Tests performed up to three months prior to anthropometric assessment were considered acceptable. The variables of interest were sex, parental consanguinity, current age, age at diagnosis (defined as the age at which parents reported a specific diagnosis of GSD or, if unavailable, the age at diagnosis as noted in the patient's first chart containing diagnostic test results and start of dietary management), first clinical manifestation (as reported by parents), laboratory parameters (current and at time of diagnosis), liver biopsy for histopathological examination or molecular analysis, and current clinical and imaging data (anthropometric assessment, liver ultrasound, and bone mineral density and body composition by dual-energy X-ray absorptiometry [DEXA]).

Anthropometric assessment consisted of weight (kg) and height (cm) measurement. Body weight was measured using digital scales with a maximum capacity of 150 kg and a resolution of 100 g, certified by the Brazilian National Institute of Metrology, Standardization, and Industrial Quality (Instituto Nacional de Metrologia, Qualidade e Tecnologia - INMETRO). Patients were weighed while nude and barefoot. Height was measured with a wall-mounted stadiometer precise to 1 mm. In adolescents, the Tanner scale was used for pubertal staging. Anthropometric measurements and classifications for age and sex were calculated in the World Health Organization's AnthroPlus software suite. The variables of interest were height-for-age and BMI-for-age Z-scores, as proposed by the Brazilian Society of Pediatrics.[8]

Liver size was measured by ultrasonography and assessed for normality on the basis of the reference sizes for children published in 2010 by Dhingra et al.[9] When objective data on liver size were missing, the sonographer's impression was used instead (normal or enlarged).

The criteria for adequacy of metabolic control were based on the European Study on Glycogen Storage Disease Type 1 (ESGSD I):[5] blood glucose > 63 mg/dL, triglycerides < 530 mg/dL, uric acid < 7 mg/dL, BMI between 0 and + 2 standard deviations (SD), and lactate > 2.5 mmol/L (the latter used as the urine lactate/creatinine ratio was unavailable). The absence of hepatic adenomas and adequate height-for-age (z-score > -2 SD) are important parameters for assessment of metabolic control adequacy, but are not part of the ESGSD I.[5]

Statistical analyses were conducted in the Statistical Package for the Social Sciences® version 20.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as means and standard deviations or as medians and interquartile ranges. Analysis of variance (ANOVA) was used for comparison of height and BMI z-scores. The significance level was set at 5%. Data were entered into a Microsoft Excel 2010 for Windows spreadsheet (Microsoft, Redmond, WA, EUA) and analyzed in SPSS 20.0 (IBM Corp., Armonk, NY, USA).

Results

Twenty-one patients were included in the study: 17 with GSDIa and four with GSDIb. Table 1 shows the sample profile at the time of diagnosis.

Table 2 presents the anthropometric and laboratory data, as well as compliance with uncooked starch therapy. Sixteen patients had excess body weight (six of 21 severely obese [BMI-for-age zscore > + 3]; six of 21 obese; four of 21 overweight). The mean BMI-for-age z-score was 2.19 (1.5 to 2.8), and the mean height-for-age z-score was -1.16 (-1.76 to -0.58); four of 21 patients had short stature, one of whom had very short stature (z-score < -3). Fig. 1 provides a graphical representation of the positive, significant correlation between height and BMI z-scores.

Table 1 Summary of findings at diagnosis among patients with glycogen storage disorder (GSD) type I (n = 21).

Patient	Sex	GSD Type	Consanguinity	Age at diagnosis (months)	Initial clinical manifestation	Method of GSD diagnosis	Lactate[b] mmol/L	Triglycerides[b] mg/dL	Cholesterol[b] mg/dL	Glucose[b] mg/dL	Uric acid[b] mg/dL	AST/ ALT[b]
1	F	Ia	+	12	N/A	Enzyme + clinical	10.1	573	259	36	9.8	137/126
2	F	Ia	+	7	Metabolic acidosis	Enzyme + clinical + DNA	4.75	1101	262	< 60	3.4	971/182
3	F	Ia	+	24	Hepatomegaly	Enzyme + clinical + DNA	12.5	714	214	< 60	7.5	N/A
4	M	Ia	-	9	Hypoglycemia	Histopathology + clinical	N/A	1646	581	113	N/A	375/348
5[a]	F	Ia	+	1	Hypoglycemia	Family history + clinical	7.81	719	N/A	31	N/A	N/A
6[a]	F	Ia	+	6	Hypoglycemia	Enzyme + clinical	7	2013	226	57	3	142/102
7	M	Ia	-	5	Hepatomegaly	Enzyme + clinical	4.6	143	155	116	3.4	31/40
8	M	Ia	+	4	Seizure	Enzyme + clinical + DNA	N/A	216	161	0	6.7	48/23
9	M	Ia	-	7	Hepatomegaly	Enzyme + clinical	1.7	366	231	77	6	26/25
10	M	Ia	-	36	Hypoglycemia	Enzyme + clinical	N/A	1695	285	48	6.3	34
11	M	Ia	-	4	Hepatomegaly	Clinical + DNA	4.4	836	318	N/A	8.1	N/A
12	F	Ia	-	48	Hepatomegaly	Enzyme + clinical	10.25	610	185	45	9	59/45
13	M	Ia	+	3	Hypoglycemia	Enzyme + clinical	N/A	218	187	98		95/63
14	M	Ia	+	132	Short stature	Enzyme + clinical	8.2	941	317	61	10	56/69
15	M	Ia	-	7	Hepatomegaly	Enzyme + clinical + DNA	N/A	940	388	32	7.3	157/130
16	F	Ia	-	84	Hepatomegaly	Histopathology + clinical	7.9	918	N/A	56	7.2	N/A
17	F	Ia	-	15	Hepatomegaly	Histopathology + Clinical	6	355	308	53	5.5	33/19
18	M	Ib	+	6	Hypoglycemia	Enzyme + Clinical	2.8	371	N/A	86	10.1	N/A
19	F	Ib	-	4	Recurrent infections	Histopathology + Clinical	1.6	266	N/A	143	8.9	N/A
20	M	Ib	-	6	Hypoglycemia	Enzyme + Clinical	N/A	123	107	N/A	4.5	26/22
21	F	Ib	-	6	Hypoglycemia	Clinical + DNA	5.3	715	196	93	6.6	51/41

All patients had clinical manifestations at the time of diagnosis or were in treatment with a pediatric gastroenterologist or medical geneticist at the time of study inclusion and had histopathological evidence of hepatic glycogen buildup and/or G6Pase activity at < 10% in liver tissue and/or presence of pathogenic mutations in the *G6Pase* gene, and absence of high levels of creatine phosphokinase.

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

[a] Patients 5 and 6 form a Sibship; patient 6 is the older sibling.

[b] Values at diagnosis; +, present; -, absent; N/A, not available. Reference ranges: lactate, 0.5–2.2 mmol/L; triglycerides, 100 mg/dL at age <10 years, ≤ 130 mg/dL at age 10–19, ≤ 150 mg/dL in adults; total cholesterol, <129 mg/dL; glucose, 60–99 mg/dL; uric acid, 2.4–7 mg/dL; AST, < 37 mg/dL; ALT, < 41 mg/dL.

Table 2 Last anthropometric and laboratory assessment of patients with glycogen storage disorder type I (n = 21).

Patient	Age (years)	Cornstarch[a] (g/kg/dose)	Weight (kg)	Height (cm)	Height Z-score	BMI	BMI Z-score	Stage of puberty	Lactate (mmol/L)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Glucose (mg/dL)	Uric acid (mg/dL)	Bone density scan	
														Z-score	BF%
1	13	2.5	32.4	130.5	-4.1	19.02	-0.13	Prepubertal	4.5	555	N/A	100	4.6	-3.1	33.2
2	5	2.1	18.9	100.5	-2.06	18.71	1.91	Prepubertal	1.6	83	101	101	4	-1.4	46.1
3	4	2.5	17.8	102.8	-1.18	16.84	0.99	Prepubertal	3.1	192	N/A	96	4.3	N/A	N/A
4	6	1	30.2	111.5	-0.95	24.29	4.64	Prepubertal	5.5	1071	242	151	5.2	-0.4	45
5	16	1.3	64.7	154	-2.46	27.28	1.88	Pubertal	2.5	276	214	52	7.6	N/A	N/A
6	17	0.6	77	150.9	-1.73	33.82	2.72	Pubertal	2.3	211	179	65	7.6	N/A	N/A
7	9	0.5	74.3	146.5	2.47	34.62	5.66	Prepubertal	0.3	N/A	115	65	2.7	N/A	N/A
8	17	1	70	161.5	-1.84	26.84	1.6	Pubertal	1	242	156	87	6.4	-1	41.3
9	21	1.3	66.9	168.5	-1.1	23.56	0.46	Adult	1.4	192	240	N/A	5.4	-1.4	22.2
10	25	1	60.3	170.5	-0.82	20.70	-0.52	Adult	6.2	809	264	92	N/A	N/A	N/A
11	20	1.4	72	170	-0.89	24.91	0.86	Adult	2.2	488	262	48	6.6	0.9	27.8
12	10	1.35	44.3	138	-0.17	23.26	2.13	Prepubertal	2.7	454	N/A	90	8.5	N/A	N/A
13	6	1.9	25.9	113.2	-1.44	20.21	2.56	Prepubertal	N/A	218	187	98	N/A	-1.3	33.9
14	16	0.8	99.3	164.5	-1.21	36.70	3.26	Pubertal	1.5	404	273	94	9	N/A	N/A
15	7	1.6	36.5	130	0.73	21.60	2.74	Prepubertal	1.6	192	N/A	85	3.9	N/A	N/A
16	10	0.8	51.9	140	0.21	26.50	2.73	Prepubertal	1.8	218	233	69	7.1	N/A	N/A
17	4	1	22.5	102.5	-1.14	21.42	3.19	Prepubertal	9.8	321	237	25	7.6	N/A	31.7
18	12	1.1	56.7	136.5	-1.92	30.43	3.02	Prepubertal	2.8	371	N/A	86	10.1	-0.6	46.2
19	11	1.6	34.4	130	-2.4	20.30	1.09	Prepubertal	0.8	159	129	86	7.1	N/A	N/A
20	7	0.9	28.7	118.5	-0.98	20.44	2.47	Prepubertal	0.5	123	107	93	3.6	0.6	30.6
21	1	[b]	11.9	75	-1.29	21.16	3.08	Prepubertal	5.3	715	196	93	6.6	N/A	N/A

BMI, body mass index.

[a] All patients were on uncooked starch therapy (four to six times/day) and none were on continuous nocturnal glucose infusion.

[b] Patient who received cornstarch irregularly.

Height-for-age and BMI-for-age Z-scores calculated in World Health Organization's Anthro and AnthroPlus. Bone density scanning performed in a Lunar iDXA (GE Healthcare) device. Bone mineral density Z-scores calculated and body composition expressed as body fat percentage (BF%). Bone mineral density Z-scores not calculated for patient 17 due to age < 5 years. N/A, not available.

Reference ranges: lactate, 0.5–2.2 mmol/L; triglycerides, ≤ 100 mg/dL at age < 10 years, ≤ 130 mg/dL at age 10–19, ≤ 150 mg/dL in adults; total cholesterol, < 129 mg/dL; glucose, 60–99 mg/dL; uric acid, 2.4–7 mg/dL.

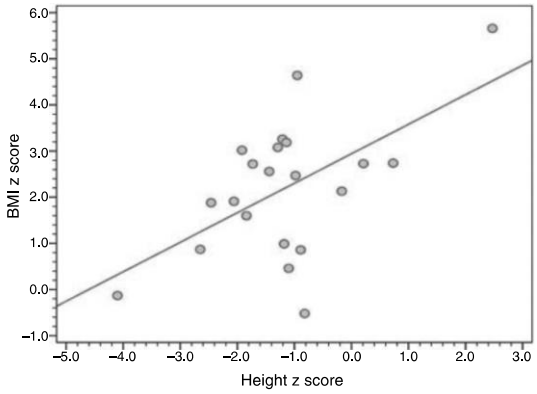

Figure 1 Correlation between BMI-for-age and height-for-age Z-scores (r = 0.561; p = 0.008).
BMI, body mass index.

Body composition was analyzed in ten patients (eight with GSDIa, two with GSDIb) (Table 2). Fourteen patients underwent abdominal ultrasonography for assessment of liver size; of these, five had a normal liver size, one of whom had a visible hepatic nodule. The eight remaining patients had hepatomegaly, and two had more than three detectable nodules.

Discussion

The characterization of the natural history of rare diseases and of the efficacy of treatments for these conditions is always hindered by small sample sizes.[10] The small number of patients is attributable not only to the rarity of these diseases, but also to underdiagnosis, particularly of cases with relatively mild clinical manifestations. Therefore, studies such as the present – the first-ever characterization of a population of GSDI patients in Brazil are paramount, given their crucial role in enabling later conduction of meta-analysis and the drawing of more robust conclusions.

Diagnosis of GSDI was delayed in this sample, confirming the initial hypothesis. According to the literature, the usual age of symptom onset in patients with GSDI is 3 months.[4] This study did not assess the variable ''age at symptom onset'' as the authors believe it to be subject to a wide range of biases, particularly recall bias. Studies have shown that earlier diagnosis and treatment onset are associated with lower odds of complications.[3] In the present sample, the earliest clinical diagnosis was established in a patient (patient 5) who developed symptoms before the 1st month of life, who had an older sister (patient 6) with a confirmed diagnosis of GSDIa. The latest diagnosis was at 132 months of age, in patient 14, who had subclinical hypoglycemia and was diagnosed after a three year investigation prompted by short stature, thus representing a somewhat attenuated phenotype of the condition. Although hypoglycemia is one of the cardinal symptoms that drive clinical suspicion of GSDI, it may sometimes go unnoticed due to use of lactic acid as a substrate for cerebral metabolism.[11] Therefore, even though symptomatic hypoglycemia is frequently reported, its absence does not rule out a diagnosis of GSDI. In 2003, Shieh et al. published a case report describing delayed diagnosis of GSDI and suggested that ''milder'' forms of the condition may occur.[11] Also in 2003, an article recommended that adolescents with unexplained hyperuricemia and hyperlipidemia should be screened for GSDI, even if hypoglycemia was absent.[12]

Regarding diagnostic procedures, most patients in the present sample underwent a liver biopsy.[13] This finding is rather surprising in view of the increased worldwide accessibility of genetic testing. The *G6PC* gene is small (12.5 kb, 5 exons) and thus easily sequenced; furthermore, the variants p.347X and p.R83C appear to be common in the Brazilian population, as reported by Reis et al. in 2011.[14] In the present sample, these mutations were found in four of ten and three of ten patients with GSDIa, respectively (data not shown). Although it is not entirely devoid of risk, blood collection for genetic assays is a far less invasive and less costly procedure than liver biopsy for histopathological examination or enzyme activity assessment. Isolated histological analysis of liver tissue without measurement of enzyme activity is not sufficient to determine the type of GSD, although it can demonstrate glycogen and fat deposition, and is valuable in the differential diagnosis of other liver diseases. Conversely, enzyme assays are available only at very few centers and are associated with a series of logistical challenges, such as tissue transport (specimens should preferably be fresh or frozen) to the reference laboratory.

The present data suggest a trend toward patients with higher height-for-age Z-scores having higher BMI-for-age Z-scores as well.[15] Although this trend was affected by outliers, it suggests that intensive dietary management leads to better growth at the expense of marked weight gain, as previously reported by Weinstein and Wolfsdorf in 2002.[6] Management of obesity in patients with GSDI is certainly a topic deserving of greater research attention.

Growth retardation is a finding of major importance in children with GSDI,[16] and short stature is common in adults with the condition. In the present sample, patients with inadequate metabolic control according to the ESGSD I[4] had the worst height-for-age Z-scores. The pathophysiology of short stature in GSDI has yet to be elucidated, but studies conducted since 2008 have shown that good metabolic control can improve growth.[17,18] Hormonal changes, variation in blood pH (due to metabolic acidosis), and hyperlactatemia may contribute to this growth deficit. According to the ESGSD I criteria,[5] half of all patients in the present sample had good metabolic control of their condition, even though some had BMI Z-scores > 2 SDs, which may account at least in part for the near-adequate growth of this population (18 of 21 patients had height-for-age Z-scores > -2 SDs).

The purpose of dietary management of GSDI is to mimic endogenous glucose production. Exogenous dextrose administration strategies for maintenance of normoglycemia have been assessed and modified in recent years. Frequent meals containing partially cooked starch, continuous nocturnal gastric drip feeding (CNGDF) of dextrose via nasogastric tube, and uncooked cornstarch (UCCS) therapy are some of the available strategies. None of the patients in this case series were on continuous nocturnal feeding; all were on uncooked cornstarch therapy (five to six doses per 24 h, including overnight). A recent meta-analysis[15] compared

several studies of UCCS (diurnal and nocturnal) with studies of CNGDF, and found both short-term and long-term improvement of metabolic control in patients given UCCS.[19] Therefore, CNGDF should be restricted to select cases, as the inconvenience of use of a feeding pump and the risk of severe hypoglycemia in case of abrupt discontinuation of feeding (e.g., due to a power outage or pump malfunction) do not outweigh the metabolic control benefits of intermittent night-time UCCS administration. A modified cornstarch formulation (Glycosade®, Vitaflo, Nestlé Health Nutrition, Vevey, Switzerland), which granted the Food and Drug Administration (FDA) approval in 2012, is an alternative that may allow patients to sleep through the night.[20]

The hepatomegaly observed in patients with GSDI may be the result of glycogen deposition and fatty liver disease secondary to increased flow of free fatty acids from the adipose tissue to the liver.[21] Routine liver ultrasound is a noninvasive diagnostic modality that can be used to assess long-term treatment success. As this study's analysis of ultrasound findings was based on a chart review, operator variability is a concern. The histology of hepatocellular adenomas in GSDI is similar to that of adenomas seen in other conditions. Several hypotheses have attempted to explain the development of adenomatous changes, such as imbalances in glucagon-to-insulin ratio, cellular glycogen overload, and proto-oncogene activation.[21] The three patients with hepatic adenomas in the present case series were 16, 17, and 25 years old (patients 5, 6, and 10 respectively). Patients 5 and 6 had poor metabolic control, with hyperuricemia and hypoglycemia despite low triglycerides and near-normal lactate levels. Patient 10, the oldest patient in this sample, also had inadequate metabolic control.

Hepatocellular adenomas may occur in 22% to 75% of adults with GSDIa, and the risk of malignant transformation is approximately 10%.[21] As most patients are under the age of 20, a low incidence of adenomas is to be expected in this population regardless of metabolic control. Thus far, there are no cases of hepatocarcinoma in this series.

Of the nine patients in whom bone density scans were performed, only one (patient 1) had low bone mass for chronological age in accordance with the 2008 Official Position Statement of the Brazilian Society for Bone Densitometry.[22] Several mechanisms have been suggested to explain the low bone mineral density observed in patients with GSDI: persistent acidosis, urinary calcium loss without adequate replacement, reduction of bone matrix (hypoglycemia leads to decreased glycosylation of bone matrix proteins), and changes in growth hormone (GH) levels.[18] Furthermore, many patients with GSDI exhibit abnormal pubertal growth,[5] and sex steroids play a major role in bone formation, particularly during puberty.[22] Some issues must be taken into account when analyzing bone mineral density in children and adolescents, such as bone maturation, sex, and stage of puberty.[23] Overall, the patients assessed in this case series had good bone mineral density despite their GSDI.

Despite the rarity of GSDI, it must not be overlooked by pediatricians. The agent used for treatment of this inborn error of metabolism is readily available at any grocery store or supermarket. Administration of cornstarch as a nutraceutical (food used for medicinal purposes) to GSDI patients leads to excessive weight gain as a side effect, due to the attendant increase in total carbohydrate intake and probably due to relative physical inactivity as well. No studies have assessed the efficacy of physical exercise in patients with GSDI, and it is not contraindicated. Therefore, physical activity – supported by a well-designed dietary prescription that takes the pre- and post-exercise periods into account – may be an effective strategy for the control of weight gain in patients whose metabolic control is otherwise satisfactory.

Greater awareness of this disorder among pediatricians should aid their search for an etiological diagnosis in cases of hypoglycemia, hepatomegaly, dyslipidemia, and short stature that might otherwise be improperly managed. Early diagnosis based on clinical and laboratory findings is feasible, easy, and affordable even where access to specialty care is limited. Nevertheless, investment in centers for expert molecular diagnosis is both warranted and necessary, as the use of molecular methods practically obviates the need for liver biopsy. Early treatment can be instituted at any health service, does not require any complex interventions, and decreases the risk of death, mainly by preventing severe hypoglycemia. Adequately treated patients can lead intellectually and socially satisfying lives with no limitations other than a special diet.

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Conflicts of interest

The authors declare no conflicts of interest.

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9.6 Apêndice 6 – Artigo publicado na Gene, 2014 - *Body composition in patients with classical homocystinuria: body mass relates to homocysteine and choline metabolism.*

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Título:

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Short Communication

Body composition in patients with classical homocystinuria: body mass relates to homocysteine and choline metabolism

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ABSTRACT

Introduction: Classical homocystinuria is a rare genetic disease caused by cystathionine β-synthase deficiency, resulting in homocysteine accumulation. Growing evidence suggests that reduced fat mass in patients with classical homocystinuria may be associated with alterations in choline and homocysteine pathways. This study aimed to evaluate the body composition of patients with classical homocystinuria, identifying changes in body fat percentage and correlating findings with biochemical markers of homocysteine and choline pathways, lipoprotein levels and bone mineral density (BMD) T-scores.
Methods: Nine patients with classical homocystinuria were included in the study. Levels of homocysteine, methionine, cysteine, choline, betaine, dimethylglycine and ethanolamine were determined. Body composition was assessed by bioelectrical impedance analysis (BIA) in patients and in 18 controls. Data on the last BMD measurement and lipoprotein profile were obtained from medical records.
Results: Of 9 patients, 4 (44%) had a low body fat percentage, but no statistically significant differences were found between patients and controls. Homocysteine and methionine levels were negatively correlated with body mass index (BMI), while cysteine showed a positive correlation with BMI ($p < 0.05$). There was a trend between total choline levels and body fat percentage ($r = 0.439$, $p = 0.07$). HDL cholesterol correlated with choline and ethanolamine levels ($r = 0.757$, $p = 0.049$; $r = 0.847$, $p = 0.016$, respectively), and total cholesterol also correlated with choline levels ($r = 0.775$, $p = 0.041$). There was no association between BMD T-scores and body composition.
Conclusions: These results suggest that reduced fat mass is common in patients with classical homocystinuria, and that alterations in homocysteine and choline pathways affect body mass and lipid metabolism.

1. Introduction

Homocysteine is a toxic amino acid formed from methionine. High levels of homocysteine are associated with an increased incidence of several diseases, such as stroke, heart failure, coronary heart disease, dementia, and bone fractures (Homocysteine Studies Collaboration, 2002; Mudd et al., 1985). There are three main pathways by which homocysteine can be removed. In the transsulfuration pathway, homocysteine is irreversibly degraded by the action of the enzyme cystathionine beta-synthase (CβS; EC 4.2.1.22). It can also be remethylated by the ubiquitous methionine synthase (MS; EC 2.1.1.13), an enzyme dependent on vitamin B12 and folate, or by the liver/kidney specific betaine–homocysteine methyltransferase (BHMT; EC 2.1.1.5) using betaine. Betaine can be either derived from the diet or formed by oxidation of choline, a key nutrient in lipid metabolism.

Abbreviations: BIA, bioelectrical impedance analysis; BMD, bone mineral density; BMI, body mass index; CβS, cystathionine beta-synthase; DXA, dual-energy X-ray absorptiometry; ESPEN, European Society for Clinical Nutrition and Metabolism; HCPA, Hospital de Clínicas de Porto Alegre; HPLC, high performance liquid chromatography; IQ, interquartile range; SPSS, Statistical Package for the Social Sciences.

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Classical homocystinuria (OMIM 236200) is an autosomal recessive inborn error of metabolism caused by a deficiency in CβS, which results in increased plasma levels of homocysteine and methionine and decreased cysteine levels. It is a rare disease, with a worldwide prevalence estimated at 1:344,000 individuals (Mudd et al., 2001). Treatment includes pharmacological doses of pyridoxine (CβS cofactor), folic acid, vitamin B12, and, in some cases, betaine and also a methionine-restricted diet (Schiff and Blom, 2012). A large study on the natural history of the disease described equal proportions of patients responsive and unresponsive to pyridoxine (Mudd et al., 1985).

In addition to the classic manifestations (ectopia lentis, thromboembolism, mental retardation, and osteoporosis), patients with classical homocystinuria are tall and have a lean biotype (Brenton et al., 1972; Mudd et al., 1985). Until recently, it was believed that bone abnormalities were responsible for this phenotype. However, growing evidence suggests that these patients have reduced fat mass, associated with alterations in choline and homocysteine pathways.

In an animal model of classical homocystinuria, a marked decrease in adipose tissue was described as being associated with low levels of cysteine (Gupta and Kruger, 2011). Betaine and choline have also been associated with body composition, weight gain and lipid metabolism, both in healthy individuals and in experimental studies (Konstantinova et al., 2008; Teng et al., 2012; Wu et al., 2012). Moreover, there is evidence that choline and homocysteine metabolisms may overlap with respect to their relation to body weight (Zeisel, 2012). Given that the amount of body fat is closely related to bone mineral density (BMD), these changes could have important clinical implications in classical homocystinuria (Reid, 2008).

Despite the evidence from animal studies, this has not been studied in patients with classical homocystinuria. The objective of this study was to evaluate the body composition of patients with classical homocystinuria, identifying changes in body fat percentage and correlating findings with biochemical markers of homocysteine and choline pathways, lipoprotein levels and BMD T-scores.

2. Subjects and methods

The present study was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA), Brazil, and the procedures were conducted after written informed consent was obtained from all subjects or their caretakers.

2.1. Study sample

Nine Brazilian patients with classical homocystinuria from 7 unrelated families were included in the study (median age = 26 years; IQ25–75 = 21–28 years). All patients had a late diagnosis (median age = 9 years; IQ25–75 = 6.25–12 years); 4 patients (44%) already had at least 3 systems compromised at diagnosis. Parental consanguinity was reported by 3/9 (33.3%) families.

At the time of study inclusion, all patients (aging 17–35 years) were receiving some type of treatment: pyridoxine (n = 7), folic acid (n = 8), betaine (n = 8), acetylsalicylic acid (n = 8), dietary methionine restriction (n = 9), and supplementation with a methionine-free formula (n = 2). However, most patients had not achieved adequate metabolic control (Table 1). Seven patients were unresponsive to pyridoxine, one was partially responsive (patient #4), and one was responsive (patient #3).

In addition, 18 healthy subjects (volunteers recruited from the institution) matched for gender and age, served as controls for bioelectrical impedance analysis (BIA) only. The controls had a median age of 25 years (IQ25–75 = 21–30 years).

The levels of homocysteine and methionine in the last 5 years (cysteine was unavailable) were obtained for 7 patients. For patient #7 these values were unavailable. Because patient #9 had a recent diagnosis, 3-year results of homocysteine and methionine measurements were obtained. Data on the last BMD measurement (T-score at the lumbar spine and femur), lipoprotein profile (triglycerides and HDL, LDL and total cholesterol) and clinical history were obtained from medical records. All patients had their diagnosis of classical homocystinuria based on the coexistence of hypermethioninemia and/or hyperhomocysteinemia and a positive cyanide-nitroprusside test, in addition to a clinical picture compatible with classical homocystinuria.

2.2. Assessment of body composition

Body composition was assessed in patients and controls in a single appointment by means of BIA (Biodynamics, 310e, Biodynamics Inc., Seattle, USA). Weight and height were measured and used to calculate BMI. BIA was performed using the tetrapolar method and following the recommendations of the European Society for Clinical Nutrition and Metabolism (ESPEN) (Kyle et al., 2004b). Based on the results obtained, body fat percentage was classified according to the cut-off points established by Heyward and Wagner (2004).

2.3. Assessment of BMD by dual-energy X-ray absorptiometry

BMD was assessed at the lumbar spine (L1–L4) and proximal and total femur by dual-energy X-ray absorptiometry (DXA) (GE—Lunar Prodigy, USA) at HCPA Department of Radiology. BMD was expressed as T-scores.

2.4. Laboratory assessment

Fasting blood was collected in EDTA tubes on the same day as BIA and anthropometry. Plasma was separated after centrifugation at 3000 rpm for 15 min. Total homocysteine, methionine and cysteine

Table 1
Results of the biochemical assessment in plasma and BMD of patients with classical homocystinuria (n = 9).

Patient	Current age (years)	Hcy	Met	Cys	Free betaine	Free choline	Total choline	Free ethanolamine	Total ethanolamine	Free DMG	T score — BMD	
		(μmol/L)			(uM)						Spine	Femur
1a	31	321.73	593.30	124.97	12.2	4.81	297	7.28	14.4	3.89	−2.6	−1.9
1b	35	186.64	88.50	354.63	229.5	9.34	208	9.55	8.7	112	0.9	−0.9
1c	26	322.23	630.50	138.82	19.2	5.53	209	8.14	12.5	2.38	−1.4	−1.3
2	22	109.76	624.60	226.49	174	10.8	295	7.9	11.8	37.75	−0.5	−0.8
3	18	10.82	110.30	354.63	31.9	8.97	216	8.76	17.0	2.87	−1.3	NA
4	17	42.71	26.08	390.81	497.5	6.31	195	7.33	11.1	146.5	−1.4	0.2
5	21	233.86	915.03	206.93	432	12.4	184	8.81	9.9	81.5	−4.5	−2.4
6	28	48.65	69.20	349.62	585	9.75	322	7.05	16.4	53	NA	NA
7	26	66.10	29.0	370.43	49.9	5.60	218	6.29	11.7	5.2	NA	NA

Hcy: homocysteine, Met: methionine. Cys: cysteine, NA: data not available, BMD: bone mineral density, DMG: dimethylglycine.
Reference values of: Hcy: 5–15 μmol/L; Met: 5–30 μmol/L; Cys: 174–378 μmol/L (Skovby, 2003). Hcy target values for the treatment of classical homocystinuria are <20 μmol/L for pyridoxine-responsive patients and <60 μmol/L for the remaining patients (Wilcken, 2006).

plasma concentrations were measured by high performance liquid chromatography (HPLC).

Free choline, ethanolamine, betaine and dimethylglycine were simultaneously assayed in plasma after deproteinization by an LC–MS/MS method adapted from Holm et al. (2003). The analytical system consisted of an Acquity UPLC system (Waters, Milford, USA) coupled with an API 4000 QTRAP mass spectrometer (AB Sciex, Framingham, USA) with an Atlantis HILIC analytical column (2.1 × 100 mm, 3 μm) (Waters, Milford, USA). Total choline and ethanolamine were measured by the same method used for free choline and ethanolamine but after acid hydrolysis (HCl 6N, 100 °C, 90 min) of the sample, releasing bound forms.

2.5. Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 17.0 for Windows®. Variables were expressed as median and interquartile range [25th–75th percentile (IQ25–75)] due to their asymmetric distribution. Spearman's correlation coefficient was used to evaluate the association between body fat percentage, biochemical markers, and BMD T-scores in patients. Between-group differences in fat mass were evaluated using the qui-square (χ^2) test. A value of $p < 0.05$ was considered significant.

3. Results

At assessment, patients had a median homocysteine level of 147.5 μmol/L (IQ25–75 = 43–299), a median methionine level of 351.5 μmol/L (IQ25–75 = 73–628), and a median cysteine level of 287.5 μmol/L (IQ25–75 = 155–354). The results of biochemical assessment and BMD measurement are shown in Table 1.

The assessment of body composition in patients is shown in Table 2. As expected, no difference was found in gender and age between patients and controls, but patients had a lower median BMI than controls (20.5 and 23.1 kg/m^2, respectively; $p = 0.027$). Of 9 patients, 4 (44%) had low body fat percentage according to BIA. Among controls, only 2 (11%) had low fat percentage, while the remaining had normal (n = 11; 61%) or high (n = 5; 28%) fat percentage. However, no statistically significant difference in the classification of fat percentage was found between patients and controls ($p = 0.138$).

Regarding homocystinuria patients only, homocysteine levels at assessment and their median values in the last 5 years correlated negatively with current BMI ($r = -0.833$, $p = 0.004$ and $r = -0.881$, $p = 0.004$, respectively), while current cysteine levels showed a positive correlation with BMI ($r = 0.912$, $p = 0.001$) (Fig. 1). Methionine levels at assessment also showed a negative correlation with BMI ($r = -0.883$, $p = 0.002$) (Fig. 1). There was a trend for correlation between total choline levels and body fat percentage ($r = 0.439$, $p = 0.07$). The remaining metabolites (free betaine, free choline, free dimethylglycine, and free and total ethanolamine) did not correlate with body fat percentage or BMI.

LDL and total cholesterol levels were normal in all patients, but 3 had low HDL levels. Total choline levels were associated with HDL and total cholesterol levels ($r = 0.757$, $p = 0.049$; $r = 0.775$, $p = 0.041$). HDL cholesterol was also associated with total ethanolamine levels ($r = 0.847$, $p = 0.016$).

Regarding BMD, T-score at the femur showed a positive correlation with cysteine ($r = 0.741$) and a negative correlation with homocysteine at assessment ($r = -0.741$), although these correlations did not reach statistical significance ($p = 0.09$). No correlation was found between BMD T-scores and BMI, fat mass and lean mass.

4. Discussion

This is the first study to evaluate the relationship between the main homocysteine and choline metabolites and body composition in patients with classical homocystinuria. Accumulating evidence showing that levels of cysteine, homocysteine, choline and betaine influence fat mass has led us to investigate this association in classical homocystinuria (Elshorbagy et al., 2008; Teng et al., 2012; Wu et al., 2012; Zeisel, 2012).

We chose BIA to assess body composition because it is a noninvasive method, with easy availability and broad clinical application. BIA uses a low-intensity electric current that passes through the body. The method is based on the concept that tissues rich in water and electrolytes are more resistant to the flow of an electric current than adipose tissue. By determining the content of total body water, it is possible to calculate the content of lean mass and fat mass (Kyle et al., 2004a; Pietrobelli and Tatò, 2005). The standard error of the estimate of body composition by BIA ranges from 3 to 5%. The main factor related to verification errors is whole body hydration status (Houtkooper et al., 1996; Kyle et al., 2004b).

In our study, the assessment of body composition showed that a high proportion of our patients with classical homocystinuria had a low body fat percentage, but no significant difference was detected between patients and controls. There are no population-based studies evaluating Brazilians' body composition through BIA or other specific method; we only found studies evaluating BMI, which does not accurately reflect body composition. Interestingly, in our study, only two patients were underweight according to BMI, which indicates a more marked decrease in adipose tissue than in total mass. One patient was obese. This patient was taking a metabolic formula and had good metabolic control, which may have contributed to this phenotype.

In a recent study conducted in South Korea, body composition of 5 well-controlled patients with classical homocystinuria was described by means of DXA (Lim and Lee, 2013). Although the authors did not describe the values for fat mass, they reported that these values were within the normal range. The fact that no abnormalities were observed

Table 2
BMI and body composition evaluated by BIA in classical homocystinuria patients (n = 9).

Patient	Sex	Age	Weight	Height	BMI		BIA	
		(years)	(kg)	(m)	(kg/m^2)	Classification	% body fat	Classification
1a	F	31	45.6	1.62	17.4	Underweight	23.5	Low
1b	M	35	62.2	1.74	20.5	Normal range	22.2	Upper
1c	F	26	46.4	1.64	17.2	Underweight	26.7	Mid
2	F	22	61.0	1.73	20.4	Normal range	16.6	Low
3	M	18	67.6	1.78	21.3	Normal range	11.9	Mid
4	M	17	65.0	1.68	23.0	Normal range	9.1	Low
5	M	21	61.4	1.80	18.9	Normal range	14.8	Mid
6	F	28	68.7	1.68	24,3	Normal range	23.6	Low
7	M	26	97.4	1.77	31.1	Obese class I	24.9	Obesity

BMI: body mass index; BIA: electrical bioimpedance. BMI was classified according to the World Health Organization (1998) criteria and the body fat percentage according to the cutoffs established by Heyward and Wagner (2004).

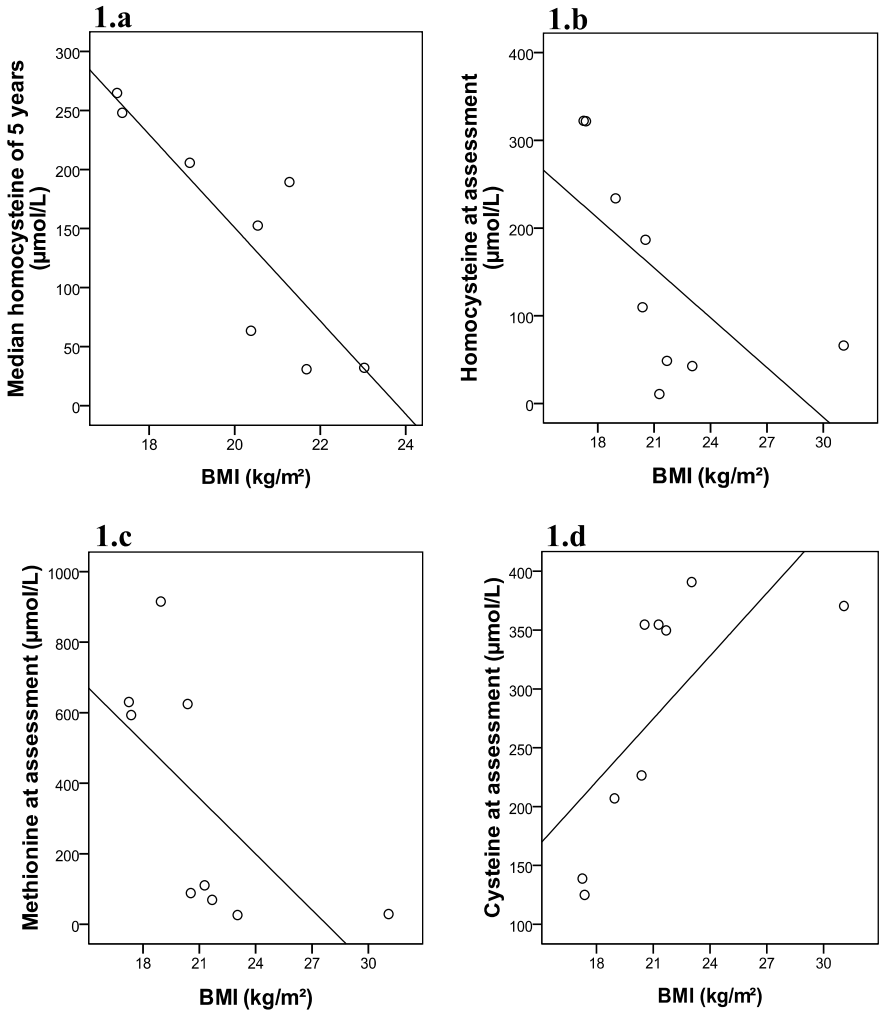

Fig. 1. Correlations between the main metabolites of homocysteine pathway and the body mass index (BMI). Solid lines represent the trend lines. 1.a) Median homocysteine values of the last 5 years correlate negatively with BMI ($r = -0.881$, $p = 0.004$). 1.b) Homocysteine levels at assessment also correlate negatively with BMI ($r = -0.833$, $p = 0.004$). 1.c) Methionine levels at assessment show a negative correlation with BMI ($r = -0.883$, $p = 0.002$). 1.d) Cysteine levels at assessment show a positive correlation with BMI ($r = 0.912$, $p = 0.001$).

in that group may be due to the following: a) as opposed to our patients, their patients had good metabolic control and a diet supplemented with cysteine, b) their sample size was even smaller than ours, and c) their study was not a controlled study.

In the present study, patients had lower BMI than controls. BMI is a measurement of total body mass. It is possible that both reductions in fat mass and in BMD observed in patients have contributed to this difference. We also found strong correlations between cysteine, methionine and homocysteine levels and BMI. A trend in the correlation between total choline levels and body fat percentage was also observed. The lack of a significant relationship between body fat and choline and its metabolites may have resulted from the small sample size, the influence of other body compartments, treatment with betaine, or limitations of the methods used. Another limitation of our study is that the biochemical variables were not evaluated in the control group. The analysis was also impaired by the lack of retrospective measures. Furthermore, it should be noted that the present sample was composed of treated patients, most of them receiving a diet supplemented with betaine. Even without good metabolic control, treatment can prevent complications and modify the natural history of the disease (Wilcken and Wilcken, 1997; Yap, 2003).

The association between cysteine and fat mass has been a recent target of studies investigating healthy individuals. In the large cohort of individuals in the Hordaland Homocysteine Study, serum concentration of cysteine showed a strong, positive and independent association with BMI and percentage of total body fat, even after adjustment for homocysteine concentration. Homocysteine, in turn, showed a negative correlation with BMI and body fat percentage. Methionine levels were not associated with body composition. The authors observed that the association between cysteine and fat mass was much stronger than that with homocysteine and concluded that this was the main determining factor for the percentage of total body fat in that population

(Elshorbagy et al., 2008). Another study performed in a large population showed that, after adjustment, methionine and homocysteine were not associated with BMI or serum lipids. Cysteine, however, was positively associated with BMI, total cholesterol and LDL-cholesterol (Elshorbagy et al., 2012).

The relation between choline levels and body fat percentage has been described in previous studies (Konstantinova et al., 2008; Teng et al., 2012). While choline is positively associated with fat mass, betaine is inversely associated. These nutrients are also related to energy expenditure and glucose, triglyceride and HDL levels (Konstantinova et al., 2008; Sparks et al., 2006; Teng et al., 2012). The positive association between HDL cholesterol and choline levels found in our study is consistent with previous observations, and may explain the low levels of HDL commonly observed in homocystinuria patients (Moat et al., 1999; Poloni et al., 2012).

In a recent study, the effect of CβS deficiency on body composition was evaluated in an animal model of classical homocystinuria. The authors observed that rats with CβS deficiency showed about 50% less fat mass than control animals, while the decrease in lean mass was small (9% in females and 14% in males). This decrease was associated with a significant decrease in cysteine levels and in the expression of hepatic *Scd-1* protein, which is a key lipogenic enzyme in the synthesis of monounsaturated fatty acids (Gupta and Kruger, 2011).

In our study, methionine levels showed a positive association with BMI. However, there is no evidence that methionine has a direct effect on body composition (Elshorbagy et al., 2011; Elshorbagy et al., 2012). A likely explanation for the correlation found in our study is that elevated methionine is merely a reflection of increased homocysteine levels, which both accumulate due to the defect. Methionine also influences the levels of cysteine, which appears to be an important mediator of body composition.

To evaluate the effect of fat mass reduction on bone health, we investigated the relationship between BMD T-scores and biochemical and body composition parameters, and found no statistical association of these parameters with bone density. However, cysteine appears to play a central role in the development of osteoporosis, since this disorder is not observed in other types of homocystinuria without cysteine deficiency (Wilcken, 2006). In the Hordaland cohort, cysteine levels were positively associated with BMD, but this association was lost when adjusted for lean mass and fat mass, demonstrating that the effect was likely mediated by body composition. Homocysteine concentration, in turn, was inversely related to bone mass in male individuals, independently of other variables (Elshorbagy et al., 2009). In our study, all patients with osteoporosis or osteopenia received a specific treatment, and this may have influenced the results obtained.

In conclusion, our results suggest that reduced fat mass is common in patients with classical homocystinuria, and that alterations in homocysteine and choline pathways may affect body mass and lipid metabolism. Furthermore, our study suggests that an effective treatment may be able to modify this phenotype. The decrease in body fat content may be one of the pathogenic mechanisms of osteoporosis in CβS deficiency; however, further studies are needed to prove this relation.

Conflict of interest

All authors confirm that they have no competing interests for declaration.

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9.7 Apêndice 7 – Artigo publicado na Ciência e Saúde Coletiva, 2015 - *Access to treatment for phenylketonuria by judicial means in Rio Grande do Sul, Brazil*.

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Título:

Access to treatment for phenylketonuria by judicial means in Rio Grande do Sul, Brazil.

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Access to treatment for phenylketonuria by judicial means in Rio Grande do Sul, Brazil

Acesso ao tratamento para fenilcetonúria por via judicial no Rio Grande do Sul, Brasil

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Abstract *Treatment of phenylketonuria (PKU) includes the use of a metabolic formula which should be provided free of charge by the Unified Health System (SUS). This retrospective, observational study sought to characterize judicial channels to obtain PKU treatment in Rio Grande do Sul (RS), Brazil. Lawsuits filed between 2001–2010 and having as beneficiaries PKU patients requesting treatment for the disease were included. Of 20 lawsuits filed, corresponding to 16.8% of RS patients with PKU, 19 were retrieved for analysis. Of these, only two sought to obtain therapies other than metabolic formula. In all the other 17 cases, prior treatment requests had been granted by the State Department of Health. Defendants included the State (n = 19), the Union (n = 1), and municipalities (n = 4). In 18/19 cases, the courts ruled in favor of the plaintiffs. Violation of the right to health and discontinuation of State-provided treatment were the main reasons for judicial recourse. Unlike other genetic diseases, patients with PKU seek legal remedy to obtain a product already covered by the national pharmaceutical assistance policy, suggesting that management failures are a driving factor for judicialization in Brazil.*

Key words *Judicialization of health, Unified Health System, Phenylketonuria*

Resumo *O tratamento da fenilcetonúria (PKU) inclui o uso de uma fórmula metabólica (FM) fornecida sem custos pelo Sistema Único de Saúde (SUS). O objetivo do estudo foi caracterizar o uso da via judicial para obter tratamento para PKU no estado do Rio Grande do Sul (RS), Brasil, através de um estudo retrospectivo e observacional, analisando ações judiciais. Foram incluídas ações judiciais arquivadas entre 2001-2010 que possuíam como beneficiários indivíduos com PKU solicitando alguma forma de tratamento para PKU. Foram localizados 20 casos, correspondendo a 16,8% dos pacientes com PKU no RS, sendo 19 obtidos para análise. Somente dois procuravam obter outras terapias que a FM. Nos outros 17 casos, uma solicitação de tratamento anterior fora concedida pela Secretaria Estadual de Saúde. Os réus incluem o Estado (n = 19), União (n = 1) e municípios (n = 4). Em 18/19 casos, os tribunais decidiram a favor dos demandantes. Violação do direito à saúde e interrupção do tratamento prestado pelo Estado foram os principais motivos para recorrer aos tribunais. Diferente de outras doenças genéticas, os pacientes com PKU buscam o meio jurídico para obter um produto já incluso na política de assistência farmacêutica nacional, sugerindo que falhas de gestão são um dos fatores desencadeantes da judicialização no país.*

Palavras-chave *Judicialização da saúde, Sistema Único de Saúde, Fenilcetonúria*

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Introduction

Brazil has a publicly funded Unified Health System (*Sistema Único de Saúde*, SUS) that aims to provide universal and free care to the Brazilian population. The SUS pharmaceutical assistance program provides for a certain group of medicines (those that are "listed") to be made available free of charge by the government. More than 500 medicines and supplies are currently listed, including those considered essential by the WHO (e.g., furosemide and prednisone), strategic medicines (e.g., thalidomide and zidovudine), and specialized medicines (such as olanzapine and infliximab), as well as supplies and medicines for hospital use[1]. Nevertheless, the use of lawsuits to obtain access to medicines is a frequent phenomenon in Brazil[2]. In the case of so-called rare diseases, such lawsuits, according to the literature, relate mainly to new technologies[3-6].

This article uses as a study model the rare genetic disorder phenylketonuria (PKU), which has an incidence of 1:12,000-16,000 in Southern Brazil[7,8]. PKU is caused by deficient activity of the hepatic enzyme phenylalanine hydroxylase (PAH). This enzyme catalyzes conversion of the amino acid phenylalanine (Phe) into tyrosine, which plays an important role in the production of the neurotransmitters dopamine and norepinephrine[9]. As a result, patients with untreated PKU have elevated plasma concentrations of Phe, which are toxic to the central nervous system and can cause mental retardation and seizures, among other manifestations[10]. PKU was the first inborn error of metabolism to be treated successfully, in a landmark study by Bickel in 1953[11]. The treatment of PKU includes lifelong administration of specific metabolic formulas, free of Phe but rich in essential amino acids, and adherence to a low-Phe diet. Both must be adjusted on an individual basis, according to the individual tolerance of each patient and target levels of Phe for each age group. In Brazil, metabolic formulas for PKU are listed in the Specialized Program for Pharmaceutical Assistance (*Componente Especializado de Assistência Farmacêutica*, CEAF) and are thus provided free of charge in accordance with the criteria established by the Brazilian Clinical Protocol and Practice Guideline for PKU (CPPG)[8]. Other treatment strategies that can be used to control Phe levels are the use of special foods (for example, foods made from low-Phe flour) and supplementation with branched long-chain amino acids (which compete with Phe, preventing its absorption and entry into the central nervous system) and BH_4, a cofactor of the PAH enzyme[12-15]; however, SUS does not cover any of these other strategies. The diagnosis of PKU is established by measuring the concentration of Phe in whole blood samples or dried blood spot testing, ideally in a neonatal screening framework, because early treatment, provided regularly and without interruption, prevents the development of mental retardation and other neurological complications[16,17]. No studies have reported on the use of legal recourse to obtain access to PKU treatment strategies in Brazil. Our hypothesis is that patients have difficulty accessing these treatments in the country due to failures in implementation of existing public policies.

Methods

This retrospective, observational study, approved by the local Research Ethics Committees, sought to characterize the use of legal recourse to obtain treatment for PKU in the State of Rio Grande do Sul (RS), Brazil**.** Located in the southernmost region of the country, RS has a population of 11,164,043, a birth rate of 11.6 per 1,000, and a per capita gross domestic product (GDP) of R$ 23,606.00[18]. The state has two public referral centers specializing in monitoring patients with PKU, at Hospital de Clínicas de Porto Alegre (HCPA) and Hospital Materno Infantil Presidente Vargas (HMIPV), both of which are public hospitals located in the capital city of Porto Alegre. At the start of data collection (2011), there were 119 known patients with PKU being monitored at these centers. Of these, 62 were treated at HCPA and 57 at HMIPV. The 2001–2010 period was chosen in an attempt to standardize data, as, in 2001, the Brazilian National Neonatal Screening Program (which includes the diagnosis of PKU) was established, and, in 2002, the first CPPG for this disease was published[19] (with an update in 2010[8]). Within this context, we identified and analyzed all lawsuits filed between 2001 and 2010 in which beneficiaries were patients with PKU who sought some form of treatment for this disease. For each lawsuit identified, variables for the period elapsed from filing of the lawsuit to the court ruling were analyzed.

Of the 119 patients followed by the two reference centers in RS, 114 patients had an indication for use of Phe-free formula (e.g., they had Phe levels at diagnosis ≥ 6 mg/dL; patients who have Phe levels at diagnosis between 2 and 6 mg/

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dL have hyperphenylalaninemia but do not require treatment), and 20 of these 114 (17.5%) had secured access to the formula by means of litigation. In RS, to request treatment by administrative means, a patient diagnosed with PKU must go to the Municipal Health Department of his or her municipality of residence with a prescription for treatment. This request undergoes technical evaluation by a healthcare professional assigned by the State and, when appropriate, the prescribed medicine is authorized for subsequent dispensation. Upon receipt of approval, the medicine is dispensed to the patient, usually once monthly; therefore, requests for treatment must be reevaluated on a quarterly basis.

The initial data about the 20 lawsuits (including lawsuit number, procedural framework, etc.) were obtained from the electronic databases of the state and federal justice systems[20,21]. After this step, the authors contacted (by email, telephone, or in person) the notaries of the counties involved and the subsections of the Brazilian Bar Association (*Ordem dos Advogados do Brasil*, OAB) at each municipality, and requested full-text copies of the legal proceedings. Using this strategy, the files of 11 cases were obtained. The remaining nine cases could not obtained due to a refusal or delay in meeting the requests for copies by county notaries (n = 3) or because the lawsuits involved children or adolescents (n = 6). An official letter was then delivered to the Internal Affairs Department of the State Court of Justice requesting access to these case files for research purposes. This strategy yielded copies of eight additional lawsuits (one request was overruled). Thus, the study sample includes 19 cases, all up to date as of the end of 2011.

Two specific forms (available upon request) were prepared by the authors for collection of data from case files. The process of creating the forms is described elsewhere by Leivas e Schwartz[22].

The first form was more extensive, including items designed to collect information about the processing of lawsuits and on the arguments advanced by the lawyers or plaintiffs in support of the filing, the defense, injunctions, blocks on government accounts, judgments, and appeals. The arguments were classified into legal, empirical (medical/research/economic), or related to administrative issues. For example, legal arguments included references to children's rights and the right to health; medical arguments included references to evidence-based medicine (scientific papers, clinical reports, and clinical guidelines related to PKU); economic arguments mentioned aspects of cost-effectiveness, scarcity of resources, lack of budgetary provisions, and impact on public budgets; and arguments related to administrative issues included references to difficulties that the State of RS faces in controlling its stocks of PKU metabolic formula and difficulties involving procurement processes.

The second form was designed for examination of questions about the plaintiffs and defendants, such as profile of the lawsuit beneficiary, source of and rationale for the prescription, type of medicine, and manner of request therefor. Prescriptions were considered adequate when they were in agreement with the CPPG for PKU published in 2002[19]. To enable proper evaluation of prescriptions, knowledge of the age and weight of the patients and their current plasma Phe levels was necessary.

Data collection was done by two legal professionals (Forms 1 and 2) and one health professional (Form 2), who subsequently met to reach a consensus instrument for each lawsuit, and whose data were entered into a database and analyzed as means and frequencies. All variables for which information was available from at least 70% of the sample were analyzed.

Results

Considering the 19 lawsuits included in the study, the average time elapsed between date of filing until 12/31/2011 was 2,117 days (approximately 6 years). The first lawsuit was filed on 01/05/2001, and the last, on 08/20/2010. The average time elapsed between filing and ruling was 648 days (approximately 1.5 years). In 17 cases, there was a report of prior administrative approval of request for PKU treatment; in the two remaining cases, this information was not available. All analyzed cases filed documentation confirming the diagnosis of PKU (prescriptions, medical reports, or statements).

Table 1 contains data on the profile of the beneficiaries of the lawsuits and the defendants, as well as on the claims made in the initial petitions. Data on the filings are shown in Table 2. According to medical reports attached to the lawsuits, the most common justification for medical prescription was the risk of developing neurological problems associated with non-treatment of PKU.

Figure 1 details the claims most frequently made by the plaintiffs in the initial application.

Table 1. Access to Phenylketonuria treatment by judicial means in Rio Grande do Sul, Brazil: characterization of the sample (n = 19 lawsuits).

Variables	N
Patient variables	
Gender (M:F)	12:7
Age	
0-2 years	4
3-11 years	12
12-18 years	1
≥ 19 years	1
N/A	1
Lawsuit variables	
Defendant	
State of Rio Grande do Sul	14
State of Rio Grande do Sul + Municipality	4
Union + State of Rio Grande do Sul	1
Author/plaintiff	
Public defender	8 (42.1)
Public prosecutor	7 (36.8)
Private attorney	4 (21.1)
Request for phenylketonuria formula	
Yes	18 (94.7)
No*	1 (5.3)
Request for special foods and/or drugs	
Yes**	2 (10.5)
No	17 (89.5)

* Request referred solely to special foods for PKU. ** One patient requested metabolic formula and anticonvulsant medication. The other patient requested infant formula for the first year of life and PKU formula. N/A = not available.

Table 2. Access to Phenylketonuria treatment by judicial means in Rio Grande do Sul, Brazil: summary of clinical information available in lawsuits (n = 19 cases).

Variables	N
Medical prescription attached to the lawsuit	18/19
Medical report attached to the lawsuit	14/19
Treatment prescribed	
Phenylketonuria formula	17/18
Special foods*	1/18
Others**	4/18
Patient weight reported in lawsuit	
Yes	3/19
No	16/19
Adequate medical prescription	
Yes	3/18
Could not be determined	15/18

* Low-Phe pasta and flour. ** In three lawsuits, the beneficiaries had been prescribed infant formula for the first year of life as well as PKU formula; in one lawsuit, the beneficiary had been prescribed anticonvulsant medication and PKU formula.

Table 3 shows that all requests for advance relief were granted. Regarding the manner in which orders were fulfilled, in all cases, the judge determined that the defendant acquire the necessary supplies for treatment of the applicants; in one lawsuit, the defendant was given the option of paying the cost of treatment to the applicant in cash. Table 4 provides detailed information on the grounds for ruling in each lawsuit.

Overall, 18 of the 19 lawsuits were contested: 15 by the state government alone, two by the state and municipal governments, and one by the federal and state governments.

Regarding the arguments used by the defense, in 12 of 18 cases, the defendant advanced arguments related to medical aspects. The most common medically based arguments were: discussions on replacement of formula provided at concentrations different from those approved at the administrative level (n = 6/12); need for submission of medical reports stating that the patient requires treatment (n = 1/12); one claim that the treatment was not included in the SUS list of supplies to be provided free of charge (claim filed prior to the 2002 CPPG) (n = 1/12); and lack of up-to-date medical reports (n = 8/12). In five cases (n = 5/18), the defendants argued economic aspects, such as lack of budgetary provision for the acquisition of the requested treatment, as well as also scarce resources (n = 1/5); impact on the public budget (n = 2/5); impact on the public budget, scarce resources, and the principle of the proviso of possibility (n = 1/5); and resources insufficient to comply with the decision (n = 1/5). In three of the 18 cases, defendants cited administrative problems related to difficulties in inventory control and delays in procurement processes as reasons for the non-availability of the formula. Regarding the legal and constitutional aspects mentioned in the disputes, the most frequent discussions and complaints concerned the fundamental right to health (n = 6/10), the principle of reserve for contingencies (n = 4/10), and the illegitimacy of the lawsuit being filed by the public prosecutor (n = 4/10). These were followed by violation of the principle of equality (n = 3/10), administrative discretion/principle of separation (n = 3/10), violation of the principle of human dignity (n = 1/10), and others (n = 1/10).

In 13 cases (n = 13/19), bank accounts of the State of RS were frozen. The sum of these assets was R$ 228,112.39 (range = R$ 1,831.32 to R$ 52,313.38). On average, two account freezes were ordered during each lawsuit; seven cases led to two freezes each, and one lawsuit involved six account freezes.

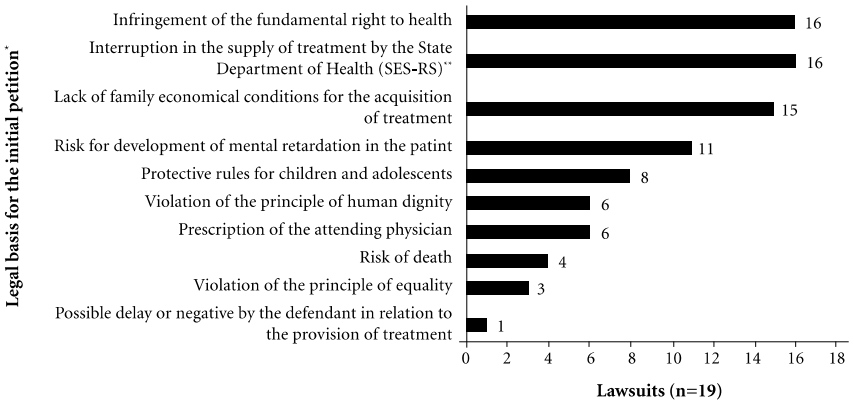

Figure 1. Access to PKU treatment by judicial means in Rio Grande do Sul, Brazil: legal basis for the initial petition.

* Medical and research-related, economic, legal, and constitutional issues found most frequently in the arguments during analysis of initial petitions. ** State Department of Health of Rio Grande do Sul, Brazil (Secretaria Estadual de Saúde, SES-RS).

Discussion

Despite the large number of policies and public lawsuits involving existing pharmaceutical assistance programs in Brazil, the practice of recourse to the courts as a means of securing supply of medicines through the SUS has provoked discussions regarding implementation of the right to health. This is a markedly Brazilian phenomenon, whereby lawsuits requesting access to medicines via the SUS are brought before the federal and state courts as a faster and more effective alternative both to obtain access to new health technologies and to ensure access to medicines that should be supplied free of charge by the SUS[23-25].

According to previous research conducted by our group, using the genetic diseases mucopolysaccharidoses and Fabry disease as models, the main cause for judicialization in the field of rare diseases in Brazil is the search for access to technologies not yet incorporated into the SUS[4-6].

In the cases studied herein, most recipients previously requested and were granted treatment by extrajudicial (administrative) means, in compliance with the criteria established in the Clinical Protocol and Practice Guideline for PKU[8]. A priori, resorting to the judicial system to obtain access to the PKU formula seems contradictory, but the data we obtained suggest that interruptions in the supply of the formula, violations of the fundamental right to health, and the economic conditions of the families of PKU patients, limiting their ability to pay for treatment out of pocket, are the key factors that led these patients to seek legal remedies to ensure access to PKU treatment. It bears stressing that, during the study period, there was no evidence of shortages of metabolic formula due to insufficient manufacturing and/or distribution by the pharmaceutical industry.

Analysis of the defendants in the analyzed lawsuits revealed that the State of RS was a defendant in all lawsuits assessed. This was already expected by the authors, as the plaintiffs had previously been granted access to treatment through the SUS via administrative means.

The limited information contained in the lawsuits precluded a more in-depth characterization of the profile of the plaintiffs. Unfortunately, data on income, occupation, and education were available only in few cases. Other data suggest that the beneficiaries of the studied cases were mostly children. The predominance of male subjects is likely due to chance, as PKU is an autosomal recessive disease and a gender difference in prevalence is not expected.

Recently, empirical studies about the judicialization of health have sought to advance the argument that this phenomenon is being taken up

Table 3. Access to Phenylketonuria treatment by judicial means in Rio Grande do Sul, Brazil: data on advance relief (n = 19).

Variables	N
Manner of compliance with court decision	
Defendant ordered to provide medicines	18
Defendant ordered to provide medicine and/or financial resources for the patient to purchase treatment	1
Periodicity of provision of the formula as set by the court	
Once monthly (enough for one month)	18
Twice yearly (enough for one semester)	1
Arguments used in ruling*	
Related to medical or research aspects	
Availability of medical reports	12
Availability of evidence**	7
Risk of death/serious harm to life associated with non-treatment	1
Risk of developing mental retardation associated with non-treatment	1
Related to legal and constitutional aspects	
Violation of the right to health	12
Violation of children's rights	6
Violation of the principle of human dignity	1
Violation of the principle of priority	1
Related to economic aspects***	0
Related to administrative issues****	0

* More than one argument may have been cited in the ruling. ** References to scientific articles, medical reports, and clinical protocols related to phenylketonuria were considered as evidence. *** References to cost-effectiveness of the requested treatment, scarcity of resources for the acquisition of supplies, lack of budgetary provisions for the procurement of supplies, and impact on the public budget were considered economic aspects. **** References to difficulties that the State of RS faces in controlling its inventory of PKU metabolic formula and difficulties with procurement processes were considered administrative aspects.

Table 4. Access to Phenylketonuria treatment by judicial means in Rio Grande do Sul, Brazil: data on court rulings (n = 18).

Variables	N
Time elapsed between lawsuit filing and ruling	
< 6 months	5
6 months to 1 year	3
1 to 2 years	6
> 2 years	4
Ruling	
Upheld*	17
Partially upheld**	1
Denied*	1
Arguments used in the ruling‡	
Related to medical and research aspects	
Availability of medical reports	13
Availability of evidence§	9
Availability of treatment alternatives	1
Related to legal and constitutional aspects	
Violation of the right to health	15
Violation of children's rights	6
Violation of the principle of human dignity	4
Reserve for contingencies/principle of proportionality	1
Administrative discretion/separation of powers	1
Violation of the principle of equality	1
Illegitimacy	1
Others‖	3
Related to administrative issues¶	0

* In one lawsuit, the ruling was upheld against the State of RS and dismissed against the County, which was cleared of the responsibility to provide PKU formula. ** In this case, the ruling determined that three cans of PKU formula be supplied, whereas the lawsuit requested four cans. ‡ More than one argument may have been cited in the ruling. § References to scientific articles, medical reports, and clinical protocols related to phenylketonuria were considered as evidence. ‖ One lawsuit discussed the legitimacy of prosecutors to plead individual rights; the other addressed the absence of a challenge to the plaintiff's claim by the State of RS; the third discussed the right to life. ¶ References to difficulties that the State of RS faces in controlling its inventory of PKU metabolic formula and difficulties with procurement processes were considered administrative aspects.

by economic elites, which goes against the constitutional principles of the SUS, such as equity. The upholding of this complex theory depends on a specific methodology being able to obtain evidence contributing to the characterization of the problem; however, said evidence remains insufficient[26]. Our study found several indications that contradict this thesis of the elites. In our sample, lawsuits were filed mainly by public defenders and by the Office of the Public Prosecutor; few lawsuits were filed by private attorneys. Data such as family income and occupation were not available in statistically significant quantities to enable proper characterization of the plaintiffs. All prescriptions originated from public university hospitals, which is not a good indicator of social class, as these facilities house referral services in patient care, research, and technology in the field of genetics, as well as providing equitable and universal access through the SUS. Hence, we cannot say whether the judicialization of PKU treatment in RS is being driven by economic elites or whether it is correlated with socioeconomic status.

A request for PKU formula was made in almost all lawsuits; in one case, there was no re-

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quest for the formula, but rather for special foods. The attempt to obtain special foods for PKU management via judicial means may have been prompted by the fact that patients or their families do not have sufficient financial resources or access to these foods, which, in the case of PKU, are imported and expensive. However, cultural issues and the difficulty of families to cope with a chronic disease that may not be socially acceptable could also be associated with poor adherence to a diet based on these special foods, prioritizing the use of judicial means to obtain the metabolic formula. In general, patients seek legal recourse to obtain medicines. However, it bears stressing that, for the treatment of PKU, access to a diet that enables control of plasma Phe levels is as important as access to the PKU formula, and combined administration of these treatment strategies enables the PKU patient to achieve acceptable Phe concentrations.

Regarding prescriptions, most beneficiaries of the lawsuits had already had prior administrative applications for the metabolic formula accepted by the State Department of Health (SES-RS), which suggests that these patients had a proper prescription for the formula. However, due to the study methodology, analysis of prescribing data for PKU metabolic formula could not determine whether the amount of formula requested or the prescription for formula were appropriate. Information on plasma Phe levels (both at the time of diagnosis and current) and patient weight, which are needed to calculate the proper dose of metabolic formula, were usually missing. Data on which type of formula was prescribed were often missing as well.

The role of the Public Prosecutor as a procedural proxy of the beneficiary was often challenged by the defendants, generating extensive discussions about the legitimacy of the prosecution in litigating for individual interests through civil action. The ability of the Public Prosecutor to take upon himself the role of guarantor of unavailable individual interests related to health issues, as well as protection of the Statute for Children and Adolescents, is settled in jurisprudence of the Superior Court of Justice (STJ)[27].

As expected, the most frequent "economic" arguments of the defense were related to the lack of budgetary provisions for the implementation of judicial decisions, the impact of these decisions on the public budget, and the violation of constitutional principles such as the proviso of possibility. The right to health was also discussed in the arguments of the defense, but only regarding the recognition by the State of its responsibility to provide adequate pharmaceutical services to citizens. In some lawsuits, the State of RS admitted its own problems and difficulties in managing inventory of PKU formula due to delays in procurement processes. These facts strongly indicate a possible reason for the interruptions in supply of the PKU formula by the State of RS, which motivated these adversely affected patients to seek access to the formula by judicial means.

Another fact that deserves attention concerns medical and research-related arguments presented by the State in legal defenses. In defense strategies, the State sought to discuss applications for replacement of treatment with PKU formula at concentrations different from those approved by the State of RS at the administrative level, requesting that the patient present evidence proving the need for treatment through medical reports; requesting updated reports; and claiming that the formula was not included in any list of medicines required to be provided by the State. Importantly, the beneficiaries of the lawsuits had already had administrative requests for treatment previously approved; as stated earlier, treatment of PKU is lifelong and consists of the administration of a specific metabolic formula, which, in Brazil, is listed in the CEAF formulary and is distributed free of charge by the State of RS in accordance with the CPPG criteria. The State demonstrated a lack of technical knowledge about PKU and about its own policies, perhaps because of miscommunication between the various sectors involved.

Analysis of advance relief is of utmost importance for understanding the phenomenon of judicialization of health in Brazil. In the cases examined, all beneficiaries had their applications for advance relief granted. As a general rule, the granting of injunctions was based on the facts alleged in the medical reports and the precepts of the right to health. No injunction mentioned the existence of a CPPG for PKU. This corroborates the findings of other studies on judicialization of health, in which judges were found to rule in favor of supplying medicines without compliance with current SUS policies for pharmaceutical care[28].

It is also important to realize that injunctive relief requires that the State of RS provide the PKU formula as requested in the initial petitions made by the beneficiaries, i.e., in the quantities and type of formula prescribed and requested. Failure to observe these decisions led to garnishment of State accounts. We found that, within the

time frame of the study, most requests for freezing of government accounts were granted. On one hand, decisions to garnish in government accounts assure immediate compliance of judicial orders for applicants, but these same decisions may have a negative impact on the performance of the State, as they prevent the State from exercising its negotiating flexibility or bargaining power through procurement processes that meet budgetary provisions. This may expose the health care system to a rise in costs corresponding to emergency purchases that bypass standard procurement processes[5,29].

At the end of the study period, almost all lawsuits had been upheld at final decision, confirming access to the formula and ensuring the individual right to health of each applicant as previously signaled by the granting of injunctions. The underlying aspects of the final rulings were similar to those used as grounds for injunctive relief, i.e., focusing on medical aspects and on the right to health.

Our data were reported to the RS State Department of Health, and some initiatives are underway in an attempt to overturn judicialization of PKU treatment, including the possibility of direct dispensation of the formula at referral centers to newly diagnosed patients. However, we believe it is essential that the State develop better inventory control strategies so as to avoid shortages of medication if a tender is impugned, among other possibilities.

Conclusions

Our data reveal that discontinuation of supply of the PKU formula is the main cause of judicialization of PKU treatment in RS, suggesting breakdowns in the management of pharmaceutical services in the State, and that the right to health is the main legal foundation for legal decisions favorable to patients/plaintiffs. Therefore, requests for access to health technologies not yet incorporated into the SUS are not always the leading cause of judicialization of rare disease treatment in Brazil.

Collaborations

LM Trevisan conceived the study, collected, analyzed and interpreted the data and wrote the paper; T Nalin and T Tonon conceived the study, collected the data and wrote the paper; LM Veiga collected and analyzed the data and wrote the paper; P Vargas interpreted the data and wrote the paper; BC Krug collected, analyzed and interpreted the data and wrote the paper; PGC Leivas conceived the study, collected, analyzed and interpreted the data and wrote the paper; IVD Schwartz conceived the study, analyzed and interpreted the data and wrote the paper. Approval of final version for publication: all authors approved the final version for publication.

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9.8 Apêndice 8 – Artigo publicado no Journal of Inborn Errors of Metabolism & Screening, 2015 - *Adherence to Treatment of Phenylketonuria: A Study in Southern Brazilian Patients.*

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Adherence to Treatment of Phenylketonuria: A Study in Southern Brazilian Patients

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Abstract

Introduction: Phenylketonuria (PKU) is caused by the deficient activity of phenylalanine hydroxylase. **Aim:** To identify the factors associated with treatment adherence among patients with PKU seen at a southern Brazil reference center. **Methodology:** A cross-sectional, outpatient-based study including 56 patients with PKU (median age, 12 years) for whom a Phe-restrict diet plus specific metabolic formula have been prescribed. Patients were considered adherent or nonadherent depending on the median phenylalanine concentration for the 12 months prior to study and target levels of phenylalanine for each age range (<13 years = ≤360 μmol/L; ≥13 years = ≤900 μmol/L). Data were collected through a review of patient's medical records and a set of interviews with patients and their relatives. **Results:** Eighteen patients (32.1%; ≥13 years, 11) were classified as treatment adherent. Among all factors analyzed, only mental retardation, living with parents, and level of maternal education were associated with adherence to treatment. **Conclusion:** Our findings reinforce the importance of the family as promoting factor for treatment adherence.

Keywords

phenylketonuria, adherence to treatment, inborn errors of metabolism

Introduction

Phenylketonuria (PKU; OMIM 261600) is an inborn error of metabolism caused by the deficient activity of the enzyme phenylalanine hydroxylase (EC1.14.16.1), which catalyzes conversion of the amino acid phenylalanine (Phe) into tyrosine. Consequently, patients develop elevated serum concentration of Phe, which is toxic to the central nervous system. Currently, most cases of PKU are diagnosed through neonatal screening programs.[1] Screening of PKU usually consists of measurement of Phe concentration in blood spotted on filter paper, preferably between the third and the seventh day of life.[2,3]

Management of PKU consists of a Phe-restricted diet—including the use of a specific metabolic formula (MF) containing amino acids and micronutrients, but free of Phe—adjusted individually according to target Phe levels for each age range,[4] which should usually be followed throughout the patient's life.[5] With early diagnosis and treatment, patients are practically asymptomatic.[1] In untreated patients, the most common clinical manifestation is mental retardation; hyperactivity, autism, pyramidal tract signs, tremor, seizures, and microcephaly are also common.[1,6]

Adherence to PKU dietary treatment is usually estimated by comparing the patient's serum Phe concentration with target levels recommended in the literature.[4] In many chronic

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conditions, treatment adherence decreases over time, and the finding that Phe concentration increases with age shows that PKU is no exception.[7] This may be due to poor understanding of dietary restrictions, decreased motivation to comply with treatment, and inability to cope with the disease. Adherence to PKU treatment may also be influenced by factors such as education, religion, socioeconomic circumstances—such as access to specific products for management of PKU, family income, and housing conditions—and also by the patient's relationship with and trust in his or her team of health care providers.[7,8] Patients with separated or divorced parents, for instance, may have higher Phe levels.[9] This study sought to identify the factors associated with treatment adherence in patients with PKU seen at a reference center in Southern Brazil.

Methods

The present study employed a cross-sectional, outpatient-based design and was approved by the local Research Ethics Committee. After informed consent forms had been signed, all necessary information was obtained through a review of patients' medical records and interviews with patients and their relatives. Data were collected over a 12-month period.

The study sample included patients with a diagnosis of PKU followed up at Medical Genetics Service-Hospital de Clínicas de Porto Alegre/Brazil outpatient PKU clinic. The PKU clinic, which has been operating since 1991, has a multidisciplinary team (consisting of physicians, a dietitian, a social worker, a psychologist, and several undergraduate and graduate students of health-related programs) and currently includes 65 patients—approximately half of the patients with PKU currently being treated for the condition in the state of Rio Grande do Sul, Brazil. Patients in the first year of life are seen at the clinic at least every month, those aged 1 to 18 years have appointments at least every 3 months, and adult patients are seen at least twice a year. Of the 65 patients, 4 presented off-diet values of Phe <360 µL/L and did not need any dietary intervention; therefore, they were not included in the study. As 5 patients declined to be included, 56 patients composed the total sample.

Classification of PKU phenotype was based on serum concentration of Phe at diagnosis (before treatment); patients with Phe levels ≥1200 µmol/L were classified as having classical PKU, and those with Phe levels between 360 and 1200 µmol/L were classified as having mild PKU. Patients for whom data on Phe levels at diagnosis were unavailable were classified as having "PKU of undefined type."[10]

Patients were classified as treatment adherent or nonadherent according to the comparison between the median Phe levels over the past 12 months of follow-up and the target Phe concentration adopted in the clinic[4] as well as to the number of Phe measurements performed in the period. Patients younger than 18 years who underwent fewer than 3 Phe measurements over the 12-month follow-up period were considered nonadherent to treatment, regardless of median Phe level; adult patients required at least 2 yearly Phe measurements to be considered adherent. Patients under the age of 13 were required to have serum Phe levels of ≤360 µmol/L (target value for this age range), and those 13 or older were required to have a median Phe level of ≤900 µmol/L (target value for this age range). The presence/absence of mental retardation was acknowledged according to the assistant physician's opinion as noted in each patient's records; no IQ tests were available.

Questionnaires

Three questionnaires (I, II, and III) devised by the PKU clinic multidisciplinary team, mostly comprising multiple-choice questions, were administered to patients and/or their relatives. These questionnaires are available as a supplementary material.

Questionnaire I was administered only to patients aged 12 or older (or, when the patient's cognitive abilities were insufficient to allow completion, to his or her parents) and contained questions on family history and socioeconomic conditions, educational level, intrafamilial variability, and access to the MF.

Questionnaires II and III comprised 12 questions each meant to assess the understanding of PKU (8 questions), the description of the PKU diet (2 questions), and main challenges to treatment adherence (2 questions). Questionnaire II should be answered by the main caretaker of patients aged younger than 12 years or of patients presenting neurological compromise, while questionnaire III should be answered by patients aged 12 or older when cognitive ability allowed. Knowledge about PKU was classified according to the answers provided to the 8 questions aiming at evaluating it (1 point was given to each correct answer): satisfactory (7-8 points), fair (4-6 points), and unsatisfactory (<4 points).

Statistical Analysis

Statistical analysis was carried out using SPSS for Windows, version 18.0 (SPSS Inc, Chicago, Illinois). Continuous variables were expressed as mean ± standard deviation or median and interquartile range. The chi-square and Fisher exact tests were used to test for association between categorical variables. Adjustment for confounding factors was accomplished with the use of Poisson regression to test for association between variables. The Mann-Whitney U test was used for comparison of independent samples. Correlation between parameters was assessed with Spearman correlation coefficient. The level of significance was $P < .05$.

Results

Of the 56 patients included (Table 1), 31 were aged 0 to 13 years, 13 were aged 13 to 18 years, and 12 were older than 18 years. Median age at inclusion was 12 years (interquartile range, 9-17 years; range, 0-56 years), and at diagnosis, 2 months (interquartile range, 1-19.2 months; range, 0.6-137 months). Forty-nine patients had been born to unrelated families, and consanguinity was present in 10 (20.4%) of 49 families. As for

Table 1. Demographic and Social Characteristics of Patients With Phenylketonuria According to Their Treatment Adherence.[a]

Characteristics	Adherent (n = 18), n (%)	Nonadherent (n = 38), n (%)	*P*
Age at inclusion (n = 56)			.088[b]
≥13 years	11 (44.0)	14 (56.0)	
<13 years	7 (22.6)	24 (77.4)	
Gender (n = 56)			.579[b]
Male	9 (29.0)	22 (71.0)	
Female	9 (36.0)	16 (64.0)	
Origin (n = 56)			1.000[c]
Rural area	2 (33.3)	4 (66.7)	
Urban area	16 (32.0)	34 (68.0)	
Housing (n = 56)			.759[c]
Owned	16 (34.8)	30 (65.2)	
Rented	1 (20.0)	4 (80.0)	
Shared	1 (20.0)	4 (80.0)	
Maternal educational achievement (in years of schooling; n = 55)[d]			.899[c]
4 years or less	5 (33.3)	10 (66.7)	
5 to 8 years	5 (29.4)	12 (70.6)	
9 to 12 years	6 (31.6)	13 (68.4)	
>12 years	2 (50.0)	2 (50.0)	
Paternal educational achievement (in years of schooling; n = 52)[e]			.695[c]
4 years or less	5 (26.3)	14 (73.7)	
5-8 years	3 (27.3)	8 (72.7)	
9-12 years	8 (44.4)	10 (55.6)	
>12 years	1 (25.0)	3 (75.0)	
Patients' educational achievement (in years of schooling; n = 56)			.667[c]
0-4 years	6 (26.1)	17 (73.9)	
5-8 years	5 (31.3)	11 (68.8)	
9-12 years	3 (50.0)	3 (50.0)	
Special education	4 (36.4)	7 (63.6)	
Economic class[f] (*n* = 50)			.500[c]
Class B	9 (37.5)	15 (62.5)	
Class C	6 (25.0)	18 (75.0)	
Class D	1 (50.0)	1 (50.0)	

[a]n = 56.
[b]Chi-square test.
[c]Fisher exact test.
[d]One patient did not answer the question on maternal educational achievement.
[e]Four patients did not answer the question on the paternal educational achievement.
[f]Economic class was established according to the Brazilian Economic Classification Criterion developed by the Brazilian Association of Research Companies (Associação Nacional de Empresas de Pesquisa), which uses acquisition of consumer goods (as a surrogate for family purchasing power) and educational achievement of the head of household. No families could be classified into class A. Six families were not classified, as they did not answer the questions meant to assess economic status.

PKU phenotype, 32 patients (58.9%) were classified as having classical PKU and 17 (28.6%) as having mild PKU; disease subtype was undefined in the 7 remaining patients.

Regarding adherence to treatment, 18 (32.1%) patients were considered adherent (median current age, 13 years; interquartile range, 7.7-17), with a median age at diagnosis of 3.0 months (interquartile range, 1.54-13.25; only 4 [22.2%] patients were diagnosed through newborn screening). Thirty-eight patients were considered nonadherent (median current age, 11.5 years; interquartile range, 9-18), with a median age at diagnosis of 2.0 months (interquartile range, 1.38-2; only 8 [21%] patients were diagnosed through newborn screening; Table 1).

There was no difference in frequency of clinical manifestations between the adherent and the nonadherent groups (data not shown). Mental retardation was the most common clinical manifestation found (Table 2).

The association between clinical manifestations of PKU and age at diagnosis, and with median serum Phe concentration in the first 12 months after diagnosis, showed that the later the diagnosis, the higher the risk of mental retardation and that the higher the concentration of Phe, the higher the risk of mental retardation, hyperactivity, and seizures (Table 2). The same analysis was performed on median Phe concentration for the 12 months preceding the inclusion in the study; the only significant association was with mental retardation (risk ratio [RR] 1.19, 95% confidence interval [CI] 1.03-1.20, p = 0.004).

Demographic and Social Characteristics

A summary of demographic and social characteristics of the study sample according to treatment adherence is shown in Table 1. Patients whose mothers had a lower educational level—namely, less than 4 years of formal education—were at a greater risk of treatment nonadherence. Adjusted analysis of the association between sociodemographic characteristics and treatment adherence showed an association between educational achievement and adherence; patients whose mothers had 4 years of formal education or less were at greater risk of nonadherence (RR 1.59, 95% CI 1.01-2.51, $P = .044$).

Origin

Mean distance between patient residence and the PKU clinic was 329.57 ± 95.45 km. Among nonadherent patients living in the countryside (n = 22), mean distance was 118.13 ± 193.08 km versus 207.06 ± 208.60 km in the treatment-adherent group living in the countryside (n = 11; $P = .792$). Also, the distance between the patient's town of origin and the PKU clinic did not correlate with median Phe concentration in the 12 months prior to study inclusion (adherent patients, n = 18, $r = -.12$, $P = .629$; nonadherent patients, n = 34, $r = .06$, $P = .72$).

Living With Relatives

Most patients (n = 38 of 55, 69.1%) lived with both parents; of these, 22 (57.9%) did not adhere to treatment. Among patients who lived with only one parent ($n = 17/55$, 30.9%), 15 (27.3%) were nonadherent ($P = .027$). Adjusted analysis of this variable revealed that living with both parents is a protective factor for treatment adherence (RRs 0.59, 95% CI 0.39-0.80, $P = .001$).

Table 2. Clinical Manifestations of Phenylketonuria and Their Association With Age at Diagnosis and With the Phenylalanine Concentration in the First 12 Months of Follow-Up.[a]

	Median Phe in the First 12 Months			Age at Diagnosis		
	RR	CI (95%)	P^b	RR	CI (95%)	P^b
Mental retardation (n = 21)	1.066	1.024-1.111	.002	1.005	1.001-1.009	.006
Learning disability (n = 18)	1.063	0.978-1.155	.149	0.990	0.979-1.002	.103
Hyperactivity (n = 9)	1.174	1.070-1.289	.001	0.989	0.977-1.101	.079
Microcephaly (n = 8)	0.870	0.727-1.042	.130	1.012	0.995-1.028	.169
Seizures (n = 8)	1.151	1.017-1.303	.026	0.988	0.974-1.002	.087
Low weight (n = 7)	0.885	0.744-1.053	.169	1.014	0.999-1.029	.066
Short stature (n = 6)	0.935	0.760-1.151	.526	1.013	0.999-1.027	.080
Aggressiveness (n = 6)	1.082	0.978-1.196	.125	1.004	0.992-1.016	.509
Attention deficit (n = 4)	1.240	1.009-1.523	.041	0.982	0.957-1.008	.170

Abbreviations: RR, relative risk; CI, confidence interval.
[a]n = 48.
[b]Poisson regression.

Table 3. A Clinical Summary of Patients With Phenylketonuria Included in this Study Who Are Siblings.[a]

Family/Patient	Current Age, yr	Gender	Adherence to Treatment	Early/Late Diagnosis	Knowledge of PKU[b]
A1	10	Male	No	Early	Fair
A2	14	Male	Yes	Early	Fair
B1	09	Male	No	Early	Fair
B2	14	Female	No	Late	Fair
B3	17	Male	Yes	Late	Unsatisfactory
C1	17	Female	Yes	Late	Unsatisfactory
C2	21	Male	No	Late	Fair
D1	8	Male	No	Late	Fair
D2	14	Male	Yes	Late	Satisfactory

Abbreviation: PKU, phenylketonuria.
[a]n = 9 patients from 4 families.
[b]According to the questionnaire devised for this research: satisfactory (7-8 points), fair (4-6 points), and unsatisfactory (<4 points).

This variable was not assessed in one patient who lived alone. There was no significant difference in treatment adherence between patients who have siblings and those who did not ($P = .394$).

Intrafamilial Variability

Of the siblings included in the study (n = 16, from 4 families), 9 diverged in compliance (from families A, B, C, and D) and are described in Table 3.

Knowledge of PKU

The questions related to knowledge of PKU were answered by 37 caretakers (questionnaire II) and by 19 patients (questionnaire III). Since we had one questionnaire per patient, we analyzed both questionnaires together. In the treatment-adherent group (n = 18 of 56), 9 (50%) questionnaires showed that the respondent had satisfactory knowledge of PKU, 6 (33.3%) showed that the respondent had fair knowledge of the condition, and 3 (16.7%) showed that the respondent had an unsatisfactory understanding of the disease. In the nonadherent group (n = 38 of 56), these numbers were 14 (41.8%), 20 (54.1%), and 3 (8.1%), respectively. There was no significant difference in knowledge of PKU between the adherent and the nonadherent groups ($P = .297$) and no association between median Phe concentration in the last 12 months and knowledge of the condition ($r = .181$, $P = .311$).

Diet

Forty-seven patients claimed to always use MF as directed by the clinical dietitian. Three patients used it as recommended only occasionally and one never did; 6 patients failed to answer the question. In the nonadherent group, 31 patients claimed to always follow recommendations regarding use of MF versus 16 patients in the adherent group ($P = .686$). Patients were then asked about MF supply in the 12 months prior to interview. Of the 54 patients or family members who answered the question, only 4 (7.4%) claimed to have received it on a regular basis during the period. One patient did not use MF at all and one failed to answer the question. Analysis of MF supply and median Phe levels over the 12 months prior to study showed that, in the group of patients who received the formula

Table 4. Reported Frequency of Challenges Associated With Treatment of Phenylketonuria.

	Adherent (n = 17/18), %	Nonadherent (n = 36/38), %	*P*
Dietary restrictions	12 (70.5)	24 (66.6)	.775[a]
Palatability of MF[b]	10 (58.8)	23 (63.8)	.723[a]
MF[b] supply issues	10 (58.8)	23 (63.8)	.723[a]
High cost of MF[b]	9 (52.9)	18 (50.0)	.842[a]
Distance to the PKU clinic	8 (47.1)	18 (50.0)	.842[a]
Cost of transportation to the PKU clinic	5 (29.4)	11 (30.5)	.933[a]
Impaired social interaction with non-PKU patients	4 (23.5)	8 (22.2)	1.000[c]
High cost of other PKU-diet foods	2 (11.8)	1 (2.8)	.238[a]

Abbreviations: PKU, phenylketonuria; MF, metabolic formula.
[a]Chi-square test.
[b]MF is funded and supplied by each state's Department of Health.
[c]Fisher exact test.

regularly (n = 4), median Phe level was 581.18 µmol/L (interquartile range, 363.2-684.1) versus 659.8 µmol/L (interquartile range, 169.5-1174.4) in patients who reported not receiving it on a regular basis ($P = .450$).

Treatment Challenges

The questions related to treatment challenges were answered by 37 caretakers (questionnaire II) and by 16 patients (questionnaire III). Since we had one questionnaire per patient, we analyzed both questionnaires together. Table 4 shows the reported frequency of challenges or difficulties associated with PKU treatment.

Only one caretaker claimed to have no difficulty in adhering to treatment. When asked whether PKU was in any way a nuisance in their lives, 8 (50.0%) of the 16 patients who answered questionnaire III replied in the affirmative. Of these 8, 3 claimed that eating out was a nuisance, one reported out-of-town follow-up as an issue, 3 complained of dietary restrictions, and one reported forgetfulness, which interfered with treatment adherence.

Discussion

In Brazil, PKU was the first condition to be included in a neonatal screening program in the 1970s; however, the first specific program establishing a nationwide screening network—the National Neonatal Screening Program (Programa Nacional de Triagem Neonatal)—was only established in 2001.[2] Phenylalanine-free MF is made available as a PKU treatment through the Brazilian Ministry of Health's Specialized Program for Pharmaceutical Assistance (Componente Especializado da Assistência Farmacêutica) and is thus state funded.[11]

A high level of treatment nonadherence was found in the present study. We used number of Phe measurements and median blood Phe concentration in the 12 months prior to study inclusion when compared to target levels as parameters for adherence assessment. These parameters are recommended as a direct and reliable measurement of adherence.[12] Adherence to PKU treatment is difficult to quantify and has been the subject of few studies; there is no single measurement to assess it, and other parameters are used as well, such as dietary recall, MF consumption, and self-reported compliance in interviews with health professionals.[13,14]

Both groups (adherent and not adherent) did not differ regarding the proportion of early (eg, through newborn screening) and late diagnosed patients and the presence of mental retardation. However, as expected, we found out a higher risk of mental retardation among individuals presenting higher concentrations of Phe after the first 12 months of follow-up and later ages at diagnosis as well, findings which reinforce the importance of neonatal screening for this disease. As higher Phe concentrations in the12 months before the inclusion in the study were also associated with the occurrence of mental retardation, our findings suggest mental retardation indeed negatively affects adherence to treatment.

Regarding patient origin and distance to the PKU clinic, most patients lived in the countryside, and although distance and transportation cost were reported as challenges associated with PKU treatment, there was no association between adherence and distance. This finding may be due to the fact that free transportation to the PKU clinic is made available to patients who live in other municipalities by their municipal governments and because the MF is provided to patients at their municipalities of origin. A study conducted at Colorado University also showed that geographic access to care does not impact adherence to PKU treatment and control of Phe concentration.[15]

The present study showed an association between treatment adherence and living with both parents; patients who did not live with both parents were therefore more likely to have higher Phe levels. A prior study conducted in Sweden found that children of divorced parents were more likely to have high Phe concentration, as found in the present study, showing that coping strategies for PKU must be viewed within a family relations context.[9] Another study conducted in Iran also showed that there was a significant increase in Phe concentration among patients whose parents were divorced.[16]

Parental knowledge of PKU is absolutely essential for proper treatment and control,[5] and the quality of control depends on parental ability to and discipline in implementing and supervising an appropriate diet.[7] In our study, most patients and/or caretakers were found to have satisfactory and fair knowledge of PKU.

A study conducted in England tested maternal knowledge and found that the higher the score of mothers of patients with PKU, the better their annual Phe control and that the mothers of patients with higher Phe concentration tended to have lower educational achievement.[7] In the present study, we indeed found an association between maternal educational achievement and treatment adherence, with patients whose mothers had lower levels of formal education being at greater risk of nonadherence.

In chronic diseases such as PKU, treatment adherence tends to decrease over time, and serum Phe concentration exceeds target limits, especially in adolescents and adults.[17,18] In the present study, most adherent patients were older than 13 years, a finding that is unexpected according to the literature. This may be explained by our choice of 900 μmol/L as the upper target for Phe levels in this age range. A recent study conducted in European centers, however, suggests that adherence is improving in adolescent and adult patients.[14]

Difficulty in living with an invisible (and possibly socially unaccepted) condition, coupled with the dearth of studies assessing the social effects of the lifestyle limitations and dietary restrictions of PKU, may be directly associated with treatment adherence.[5,13] We found dietary restriction to be one of the challenges most frequently reported by the patients and family members interviewed in this study, as was obtaining an adequate supply of MF, which is a key component of PKU treatment. Identifying factors associated with dietary adherence, assessing knowledge of therapeutic regimen and treatment, and highlighting the importance of family relations in maintaining adherence to an appropriate diet play an essential role in supporting families and making treatment more effective. Interventions such as home visits, educational materials, games, courses, and cooking classes in which the food issue is given due importance have all been described as having positive effects on adherence to PKU treatment.[5,7,19,20]

Our data confirm that siblings can show variability in treatment adherence. Although our sample of siblings is small, it does not suggest that there is an association between gender, early/late diagnosis, and knowledge of PKU. However, an association appears to exist between current age and adherence (the older the patient, the better the adherence)—of the 4 families assessed, the adherent patient was the older one in 3, a fact that may be explained by the cutoffs adopted to classify treatment adherence according to patient's age.

One of the limitations of our study was the absence of more diversified questions for assessing knowledge of PKU. In future studies, we intend to assess family stance on religion, which was not addressed at the present time, and factors associated with intrafamilial variability in adherence. Other limitation was the broad heterogeneity of sample's age, since the oldest patients have actually been treated since times when objectives and circumstances were different. These changes (Phe target level, frequency of controls, health staff, and treatment out of an organized screening program) must influence the adherence and have not been analyzed in our study.

We conclude that the issue of adherence to PKU treatment still requires further studies and that the challenges associated with PKU treatment should be discussed and addressed jointly by families and health professionals so that the most effective interventions can be identified for each case. As this was the first study of its kind to be carried out on a sample of Brazilian patients with PKU, we hope its findings can contribute to improve treatment adherence and quality of life in this population. We suggest all nonadherent patients should be periodically reevaluated in order to identify the reasons for nonadherence and to improve the educational and nutritional skills of the family.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

The online data supplements are available at http://iem.sagepub.com/supplemental.

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9.9 Apêndice 9 – Artigo publicado no Jornal de Pediatria, 2015 - *Maple syrup urine disease in Brazil: a panorama of the last two decades*

Artigo publicado no Jornal de Pediatria, 2015

Título:

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ORIGINAL ARTICLE

Maple syrup urine disease in Brazil: a panorama of the last two decades☆,☆☆

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KEYWORDS
Maple syrup urine disease;
MSUD;
Inborn errors of metabolism;
Diagnosis

Abstract
Objective: To characterize a sample of Brazilian patients with maple syrup urine disease (MSUD) diagnosed between 1992 and 2011.
Methods: In this retrospective study, patients were identified through a national reference laboratory for the diagnosis of MSUD and through contact with other medical genetics services across Brazil. Data were collected by means of a chart review.
Results: Eighty-three patients from 75 families were enrolled in the study (median age, 3 years; interquartile range [IQR], 0.57–7). Median age at onset of symptoms was 10 days (IQR 5–30), whereas median age at diagnosis was 60 days (IQR 29–240, p = 0.001). Only three (3.6%) patients were diagnosed before the onset of clinical manifestations. A comparison between patients with

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(n = 12) and without (n = 71) an early diagnosis shows that early diagnosis is associated with the presence of positive family history and decreased prevalence of clinical manifestations at the time of diagnosis, but not with a better outcome. Overall, 98.8% of patients have some psychomotor or neurodevelopmental delay.
Conclusion: In Brazil, patients with MSUD are usually diagnosed late and exhibit neurological involvement and poor survival even with early diagnosis. We suggest that specific public policies for diagnosis and treatment of MSUD should be developed and implemented in the country.

PALAVRAS-CHAVE
Doença da urina de xarope de bordo;
DXB;
Erros inatos do metabolismo;
Diagnóstico

Doença da urina de xarope de bordo no Brasil: um panorama das últimas duas décadas

Resumo
Objetivo: Caracterizar uma amostra de pacientes brasileiros com a doença da urina de xarope de bordo (DXB) diagnosticados entre 1992 e 2011.
Métodos: Neste estudo retrospectivo, os pacientes foram identificados por meio de um laboratório de referência nacional para o diagnóstico de DXB e por meio do contato com outros serviços de genética médica no Brasil. Os dados foram coletados por meio de uma revisão de prontuários.
Resultados: 83 pacientes de 75 famílias foram incluídos no estudo (idade média: 3 anos; intervalo interquartil (IQR): 0,57-7). A idade média no surgimento dos sintomas era de 10 dias (IQR: 5-30), ao passo que a idade média no diagnóstico era de 60 dias (IQR: 29-240; p = 0,001). Somente três (3,6%) pacientes foram diagnosticados antes do surgimento de manifestações clínicas. Uma comparação entre pacientes com (n = 12) e sem (n = 71) um diagnóstico precoce mostra que o diagnóstico precoce está associado à presença de histórico familiar positivo e à redução na prevalência de manifestações clínicas no momento do diagnóstico, porém sem melhor resultado. Em geral, 98,8% dos pacientes têm algum atraso no desenvolvimento psicomotor ou neurológico.
Conclusão: No Brasil, os pacientes com DXB normalmente recebem um diagnóstico tardio e exibem um envolvimento neurológico e baixa sobrevivência, mesmo com um diagnóstico precoce. Sugerimos que políticas públicas específicas para o diagnóstico e tratamento da DXB sejam desenvolvidas e implementadas no país.

Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive genetic disorder caused by deficient activity of the branched-chain alpha-keto acid dehydrogenase complex (BCKDC). Deficiency of this enzyme complex leads to high levels of the branched-chain amino acids (BCAA) leucine, valine, and isoleucine. Leucine and its keto analog 2-oxoisocaproic acid are particularly toxic to the central nervous system (CNS). Although the incidence of MSUD worldwide is usually estimated as being 1:185,000 newborns (NB),[1] data retrieved from newborn screening suggest this rate can be higher; in Germany, for instance, the incidence is estimated at 1:133,000 NB,[2] and in some Mennonite and Pennsylvania Dutch communities in the United States, it may be as high as 1 in 200 live births.[3]

Neonatal screening by tandem mass spectrometry (MS/MS), also known as expanded newborn screening, enables diagnosis of MSUD while the patient is still asymptomatic, as well as early treatment onset—two essential factors in improving the clinical course.[3] Before the introduction of expanded newborn screening, the severe form (classical MSUD) was believed to account for 75-80% of cases,[4] but recent data suggest the milder forms of MSUD can account for up to 50% of diagnosed cases.[5] In the classical form, symptoms first occur between the 4th and 7th day of life, and often include respiratory changes, encephalopathy, a characteristic odor, seizures, and coma.[6] In the acute phase, prompt, aggressive treatment to reduce leucine levels is required, which should consist of a high-rate glucose infusion to stimulate insulin secretion and suppress protein catabolism. If this fails, invasive interventions such as peritoneal dialysis, hemodiafiltration or hemodialysis may be required. During the maintenance phase, treatment usually consists of dietary BCAA restriction and supplementation with thiamine and a BCAA-free formula,[6–8] although liver transplantation is a good alternative.[9–11]

The Brazilian Public Newborn Screening Program was implemented in 2001 and does not include screening for MSUD. The BCAA-free formula, a high-cost product, is not provided by the public Brazilian Unified Health System (Sistema Único de Saúde, SUS). Furthermore, the laboratory tests required for diagnosis of this condition are also not provided through the SUS, and are only available at a few select university centers or private medical laboratories. Regarding liver transplantation, there is no coordinated countrywide

network aiming to improve liver transplantation conditions for patients with metabolic disorders. Finally, there are no data on the prevalence of this disease in Brazil.

The objective of this study was to outline the profile of Brazilian patients with MSUD from 1992 to 2011 so as to contribute to the consolidation of specific public policies for MSUD in the country.

Methods

This retrospective, multicenter, longitudinal study was approved by the local Institutional Review Board.

Patients were identified from the records of the Inborn Errors of Metabolism Laboratory of the Medical Genetics Service, a university-based service that serves as a nationwide referral center for the diagnosis and treatment of inborn errors of metabolism, and from the records of the Inborn Errors of Metabolism Hotline (Serviço de Informações sobre Erros Inatos do Metabolismo - SIEM) run by the same Medical Genetics Service.[12] This laboratory probably accounts for most MSUD diagnoses made in the country; the necessary workup is provided at no cost to the patient or referring physician, and is usually covered by research funding. Quantitation of BCAAs by high-performance liquid chromatography (HPLC) and urine organic acid analysis have been available at the laboratory since 1994; automatic amino acid analysis and MS/MS are also currently available, but alloisoleucine detection is no longer performed. The SIEM is a toll-free telephone hotline, established in 2001, that provides information to physicians and other healthcare providers involved in the diagnosis and treatment of patients with suspected or confirmed IEMs.

To be included in the study, a patient should present: 1) a significant increase in blood BCAA levels, on more than one measurement, as determined by a gold-standard method (HPLC-based BCAA quantitation or automatic amino acid analyzer or MS/MS); and 2) a biochemically confirmed diagnosis of MSUD, established between 1992 and 2011.

Data collection forms were filled out for each patient by their attending physician or one of the study investigators by means of a review of available patient records and charts. For deceased patients, the date of the last available record was considered the date of study enrollment.

Definition of study variables

Diagnosis was considered ''early'' if the patient had been diagnosed before the 15th day of life. The duration of disease until diagnosis was defined as the time elapsed between the onset of clinical manifestations and the biochemical diagnosis of MSUD. Presence and severity of psychomotor and neurodevelopmental delay were assessed on the basis of the impressions of each patient's attending neurologist or pediatrician. MSUD was classified into variants according to the criteria usually cited in the literature.[1]

Statistical analysis

All statistical analyses were carried out in the Statistical Package for the Social Sciences (SPSS®, Statistics for Windows, Chicago, USA) 18.0 software environment. Variables were only taken into account for analysis if data were available for at least 60% of the sample.

For descriptive analysis, data were expressed as absolute and relative frequencies. Asymmetrically distributed continuous variables were expressed as medians and interquartile ranges. The chi-square test and Fisher's exact test were used to determine associations between categorical variables. The Kruskal–Wallis and Mann–Whitney U tests were used to compare the medians of different characteristics. The significance level was set at 5%.

Results

One hundred and nineteen patients with clinical or laboratory evidence of MSUD (''potential MSUD'' patients) were identified, 83 of whom met the inclusion criteria. Of these, 48 were alive at the time of the study, 20 died before the start of the study, and 15 lacked information regarding survival.

The patients enrolled in the study came from all five regions of Brazil. The median age at inclusion was 3 years (IQR 0.57–7.00 years; range, 30 days–23 years). Forty-six (55.4%) were male, 75 (90.4%) were unrelated and 14 (18.7%) had a family history of MSUD. Consanguinity was reported in 17 families (22.7%).

Diagnosis

The median age at diagnosis was 60 days (IQR 29–240 days; range, 7 days–10 years). The median leucine level at diagnosis was 1,693 μmol/L (IQR 965–2,836 μmol/L; reference range, 80–200 μmol/L).

Eighty patients (96.4%) had clinical manifestations of MSUD at the time of diagnosis (median age at symptom onset, 10 days; IQR 5–30 days; range, 1 day–2 years). The most common manifestations were seizures (51.2%) and hypoactivity (50%). Other presenting symptoms included poor feeding, poor sucking and changes in respiratory pattern (48.7% each), hypotonia (48.2%), characteristic odor (42.5%), lethargy (41.2%), metabolic acidosis (31.2%), vomiting (30.0%), and encephalopathy (20.0%). The characteristic odor of MSUD was reported by health care providers as a strong, ''soy sauce-like,'' ''caramel-like,'' or sweet scent, which was most detectable in patients hospitalized due to metabolic decompensation. There was a statistically significant difference between median age at symptom onset and median age at diagnosis ($p = 0.001$).

Fig. 1 shows the distribution of the number of diagnoses per year, revealing an upward trend in diagnoses over the course of the study period. However, comparison of median age at diagnosis between 1992 and 2001 (90 days; IQR 36–270; $n = 31$) and between 2002 and 2011 (53 days; IQR 20–202; $n = 52$) revealed no statistically significant difference ($p = 0.053$). The median time elapsed between symptom onset and diagnosis was 60 days (IQR 28–240) over the first decade of the study and 37 days (IQR 9–180) during the second decade ($p = 0.075$). Considering all MSUD patients who were alive as of 2011 ($n = 48$), 13 had been diagnosed between 1992 and 2001 and 35 between 2002 and 2011.

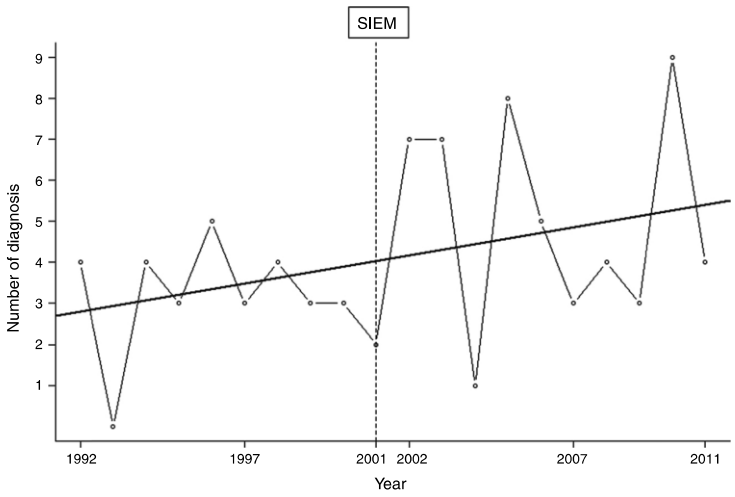

Figure 1 Number of MSUD diagnoses in Brazil and trendline, 1992-2011.
SIEM is a toll-free telephone hotline, established in 2001, that provides information to physicians and other healthcare providers involved in the diagnosis and treatment of patients with suspected or confirmed IEMs.

Table 1 Influence of early diagnosis on the course of MSUD[a].

	Early diagnosis (n = 12)	Late diagnosis (n = 71)	p
Positive family history (n = 14/83)	5/12 (41.6%)	9/71 (12.6%)	0.034
Severity of developmental delay (n = 58/83)			
None (n = 1)	1/12 (8.3%)	0/46 (0%)	0.230
Severe (n = 15)	2/12 (16.7%)	13/46 (28.3%)	
Moderate (n = 20)	4/12 (33.3%)	16/46 (34.8%)	
Mild (n = 22)	5/12 (41.7%)	17/46 (37%)	
Leucine ≥ 1000 mol/L at diagnosis (n = 53/73)	9/12 (75%)	44/61 (72.1%)	0.716
Symptoms present at diagnosis (n = 80/83)	9/12 (75%)	71/71 (100%)	0.02
Diagnosis period (n = 83/83)			
1992-2001 (n = 31)	2/12 (16.7%)	29/71 (40.8%)	0.195
2002-2011 (n = 52)	10/12 (83.3%)	42/71 (59.1%)	
Survival as of 2011 (n = 68/83)			
Alive (n = 48)	9/12 (75%)	39/56 (69.6%)	0.866
Dead (n = 20)	3/12 (25%)	17/56 (30.4%)	

[a] Early diagnosis: diagnosis of MSUD made before the 15th day of life.

Only 12 patients had been diagnosed early. In three of these cases, diagnosis was made before symptom onset as a result of neonatal screening at a private laboratory; at the time of writing, one of these patients is 4 years old and has normal neurological and psychomotor development, and the other two patients, aged 1 year and 6 years, have mild and moderate psychomotor and neurodevelopmental delays respectively. Table 1 presents a comparison between patients with and without an early diagnosis of MSUD.

Clinical manifestations

The most common clinical manifestations at the time of patient enrollment were psychomotor and neurodevelopmental delay (98.8%) and poor nutritional status (74.7%). Two patients were overweight.

Median age at diagnosis was not significantly associated with severity of developmental delay (n = 58/83; p = 0.31), nor were elevated leucine levels (n = 57/73; p = 0.961).

Seventy-three (88.0%) patients had classic MSUD (median age at diagnosis, 60 days; IQR 27.5–180 days), eight (9.6%) had intermediate MSUD (median age at diagnosis, 257 days; IQR 33.7–668 days), and two (2.4%) had intermittent MSUD (age at diagnosis, 6 and 7 years respectively). For patients with the intermediate form, the most common clinical manifestations at diagnosis were food avoidance and respiratory abnormalities, while for patients with the intermittent form, those manifestations were seizures and respiratory abnormalities. All patients with the

intermediate/intermittent forms were alive at the time of writing.

Treatment

Fifty-eight (69.9%) patients were being managed by neurologists, 56 (67.5%) by medical geneticists, 49 (59.0%) by pediatricians, and 46 (55.4%) by dietitians. Other professionals involved in patient management and follow-up included neonatologists, gastroenterologists, physician nutrition specialists, speech and language pathologists, and physical therapists.

The patients in our sample received follow-up at 16 treatment centers, with a median of five patients per center (IQR 1.75–6.5). Use of an MSUD-specific metabolic formula was reported in 62 of 73 patients (74.7%). Three patients had undergone liver transplantation; in two cases, the procedure was performed in Brazil. Thirty-seven patients (59.7%) received the metabolic formula regularly (median age, 5 years; IQR 1-7.5 years); those who reported failures in formula supply had a median age of 2 years (IQR 0.5-5.00). Median time elapsed between diagnosis and receipt of the formula was 17.5 days (IQR 5.75–30 days). There was no significant association between severity of developmental delay and regularity of formula supply (n = 40/62, p = 0.074).

Deaths

Of the patients for whom data were available (n = 68), 20—all with classic MSUD—died before the start of the study. Median age at death was 225 days (IQR 127.5–365 days). There was no statistically significant correlation between fatal outcome and leucine levels at diagnosis (p = 0.568).

Discussion

To the best of our knowledge, this is the first study to outline a profile of Brazilian patients with MSUD. The current Brazilian population is estimated at 190,732,694, with 2,944,928 live births occurring per year.[13] Therefore, considering a mean incidence of MSUD of 1:100,000 in the country, we would expect approximately 300 new diagnoses of the disease over a 10-year period—a much lower estimate than the sample actually included in the study. This suggests that MSUD is underdiagnosed in the country.

MSUD meets most of the Wilson and Jungner[14] criteria for screening: for instance, there is a recognizable latent or early symptomatic stage and an accepted treatment for patients with recognized disease. In countries where MSUD is included in neonatal screening, patients are usually diagnosed before the 10th day of life.[3,4] Conversely, in countries where MSUD is not included in public neonatal screening programs, such as Brazil, diagnosis is usually delayed, occurring at ages similar to those reported for our sample.[15,16] The predominance of classic MSUD and symptomatic patients in our sample could be also due to the non-inclusion of MSUD in the public neonatal screening program, as the literature suggests that newborn screening enables earlier diagnosis of milder forms of the condition.[5]

As expected, patients with a positive family history were tested earlier than patients with no family history of MSUD; this was probably due to genetic counseling of families who had already had one child with the condition and were thus aware of the risk of recurrence and the need for early investigation.

Our study found an upward trend in the number of MSUD diagnoses over the past decade, which coincided with the establishment of the SIEM hotline and the implementation of a public neonatal screening program by the Brazilian Ministry of Health.[17] The reasons behind this trend are unknown, but it may reflect greater awareness of IEMs in general by health care providers, as well as greater awareness of the early clinical manifestations of these conditions. Nevertheless, the increase was not statistically significant, and there was no significant difference in age at diagnosis between the two periods, which corroborates our belief that a substantial portion of MSUD patients continue to die undiagnosed and untreated in Brazil. A similar situation is seen in Malaysia, where newborn screening does not include MSUD: the diagnosis is often late and MSUD appears to be less prevalent than expected.[18]

Psychomotor and neurodevelopmental delays were detected in practically all patients in the sample. Just over half of patients who received the MSUD-specific metabolic formula reported that the formula was supplied regularly. However, most patients exhibited inadequate nutritional status. It bears stressing that MSUD patients should always be followed by nutritional care providers, and only half of the patients in our sample had the support of a registered dietitian.[19] Most patients who received metabolic formula, however, were monitored by dietitians (data not shown). Neurologists were the professionals most often responsible for patient follow-up, which may be secondary to the high frequency of developmental delay in this sample.

In Brazil, time between diagnosis and receipt of the metabolic formula is long and variable. When patients were diagnosed in the acute stage of the disease, during a hospital stay due to metabolic decompensation, they were likely to be started on metabolic formula on the date of diagnosis (if the formula is available at the hospital of admission, of course). Conversely, patients who were diagnosed at a non-acute stage of the disease and treated on an outpatient basis were likely to receive the formula only much later; in fact, these patients usually secure access to the metabolic formula through litigation. Again, it bears stressing that use of the BCAA-free formula is essential, as it ensures the amount of protein required for proper growth and development.[1] Recently, studies have been conducted in rats with newly induced classical and intermediate MSUD to assess the consequences of rapid BCAA buildup and assess potential treatment options, such as norleucine.[20]

Leucine levels at diagnosis were high, with a median value of 1,693 μmol/L. Leucine levels in excess of 1,000 μmol/L are considered critical, as they may produce irreversible damage or even death.[3,21,22] However, there was no significant association between severity of developmental delay and leucine levels at diagnosis. This may be attributable to the fact that long-term metabolic control is considered a more decisive determinant of psychomotor and cognitive development than leucine levels at diagnosis.[22]

In this study, the advantage of early diagnosis appears to have been lost due to a lack of short- and long-term clinical management. As reported for Filipino patients,[15] no clinical protocol for management of acute-stage MSUD is available in Brazil, and patients do not receive the metabolic formula reliably. Conversely, in a study by Morton et al. [4] in which patients had access to the formula and a clinical protocol was followed in the acute stage of the disease, the overall outcome was better and patients achieved more adequate development.

In light of the recent adoption of a specific public policy for diagnosis and management of rare diseases in Brazil,[23,24] we suggest the following steps should be taken to further improve the quality of life of MSUD patients in the country: a) expand the public newborn screening program to include MSUD among the identified disorders; b) develop the ability to conduct alloisoleucine testing locally; c) make the specific metabolic formulas available to all patients, without the need for legal intervention; d) establish a national center specializing in liver transplantation for metabolic disorders; and e) establish a network of multidisciplinary teams comprising physicians, nurses and dietitians specialized in the treatment of inborn errors of metabolism to develop national protocols for MSUD management. The creation of the Brazilian MSUD Network (http://redexaropedobordo.com.br/), established in 2010 to promote education on the diagnosis and management of MSUD and supported by a Brazilian research agency and by the Ministry of Science and Technology, is one of the first steps toward this goal.

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Conflicts of interest

The authors declare no conflicts of interest.

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9.10 Apêndice 10 – Capitulo de livro no Manual de Genética para atenção primária à saúde, 2013 - *Erros Inatos do Metabolismo e Triagem Neonatal*

Capitulo de livro no Manual de Genética para atenção primária à saúde, 2013

Título:

Erros Inatos do Metabolismo e Triagem Neonatal.

Autores:

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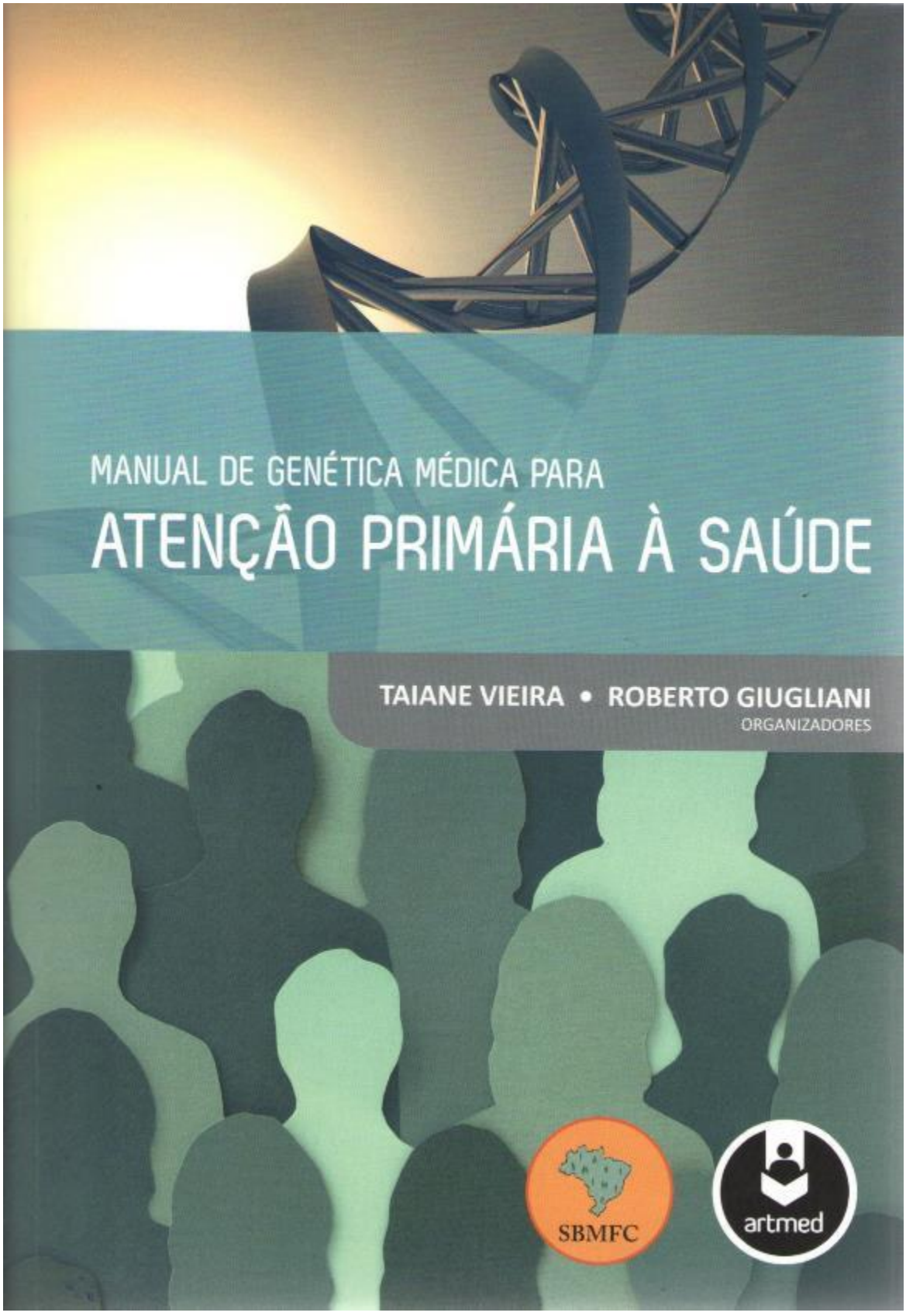
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Erros Inatos do Metabolismo e Triagem Neonatal

Erros inatos do metabolismo

Archibald Garrod, em 1908, constatou que irmãos, filhos de pais normais e consanguíneos, excretavam altas quantidades de ácido homogentísico e que frequentemente um ou mais irmãos eram afetados, inclusive nos casos em que seus pais ou parentes não apresentavam a doença. Garrod e Bateson observaram maior incidência de consanguinidade entre os pais dos indivíduos afetados e, segundo as leis de Mendel, propuseram que esse distúrbio era compatível com padrão de herança autossômica recessiva. Após essas observações, chegou-se ao conceito atual de que algumas doenças metabólicas ocorrem devido à deficiência de uma determinada enzima responsável por uma rota metabólica, fundamental para o funcionamento normal do nosso organismo. Por esse processo ser hereditário, foi denominado erro inato do metabolismo (EIM).[1]

Os EIMs são um grupo de doenças genéticas causadas pela função deficiente de proteínas (em geral, enzimas) envolvidas no processo de síntese, degradação, transporte ou armazenamento de moléculas no organismo (aminoácidos, carboidratos, ácidos graxos e outras) (**Fig. 7.1**). A manifestação clínica geralmente decorre do acúmulo de substratos tóxicos ou da falta de produtos vitais para o funcionamento adequado do organismo. O acúmulo pode ser agudo ou crônico, levando a diferentes formas de manifestação clínica. Sabe-se que a deficiência da enzima pode ser total ou parcial, ocasionando quadros clínicos mais graves ou mais leves.[1]

Os EIMs são ocasionados pelo acúmulo do substrato e/ou deficiência do produto da reação, secundários à deficiência da enzima envolvida e/ou de seu cofator. Em muitos casos, há o desvio para uma rota alternativa, e esse produto C poderá ser o responsável pelos danos matabólicos (ver **Fig. 7.1**).

Quando os EIMs são mencionados, imagina-se que sejam extremamente raros e que ocorram em pequenos segmentos da população. Contudo, segundo Martins e colaboradores,[2] são descritas mais de 500 doenças classificadas como EIMs, sendo raras apenas ocasionalmente, mas com frequência global de 1:2.500 nascidos vivos.

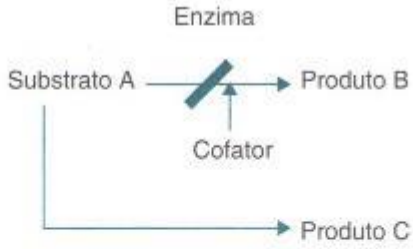

Figura 7.1 Reação enzimática.

Os EIMs ocorrem em todos os grupos étnicos e existem em todos os continentes,[3] já tendo sido descritos em todas as áreas do metabolismo humano normal.[1]

Classificação

Grupo 1 – Doenças que causam intoxicação

Nesse grupo estão incluídos EIMs intermediários que podem causar intoxicação aguda ou progressiva pelo acúmulo de compostos que se formam próximos ao local do bloqueio metabólico. São exemplos desse grupo: fenilcetonúria, doença da urina do xarope de bordo, acidúrias metilmalônica, propiônica e isovalérica, galactosemia, doença de Menkes, doença de Wilson e porfirias.[4]

Grupo 2 – Doenças que envolvem o metabolismo energético

Os EIMs incluídos nesse grupo relacionam-se com sintomas causados pela deficiência de produzir ou utilizar energia no fígado, no cérebro, no músculo e em outros tecidos. Podem ser divididos ainda em defeitos energéticos mitocondrial e citoplasmático. São exemplos desse grupo: glicogenoses, acidemias láticas, doenças mitocondriais e defeito de oxidação de ácidos graxos.[4]

Grupo 3 – Doenças que envolvem acúmulo de moléculas complexas

Os EIMs incluídos nesse grupo têm como característica o distúrbio na síntese ou no catabolismo de moléculas complexas. São exemplos desse grupo: mucopolissacaridoses, doenças dos peroxissomos, mucolipidoses e lipofuscinoses.[4]

Sinais clínicos

Alguns sintomas são mais frequentemente relatados nos EIMs, como letargia, vômitos, coma, convulsões, retardo progressivo do desenvolvimento psicomotor, dificuldade em alimentar-se, acidose e alcalose metabólica, hepato e/ou esplenomegalia, hipotonia, malformações osteoarticulares e deficiência mental. Contudo, as mani-

festações clínicas dos EIMs podem ser inespecíficas e variadas. Essa variação ocorre principalmente pela diferença no grau de deficiência enzimática, do tecido ou do metabolismo que está afetado. Além disso, os sinais clínicos podem iniciar em várias fases da vida: neonatal, infantil, juvenil ou adulta.[1]

Tratamento

Podem ser utilizadas como estratégias de tratamento para os EIMs:

- Restrição dietética do substrato (p. ex., restrição de proteína e galactose);
- Remoção do metabólico tóxico (p. ex., diálise e uso de carnitina);
- Reposição do produto deficiente (p. ex., uso de biotina);
- Estímulo da atividade da enzima deficiente (p. ex., uso de cofatores vitamínicos);
- Terapia de reposição enzimática;
- Transplante de órgãos;
- Terapia gênica.

Contudo, o tratamento de cada EIM é individualizado e relacionado ao metabolismo que está alterado. Além disso, muitos EIMs permanecem sem tratamento específico.[4]

Diagnóstico

O diagnóstico dos EIMs pode ser pré-sintomático, em que há ausência de sequela ou esta é mínima; ou, ainda, o diagnóstico pode ser sintomático, no qual as sequelas já podem estar estabelecidas e ser irreversíveis. De preferência, as doenças metabólicas deveriam ser diagnosticadas precocemente, ainda no seu período assintomático, por meio de testes de triagem neonatal, contudo, nem todas as doenças são passíveis de triagem neonatal, pois devem ser consideradas as características da doença, a sensibilidade e os custos do teste a ser realizado.

Exemplos de erros inatos do metabolismo

Fenilcetonúria

A fenilcetonúria (PKU) é um erro do metabolismo dos aminoácidos, causada pela deficiência da enzima fenilalanina hidroxilase (PAH), que converte a fenilalanina (Phe) em tirosina. A PKU foi descrita pela primeira vez por Asbjørn Følling, em 1934, ao observar dois irmãos com deficiência mental.[5] A prevalência da deficiência de PAH é de aproximadamente 1:12.000, mas é possível encontrar números de 1:1.000.000

na Finlândia e 1:4.200 na Turquia, mostrando, assim, a variação da prevalência entre diferentes populações.[6] Segundo Carvalho,[7] a prevalência da PKU no Brasil é de 1:24.780 habitantes. Na Região Sul do Brasil, segundo Jardim e colaboradores,[8] essa razão é de 1:12.500 recém-nascidos vivos. O diagnóstico da PKU deve ser feito no período neonatal por meio do teste do pezinho.[6] A PKU pode ser dividida em PKU clássica, PKU leve e hiperfenilalaninemia não PKU, relacionada à atividade enzimática residual.[9]

Ao nascimento, o paciente com PKU parece normal. Alguns sintomas podem ser vistos no início da vida, como irritabilidade, eczema e odor incomum. A manifestação mais importante nesses pacientes é a deficiência mental.[10] O nível de Phe sanguínea está diretamente relacionado ao grau de deficiência mental e lesão neurológica, que também se correlacionam com a idade em que o tratamento foi iniciado.[2]

O tratamento da PKU é preconizado em uma dieta de baixa concentração de Phe, capaz de manter baixa concentração plasmática desse aminoácido. Como a Phe é um aminoácido essencial, é necessário que o seu consumo seja suficiente para satisfazer as necessidades do crescimento.[10] A dieta restrita em Phe deve ser iniciada precocemente, de preferência no primeiro mês de vida. Assim, os aspectos clínicos indesejáveis da hiperfenilalaninemia persistente serão evitados. Os resultados são bem menos favoráveis quando o tratamento é iniciado tardiamente.[11]

Doença da urina do xarope de bordo

A doença da urina do xarope de bordo, também conhecida como leucinose, é um EIM causado pela deficiência da atividade do complexo da desidrogrenase dos α-cetoácidos de cadeia ramificada dependente de tiamina (CACRs), que é composto pelas subunidades E1αβ, E2 e E3. A deficiência desse complexo é responsável pelo aumento nos fluidos fisiológicos dos aminoácidos de cadeia ramificada leucina, valina e isoleucina (AACRs), bem como dos seus respectivos α-cetoácidos. O acúmulo desses aminoácidos afeta principalmente o sistema nervoso central. A doença da urina do xarope de bordo é herdada de forma autossômica recessiva, e sua incidência mundial é estimada em 1:185.000 nascidos. Em relação à época do diagnóstico, ele pode ser realizado em período pré-sintomático (teste de triagem neonatal – não disponível na rede pública) ou sintomático.[12]

Os fenótipos clínicos associados são classificados como forma clássica, intermediária, intermitente, responsiva à tiamina e deficiência de lipoamida desidrogenase (da subunidade E3), dependendo, entre outros, da idade de início e da gravidade da doença; dessa forma, as manifestações clínicas dos pacientes são variadas.[12,13]

O tratamento é dividido em tratamento da fase aguda (fase de descompensação metabólica) e da fase de manutenção.[13] O tratamento da fase aguda da doença visa à correção da hipoglicemia e da acidose metabólica associadas, sendo baseado em três pontos: eliminação dos metabólicos tóxicos, suporte nutricional e indução do anabolismo. O tratamento a longo prazo consiste em restrição dietética de proteínas e dos AACRs.[12]

Glicogenoses

As doenças de depósito de glicogênio são causadas por anormalidades de enzimas que regulam a síntese e a degradação do glicogênio. Essas doenças são classificadas em 12 tipos diferentes, nomeadas de acordo com o defeito enzimático específico e os órgãos afetados.[14] Subtipos vêm sendo descritos, devido a diferentes características clínicas, bioquímicas e genéticas.[15] A frequência global das glicogenoses é de 1:20.000 a 1:25.000 nascidos vivos.[16] Para confirmar o diagnóstico de glicogenose é realizada biópsia hepática e estudo enzimático ou análise de mutação.[17] A glicogenose tipo 1 representa cerca de 25% das glicogenoses.[18]

A manifestação primária das glicogenoses hepáticas é a hipoglicemia associada a hepatomegalia, "face de boneca", fadiga, aumento de triglicerídeos, ácido úrico e lactato (**Fig. 7.2**). Já entre as glicogenoses musculares, predominam sintomas de fraqueza muscular e cãibras.[14]

O tratamento padrão da glicogenose consiste em evitar o acúmulo de glicogênio hepático, restringindo o uso de açúcares simples, como glicose, maltose, sacarose, frutose e dextrose. O tratamento é basicamente dietético e, para a manutenção da glicemia estável, utiliza-se o amido cru (maisena), que promove uma lenta degradação e manutenção da glicemia plasmática. A dieta deve ser frequente (a cada 3 a 4 horas, durante as 24 horas do dia).[18] Em muitos casos, é necessária a infusão contínua noturna de uma dieta específica calculada para cada paciente.[14] As glicemias devem ser monitoradas frequentemente e diariamente com o hemoglicoteste, e a dose de amido é calculada conforme os níveis de glicose no sangue, sendo uma média de 1 a 2 g/kg/dia, a cada 4 horas. Em caso de hipoglicemia, é necessário tratar imediatamente com glicose intravenosa ou via oral até o restabelecimento da glicemia.

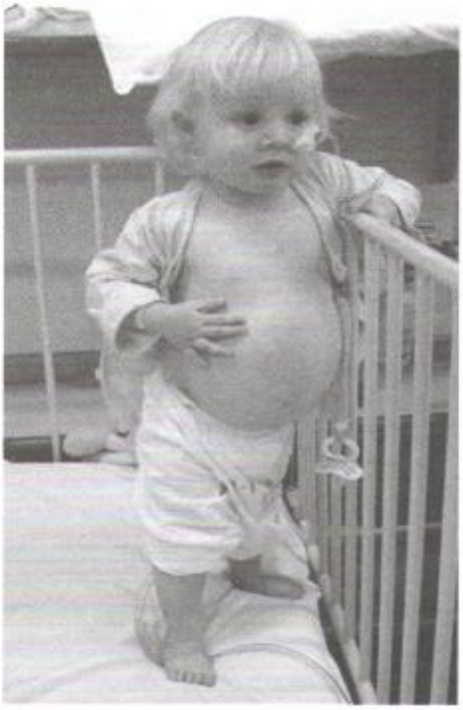

Figura 7.2 Paciente com Glicogenose, observe a face de boneca e hepatomegalia.
Fonte: Foto cedida por Carolina Fischinger Moura de Souza.

Mucopolissacaridoses

As mucopolissacaridoses (MPSs) são doenças de depósito lisossômico caracterizadas pela degradação deficiente dos mucopolissacarídeos (glicosaminoglicanos). Esse acúmulo é causado pela deficiência na atividade de uma enzima. Já foram descritos sete tipos de MPSs, incluindo ainda alguns subtipos dentre esses.[19] A frequência das MPSs é de 1:25.000 nascimentos. O diagnóstico das MPSs é realizado por meio da quantificação e determinação da excreção de glicosaminoglicanos na urina, seguido da confirmação por meio da medida da atividade enzimática, específica para cada tipo de MPS.[17]

Podem ser descritas, nessa doença, manifestações clínicas como hepatomegalia, esplenomegalia, alterações esqueléticas, face característica (infiltrada e aspecto grosseiro), alteração de crescimento, mãos em garra, infecções de vias aéreas de repetição, hérnia inguinal e umbilical, retardo de crescimento e malformações cardíacas (**Fig. 7.3**). Dependendo do tipo e da gravidade da MPS, as manifestações podem iniciar na infância ou até mesmo na idade adulta.[19] A deficiência mental e de desenvolvimento pode estar presente em alguns tipos de MPS. O tratamento visa à melhora dos sintomas clínicos e para a MPS I, II e VI, está disponível a terapia de reposição enzimática. Quando o diagnóstico é precoce, algumas formas de MPS são passíveis de transplante de células hematopoiéticas.[19]

Aconselhamento genético para EIM

O aconselhamento genético (AG) é um processo de comunicação ou informação acerca da ocorrência de uma situação de causa ou predisposição genética, seus possíveis mecanismos etiológicos, riscos de recorrência, implicações e possibilidades atuais e

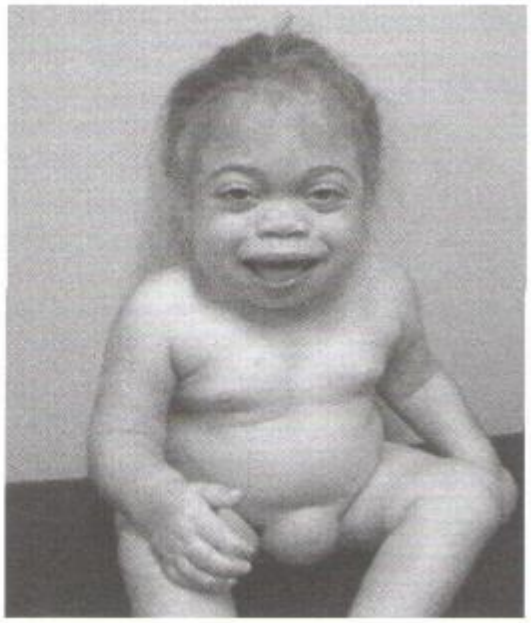

Figura 7.3 Paciente com Mucopolissacaridose, observe as características faciais típicas, abdome globoso devido à hepatoesplenomegalia, hérnia umbilical e contraturas articulares.

Fonte: Foto cedida por Carolina Fischinger Moura de Souza.

futuras de prevenção ou tratamento. O AG tem como base a verdade, a imparcialidade e a confidencialidade. O AG deve preceder qualquer teste de diagnóstico pré-natal e a testagem de indivíduos que estão sob risco de ter ou desenvolver uma doença.

A maioria dos EIMs segue o padrão de herança autossômica recessiva.[20] As doenças recessivas expressam-se apenas em homozigotos que, portanto, devem ter herdado um alelo mutante de cada genitor (pai e mãe). Desse modo, o risco dos pais terem outro filho com a mesma doença é de 25% (¼). Considera-se sempre que os pais são portadores obrigatórios. A consanguinidade dos genitores de um paciente com uma doença genética é uma forte evidência em favor da herança autossômica recessiva. Como a maioria dos EIMs segue esse padrão de herança, diante de uma criança com alterações metabólicas, como hipoglicemia, alterações na função hepática, acidose ou alcalose metabólica, hiperlactacidemia, entre outras, deve-se pensar em doenças metabólicas hereditárias.

O padrão de herança autossômica dominante também ocorre em algumas doenças metabólicas hereditárias, mas é mais raro. Nesses casos, há uma história familiar positiva na verticalidade, ou seja, pai e mãe afetados pela mesma doença. A expressividade pode ser variável, mas a alteração genética está presente em um dos genitores, que transmite o gene alterado. Cada filho desse casal tem uma chance de 50% de receber o alelo anormal do genitor afetado e, portanto, ser afetado, e uma chance de 50% de receber o alelo normal e, assim, não ser afetado.[20]

Há também a possibilidade de a herança ser ligada ao X. Nesse caso, a alteração genética (mutação) está no cromossomo X. Há uma tendência de as mulheres portadoras não apresentarem sintomas ou, se apresentarem, eles são mais brandos. As mulheres são consideradas portadoras, e os homens são afetados. A incidência do fenótipo é maior nos homens.[20]

De uma forma geral, a elaboração do heredograma pode auxiliar no diagnóstico de algumas doenças metabólicas somente pelo padrão de herança. Os critérios clínicos são muito importantes para a elaboração das hipóteses diagnósticas, mas a história familiar pode auxiliar de maneira significativa.[21]

Triagem neonatal

Na prática da saúde pública, o estabelecimento de um papel para a genética começa com a avaliação das necessidades da população, em termos de carga das doenças, e com o desenvolvimento de políticas apropriadas, assegurando o acesso aos serviços de saúde aos indivíduos, famílias e comunidades. Desde os anos 1960, a triagem neonatal (TN) tornou-se um programa especialmente focado na genética e na saúde pública. Devido à contínua descoberta de que doenças monogênicas com alterações metabólicas levam à deficiência mental e a outras morbidades e deficiências, programas de triagem mandatórios em saúde pública foram desenvolvidos, dada a evidência de que a detecção precoce dessas condições podem salvar vidas – por exemplo, no caso da anemia falciforme – e prevenir a deficiência mental, como no caso da PKU.[22]

A TN é um dos vários programas de triagem populacional existentes. Atualmente, é empregada para o diagnóstico precoce (no período neonatal, i.e., entre 0 e 28 dias

de vida) de doenças genéticas, geralmente EIMs, podendo incluir também doenças infecciosas e outras doenças genéticas.[23]

O Programa Nacional de Triagem Neonatal (PNTN) foi instituído em 2001 (Portaria and (25) GM/MS nº 822/GM, de 6 de junho de 2001) e prevê o diagnóstico, o tratamento e o aconselhamento genético dos pais de recém-nascidos com PKU, hipotireoidismo congênito, hemoglobinopatias e fibrose cística.[24] Esse programa tem uma conotação populacional importante, embora ainda necessite, para alcançar pleno êxito, de uma maior participação da comunidade e das universidades.[25]

Em virtude dos diferentes níveis de organização das redes assistenciais existentes nos Estados, da variação percentual de cobertura da triagem dos nascidos vivos que vinha sendo realizada no País e da diversidade das características populacionais existentes no Brasil, optou-se em implantar o PNTN em fases, conforme descrito a seguir:

Fase I Triagem, confirmação diagnóstica, acompanhamento e tratamento da PKU e hipotireoidismo congênito.

Fase II Triagem, confirmação diagnóstica, acompanhamento e tratamento da PKU, hipotireoidismo congênito + doenças falciformes e outras hemoglobinopatias.

Fase III Triagem, confirmação diagnóstica, acompanhamento e tratamento da PKU, hipotireoidismo congênito, doenças falciformes e outras hemoglobinopatias + fibrose cística.

O Ministério da Saúde define TN como uma ação preventiva que permite fazer o diagnóstico de diversas doenças congênitas ou infecciosas, assintomáticas no período neonatal, a tempo de se interferir no curso da doença, permitindo, dessa forma, a instituição do tratamento precoce específico e a diminuição ou eliminação das sequelas associadas a cada doença.[25]

Para que uma doença seja considerada importante para um procedimento de triagem, certos critérios devem ser observados:

- Não apresentar características clínicas precoces;
- Permitir a realização de um teste de identificação com especificidade e sensibilidade altas (confiável);
- Ser um programa economicamente viável;
- Ter um programa logístico para acompanhamento dos casos detectados até o diagnóstico final;
- Estar associado a uma doença cujos sintomas clínicos possam ser reduzidos ou eliminados por meio de tratamento;
- Ter estabelecido um programa de acompanhamento clínico com disponibilidade dos quesitos mínimos necessários ao sucesso do tratamento.

É importante lembrar que a qualidade das amostras coletadas e a acurácia das informações demográficas associadas, assim como o período ideal da coleta e pronti-

dão nos resultados, além da inclusão de todos os neonatos na testagem, são aspectos fundamentais para o sucesso do programa.[26]

Além das doenças incluídas no PNTN, várias outras são passíveis de diagnóstico no período neonatal. A **Tabela 7.1** descreve as principais doenças que podem ser triadas no teste do pezinho, suas características clínicas e frequências.

Tabela 7.1 Doenças triadas pelo teste do pezinho

Doença	Teste	Frequência	Informações sobre a doença
Fenilcetonúria e outras aminoacidopatias	Dosagem quantitativa	1:2.600	A fenilalanina (Phe) se acumula pela deficiência da enzima fenilalanina hidroxilase (PAH). Sem tratamento, as crianças desenvolvem deficiência mental.
Hipotireoidismo congênito	Dosagem quantitativa de TSH e T4	1:3.000	Ocorre pela incapacidade de produzir quantidades normais de hormônio da tireoide. A falta de tratamento leva ao atraso do desenvolvimento neuropsicomotor.
Hiperplasia suprarrenal congênita	Dosagem 17α-OH--progesterona	1:10.500	Ocorre devido a um defeito genético que leva a um bloqueio da rota metabólica envolvida na síntese do cortisol e dos mineralocorticoides.
Fibrose cística	Quantificação da tripsina imunorreativa (ITR), pesquisa das mutações. (p. ex., ΔF508)	1:3.100	Ocorre devido a um distúrbio no transporte de cloreto pelas membranas epiteliais. As manifestações podem incluir íleo meconial, infecções frequentes, diarreia e peso e altura baixos.
Galactosemia	Detecção de galactose e galactose 1-fosfato uridil transferase	1:25.000	É um erro inato do metabolismo dos carboidratos relacionado à deficiência enzimática na rota da metabolização da galactose.
Deficiência de biotinidase	Dosagem da atividade da biotinidase	1:20.000	A deficiência da biotinidase provoca a deficiência de múltiplas carboxilases. Pode causar crises convulsivas, lesões de pele e alterações metabólicas graves.
Deficiência de glicose-6-fosfato desidrogenase (G6PD)	Dosagem da atividade da G6PD	1:40	Ocorre pela deficiência da enzima G6PD. Pode provocar icterícia neonatal e/ou anemia hemolítica.
Acidemias orgânicas e defeitos da oxidação dos ácidos graxos	Perfil Tandem de acilcarnitinas	1:2.200	As acidemias orgânicas são doenças que envolvem o metabolismo de aminoácidos. É a principal patologia relacionada aos defeitos da oxidação dos ácidos graxos.

Lembre-se:

- De forma isolada, cada EIM é extremamente raro, entretanto são conhecidas mais de 500 doenças classificadas como EIMs, apresentando uma frequência bastante elevada em conjunto.
- Os EIMs podem ter sinais e sintomas inespecíficos e variados e manifestar-se em várias fases da vida (neonatal, infantil, juvenil ou adulta).
- A elaboração do heredograma pode auxiliar no diagnóstico de alguns EIMs somente pelo padrão de herança. Os critérios clínicos são muito importantes para a elaboração das hipóteses diagnósticas, mas a história familiar pode auxiliar significativamente.

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