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

FENILCETONÚRIA, NEOFOBIA ALIMENTAR E SENTIDOS QUÍMICOS DE PACIENTES ACOMPANHADOS NO AMBULATÓRIO DE TRATAMENTO DE ERROS INATOS DO METABOLISMO DO HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

TÁSSIA TONON

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TÁSSIA TONON

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

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APRESENTAÇÃO

Conforme formato requerido pelo Programa de Pós-Graduação em Medicina: Ciências Médicas da Universidade Federal do Rio Grande do Sul, esta tese está dividida em: Introdução, Revisão da Literatura, Marco Conceitual, Justificativa, Objetivos, Referências Bibliográficas, Artigos científicos, Conclusões, Perspectivas e Apêndices.

Este trabalho foi desenvolvido no Serviço de Genética Médica, em parceria com o Serviço de Otorrinolaringologia, ambos do Hospital de Clínicas de Porto Alegre (HCPA), e financiado pelo Fundo de Incentivo à Pesquisa e Eventos do HCPA. A aluna recebeu bolsa de estudos concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. A pesquisa apresentada neste trabalho está incluída em um estudo aprovado em seus aspectos éticos e metodológicos pelo Comitê de Ética em Pesquisa do Grupo de Pesquisa e Pós Graduação do HCPA, sob número 2015-0072.

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RESUMO

Introdução: A Fenilcetonúria (PKU) é uma doença genética, que possui uma importante história médica, visto ser o primeiro erro inato do metabolismo identificado por meio da triagem populacional, iniciando uma nova era no diagnóstico e tratamento das doenças genéticas. A terapia dietética com restrição do aporte de fenilalanina (Phe) continua sendo a base do tratamento para a PKU, requerendo uma redução da ingestão de proteínas naturais e substituindo-a por uma fonte proteica isenta de Phe, a fórmula metabólica. A adequação necessária para a manutenção dos níveis sanguíneos adequados de Phe exige frequente modificação para responder ao crescimento, aos estágios da vida, às doenças concomitantes e às comorbidades. Destaca-se a importância de monitorar os níveis de Phe e tirosina nos pacientes e assegurar que outras necessidades nutricionais também estejam sendo atendidas. Na PKU, torna-se um desafio a adesão ao tratamento dietético sem que haja prejuízos no comportamento alimentar e alterações na percepção dos sentidos químicos.

Objetivos: Investigar aspectos relacionados ao comportamento alimentar (estudo I) e sistema sensório (estudo II) de pacientes brasileiros com PKU e de controles saudáveis.

Métodos: Estudo transversal, controlado, que inclui pacientes com diagnóstico de PKU em tratamento dietético e uso de fórmula metabólica, acompanhados no Ambulatório de Tratamento de Erros Inatos do Metabolismo no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. Também, foram incluídos indivíduos controles, hígidos, que foram pareados por sexo e idade. A avaliação da neofobia alimentar foi baseada em um questionário, validado para o português do Brasil, que objetiva investigar o medo de experimentar novos alimentos. Uma alta média no escore indica menor vontade, ao passo de que um menor escore indica maior vontade de experimentar novos alimentos. Para a avaliação da percepção olfativa, foi utilizado o teste *Sniffin' Sticks*, o qual avalia a percepção olfativa através de canetas impregnadas com odores. Já a percepção gustativa foi avaliada através do teste *Taste Strips*, baseado no reconhecimento dos gostos amargo, azedo, doce e salgado através da identificação de tiras colocadas em contato com a língua. As variáveis clínicas e de tratamento foram coletadas através de revisão de prontuário.

Resultados: Vinte e cinco pacientes (média idade: 19,3 ± 4,7 anos, sexo feminino= 13) e 25 controles (média de idade 19,9 ± 4,9 anos, p=0,676, sexo feminino= 13) foram avaliados. A média de idade no início do tratamento dos pacientes com PKU foi de 52,8 ± 29,7 dias. A média de Phe ao diagnóstico foi de 1360.28 ± 671,31 µmol/L (22,48 ± 11,09 mg/dL), e do ano anterior aos testes executados foi de 710,5 ± 346,4 µmol/L (11,74 ± 5,72 mg/dL). Nenhum paciente analisado recebeu aleitamento materno exclusivo até os 6 meses de vida. Em relação aos controles, o aleitamento materno exclusivo até os 6 meses de idade foi seguido em 21 indivíduos (p <0,001). No estudo I, a média do escore que avaliou a fobia alimentar foi maior nos pacientes (47,2 ± 9,7) quando comparado aos controles (29,4 ± 12,5, p <0,001). Foi encontrada associação estatisticamente significativa entre o sexo feminino com a neutralidade, a qual parece proteger para a neofobia alimentar. No estudo II, foram encontrados escores da percepção olfativa (9,3 ± 1,6) e gustativa (10,0 ±2,5) menores nos pacientes quando comparado aos controles (olfativa: 10,3 ± 1,6, p= 0,039) e (gustativa: 11,9 ± 2,0, p= 0,004), respectivamente. Também, os escores para os sabores amargo e salgado foram menores nos pacientes quando comparado aos controles (p= 0,008 e p= 0,020, respectivamente).

Conclusão: Nossos dados sugerem que a neofobia alimentar e a diminuição da percepção dos sentidos químicos em pacientes com PKU é prevalente. A manutenção do controle metabólico durante toda a vida sem que haja prejuízos para o comportamento alimentar e alterações sensoriais, torna-se um desafio no tratamento dos pacientes com PKU. Ressalta-se neste estudo a importância da equipe multidisciplinar acompanhar de perto estas questões a fim de minimizar os efeitos gerados no tratamento da PKU e como consequência aumentar a qualidade de vida dos pacientes. Acreditamos que a introdução de terapias adjuvantes possam contribuir na tentativa de maximizar as escolhas alimentares permitindo maior exposição a novos e diferentes alimentos.

Palavras-chave: Fenilcetonúria; Neofobia alimentar; Comportamento alimentar; Erros Inatos do Metabolismo; Olfato; Paladar; Fenilalanina.

ABSTRACT

Introduction: Phenylketonuria (PKU) is a genetic disease that has an important medical history, since it is the first inborn error of metabolism identified through neonatal screening, initiating a new era in the diagnosis and treatment of genetic diseases. Restricted phenylalanine (Phe) dietary therapy continues to be the basis of treatment for PKU, requiring a reduction in the intake of natural proteins and replacing it with a Phe-free protein source, the metabolic formula. The adequacy necessary to maintain adequate blood levels of Phe requires frequent modification to respond to growth, stages of life, concomitant diseases and comorbidities. It is important to monitor the levels of Phe and tyrosine in patients and to ensure that other nutritional needs are also being met. In the PKU, it becomes a challenge to adhere to dietary treatment without any loss in eating behavior and changes in the perception of the chemical senses.

Objectives: To investigate aspects related to eating behavior (study I) and sensory system (study II) of Brazilian patients with PKU and healthy controls.

Methods: This was a controlled, cross-sectional study that included patients with a diagnosis of PKU in diet therapy and use of metabolic formula, followed at the Ambulatory of Treatment of Metabolic Inborn Errors at the Medical Genetics Service of the Clínicas Hospital, Porto Alegre. Also, healthy controls were included, that were paired by sex and age. The evaluation of food neophobia was based on a questionnaire, validated for Brazilian Portuguese, which aims to investigate the fear of trying new foods. A high mean in the score indicates lower will, while a lower score indicates a greater willingness to try new foods. For the evaluation of olfactory perception, the Sniffin 'Sticks test was used, which evaluates olfactory perception through pens impregnated with odors. The taste perception was evaluated through the Taste Strips test, based on the recognition of bitter, sour, sweet and salty tastes through the recognition of strips placed in contact with the tongue. The clinical and treatment variables were collected through a chart review.

Results: Twenty-five patients (mean age: 19.3 ± 4.7 years, female = 13) and 25 controls (mean age 19.9 ± 4.9 years, p = 0.766, female =13) were evaluated. The mean age at the start of treatment of patients with PKU was 52.8 ± 29.7 days. The mean Phe at diagnosis was 1360.28 ± 671.31 µmol/L, and the year before the tests performed was 710.5 ± 346.4 µmol/L. No patient underwent exclusive breastfeeding

until 6 months of age. Regarding controls, exclusive breastfeeding up to 6 months of age was followed in 21 subjects (p <0.001). In study I, the mean score for food neophobia was higher in patients (47.2 ± 9.7) than in controls (29.4 ± 12.5, p <0.001). The only variable that showed significance was the female association with neutrality, which seems to protect against food neophobia. In the second study, olfactory (9.3 ± 1.6) and gustatory (10.0 ± 2.5) lower scores were found in patients when compared to controls (olfactory: 10.3 ± 1.6, p = 0.039) and (gustative: 11.9 ± 2.0, p = 0.004), respectively. Also, the scores for the bitter and salty flavors were lower in the patients when compared to the controls (p = 0.008 and p = 0.020, respectively).

Conclusion: Our data suggest that food neophobia and decreased perception of chemical sensations in patients with PKU is prevalent. Maintaining life-long metabolic control without impairing eating behavior and sensory changes becomes a challenge in the treatment of patients with PKU. This study emphasizes the importance of the multidisciplinary team closely monitor these issues in order to minimize the effects generated in the treatment of PKU and as a consequence increase the quality of life of the patients. We believe that the introduction of adjuvant therapies may contribute in an attempt to maximize food choices allowing greater exposure to new and different foods.

Keywords: Phenylketonuria; Food Neophobia; Food behavior; Inborn Errors of Metabolism; Smell; Taste; Phenylalanine.

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LISTA DE ABREVIATURAS E SIGLAS

BH$_4$ - Tetrahidrobiopterina

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

DHPR - Diidropteridina Redutase

EIM - Erros Inatos do Metabolismo

GSD - Glicogenoses Hepáticas

GTP - Guanosina Trifosfasto;

GTPCH - Guanosina Trifosfato Cicloidrolase

HCPA – Hospital de Clínicas de Porto Alegre

HPA - Hiperfenilalaninemia

LNAA - Large Neutral Amino Acids

PAH - Fenilalanina Hidroxilase

PAL - Fenilalanina Amonia Liase

PCD - Pterina 4α-carbinolamina Desidratase

PHE - Fenilalanina

PKU - Fenilcetonúria

PNTN - Programa Nacional de Triagem Neonatal

PTPS - Piruvoil Tetraidrobiopterina Sintase

SGM - Serviço de Genética Médica

SR - Sepiapterina Redutase

SRTN - Serviço de Referência em Triagem Neonatal

SUS - Sistema Único de Saúde

TYR - Tirosina

SUMÁRIO

1. INTRODUÇÃO

A Fenilcetonúria (PKU) é um erro inato do metabolismo (EIM) dos aminoácidos, causada pela deficiência da enzima fenilalanina hidroxilase (PAH) que tem como função converter o aminoácido fenilalanina (Phe) no aminoácido tirosina (Tyr). A incidência varia entre as diferentes nações; na Europa, a prevalência é de aproximadamente 1:10.000 recém nascidos (LOEBER 2007), já no Brasil, o último levantamento realizado verificou 1:24.780 recém-nascidos (CARVALHO et al 2003). O rastreamento, o diagnóstico, a confirmação e o tratamento no Brasil são preconizados pelo popular "teste do pezinho", no âmbito do Programa Nacional de Triagem Neonatal, instituído em 2001 (BRASIL 2002). Ao nascimento o paciente tem aparência normal, sendo que alguns sintomas podem ser detectados nos primeiros dias de vida, entretanto, a manifestação mais importante nesses pacientes é a deficiência mental (NYHAN E OZAND 1998; JAHJA 2017).

Devido ao diagnóstico e tratamento precoces, têm se evitado a deficiência intelectual de muitas crianças com PKU nos últimos anos (BLAU et al 2010); para que isto aconteça, uma dieta restrita em Phe deve ser seguida, juntamente com o uso de fórmula metabólica isenta deste aminoácido, que contém uma mistura de micronutrientes e aminoácidos livres (PLASENCIA et al 2009), sendo esta a principal fonte proteica da dieta. A amamentação em combinação com a fórmula metabólica deve ser incentivada, visto que fornece vantagens nutricionais e psicológicas (MACDONALD et al 2006).

Os alimentos modificados com baixo teor de proteína são importantes para o manejo dietético na PKU. Estes mimetizam alimentos ricos em proteínas e ajudam a aumentar a variedade da dieta, bem como a normalizar a aparência dos alimentos com baixo teor em Phe (VOCKLEY et al 2014). No Brasil, esses alimentos não são cobertos pelo Sistema Único de Saúde (SUS); tornando seu acesso difícil devido ao alto custo comparado com o das versões não modificadas. Em relação às fórmulas metabólicas, estas possuem odor e paladar desagradáveis (no Brasil os pacientes não participam do processo de escolha de qual marca será fornecida), e sua ingestão, contrariando as orientações nutricionais, geralmente é feita em uma única dose ao dia, prejudicando sua utilização biológica e aumentando os efeitos adversos, tais como náuseas, vômitos, tonturas e diarreia (VAN WEGBERG 2017).

Apesar do tratamento ser bem-sucedido, a pobre palatabilidade é responsável pela falta de adesão da mesma, principalmente por adolescentes e adultos (BLAU e LONGO 2015; VAN WEGBERG 2017). O tratamento da PKU é uma das mais restritivas dietas conhecidas e a adesão adequada torna-se estressante tanto para os pacientes quanto para suas famílias. Assim, acredita-se que uma dieta extremamente rígida, de início precoce, afete negativamente não somente o comportamento alimentar, como o paladar, produto sensorial da percepção olfativa e gustativa (GUYTON e HALL 2011), podendo suscetibilizar o desenvolvimento de dificuldades alimentares (ANTISDEL e CHRISLER 2000). A literatura aponta que problemas de comportamento alimentar são mais comuns em crianças com PKU quando comparada a crianças sem nenhuma doença metabólica (MACDONALD et al 1996; CRONE et al 2005; EVANS et al 2016), porém destaca-se a escassez de estudos que investiguem de forma específica a relação entre os sentidos químicos e o comportamento alimentar em pacientes com PKU.

2. REVISÃO DE LITERATURA

2.1 Estratégias para localizar e selecionar as informações

Esta revisão da literatura está focada nos aspectos relacionados à neofobia alimentar e sentidos químicos em pacientes com PKU. As estratégias de busca envolveram as seguintes bases de dados: PubMed e SciELO, no ano de 1977 a janeiro de 2019. Foram realizadas buscas através dos seguintes descritores: fenilcetonúria (phenylketonuria), erros inatos do metabolismo (inborn errors of metabolism), comportamento alimentar (feeding behaviour), neofobia alimentar (food neophobia), sentidos químicos (chemical senses), hiposmia (hyposmia), hipogeusia (hypogeusia) e percepção do olfato e paladar (taste and smell perceptions). A busca limitou-se a artigos escritos em português e em inglês.

Referente à combinação dos termos "fenilcetonúria" e "comportamento alimentar", foram identificados 118 artigos (figura 1) e dos termos "fenilcetonúria" e "sentidos químicos", foram identificados 29 artigos (figura 2).
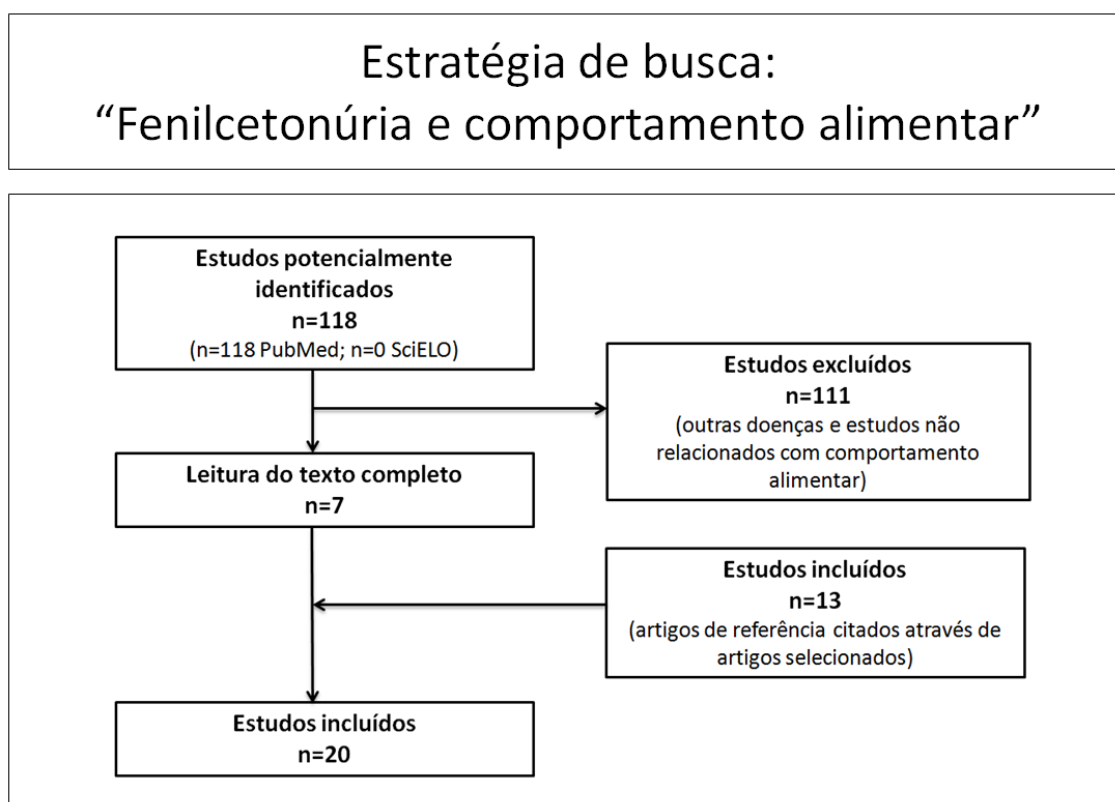
Figura 1. Fluxograma da estratégia de busca na seleção de informações sobre Fenilcetonúria e comportamento alimentar.
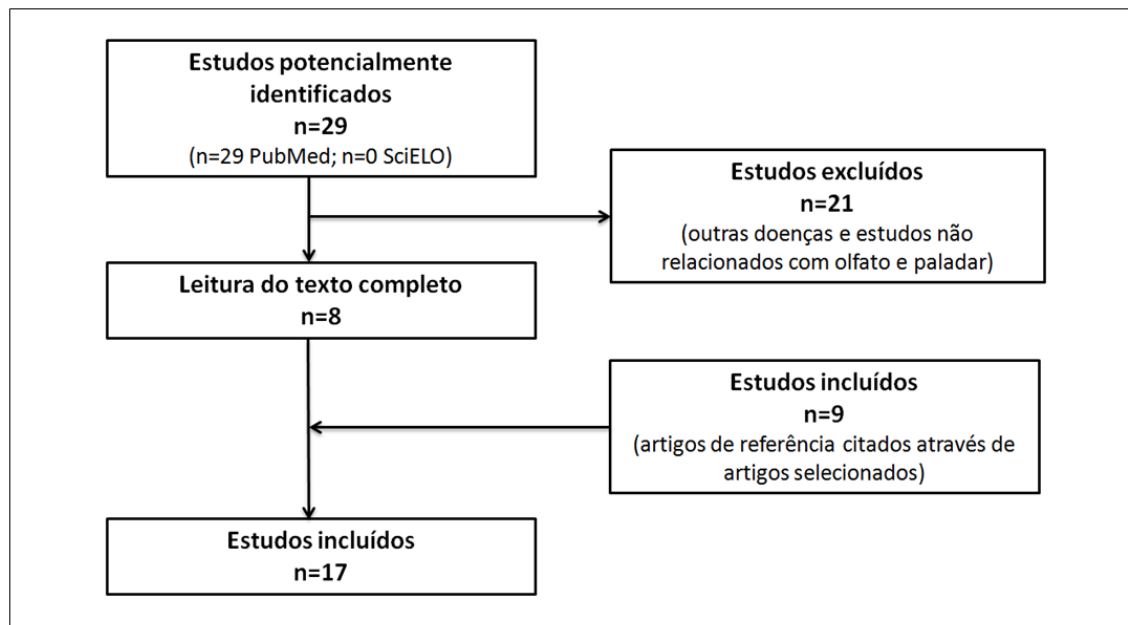
Figura 2. Fluxograma da estratégia de busca na seleção de informações sobre Fenilcetonúria e sentidos químicos.

2.2 Fenilcetonúria

Aspectos históricos

A PKU foi identificada pela primeira vez por Asbjörn Fölling, em 1934, ao observar dois irmãos com déficit mental que apresentavam corpos de fenilcetona na urina. Nove anos depois, Bickel relatou a eficácia de uma dieta pobre em Phe em uma criança com PKU (VAN WEGBERG et al 2017). A PKU, além de ser uma das primeiras doenças neurogenéticas identificadas, foi o primeiro EIM tratado com sucesso (ZSCHOCKE e HOFFMANN 2004), e a primeira doença a se beneficiar de triagem neonatal através de um teste simples desenvolvido por Guthrie em meados dos anos 1960 (VAN WEGBERG et al 2017).

Epidemiologia

Mundialmente, a incidência da doença varia entre as diferentes nações e os diferentes grupos étnicos, sendo esta mais prevalente na população caucasóide. Segundo Loeber (2007), na Europa a prevalência média é de 1:10.000 recém-nascidos. As maiores taxas são descritas em países como Turquia (1:2.600 nascimentos) e Irlanda (1:4.500), e as menores na Finlândia, Japão e Tailândia (1:200.000, 1:143.000 e 1:212.000, respectivamente) (WAISBREN et al 2007). Na América do Sul, a incidência é estimada em 1:20.000 nascimentos. No Brasil, o último estudo estimou em 1:24.780 nascimentos (CARVALHO et al 2003). Contudo, esses dados se referem a 18 dos 27 estados brasileiros, não incluindo os estados do Amazonas, Amapá, Mato Grosso, Pará, Piauí, Rio Grande do Norte, Roraima, Sergipe e Tocantins. No sul do Brasil, a primeira incidência relatada foi através do estudo de Jardim et al (1992), estimando em 1:12.500 recém-nascidos. A incidência mais recente disponibilizada na literatura é a de Pinto et al (2005), a qual aponta em 1:16.229 recém-nascidos.

Aspectos genéticos

A PKU é uma doença rara, autossômica recessiva causada por variantes (60% do tipo missense) no gene que codifica a enzima Fenilalanina Hidroxilase (PAH, EC 1.14.16.1). A PAH tem como função converter o aminoácido Phe em Tyr (figura 1), por meio de uma reação dependente de BH_4, ferro e oxigênio molecular (BLAU et al 2010). O gene codificador da enzima está localizado no braço longo do

cromossomo 12 (região q22-24.1), sendo descritas aproximadamente 1.083 (última atualização em 22/11/2018) mutações diferentes causadoras da doença (http://www.biopku.org/home/pah.asp), o que corresponde a uma gama enorme de fenótipos e, portanto, a uma grande variedade de manifestações clínicas. Contudo, a variabilidade entre os pacientes não depende apenas dos fatores genéticos; fatores ambientais e estilos de vida diferentes, como a idade no início do tratamento e o grau de controle da dieta, também contribuem para estas variações (BRASIL 2013).
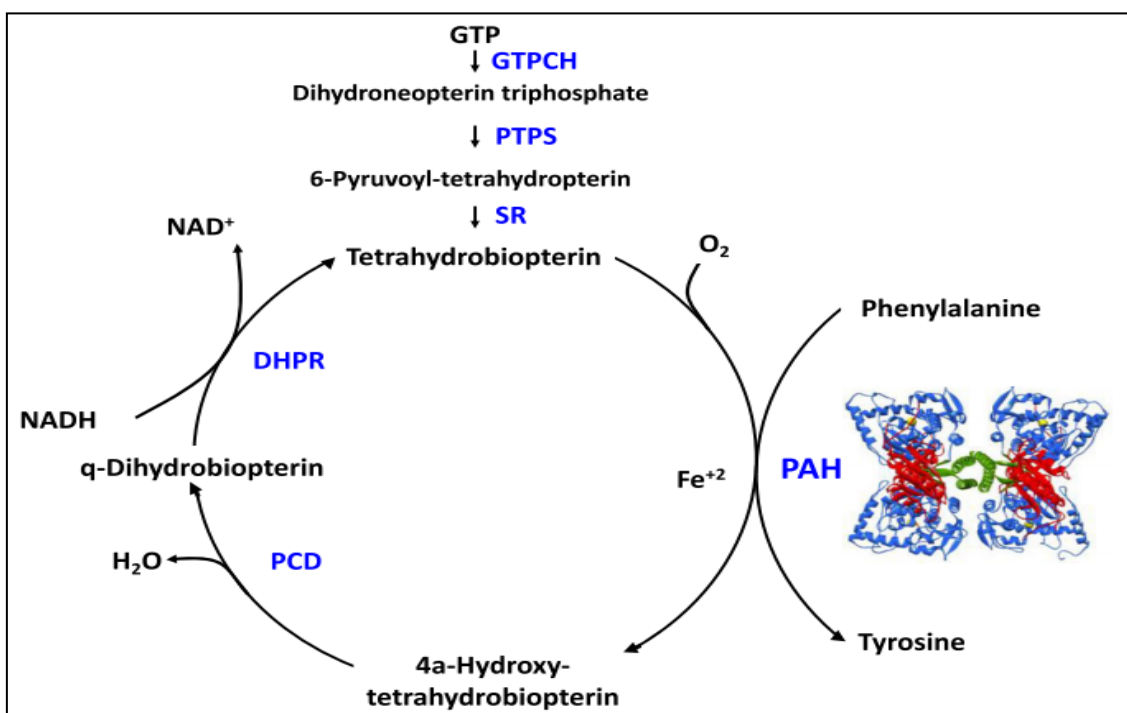
Figura 3- Sistema de Hidroxilação da Fenilalanina: BH4: Tetrahidrobiopterina; DHPR: Diidropteridina Redutase; GTP: Guanosina Trifosfasto; GTPCH: Guanosina Trifosfato Cicloidrolase; Phe: Fenilalanina; PAH: Fenilalanina Hidroxilase; PCD: Pterina 4α-carbinolamina Desidratase; PTPS: Piruvoil Tetraidrobiopterina Sintase; SR: Sepiapterina Redutase (VAN WEGBERG et al 2017).

O genótipo dos pacientes não é essencial para o diagnóstico da doença (VAN WEGBERG et al 2017), mas pode determinar o grau da disfunção proteica, atividade residual da enzima PAH e o fenótipo metabólico. Também, auxilia na predição ou exclusão da responsividade a tetrahidrobiopterina (BH_4) (BURGARD et al 2017). Os alelos conhecidos por serem responsivos ao tratamento com BH_4 estão listados no banco de dados BIOPKU (http://www.biopku.org/home/biopku.asp).

O diagnóstico pré-natal da PKU é viável e o aconselhamento genético depende de muitos aspectos, incluindo questões éticas, religiosas e legais em cada país (VAN WEGBERG et al 2017).

Aspectos bioquímicos

A Phe é um aminoácido essencial para o organismo. É utilizada principalmente para a síntese de proteína tecidual (10%) e formação de Tyr (90%), esta última responsável pela biossíntese de neurotransmissores como a dopamina e norepinefrina (ELSAS e ACOSTA, 2003; LEHNINGER, 2004). Quando a hidroxilação para Tyr é impedida, a Phe acumulada é transaminada com piruvato, produzindo o metabólito fenilpiruvato, uma fenilcetona tóxica ao cérebro (BLAU et al 2010).

A Phe compete com outros aminoácidos neutros na barreira hematoencefálica, inibindo-os e levando à redução de tirosina cerebral e triptofano. A redução destes aminoácidos limita a produção de serotonina, dopamina, noradrenalina e adrenalina. Embora as consequências da diminuição destes neurotransmissores sejam claramente definidas, os efeitos agregados permanecem obscuros (VAN WEGBERG et al 2017).

Classificação dos tipos de PKU

De acordo com o último guideline, publicado em 2017, não existe ainda literatura disponível consensual quanto à classificação do fenótipo (VAN WEGBERG et al 2017). Segundo Blau et al (2010), a PKU pode ser dividida em Clássica, Leve ou Hiperfenilalaninemia (HPA). Essa classificação é baseada nos níveis de Phe antes de qualquer intervenção dietética/tratamento: níveis de Phe acima de 1.200µmol/L (20 mg/dL) configuram a PKU Clássica; níveis de Phe entre 600-1.200µmol/L (10 mg/dL-20 mg/dL) a PKU Leve; e níveis de Phe entre 120-600µmol/L (2 mg/dL-10 mg/dL) a HPA. Há ainda outras formas de classificação, ligadas à tolerância de Phe da dieta e à razão Phe/Tyr.

Diagnóstico

O diagnóstico da PKU deve ser feito no período neonatal, sendo que o seu rastreamento no Brasil é realizado pelo "teste do pezinho", no âmbito do Programa Nacional de Triagem Neonatal (PNTN), do Ministério da Saúde (BRASIL 2013). O rastreamento da PKU atende a todos os critérios de seleção definidos por Wilson e Jungner em 1967, justificando os custos e a infra-estrutura necessários para a realização do teste de triagem (VAN WEGBERG et al 2017). Para a realização do teste, uma gota de sangue deve ser coletada do calcanhar do recém-nascido, em

papel filtro, após aporte proteico (aleitamento materno e/ou fórmulas infantis), a partir das 48 horas de vida até, no máximo, o 5º dia de vida (BRASIL 2016).

Os métodos laboratoriais utilizados para medir os níveis de Phe são: espectrometria de massa em tandem, cromatografia líquida de alto desempenho, cromatografia gasosa e testes enzimáticos e fluorimétricos. Um resultado positivo de rastreamento ocorre quando os níveis de Phe estão acima do ponto de corte, ou seja, maiores que 120 μmol/L (2 mg/dL), o qual deve ser confirmado por uma segunda análise quantitativa dos aminoácidos Phe e Tyr (NYHAN e OZAND 1998; BRASIL 2013). Nos casos confirmados de PKU, a Tyr encontra-se normal ou diminuída (BLAU et al 2010). No Brasil, o diagnóstico, o tratamento e o acompanhamento dessa doença são preconizados desde 2001 por meio do PNTN (BRASIL, 2002). Atualmente, são cadastrados no Brasil 30 Serviços de Referência em Triagem Neonatal.

De acordo com Burgard et al (2017), o diagnóstico diferencial para as diversas formas das Deficiências de BH_4 deve ser realizado em todas as crianças com HPA, sendo usualmente feito através da análise de pterinas urinárias e determinação da enzima Diidropterina Redutase em sangue total impregnado em papel filtro. Entretanto, no Brasil, o diagnóstico diferencial bioquímico não é oferecido pelo PNTN e nenhum laboratório particular executa o mesmo.

Aspectos Clínicos

Ao nascimento o paciente tem aparência normal, sendo que alguns sintomas podem ser detectados nos primeiros dias de vida, como irritabilidade, eczema e odor incomum. Entretanto, a manifestação mais importante nesses pacientes é a deficiência mental (NYHAN e OZAND 1998; JAHJA 2017). Outros sintomas descritos são a diminuição da pigmentação de cabelo, pele e íris, o crescimento reduzido, a microcefalia e o comprometimento neurológico. Também podem ser encontrados problemas comportamentais que incluem hiperatividade, agressividade e ansiedade (WALTER et al 2006). Nos últimos anos, o diagnóstico precoce e o tratamento imediato têm evitado a deficiência intelectual de muitas crianças com PKU (BLAU et al 2010).

Início do tratamento e tratamento para toda a vida

Em 1990, Smith e colaboradores relataram que a cada 4 semanas de atraso no início do tratamento há aproximadamente declínio de 4 pontos do QI (SMITH et al 1990). O tratamento deve ser iniciado o mais precoce possível, idealmente até o 10º dia de vida (VAN WEGBERG et al 2017), nunca ultrapassando os 20 dias de vida do recém nascido (BURGARD et al 2017).

Há unanimidade na literatura de que pacientes com níveis de Phe acima de 600 μmol/L devem ser tratados, ao passo de que indivíduos com níveis abaixo de 360 μmol/L devem permanecer sem tratamento, mas a monitorização deve ser seguida, no mínimo, durante o primeiro ano de vida (SCHULZE et al 2002).

O objetivo principal do tratamento é o funcionamento adequado do sistema neurocognitivo e psicossocial (VAN WEGBERG et al 2017). Para que isto aconteça, uma dieta restrita em Phe, sugerida desde os trabalhos pioneiros de Bickel, em 1953, deve ser seguida. Na tabela 1 são apresentados níveis alvo de Phe de acordo com a idade dos pacientes, segundo o último Protocolo Clínico e Diretrizes Terapêuticas (PCDT) (Brasil 2013).

Tabela 1 - Níveis Alvo de Phe Preconizados

Idade	Phe alvo (μmol/L)	Phe alvo (mg/dL)
0 – 13 anos	120 - 360	2 - 6
> 13 anos		
permitido	120 - 900	2 - 15
desejável	120 - 600	2 - 10
gestação	120 - 360	2 - 6

Fonte: Adaptado de BLAU e BLASKOVICS (1996).

Contudo, como a alimentação desses pacientes torna-se pobre de proteínas de alto valor biológico, a dieta é complementada com a utilização de fórmula metabólica isenta de Phe, que contém uma mistura de micronutrientes e aminoácidos livres (PLASENCIA et al 2009). A fórmula metabólica deve ser considerada um medicamento para os pacientes com PKU (VOCKLEY et al 2014).

Segundo o último consenso europeu, publicado em 2017, a amamentação em combinação com fórmula metabólica deve ser incentivada, pois fornece várias vantagens nutricionais e psicológicas. O leite materno é pobre em Phe (46 mg / 100

mL), contém ácidos graxos poli-insaturados de cadeia longa e muitos compostos bioativos. Além de ser conveniente, reduz o número de mamadeiras e fornece à mãe algum controle sobre o processo de alimentação (MACDONALD et al 2006; VAN WEGBERG et al 2017). Alguns estudos relataram controle metabólico de Phe e crescimento adequados com seu uso (MOTZFELDT et al 1999; VAN RIJIN et al 2003; BANTA-WRIGHT et al 2012).

Os alimentos modificados com baixo teor de proteína constituem outra categoria de alimentos médicos importantes para o manejo dietético na PKU, sendo fonte de calorias e tornando os pacientes mais saciados. Estes alimentos mimetizam alimentos ricos em proteínas e ajudam a aumentar a variedade da dieta, bem como a normalizar a aparência dos alimentos com baixo teor em Phe (VOCKLEY et al 2014). No Brasil, esses produtos não são cobertos pelo SUS; tornando seu acesso difícil devido ao alto custo comparado com o das versões não modificadas.

Em termos mundiais, existe uma ampla variação nas diretrizes nacionais em relação aos níveis de Phe para iniciar o tratamento dietético, variando de 400 µmol/L (6,6 mg/dL) no Reino Unido e 360 µmol/L (6 mg/dL) nos Estados Unidos, para 600 µmol/L (10 mg/dL) na Alemanha e na França. Não existe, até o momento, uma base científica para essas diferenças (BURGARD et al 2017). No Brasil, segundo o manual de Protocolos Clínicos e Diretrizes Terapêuticas, crianças com níveis maiores ou iguais a 600 µmol/L (10 mg/dL) devem iniciar a dieta o mais precoce possível. Níveis persistentes entre 480-600 µmol/L (8-10 mg/dL) pelo menos em 3 dosagens consecutivas semanais, em dieta normal, também indicam tratamento (BRASIL 2013).

No Brasil, o tratamento dos pacientes com PKU, conforme definido pelo PNTN, deve ser realizado em centros de atendimento estaduais especializados (Serviços de Referência em Triagem Neonatal – SRTN), incluindo também o aconselhamento genético. O acompanhamento dos pacientes deve ser feito por equipe multidisciplinar com composição mínima de médico e nutricionista especializados (BRASIL 2013).

Nos últimos anos, estudos demonstraram que não é seguro interromper o tratamento durante a infância, pré-adolescência e até mesmo na vida adulta (AZEN et al 1991; CABALSKA et al 1997; WEGLAGE et al 2013). Em relação à população de adultos com PKU nascidos antes dos testes de rastreamento (que são

atualmente incapacitados intelectualmente e que possuem problemas de comportamento), a dieta é extremamente recomendável, pois auxilia na melhora do comportamento, propiciando melhor qualidade de vida tanto aos pacientes quanto aos seus cuidadores (BLAU et al 2009; BRASIL 2013).

Monitoramento

O nível de Phe no sangue é o principal marcador do controle metabólico na PKU (FONNESBECK et al 2013). Valores flutuantes durante 24 horas parecem estar mais relacionadas à administração desigual de fórmula metabólica isenta de Phe, ao invés do estado de jejum, pós-prandial ou distribuição desigual de proteína natural (MACDONALD et al 1996; VAN SPRONSEN et al 1996; WAISBREN et al 2007; ALBRECHT et al 2009).

O manejo dietético necessário para a manutenção de níveis sanguíneos adequados de Phe exige frequente modificação para responder ao crescimento, aos estágios da vida, às doenças concomitantes e às comorbidades. É importante monitorar os níveis sanguíneos de Phe e Tyr e assegurar que outras necessidades nutricionais também sejam atendidas. O suprimento adequado de ácidos graxos essenciais, vitaminas e minerais constitui uma preocupação especial em pacientes que consomem alimentos médicos que podem não conter quantidades apropriadas às suas circunstâncias individuais (VOCKLEY et al 2014).

No Brasil, segundo o PCDT, o monitoramento dos níveis séricos de Phe deve ser feito a cada 15 dias nos pacientes de até 1 ano de idade e nas mulheres grávidas, e mensalmente nos demais. Salienta-se que essa recomendação pode ser adaptada às necessidades dos pacientes. Além disso, Vockley et al (2014) recomendam exames como albumina, hemograma completo, ferritina, 25-OH-vitamina D, vitamina B12, zinco, cobre, selênio, vitamina A e ácido fólico nos pacientes em tratamento dietético. Sugerem também que a mineralização óssea pode ser insuficiente por conta da baixa ingestão de cálcio de fontes naturais de laticínios (VOCKLEY et al 2014). O recente consenso Europeu recomenda também que sejam realizadas dosagens anuais de homocisteína e/ou ácido metilmalônico e do hormônio da paratireoide (VAN WEGBERG et al 2017).

Tratamentos farmacológicos e terapias emergentes

Apesar do tratamento com dieta restrita em Phe ser bem-sucedido, a pobre palatabilidade é responsável pela falta de adesão da mesma, principalmente por adolescentes e adultos (BLAU e LONGO 2015). As fórmulas metabólicas possuem odor e paladar desagradáveis, e sua ingestão, contrariando as orientações nutricionais, geralmente é feita de uma só vez, prejudicando sua utilização biológica e aumentando os efeitos adversos, tais como náuseas, vômitos, tonturas e diarreia (VAN WEGBERG 2017).

Para auxiliar os pacientes em razão das dificuldades impostas pela dieta, novas terapias surgiram ao longo dos anos, a saber:

- Suplementação de *Large Neutral Amino Acids* (LNAA): reduz a concentração de Phe no cérebro, mesmo com níveis plasmáticos elevados, através de competição do mesmo transportador da Phe e de outros aminoácidos neutros (REGIER et al 2017).

- Administração da enzima fenilalanina amônia liase (PAL): a PAL é uma enzima autocatalítica que não requer um cofator, presente apenas em bactérias, fungos e algumas plantas. Nessa terapia, a enzima PAL converte a Phe em quantidades metabolicamente insignificantes de amônia e de ácido trans-cinâmicos - um metabólico inofensivo que posteriormente é convertido em ácido benzóico e rapidamente excretado na urina como ácido hipúrico. Existem muitos desafios para a utilização desta enzima como tratamento, mas a mesma tem demonstrado resultados promissores em estudos clínicos (LONGO et al 2014).

- Terapia gênica: tem sido explorada em modelos animais, demonstrando resultados positivos (EMBURY et al 2007). A técnica possibilitou também o aumento dos níveis neurológicos de dopamina e serotonina a quantidades normais em camundongo com PKU (YAGI et al 2012).

- Suplementação com BH_4: pacientes com PKU podem ser sensíveis ao BH_4 quando possuem atividade residual da enzima PAH, aumentando a tolerância à PHE da dieta (SCALA et al 2015). Dicloridrato de Sapropterina *(Kuvan®, Biomarn Pharma)* é uma forma sintética oral do BH_4. Ensaios clínicos de fase II e III têm demonstrado que o *Kuvan®* é uma terapia segura e eficaz em pacientes com PKU nas formas leve e moderada, e também em alguns pacientes com PKU nas formas mais graves (BURNETT et al 2007).

No Brasil, o BH$_4$ é aprovado pela ANVISA, mas o seu fornecimento não é custeado pelo sistema público. A relação custo-efetividade do BH4 não está estabelecida, especialmente quando o tratamento dietético e a fórmula metabólica ainda são necessários. A mais recente definição de responsividade é "estabelecer um aumento na tolerância à proteína natural ≥100%, permanecendo as concentrações sanguíneas de Phe dentro da faixa alvo" (VAN WEGBERG et al 2017).

Adesão

A baixa adesão ao tratamento é comumente relatada na maioria dos tratamentos de doenças crônicas. Aconselhamento e educação são frequentemente recomendados nos serviços de saúde. Contudo, pesquisas indicam que embora o conhecimento seja necessário para adesão ao tratamento, este não é um forte preditor de adesão e consequente controle metabólico (WAISBREN et al 1997; SINGH et al 2000; BEKHOF et al 2003; DURHAM-SHEARER et al 2008; MACDONALD et al 2008). Em outro modelo de doença crônica, mudanças nas atitudes e motivação podem ser mais eficazes na adesão ao tratamento (NORRIS et al 2001). Fornecer cuidado personalizado com adequado aconselhamento e apoio é recomendado, ao invés do julgamento da má adesão (VAN SPRONSEN e BURGARD 2008).

Já é descrito que quando o paciente atinge a idade adulta a adesão torna-se inferior, pois esse grupo de pacientes tende a diminuir a frequência nas consultas de acompanhamento, e o reengajamento torna-se um desafio. Atingir essa coorte exige esforços dos serviços, e uma estratégia proposta é o estímulo ao acompanhamento clínico através das mídias sociais (BURTON e LEVITON 2010).

Um estudo realizado em nosso serviço mostrou que 32,1% dos pacientes foram classificados como aderentes ao tratamento. Entre todos os fatores analisados, apenas deficiência intelectual, morar com os pais e o nível de escolaridade materna foram associados à adesão ao tratamento (VIEIRA et al 2015).

Viver com uma restrição alimentar severa por toda a vida pode afetar negativamente o comportamento alimentar, suscetibilizando o desenvolvimento de perturbações alimentares (ANTISDEL e CHRISLER 2000). A adesão adequada da dieta torna-se estressante tanto para os pacientes quanto para suas famílias. A

literatura aponta que problemas de comportamento alimentar são mais comuns em crianças com PKU quando comparada a crianças sem nenhuma doença metabólica; estes problemas parecem estar mais associados com o manejo do comportamento alimentar do que a condição intrínseca a PKU (MACDONALD et al 1997; CRONE et al 2005; EVANS et al 2016).

2.3 Comportamento Alimentar

Estudos relatam que mais de 50% das mães de crianças saudáveis preocupam-se com alguma dificuldade alimentar de pelo menos um de seus filhos (SAARILEHTO et al 2004; CARRUTH et al 2004). As queixas em relação aos problemas alimentares englobam uma ampla gama: de "enjoado para comer" (picky eating) a formas mais severas (KERZNER et al 2009).

A literatura mostra 3 tipos anormais de comportamento alimentar em crianças, a citar: as que comem pouco, as com alimentação restrita do número de alimentos e as que possuem medo de comer. As recentes classificações não identificaram o comportamento parental anormal como uma subcategoria de problemas alimentares (KERZNER 2009). De acordo com Davies et al (2015), as dificuldades alimentares devem ser vistas numa relação entre a pessoa que alimenta com o alimentado. As dificuldades alimentares, muitas vezes, surgem durante a transição de fases da criança; da amamentação para mamadeira ou xícara, quando alimentação complementar for introduzida ou quando se inicia a alimentação de forma autônoma (COULTHARD et al 2009; DISANTIS et al 2011).

A seguir são descritas as nomenclaturas das dificuldades alimentares:
- Neofobia alimentar: Definida como rejeição de alimentos que são novos ou desconhecidos. Exposições repetidas parecem resolver o problema (DOVEY et al 2008).
- Seletividade alimentar: inclui crianças exigentes, com falta de apetite. Geralmente um problema leve ou transitório. Embora não seja considerada uma "condição médica", requer atenção (CHATOOR 2002; DOVEY et al 2008).

- Transtorno alimentar: condição grave em que existe um distúrbio restritivo de ingestão alimentar, resultando em consequências emocionais, nutricionais e orgânicas (MANIKAM e PERMAN 2000; CHATOOR 2002).
- Dificuldades alimentares: termo que simplesmente sugere que há um problema de alimentação (SAARILEHTO et al 2004).

Efeitos das experiências iniciais do sabor: do líquido amniótico aos primeiros alimentos

- Líquido amniótico e leite materno

As preferências alimentares por sabores como o sal, o doce e o amargo podem ser modificadas através de repetidas exposições aos sabores provenientes do líquido amniótico, leite materno, fórmulas infantis e alimentos sólidos (MENNELLA 2014). Uma grande variedade de sabores ingeridos (frutas, hortaliças, temperos) ou inalados (tabaco, perfumes) pela mãe são transmitidos via líquido amniótico e leite materno (aumentando significativamente a intensidade do sabor no leite materno horas após o consumo), e já se sabe que a exposição a esses sabores modulam sua aceitação na hora da amamentação, uso de fórmula e introdução alimentar (MENNELLA 2007).

As experiências sensoriais através do leite materno em crianças cujas mães consomem uma variedade de alimentos pode explicar porque as crianças que foram amamentadas tendem a ser menos seletivas (GALLOWAY et al 2003) e mais dispostas a experimentar novos alimentos durante a infância (SKINNER et al 2002; COOKE et al 2004).

Crianças que foram amamentadas por pelo menos 6 meses, demonstraram níveis mais baixos de neofobia alimentar quando comparados a crianças com um tempo de amamentação menor (GALLOWAY et al 2003).

- Fórmulas infantis

Em contraste com as variadas e ricas experiências sensoriais que o leite materno proporciona, as fórmulas infantis são consideradas monótonas. Além disso, há diferenças nos sabores entre os diferentes tipos de fórmulas (leite de vaca, leite de soja, hidrolisado parcial, hidrolisado de proteína), e os recém nascidos alimentados por estas aprendem a preferir seus sabores (MENNELLA et al 2009; MENNELLA et al 2011).

- Introdução alimentar

É ainda durante o primeiro ano de vida que as crianças fazem a transição de uma dieta líquida para uma dieta mista, composta de leite materno/fórmula e uma variedade de alimentos complementares, com uma ampla gama de sabores e texturas (MENNELLA et al 2014).

Devido à escassez de pesquisas, muitas práticas na hora da introdução alimentar se fazem de forma errônea. Um exemplo disso é de que as crianças não devem ter nenhuma experiência alimentar com frutas antes de serem introduzidas aos vegetais verdes de sabor amargo, pois suas preferências doces inerentes irão interferir na aceitação de alimentos não doces. Até o momento, nenhum dado suporta essa afirmação (GERRISH et al 2001).

Independentemente das experiências nos primeiros meses de vida, as crianças aprendem sobre alimentos complementares através de repetidas exposições a novos sabores e texturas. Isso, por sua vez, promove disposição para comer não apenas os alimentos introduzidos como também novos. A introdução de um novo alimento juntamente com um já familiarizado pode ser uma ótima combinação para uma melhor aceitação (FORESTELL e MENNELLA 2007).

Embora a reatividade dos bebês durante a alimentação esteja relacionada ao consumo, este é governado por diferentes substratos neurais. Assim, as crianças podem continuar com expressões faciais de desagrado (careta) mesmo aumentando a ingestão desses alimentos através de exposições repetidas (FORESTELL e MENNELLA 2007). Portanto, os cuidadores devem observar que as expressões faciais feitas durante a alimentação muitas vezes não correspondem à real disposição em ingerir determinado alimento. O objetivo, nesse período crítico, é acostumar as crianças a uma dieta variada que atenda às necessidades nutricionais de crescimento e desenvolvimento, e que lhes proporcione oportunidades de aprender a gostar de uma variedade de alimentos saudáveis (MENNELLA 2014).

A literatura mostra que uma única exposição repetida a um novo alimento aumenta significativamente a ingestão de alimentos semelhantes em bebês, em contraste com crianças na fase pré-escolar que podem levar de 6 a 10 exposições repetidas antes de aceitar um alimento (ANZMAN-FRASCA et al 2012). Isso sugere que a neofobia alimentar está relacionada com a idade (EVANS et al 2016).

Outro componente importante é a textura dos alimentos. Evidências suportam que o tempo de exposição a diferentes texturas também torna-se uma ferramenta importante na aceitação do novo alimento. Um estudo mostrou que bebês de 6 a 12 meses de idade, expostos a uma variedade de texturas de maçã (purê, picada e granulada), prefeririam maior complexidade de texturas quando testados posteriormente (LUNDY et al 1998).

Mais estudos são necessários para analisar se há um período ideal no qual a falta de exposição a uma variedade de texturas e sabores dificulta a aceitação de determinados alimentos a longo prazo. Muitas doenças crônicas que afligem a sociedade moderna, como obesidade, diabetes e hipertensão, derivam em grande parte de escolhas alimentares precárias, as quais se relacionam com experiências de sabores e alimentos do início da vida (MENELLA 2014).

Outros determinantes de neofobia alimentar

- Estado Nutricional:

Sabe-se que a auto-percepção da aparência física e peso corporal podem afetar o comportamento alimentar e o estado nutricional, especialmente em meninas jovens. O medo do ganho de peso, principalmente na recente era digital, pode explicar a rejeição a alimentos desconhecidos (ROßBACH et al 2016). Estudos em crianças mostraram associação de neofobia alimentar com sobrepeso (FINISTRELLA et al 2012) e baixo peso (EKSTEIN et al 2010).

- Sexo e Idade:

Hursti e Sjööden (1997) relataram maior neofobia alimentar em meninos hígidos quando comparado a meninas. Também foi observada maior neofobia alimentar nos pais das crianças em relação as mães. Em um recente estudo alemão, os adolescentes avaliados não diferiram em relação aos níveis de fobia alimentar entre meninos e meninas (ROßBACH et al 2016), mas apontam que os níveis de fobia alimentar entre os diferentes sexos são dependentes da idade. A literatura mostra que na infância e vida adulta os homens parecem ser mais neofóbicos, enquanto Cooke e Wardle (2005) acreditam que na adolescência o peso corporal e a

aparência física são tópicos mais relevantes para as meninas, tornando-as mais neofóbicas do que os meninos.

2.4 Sentidos Químicos

A relação dos sentidos com o comportamento alimentar

Segundo Netto (2007), o comportamento alimentar, incluindo apetite, escolhas alimentares e ingestão de nutrientes, é influenciado diretamente pelo desempenho do olfato e gosto, que juntos formam o paladar. Entre a importância desses sentidos, destacam-se:

- sinais quimiosensoriais que preparam o organismo para a digestão do alimento por causar secreções salivares, gástricas, pancreáticas e intestinais, as quais são denominadas respostas da fase cefálica;
- detecção e distinção das qualidades nutricionais exigidas entre os alimentos de aparência duvidosa;
- seleção de uma dieta nutritiva;
- sinais que mantém e concluem a ingestão, e portanto, desempenham uma grande função na quantidade de alimento que é consumido;
- sensações gustativas que induzem o sentimento de saciedade.

Os sentidos da gustação e olfação permitem que separemos os alimentos indesejáveis ou mesmo letais dos que nos dão prazer e nutrição. A gustação é sentida através dos botões gustatórios presentes na boca e, juntamente com a olfação, contribui intensamente para a percepção do paladar. A importância do paladar reside no fato de que ele permite à pessoa selecionar substâncias específicas, de acordo com seus desejos e necessidades metabólicas (GUYTON e HALL 2011).

De acordo com Guyton e Hall (2011), treze receptores químicos já foram identificados nas células gustatórias, a citar: dois receptores para sódio, dois receptores para potássio, um receptor para cloreto, um receptor para adenosina, um receptor para inosina, dois receptores para doce, dois receptores para amargo, um receptor para glutamato e um receptor para o íon hidrogênio; os quais agrupam-se em cinco categorias gerais chamadas sensações primárias da gustação: azedo, salgado, amargo, doce e umami. No entanto, com a descoberta recente do gosto

umami, os estudos concentram-se na percepção gustativa de quatro qualidades: azedo, salgado, amargo e doce.

Exposições precoces, tanto biológicas quanto sociais, explicam as trajetórias de saúde na vida adulta anos mais tarde. Com os alimentos, essa questão não é diferente. A ingestão excessiva de alimentos ricos em sal e açucares refinados no início da vida impacta as preferências alimentares a longo prazo e predispõe a uma série de doenças crônicas como obesidade, síndromes metabólicas e doenças cardiovasculares, razão pela qual já se sabe que as intervenções preventivas devem se concentrar no início da vida (MENNELLA 2014).

As crianças têm sistemas sensoriais que detectam e preferem os alimentos ricos em calorias e minerais de gosto doce ou salgado, enquanto rejeitam os alimentos potencialmente tóxicos de gosto amargo. As experiências sensoriais iniciais se moldam e determinam o sabor e as preferências alimentares (MENNELLA 2014).

Embora os humanos tenham respostas inatas e positivas ao açúcar e sal, e negativas ao amargo, existem também diferenças individuais geneticamente determinadas a fim de garantir que as crianças não sejam restritas a uma determinada faixa de alimentos. Os sabores anatomicamente são bem desenvolvidos ao nascimento, e desempenham decisões importantes ao longo da vida, como rejeitar ou aceitar uma substância estranha (MENELLA 2014). Além disso, eles informam ao sistema gastrointestinal sobre a qualidade e a quantidade de nutrientes ou toxinas presentes (SCLAFANI 2007).

Os gostos possuem a seguinte biologia básica:

- Doce:

O gosto doce não é induzido por categoria única de substâncias químicas. Alguns tipos provocam este sabor: açucares, glicóis, alcoóis, aldeídos, cetonas, amidos, ésteres, alguns aminoácidos, algumas proteínas pequenas, ácidos sulfônicos, ácidos halogenados e sais inorgânicos de chumbo e berílio (GUYTON e HALL 2011).

A preferência pelo doce, presente no início da vida, é notavelmente conservada em primatas. Antes do nascimento os bebês já são capazes de detectar o sabor, sendo que quando sua cavidade oral é exposta a uma solução doce, relaxam a face e muitas vezes sorriem (ROSENSTEIN e OSTER 1988). O sabor

doce também é capaz de atenuar as expressões de dor em uma criança (BLASS e WATT 1999), e sua capacidade analgésica tem sido investigada em bebês prematuros e em menor escala em crianças (MENELLA et al 2010). Inclusive, já se sabe que o sabor doce é eficaz na redução do choro espontâneo e da dor em bebês prematuros durante uma série de procedimentos dolorosos (como por exemplo, punção venosa) (HARRISON et al 2010).

A preferência pelo sabor adocicado é universal e evidente entre todas as crianças (DESOR e BEAUCHAMP 1987), permanecendo durante toda a infância e início da adolescência, diminuindo a preferência na vida adulta.

- Amargo

O gosto amargo, assim como o doce, não é induzido por categoria única de substâncias químicas. Duas classes particulares de substâncias destacam-se como indutoras: substâncias orgânicas de cadeia longa contendo (1) nitrogênio e (2) alcalóides. Os alcalóides incluem muitos dos fármacos utilizados em medicamentos, como cafeína, estricnina e nicotina (GUYTON e HALL 2011).

A detecção e rejeição do sabor amargo são evidentes logo após o nascimento (STEINER et al 2001). Respostas inatas têm efeitos na aceitação inicial de alimentos como vegetais verde-escuros. Em modelos animais, a falta de exposição precoce ao amargo afeta o desenvolvimento do sistema gustativo, enquanto a exposição repetida ao sabor no início da vida modifica as preferências alimentares na vida adulta (HARDER et al 1989).

Deve-se ressaltar que a sensibilidade para o gosto amargo é muito maior do que para todos os outros gostos. Isso pode ser explicado pelo fato de que muitas toxinas perigosas têm este sabor (GUYTON e HALL 2011).

Ao examinar o gosto dos aminoácidos, nota-se que a maioria na forma isômera L tem gosto amargo (Tabela 2).

Tabela 2 Gosto dos aminoácidos

NÚMERO	AMINOÁCIDOS (FORMA L)	GOSTO
1	Histidina	amargo
2	Metionina	Amargo + doce
3	Valina	Amargo + doce
4	Arginina	amargo
5	Isoleucina	amargo
6	Triptofano	amargo

7	Fenilalanina	amargo
8	Leucina	amargo
9	Tirosina	amargo
10	Alanina	Doce +umami
11	Glicina	Doce +umami
12	Serina	Doce +umami
13	Treonina	Doce +azedo
14	Lisina	Doce+amargo
15	Prolina	Doce+amargo
16	Aspartato	Ácido
17	Glutamato	Ácido + umami
18	Asparagina	Ácido + umami
19	Glutamina	Ácido + umami
20	Cisteína	Amargo + doce

Adaptado de KAWAI et al 2012.

- Salgado:

O gosto salgado é provocado por sais ionizados, principalmente pela concentração de íons sódio (GUYTON e HALL 2011). As crianças também preferem concentrações maiores de sabor salgado quando comparado a adultos (BEAUCHAMP e MORAN 1984; BEAUCHAMP et al 1986; BERTIO et al 1986). Contudo, diferentemente do doce, os recém-nascidos geralmente não reagem a concentrações moderadas de sal (ROSENSTEIN e OSTER 1988). A detecção do gosto salgado se desenvolve mais tarde, entre 2 e 6 meses de vida (BEAUCHAMP et al 1986).

Curiosamente, a preferência pelo sal é diretamente afetada por experiências pré-natais (CRYSTAL e BERNSTEIN 1995; STEIN et al 2006). Bebês nascidos de mulheres que tiveram enjôos matinais moderado a grave apresentaram ingestão significativamente maior de solução salina quando comparado a bebês cujas mães relataram não ter enjôos matinais ou tê-los apenas de forma leve (CRYSTAL e BERNSTEIN 1998).

- Azedo

O gosto azedo é ocasionado pelos ácidos, isto é, pela concentração do íon hidrogênio; e sua intensidade é proporcional a concentração desse íon, ou seja,

quanto mais ácido o alimento, mais forte se torna a sensação do sabor azedo (GUYTON e HALL 2011).

- Umami

Já o umami, palavra japonesa que significa "delicioso", designa a sensação de gosto prazeroso, qualitativamente diferente do azedo, do salgado, do doce e do amargo. Umami é o gosto predominante dos alimentos que contém L-glutamato, tais como caldos de carne e queijos amadurecidos (GUYTON e HALL 2011).

Já a olfação é pouco desenvolvida nos seres humanos quando comparados com os animais. As células receptoras para a sensação da olfação são as células olfatórias, que tratam-se de neurônios bipolares derivados originalmente do sistema nervoso central. Existem pelo menos 100 sensações primárias olfatórias, em contraste com apenas três sensações primárias de cor, detectadas pelos olhos, e somente cinco sensações primárias gustatórias, detectadas pela língua. A olfação, mais ainda que a gustação, tem a qualidade afetiva de ser agradável ou desagradável. Por isso, para seleção dos alimentos, o sentido da olfação é ainda mais importante que a gustação (GUYTON e HALL 2011).

Sentidos químicos e os erros inatos do metabolismo

Muitas crianças com distúrbios metabólicos hereditários do metabolismo protéico dependem de tratamentos dietéticos complexos. Embora a prioridade seja manter o controle metabólico, com crescimento adequado, existem muitas barreiras que impedem esses objetivos (EVANS et al 2012).

Um estudo publicado recentemente avaliou os sentidos químicos de 22 pacientes com Glicogenoses Hepáticas (GSD), todos com idade ≥11 anos, sendo que 40,9% apresentaram hiposmia, ou seja, menor percepção do sentido olfatório, e 18,2% apresentaram hipogeusia, definida como menor percepção do sentido gustatório. Segundo Martinez et al (2018), a diminuição da percepção olfatória foi associada à ingestão seletiva de alimentos, visto que a GSD é uma doença genética de tratamento nutricional.

Com base no exposto, entende-se que os sentidos químicos estão relacionados e interferem na alimentação. Na PKU, um estudo de Evans et al (2015) verificou as preferências alimentares de pacientes comparados com um grupo

controle sem doença metabólica. O mesmo estudo avaliou se os pacientes com PKU, através de um questionário aplicado em seus pais, apresentavam neofobia alimentar. Trinta e cinco crianças com PKU (4-13 anos de idade) e 35 controles pareados por sexo e idade foram avaliados. Tanto os pacientes como os controles preferiram o sabor doce comparado ao azedo, amargo e salgado. Além de preferir o sabor doce, houve evidência para sugerir que as crianças com PKU preferem comida salgada (especificamente hortaliças) quando comparadas aos controles. Segundo os autores, a administração precoce e persistente de fórmula metabólica de sabor amargo não está associada com o "taste imprinting" (que permite que crianças sem a doença prefiram alimentos que foram extensivamente expostos quando crianças), em pacientes com PKU. Também concluíram que os pacientes com PKU apresentam mais neofobia alimentar quando comparado com os controles.

Embora esteja bem estabelecido que o início precoce do tratamento da PKU está associado a um melhor desfecho, é possível que as práticas alimentares destes afetem o desenvolvimento alimentar, o estado nutricional e a percepção dos sentidos químicos.

3. MARCO CONCEITUAL
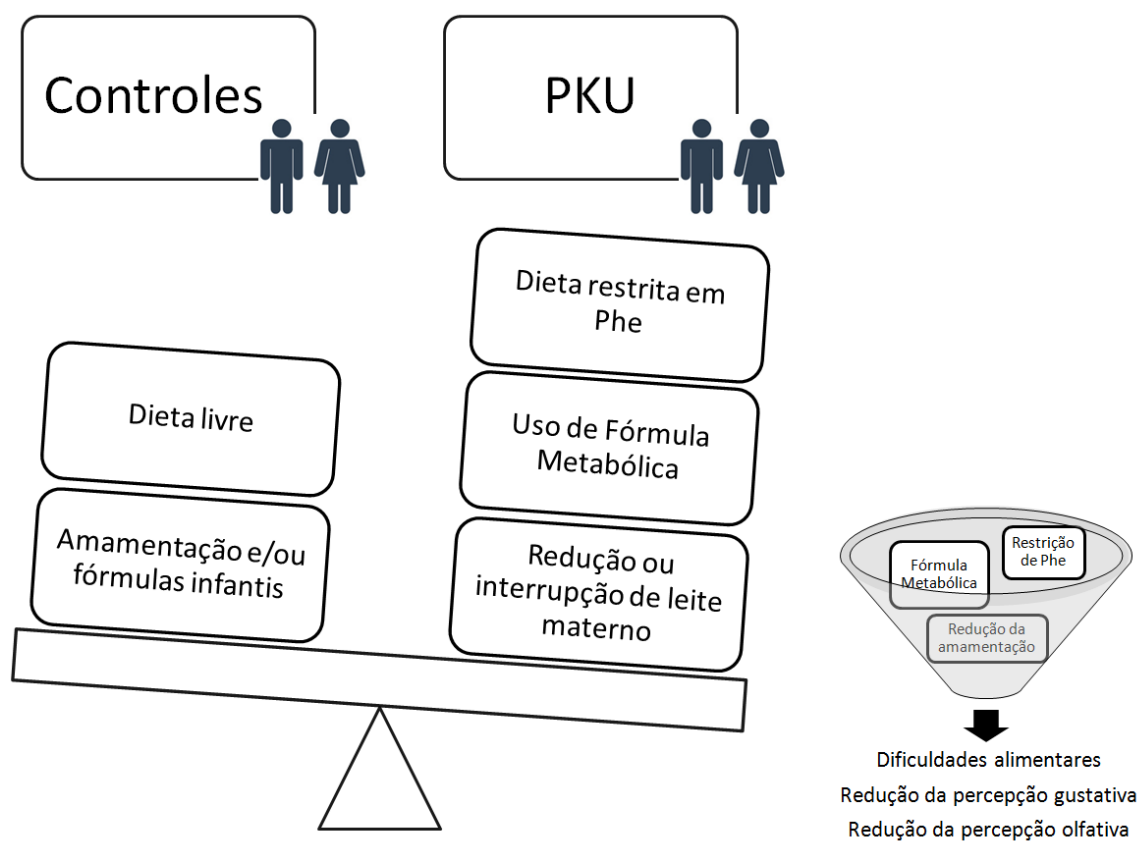
Figura 4. Mapa conceitual do estudo: representação esquemática da relação entre o tratamento da PKU, as dificuldades alimentares e os sentidos químicos.

4. JUSTIFICATIVA

O Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (SGM-HCPA) é um centro de diagnóstico, tratamento e acompanhamento de pacientes com PKU, amplamente reconhecido pela excelência na prestação de serviços. O acompanhamento dos pacientes é realizado por equipe multidisciplinar com grande experiência e formação no tratamento desta condição.

A PKU é um erro inato do metabolismo, relativamente frequente, rastreada mundialmente através dos programas de triagem neonatal, que permitem o diagnóstico precoce a fim de evitar manifestações clínicas importantes.

No Brasil, devido às políticas públicas atuais, o tratamento da PKU baseia-se exclusivamente em uma dieta restrita em Phe, suplementada com fórmula metabólica isenta deste aminoácido, enriquecida de micronutrientes e outros aminoácidos livres, compondo a principal fonte proteica da dieta.

Apesar desse tratamento ser o padrão-ouro, em países mais desenvolvidos os pacientes com PKU já se beneficiam de terapias adjuvantes, como, por exemplo, o uso do BH_4. Também, é ofertado diversas opções de alimentos com baixo teor de Phe (importantes para o manejo e variedade dietética) e variados tipos de fórmula metabólica (de acordo com a escolha dos pacientes). Ainda assim, um estudo publicado em 2016 pelo grupo da renomada nutricionista Anita MacDonald (Reino Unido), através de um questionário aplicado nos pais de crianças com PKU e nos pais de crianças controle, concluiu que os pacientes sentem-se mais apreensivos com a tentativa de experimentar novos alimentos.

Diante do exposto, motivamo-nos a pesquisar sobre o comportamento alimentar dos pacientes e seus sentidos químicos, até o momento inéditos na literatura de PKU e associar a variáveis clínicas e de tratamento.

Dessa forma, destaca-se a importância da realização desse estudo, visando à identificação de possíveis alterações para o oferecimento de intervenções futuras nessa população, a fim de melhorar o tratamento e posterior qualidade de vida dos pacientes.

5. OBJETIVOS

5.1 Objetivo Geral

1) Investigar aspectos relacionados ao comportamento alimentar e sistema sensório de pacientes brasileiros com PKU.

5.2 Objetivos específicos

1) Comparar a presença de neofobia alimentar em pacientes com PKU e em controles saudáveis;

2) Verificar os possíveis fatores associados à PKU com a neofobia alimentar;

3) Comparar a percepção dos sentidos químicos (gustativa e olfativa) em pacientes com PKU e em controles saudáveis;

4) Verificar os possíveis fatores associados à PKU com a percepção dos sentidos químicos (gustativa e olfativa).

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FOOD NEOPHOBIA IN PATIENTS WITH PHENYLKETONURIA

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Background: In phenylketonuria (PKU), only a limited range of natural foods -such as fruits, vegetables, oils, and sugars - can be consumed without restrictions. Evidence and information are limited about the clinical and treatment variables of PKU patients with the presence of food neophobia, characterized by an unwillingness to eat novel food. The present study aimed to compare the presence of food neophobia in patients with PKU and in non-PKU controls and to elucidate the factors possibly associated with this condition. **Methods:** This cross-sectional case-control study used a convenience sampling strategy to recruit patients diagnosed with PKU and healthy controls matched by sex and age. Patients and controls were invited to complete the Food Neophobia Scale (FNS) questionnaire, and clinical and treatment data were collected through a review of medical records. **Results:** Overall, 25 patients (mean age 19.3 ± 4.7 years, 13 women) and 25 controls (mean age 19.9 ± 4.9 years; p= 0.676, 13 women) were evaluated. The mean age at treatment onset among patients with PKU was 52.8 ± 29.7 days. The mean phenylalanine (Phe) level in the 12 months preceding study inclusion was 710.5 ± 346 µmol/L. The mean FNS score of patients with PKU was significantly higher (47.2 ± 9.7) than that found in controls (29.4 ± 12.5, p<0.001). Food neophobia appeared to be more associated with male sex (p=0.039). **Conclusions:** Food neophobia is frequent in patients with PKU. It does not appear to be associated with female sex or with Phe levels.
Keywords: Phenylketonuria, food neophobia, inborn errors of metabolism.

Introduction

Phenylketonuria (PKU) is an inborn error of metabolism with an autosomal recessive inheritance pattern caused by the presence of mutations in the gene that codes for phenylalanine hydroxylase (PAH, EC 1.14.16.1). PAH catalyzes the conversion of phenylalanine (Phe) to tyrosine by means of a reaction dependent on tetrahydrobiopterin, iron, and molecular oxygen[1]. In untreated patients, serum Phe concentrations are raised to neurotoxic levels, resulting in progressive, irreversible neurological impairment[2,3]. The worldwide prevalence of PKU is estimated at 1 per 10,000 live births[4]. In Brazil, the most recent survey found a prevalence of 1 per 24,780 newborns[5].

The treatment consists of a Phe-restricted diet and supplementation with a specific metabolic formula to ensure adequate intake of other amino acids and micronutrients. If a strict diet is started early (ideally before 10 days of life) and maintained carefully, normal development can be expected[2]. Although nutritional management is effective, only a very limited range of natural foods can be offered without any restrictions; these include fruits, vegetables, fats, and sugars[6]. In Brazil, medical foods with low Phe content are not provided by the public health system. Although they can be bought independently, their out-of-pocket cost is high and availability is limited, contributing to a lack of variety in the diet of PKU patients.

According to MacDonald and colleagues (1994)[6], it is important to support the development of normal eating behavior by patients with PKU, within the limits of dietary management, so that all their nutritional needs are met[6]. Food neophobia, defined as an unwillingness to eat novel foods[7], may further limit the variety of foods and, consequently, nutrients ingested by patients with PKU[6]. In recent years, neophobic eating behavior in the general population has been investigated since it can affect dietary preferences and it's quality, and variety, thus increasing the risk of developing several chronic diseases[8].

However, to date, little is known about the relationship between the presence of food neophobia in patients with PKU and clinical and treatment-related variables. Within this context, the present study aimed to compare the presence of food neophobia in patients with PKU and in non-PKU controls and to elucidate the factors possibly associated with this condition.

Methods

This was a cross-sectional, controlled study. A convenience sampling strategy was used to enroll patients with PKU followed up at the Outpatient Metabolic Disorders Clinic of the Medical Genetics Service at Hospital de Clínicas of Porto Alegre (HCPA), Brazil. The study protocol was approved by the local Research Ethics Committee. Seventy-five patients with PKU are followed at the HCPA clinic. The inclusion criteria were age ≥9 years (deemed as the minimum for understanding the applied questionnaire), current nutritional management with Phe-free metabolic formula, and absence of any intellectual impairment. Thirty patients met the inclusion criteria; of these, 25 agreed to participate in the study. The sample also included 25 healthy controls age and gender matched, included in the period from 2016 to 2018.

Clinical and demographic information, such as date of birth, gender, mean age at diagnosis, mean age at introduction of solid foods, and mean Phe levels at diagnosis and during the year prior to study inclusion were obtained by a chart review. PKU subtype was classified as mild or classical as described in Nalin and colleagues (2010)[9]. Good metabolic control was defined as in the latest European guideline[2]; for patients <13 years of age, a mean Phe level <360 µmol/L on at least three measurements obtained in the 12 months prior to study enrollment was considered satisfactory. For patients over 13 years of age, mean Phe levels <600 µmol/L were required. Data on breastfeeding and smoking were collected through a specific questionnaire.

Weight and height were used to calculate the Body Mass Index (BMI), which was expressed as the z-score for age and sex; patients were then classified as underweight, normal weight, overweight or obese, according to the World Health Organization criteria (2007)[10].

Food neophobia was evaluated through the Food Neophobia Scale (FNS), a questionnaire first developed in English by Pliner and Hobden (1992)[7] and later translated into Portuguese and validated for use in Brazil by Previato (2015)[8]. The FNS consists of a 10-item self-report questionnaire. A high average score (obtained by adding the individual scores of each item on a Likert-type scale) indicates less willingness to try new foods (neophobia), while a low average score indicates greater willingness to try new foods (neophilia). Individuals with scores ≤16.4 were classified

as neophilic, those with scores between 16.5 and 38.5 were considered neutral, and those with scores >38.6 were classified as neophobic[8].

Additionally, patients and controls were asked about their self-perception when rejecting new foods, through the following item: "Do you reject new foods?"

Statistical analyses were carried out in *Statistical Package for Social Sciences*, Version 18.0 (SPSS Inc., Chicago, IL). Descriptive analysis consisted of absolute frequencies. Continuous variables were expressed as mean and standard deviation. Student's *t*-test and the chi-square test for paired or independent samples were used. For the univariate analyses presented in the chart, Fisher's exact test was used. The level of significance was set at 5%.

Results

Fifty participants (25 patients and 25 controls) were included in this study (Table 1). The mean Phe levels at diagnosis of the patients were 1360.3 ± 671.3 µmol/L and in the 12 months prior to the study were 710.5 ± 346.4 µmol/L (range, 215.1-1408.9). The mean age at treatment onset was 52.8 ± 29.7 days.

According to the FNS, 20 patients and 7 controls were classified as neophobic (p= 0.001); 5 patients and 15 controls as neutral (p= 0.001); and 3 controls and no patients as neophilic (p< 0.001). The mean FNS score was 47.2 ± 9.7 for patients with PKU and 29.4 ± 12.5 for controls (p< 0.001).

Figure 1 shows the possible factors associated with food neophobia in patients with PKU. Female sex was the only factor significantly (p= 0.039) associated with neutrality (in this case, as a protective factor against the occurrence of food neophobia).

Discussion

In this study, a high prevalence of food neophobia was found among patients with PKU. Food neophobia does not appear to be associated with female gender, and Phe levels do not appear to be associated with food neophobia in patients with PKU.

According to Knaapila (2007)[11], from an evolutionary point of view, food neophobia is a protective phenomenon, designed to prevent humans from eating foods that are potentially harmful to health. Thus, it is unsurprising that, in our

sample, children with PKU were classified as neophobic, corroborating with the findings of Evans and colleagues (2016)[12], since these children are instructed from birth to avoid foods with high protein content, which are potentially harmful to their health.

In the past, these restrictions were recommended to PKU patients only until early adulthood, which allowed a greater variety in their choice of foods after the treatment period. Over the years, however, guidelines have been updated, and currently recommend "diet for life"[2]. In the present study, food neophobia was not only a characteristic of children, as described by Evans and colleagues (2016)[12]; it persisted even into adulthood.

Theoretically, because it allows intake of fruits and vegetables (which do not affect metabolic control) at will, the PKU diet is considered a healthy one. However, it is among the most restrictive of all known nutritional therapies. Given the central role of food in the life of a developing child, extremely strict dietary therapies can adversely affect the eating behaviors and nutritional status of these patients[13].

Few studies have addressed this issue, but patients with PKU are known to be apprehensive when trying new foods, limiting themselves to the intake of few foods that are within their dietary restrictions. The aforementioned study by Evans and colleagues (2016)[12] evaluated 35 children with PKU (age 4-13 years) through a parental-report questionnaire about their children dietary habits. We believe that our study is novel since we evaluated patients directly, and was the first, to our knowledge, to investigate the potential association between presence of food neophobia in patients with PKU and metabolic control, as well as other variables that can influence food choices in this population. In our study, diagnosis was late in the majority of patients (mean age of treatment onset was 52.8 ± 29.7 days), unlike in the Evans and colleagues (2016)[12] population, which was diagnosed by neonatal screening.

Some Determinants of Food Neophobia

Sex and Age:

Hursti and Sjööden (1997)[14] reported higher rates of food neophobia in healthy boys when compared to girls (n=722, age 7-17 years). Greater food neophobia was also observed in the children's fathers as opposed to their mothers. In a recent

German study which evaluated adolescents, food phobia levels did not differ between boys and girls[15], but the authors noted that food phobia levels between the sexes were age-dependent. The literature shows that, in childhood and adulthood, men appear to be more neophobic, whereas Cooke and Wardle (2005)[16], who evaluated 1,291 children ages 4 to 16, believe that in adolescence body weight and physical appearance are more relevant topics for girls, making them more neophobic than boys. The present study is consistent with the literature, evincing a higher level of food neophobia in boys than in girls.

Phe Levels

This was the first study to associate Phe levels with food neophobia in patients with PKU. Good metabolic control—i.e., having Phe levels within reference range— was not a determinant of greater rejection of novel foods. Regarding the Phe level at diagnosis, we were unable to verify any significant influence of Phe >1200 μmol/L on later food neophobia; however, the sample size may have limited the power of this analysis. No other study in the literature has sought comparative associations between food neophobia and Phe levels.

Breastfeeding and Diet

Another potential determinant of food neophobia is the duration of the breastfeeding period. Babies who are breastfed for longer discover new flavors as early as the first days of life, as flavors from the mother's diet are conveyed to them via the breast milk[17]. Children who were breastfed for at least 6 months demonstrated lower levels of food neophobia compared to children with shorter breastfeeding times[18]. In addition, exclusive breastfeeding may be associated with reduced food neophobia, as early introduction of food may lead to increased gastrointestinal discomfort and food allergies[18].

The World Health Organization and the Brazilian Ministry of Health recommend exclusive breastfeeding for 6 months, with complementary feeding for 2 years or longer[19,20]. However, in patients with PKU, the Phe-free metabolic formula should ideally be introduced as early as the first month of life, substantially reducing their breast milk intake after diagnosis and thereby modifying their taste perceptions into childhood. The Phe-restricted diet for PKU patients is rife with obstacles, such as

limited food variety, the unpalatability of the metabolic formula (which tastes strong and bitter), and the rigid dietary management routine. MacDonald and colleagues (1997)[13] associates the food neophobia found in children with PKU to factors such as an innate fear of eating foods they consider "unsafe", limited food choices, palatability issues, and a lack of appetite due to use of the metabolic formula.

We believe that use of the metabolic formula; the significant, early reduction of breast milk intake; and the limited choice of foods are all factors that trigger food neophobia in patients with PKU. In Brazil, these issues are compounded by the fact that medical foods with low Phe content are not provided by the public health system. Although they can be bought independently, their out-of-pocket cost is high and availability is limited, contributing to a lack of variety in the diet of PKU patients. Furthermore, problems related to the metabolic formula are frequent, such as shortages of government supply and the inability to choose more palatable formulations, making treatment even more difficult.

Conclusions

Long-term good metabolic control—i.e., having Phe levels within reference range—without impairing dietary behavior is a challenge for patients, their families, and healthcare providers. We believe that the introduction of adjuvant therapies could contribute to better management of Phe levels in patients, allowing greater exposure to novel foods. The importance of developing normal eating behavior within the constraints of a Phe-restricted diet, encouraging intake a wide range of flavors to maximize healthy food choices and to promote better social interaction, cannot be underestimated. Education strategies involving programs to promote safe and healthy diets and dietary practices should be encouraged at facilities that serve PKU patients and their families.

Acknowledgments

Transparency Declaration:

The lead author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported. The reporting of this work is compliant with STROBE guidelines. The lead author affirms that no important aspects of the study have been omitted and that any discrepancies from the study as planned have been explained.

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Table 1: Demographic and clinical characteristics of patients with phenylketonuria and controls

	Patients (n= 25)	Controls (n=25)	p
Gender (F/M)	13/12	13/12	NS
Current age (years)**	19.3 ± 4.7	19.9 ± 4.9	0.676
Breastfeeding	None	21	<0.001
Smoking	None	None	NS
Food neophobia	15	4	0.003
Classical/Mild PKU	13/12	-	NS
Good metabolic control	10	-	NS

PKU= phenylketonuria; F= female: M: male; Breastfeeding= exclusive up to 6 months of age; Smoking= no patient declared to be a smoker; Food neophobia= self-perception when rejecting new foods; Good metabolic control= for patients <13 years of age, a mean Phe level of <360 μmol/L; for patients >13 years of age, a mean Phe levels <600 μmol/L; NS= non significant.
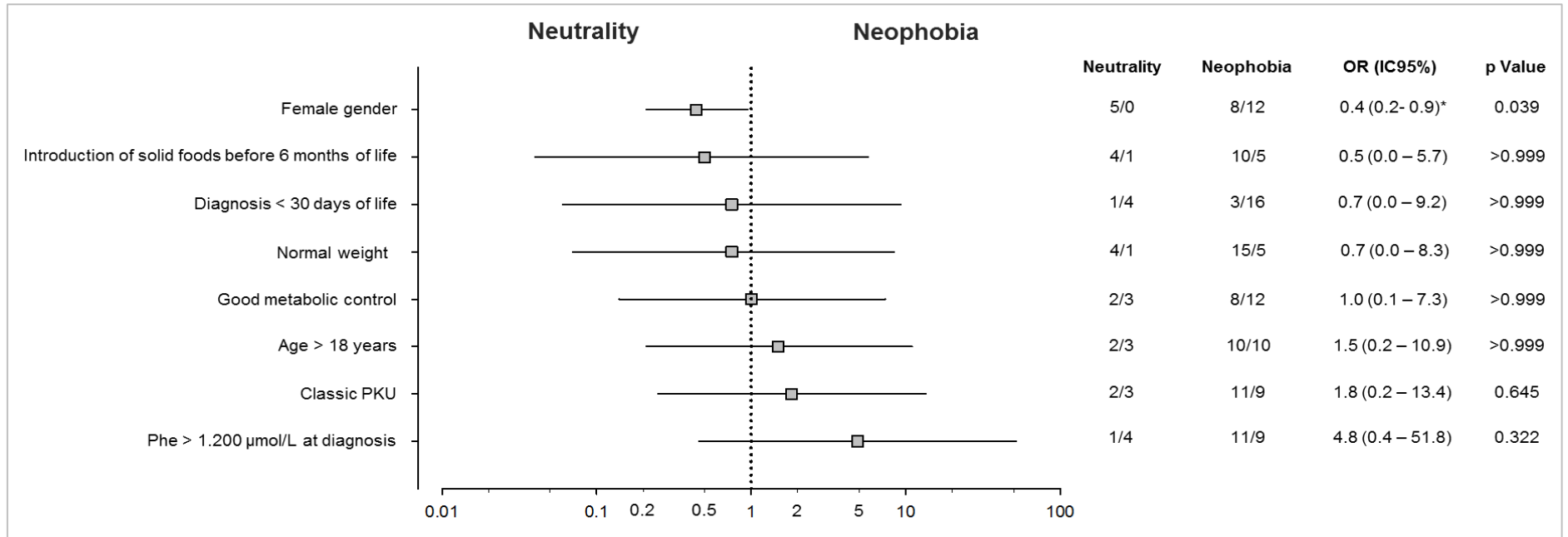
Figure 1 - Potential factors associated with food neophobia in patients with PKU. Univariate analysis representing the association between patients with PKU and the factors involved with food neophobia. Odds ratios are represented by a log scale with their respective confidence intervals (95%) and statistical significance (p) obtained by Fisher's exact test. * Values estimated by the likelihood ratio with Agresti correction.

7. Artigo II – Em elaboração (artigo com as normas da revista "JIMD Reports")

FENILCETONÚRIA: EFEITO DO TRATAMENTO SOBRE AS PERCEPÇÕES GUSTATIVA E OLFATIVA

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Introdução: Na Fenilcetonúria (PKU), pouco se sabe sobre o impacto que a dieta altamente restritiva em Fenilalanina (Phe) e uso diário de fórmula metabólica podem causar sobre o paladar e olfato dos pacientes. O presente estudo teve como objetivo avaliar as percepções olfativa e gustativa de pacientes brasileiros com PKU e associa-las a marcadores da doença. **Métodos**: Estudo tranversal, controlado, o qual incluiu pacientes com diagnóstico de PKU em tratamento dietético e controles hígidos, pareados por sexo e idade. A percepção olfativa foi avaliada através do teste *12-item Sniffin' Sticks* e a percepção gustativa foi avaliada através do teste *Taste Strips*. **Resultados:** Vinte e cinco pacientes (média idade: 19,3 ± 4,7 anos; sexo feminino= 13) e 25 controles (média de idade 19,9 ± 4,9 anos, p=0,676; sexo feminino= 13) foram avaliados. A média de idade do início do tratamento dos pacientes com PKU foi de 52,8 ± 29,7 dias. Os escores de percepção olfativa e gustativa encontrado nos pacientes (9,3 ± 1,6 e 10,0 ± 2,5, respectivamente) foram menores do que aqueles encontrados nos controles (10,3 ± 1,6, p= 0,039; e 11,9 ± 2,0, p= 0,004, respectivamente). Além disso, os escores para os sabores amargo e salgado foram menores nos pacientes quando comparado aos controles (p= 0,008 e p= 0,020). Nenhum paciente analisado recebeu aleitamento materno exclusivo até os 6 meses de vida. Em relação aos controles, o aleitamento materno exclusivo até os 6 meses de idade foi seguido em 21 indivíduos (p <0,001). Em relação aos pacientes, foi encontrada correlação entre os níveis de Phe ao diagnóstico com a

menor percepção ao sabor amargo (p= 0,036 r= -0,421). **Conclusões:** Os menores escores de percepção olfativa e gustativa encontrados em pacientes com PKU podem estar relacionados à falta de estímulos causados pela dieta altamente restritiva, privação de aromas oriundo do leite materno e uso precoce de fórmula metabólica.

Palavras-chave: Fenilcetonúria; Percepção olfativa; Percepção Gustativa; Erros Inatos do Metabolismo.

Introdução

A Fenilcetonúria (PKU) é um erro inato do metabolismo dos aminoácidos, causada pela atividade deficiente da enzima Fenilalanina Hidroxilase, necessária para converter fenilalanina (Phe) em tirosina (van Wegberget al 2017). O tratamento, que deve ser iniciado idealmente antes dos 10 dias após o nascimento e mantido por toda a vida, consiste em manejo nutricional através de uma dieta altamente restrita em Phe e suplementada por uma mistura de aminoácidos sintéticos (fórmula metabólica), isenta em Phe, sendo esta a principal fonte proteica e de micronutrientes da dieta dos pacientes (Evans et al 2016). O objetivo do tratamento é manter as concentrações plasmáticas de Phe dentro dos valores preconizados, que suportam o crescimento e o desenvolvimento físico e neurológico adequados (MacLeod and Ney 2010).

A prescrição dietética individualizada é necessária para atender às necessidades nutricionais de cada paciente. Um importante desafio na PKU é a necessidade permanente de adesão à dieta altamente restritiva em Phe suplementada com as fórmulas metabólicas de gosto e cheiro desagradáveis (Owada et al 2000). O gosto amargo e não palatável da fórmula metabólica é explicado pelos aminoácidos que compõe a mesma, como, por exemplo, a isoleucina, a leucina, o triptofano e a histidina (Evans et al 2016). No Brasil, a fórmula metabólica é reembolsada pelo sistema público de saúde. Contudo, os pacientes não participam do processo de escolha de qual marca será disponibilizada.

A restrição alimentar dos pacientes com PKU é uma das mais rígidas dietas conhecidas (Mac Donald et al 1994), e inclui a exclusão completa de alimentos de origem animal, leguminosas, oleaginosas, e alguns cereais. Até o momento, pouco se sabe sobre o impacto destas restrições dietéticas sobre os sentidos químicos

desses pacientes. Dentre as importâncias dos sentidos da gustação e olfação, está a habilidade em separarmos os alimentos indesejáveis ou mesmo letais dos que nos dão prazer e nutrição. O paladar é resultante da combinação do gosto, do olfato e da sensação trigeminal (tato e temperatura) (Guyton and Hall 2011). Entre as percepções básicas dos sabores, o salgado e doce são considerados inatos, geneticamente ligados e evidentes ao nascimento (Anliker et al 1991). Segundo Evans e colaboradores (2016), na PKU, uma importante influência sobre o desenvolvimento de preferências alimentares ocorre no início da administração de fórmula metabólica, podendo ser "gravada" uma preferência por sabores amargos.

Crianças que receberam fórmulas de hidrolisado de proteína, cujo sabor se assemelha às fórmulas para PKU, são mais propensas a preferir alimentos de gosto salgado, amargo e azedo, quando comparado a crianças que receberam leite materno ou fórmulas mais doces à base de leite (Beauchamp and Mennella 2011). Assim, é possível que pacientes com PKU tenham diferentes percepções olfativas e gustativas devido à introdução de fórmula metabólica e privação de vários alimentos por conta da dieta restrita em Phe nos primeiros meses de vida. O objetivo do presente estudo é avaliar a percepção olfativa e gustativa de pacientes brasileiros com PKU e associar a marcadores da doença.

Metodologia

Trata-se de um estudo transversal, controlado, que incluiu pacientes acompanhados no Ambulatório de Tratamento de Distúrbios Metabólicos do Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, e que foi aprovado pelo Comitê de Ética em Pesquisa local. Para ser convidado a participar do estudo, o paciente deveria ter idade ≥9 anos (idade mínima para compreensão dos testes aplicados), estar em tratamento dietético com uso da fórmula metabólica isenta em Phe e não possuir déficit intelectual. Vinte e cinco pacientes aceitaram participar do estudo. A amostragem também incluiu 25 controles hígidos sem qualquer doença metabólica, pareados por sexo e idade. Os dados foram coletados no período de 2016 a 2018.

Informações relativas à caracterização clínica dos pacientes, tais como, Phe ao diagnóstico e média de Phe do ano anterior ao teste foram obtidos por meio de revisão de prontuário. A definição de bom controle metabólico seguiu o preconizado pelo último *guideline* europeu (van Wegberg et al 2017); para pacientes <13 anos de

idade, foi considerado como bom controle metabólico uma média de Phe <360µmol/L nos 12 meses anteriores ao estudo (mínimo três dosagens). Já para os pacientes ≤ 13 anos de idade, a média dos níveis de Phe deveria ser <600µmol/L. O tipo de PKU foi classificado como Leve ou Clássica de acordo com Nalin et al (2010). Tabagismo e dados de histórico de amamentação foram também coletados através de questionário específico.

A partir do peso e estatura, foi calculado o Índice de Massa Corporal (IMC) dos pacientes. O IMC foi expresso em escore-z e classificado, de acordo com idade e sexo, em desnutrição, eutrofia, sobrepeso e obesidade, segundo critérios da Organização Mundial da Saúde (2007).

Procedimento

A percepção olfativa foi avaliada através do teste *12-item Sniffin' Sticks* (Burghart, Wedel, Germany) (Hummel et al 2001), baseado na identificação de cheiros através de aparelhos semelhantes a canetas impregnadas de odores. De modo geral, para cada uma das 12 canetas, os participantes deveriam escolher a resposta correta a partir de uma lista de quatro palavras, entre as quais encontrava-se a correspondente ao odor impregnado na caneta. A soma da pontuação representa um escore entre 0 a 12, o qual determina a triagem de normosmia – percepção olfativa normal - (escore ≥10), hiposmia – diminuição da percepção olfativa - (escore ≥6 – 9) ou anosmia – perda total da percepção olfativa - (escore <5). Pacientes com triagem positiva para hiposmia ou anosmia foram convidados para uma avaliação otorrinolaringológica, a fim de verificar possíveis defeitos anatômicos das vias aéreas, as quais poderiam afetar o resultado do teste. Também, através de uma investigação subjetiva do médico otorrinolaringologista, foi questionado sobre a percepção olfativa dos pacientes através da seguinte pergunta: "O que você acha da sua percepção olfativa?".

A percepção gustativa foi avaliada através do teste *Taste Strips*, (Burghart, Wedel, Germany). O kit utilizado consiste em 18 tiras de papel absorvente impregnadas em quatro diferentes concentrações dos quatro principais sabores (doce, amargo, azedo e salgado), e também de duas tiras onde não são adicionados sabores. As tiras foram colocadas em contato com o terço anterior dos lados direito e esquerdo da língua, bem como na região central. A cada troca de tira, os participantes foram instruídos a lavarem a boca com água. As tiras foram utilizadas nas concentrações conforme indicação do fabricante (da menor concentração para

maior concentração, sendo os sabores intercalados). A soma da pontuação de todos os estímulos representa um escore entre 0 e 16, a qual determina a triagem de normogeusia – percepção gustativa normal - (escore ≥ 10-16) ou hipogeusia – diminuição da percepção gustativa - (escore ≤ 9). As pontuações para a identificação do tipo específico de cada sabor variam de 0-4 pontos.

A análise estatística foi realizada através do Programa *Statistical Package for Social Sciences, versão 18.0* (SPSS® Inc, Chicago, IL). A análise descritiva foi realizada com o fornecimento das frequências absolutas. As variáveis contínuas foram apresentadas como média e desvio-padrão. Foi utilizado o teste t de Student e Qui-quadrado para amostras pareadas ou independentes e correlação de Pearson. O nível de significância considerado foi de 5%.

Resultados:

Vinte e cinco pacientes (sexo feminino= 13) e 25 controles (sexo feminino= 13) participaram do estudo. A média de idade dos pacientes foi de 19,3 ± 4,7, e dos controles de 19,9 ± 4,9 anos (p= 0,676). A média de Phe ao diagnóstico foi de 1360,28 ± 671,31 µmol/L, e nos 12 meses anteriores ao teste foi de 710,5 ± 346,4; range= 215,1-1408,9 µmol/L. A média da idade no início do tratamento foi de 52,8 ± 29,75; range= 11-134 dias. Dados demográficos e de caracterização dos pacientes são apresentados na Tabela1.

Devido ao tratamento para PKU e introdução da fórmula metabólica, nenhum dos 25 pacientes analisados recebeu aleitamento materno exclusivo até os 6 meses de vida (tabela 1). Em relação aos controles, o aleitamento materno exclusivo até os 6 meses de idade foi seguido em 21 indivíduos (p<0,001). Atualmente, dezessete pacientes (68%) fazem uso de açúcar, maltodextrina ou outros saborizantes com o intuito de mascarar o gosto da fórmula metabólica.

Em relação à percepção olfativa, a média do escore dos pacientes foi significativamente mais baixa 9,36 ±1,6 quando comparada com a pontuação dos controles 10,32 ±1,6 (p= 0,039). Entre os pacientes, 52% apresentaram escores médios abaixo do ponto de corte, indicando triagem positiva para hiposmia, e entre os controles esse número foi de 28%. Nenhum paciente ou controle apresentou escores indicativos de anosmia.

Para a percepção gustativa, a pontuação média do escore dos pacientes com PKU foi significativamente menor – 10,00 ±2,5 - quando comparada com a dos

controles – 11,96 ±2,0 (p= 0,004). Entre os pacientes, 44% apresentaram escores médios abaixo do ponto de corte, indicando triagem positiva para hipogeusia, e em relação aos controles esse número foi de 12%. A tabela 2 apresenta o escore médio dos pacientes e controles em relação a cada um dos sabores testados. Nela podemos observar que os escores dos sabores amargo e salgado foram significativamente menores nos pacientes quando comparado aos controles. Também, os menores escores encontrados para o sabor amargo nos pacientes com PKU foram correlacionados com os altos níveis de Phe ao diagnóstico (p= 0,036 r= -0,421).

Não foi observada correlação entre as variáveis hiposmia e hipogeusia com os níveis de Phe ao diagnóstico, níveis de Phe do ano anterior ao teste, controle metabólico, tipo de PKU e estado nutricional (dados não apresentados).

O exame clínico e de nasofibroscopia, realizado por um médico otorrinolaringologista, foi obtido em 5 dos 13 pacientes com triagem positiva para hiposmia. Alterações anatômicas não relacionadas à percepção do olfato foram encontradas, como desvio de septo, em todos os pacientes avaliados. Em 2 casos foi relatado queixa de percepção reduzida de odor (tabela 1).

Discussão

Este foi o primeiro estudo a avaliar as percepções olfativa e gustativa de pacientes com PKU e associar a marcadores da doença. Nossos achados inéditos indicam alta prevalência de hiposmia e hipogeusia em pacientes com PKU quando comparada com controles saudáveis. Além disso, foi observado menor escore da percepção gustativa dos sabores amargo e azedo dos pacientes quando comparado com os controles. Os altos níveis de Phe ao diagnóstico parecem interferir na percepção do sabor amargo.

Segundo a literatura, experiências aromáticas precoces, que ocorrem ainda na vida intra-uterina, podem influenciar na aceitação de alimentos após a amamentação, visto que o aroma placentário muda em função das escolhas alimentares da mãe (Mennella 2014). A aprendizagem do sabor continua após o nascimento como consequência da exposição a fórmulas infantis e/ou leite materno, seguido pela introdução de alimentos sólidos. A amamentação permite, ao contrário da alimentação com uso de fórmula infantil, uma rica fonte de variação de experiências de sabor. Crianças que receberam hidrolisados de proteínas de sabor

amargo durante a infância são mais propensos a preferir alimentos salgados, amargos e azedos, quando comparado a crianças que receberam fórmulas mais doces à base de leite (Beauchamp and Mennella 2011). No presente estudo, podemos observar que o uso de fórmula metabólica nos pacientes, a diminuição considerável da oferta de leite materno ou até mesmo a interrupção do mesmo, e a dieta restrita de vários alimentos provavelmente alteraram as percepções olfativas e gustativas dos pacientes com PKU.

A literatura apresenta apenas um artigo (Martinez et al 2018) sobre a relação do paladar e olfato em pacientes com algum tipo de erro inato do metabolismo, em que o tratamento seja através de uma dieta altamente restritiva. No estudo, 22 pacientes com Glicogenoses Hepáticas (GSD), com idade ≥11 anos foram avaliados. Destes, 40,9% dos pacientes tiveram triagem positiva para hiposmia e 18,2% tiveram triagem positiva para hipogeusia, sendo que a maior dificuldade foi em relação ao reconhecimento do sabor azedo, fato este explicado pela falta de estímulos causados pela dieta restritiva de frutas, sendo uma das principais fontes de sabor cítrico. Assim como a PKU, o tratamento da GSD é nutricional, carente de carboidratos (frutose, maltose, glicose, lactose e galactose), com a suplementação de amido de milho cru várias vezes ao dia (Weinstein and Wolfsdorf 2002; Derks and Smit 2015). Contudo, os pacientes com GSD são diagnosticados mais tardiamente quando comparado com os pacientes PKU (a GSD não está incluída no programa de triagem neonatal do Brasil), fato que justifica maior prevalência de hiposmia (52%) e hipogeusia (44%) de nosso estudo.

Segundo Martinez et al., 2018, existe uma alta prevalência de dificuldades alimentares e distúrbios miofuncionais orofaciais em pacientes com GSD, associados à diminuição da percepção do paladar e olfato. Os autores acreditam que as várias experiências sensoriais da alimentação na infância desempenham um importante papel na promoção de hábitos alimentares adequados e agradáveis.

Nosso estudo está de acordo com os achados de Martinez et al (2018), onde a percepção olfativa e gustativa pode ser reduzida pela falta de estímulos causados pela dieta restritiva. Este fato pode ser corroborado através de nosso estudo, que analisou detalhadamente os sentidos químicos através de uma avaliação otorrinolaringológica feita nos pacientes PKU que apresentaram triagem positiva para hiposmia, onde não evidenciou nenhum defeito anatômico que pudesse interferir nas dificuldades sensoriais apresentadas.

A fórmula metabólica para pacientes com PKU é considerada impalatável para indivíduos não expostos, ou seja, indivíduos sem a doença (Beauchamp and Mennella 2011). Contudo, um estudo feito por Owada e colaboradores (2000) verificou que crianças com PKU preferem o sabor desagradável da fórmula metabólica quando comparada a uma nova formulação ofertada (considerada mais palatável por indivíduos que não tem a doença). No Brasil, os pacientes não participam do processo de escolha de qual marca será fornecida e, muitas vezes, o sabor ofertado prejudica não somente a palatabilidade como também a adesão ao tratamento, forçando o uso exagerado de açúcar, adoçantes e outros sabores que são projetados para mascarar o sabor da fórmula. De acordo com Evans e colaboradores (2016), crianças com PKU preferem o sabor doce quando comparado com crianças sem a doença e, como consequência, a ingestão de doces, biscoitos e refrigerantes acaba sendo maior nessa população.

De acordo com Guyton e Hall (2011), a sensibilidade para o gosto amargo é muito maior do que para todos os outros gostos, fato justificado devido ao sabor amargo que muitas toxinas perigosas apresentam. Acreditamos que a menor percepção gustativa do sabor amargo relatada em nosso estudo em pacientes fortemente expostos a fórmula metabólica seja devido a uma "saciedade sensorial específica" (sensory-specific satiety; Rolls et al 2000), que torna menos responsiva a um sabor ao qual foi extensivamente exposto. Evans e colaboradores (2016) acreditam que o "taste imprinting", o qual permite que crianças sem a doença prefiram alimentos que foram extensivamente expostos quando crianças, não exista nos pacientes com PKU, visto que estes preferem sabores adocicados (devido ao tratamento, sabores não estimulados até a introdução de alimentos sólidos). Mais estudos são necessários, mas nossos resultados corroboram e complementam essas ideias, visto que a falta de "taste imprinting" acarreta uma menor percepção dos sabores que foram fortemente expostos logo após o nascimento. Nota-se que este fato pode ser agravado, quando os níveis de Phe ao diagnóstico são extremamente altos, necessitando de uma dieta ainda mais rigorosa já nos primeiros dias de vida.

Embora a literatura mostre que crianças com PKU preferem mais o sabor doce quando comparadas a crianças controles sem a doença, há evidencias de que estas também preferem mais os alimentos salgados, (principalmente os vegetais), fato que pode ser associado ao uso de fórmula metabólica (Evans et al 2016).

Assim, novamente associamos a menor percepção gustativa pelo sabor salgado à "saciedade sensorial específica", que torna menos responsiva a um sabor ao qual é repetidamente exposto.

Nossos achados também podem estar associados com a percepção olfativa e gustativa reduzida associados à presença de dificuldades alimentares já relatadas na literatura desde 1994 por MacDonald e colaboradores. De acordo com Evans et al (2016), uma coorte de 35 crianças com PKU mostrou-se mais apreensiva com a tentativa de ingerir alimentos que são novos e desconhecidos quando comparado com as 35 crianças controles do estudo. Essa associação também vai de acordo com o estudo de Martinez et al., 2018, em que as experiências sensoriais gustativas e olfativas, juntamente com as táteis (repletas de diferentes texturas e espessuras), ainda no período da infância desempenham um papel importante na promoção de hábitos alimentares adequados e agradáveis. No estudo, Martinez e colaboradores (2018) verificaram associação da percepção olfativa reduzida com seletividade alimentar.

Acreditamos que avaliações sensoriais devem fazer parte da rotina dos pacientes, a fim de identificar possíveis alterações dos sentidos químicos causados pelo rígido tratamento dos pacientes com PKU, principalmente daqueles com níveis de Phe altos ao nascimento e que necessitam de uma dieta mais rigorosa. As consequências destas alterações podem modificar as escolhas alimentares dos pacientes e futuramente exacerbar uma situação de doença nutricional, como perda de peso, por exemplo.

Nossos achados indicam alta prevalência da percepção reduzida do olfato e paladar em pacientes brasileiros com PKU, nos levando a crer que esses resultados sejam relacionadas à falta de estímulos causados pelo uso de fórmula metabólica e dieta altamente restritiva desde o nascimento, com consequente privação de aromas oriundo do leite materno.

AGRADECIMENTOS

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Tabela 1: Características demográficas e clínicas de pacientes com Fenilcetonúria (n=25).

Paciente	Gênero	Idade	Phe diagnóstico	Tipo PKU	Tratamento	Aleitamento materno	SST	Avaliação Otorrinolaringológica	TS	Fumante	Fumante passivo	Bom Controle Metabólico
1	F	15,0	1996,5	Clássica	Dieta+FM	Não	7	Sim*	8	Não	Sim	Sim
2	M	14,9	1022,5	Leve	Dieta+FM	Não	7	Sim	14	Não	Sim	Sim
3	M	15,8	1845,3	Leve	Dieta+FM	Não	9	Não	10	Não	Sim	Sim
4	F	16,2	1070,9	Leve	Dieta+FM	Não	6	Sim	6	Não	Sim	Não
5	F	24,3	1633,5	Clássica	Dieta+FM	Não	11	-	12	Não	Não	Sim
6	M	20,0	2051,0	Clássica	Dieta+FM	Não	10	-	8	Não	Não	Não
7	M	16,9	2268,8	Clássica	Dieta+FM	Não	10	-	11	Não	Sim	Não
8	F	19,2	1724,3	Clássica	Dieta+FM	Não	10		8	Não	Não	Não
9	M	26,2	2946,4	Clássica	Dieta+FM	Não	8	Sim	10	Não	Não	Não
10	F	20,2	1923,9	Clássica	Dieta+FM	Não	9	Não	9	Não	Não	Não
11	F	18,5	901,5	Clássica	Dieta+FM	Não	11	-	12	Não	Não	Não
12	F	9,3	1784,8	Clássica	Dieta+FM	Não	9	Não	10	Não	Não	Não
13	F	18,4	605,0	Leve	Dieta+FM	Não	9	Não	9	Não	Sim	Sim
14	M	15,8	871,2	Leve	Dieta+FM	Não	11	-	5	Não	Não	Não
15	M	17,3	1681,9	Leve	Dieta+FM	Não	7	Sim*	8	Não	Não	Sim
16	F	21,6	901,5	Leve	Dieta+FM	Não	11	-	12	Não	Não	Sim
17	M	16,4	490,1	Leve	Dieta+FM	Não	9	Não	9	Não	Não	Sim
18	M	27,7	2099,4	Clássica	Dieta+FM	Não	12	-	7	Não	Não	Não
19	M	21,8	738,1	Clássica	Dieta+FM	Não	10	-	10	Não	Sim	Não
20	F	17,6	1179,8	Leve	Dieta+FM	Não	10	-	13	Não	Não	Não
21	M	21,7	847,0	Clássica	Dieta+FM	Não	11	-	9	Não	Não	Não
22	F	16,6	405,4	Leve	Dieta+FM	Não	11	-	15	Não	Não	Sim
23	F	25,2	1028,5	Leve	Dieta+FM	Não	8	Não	11	Não	Sim	Não
24	M	30,6	1645,6	Clássica	Dieta+FM	Não	8	Não	10	Não	Não	Não
25	F	17,0	344,9	Leve	Dieta+FM	Não	11	-	14	Não	Não	Sim

Gênero= F: feminino, M: masculino; Phe ao diagnóstico em µmol/L; Idade= em anos; Tratamento= FM: fórmula metabólica; SST: Sniffin' Sticks Test; Avaliação otorrinolaringológica: avaliação clínica + nasofibroscopia (*dois casos foi relatado queixa de percepção reduzida de odor); TS= taste strips; Fumante passivo= pais fumam; Bom controle metabólico= pacientes <13 anos de idade: média de Phe <360µmol/L nos 12 meses anteriores ao estudo, pacientes ≤ 13 anos de idade: média de Phe <600µmol/L. O tipo de PKU foi classificado como Leve ou Clássica de acordo com Nalin et al., (2010).

Tabela 2. Escores da escala "Taste Strips" usada para avaliar a percepção gustativa em termos de 4 qualidades: doce, azedo, salgado e amargo, de pacientes com Fenilcetonúria e controles hígidos.

	Pacientes (n=25)	Controles (n=25)	p*
Doce	3,40 ± 0,5	3,48 ± 0,5	0,629
Azedo	2,36 ± 0,9	2,52 ± 0,6	0,505
Salgado	2,40 ± 1,2	3,12 ± 0,7	**0,020**
Amargo	1,84 ±1,3	2,84 ± 1,1	**0,008**

p*: T test

8. CONSIDERAÇÕES FINAIS

Destaca-se o caráter inovador deste trabalho, visto ser o primeiro que avaliou a neofobia alimentar de pacientes com PKU, através de um questionário auto-aplicável, e que associou os achados com os marcadores de tratamento e características clínicas.

Também foi o primeiro estudo que avaliou os sentidos químicos dos pacientes com PKU, associando a marcadores de tratamento, a características clínicas e ao comportamento alimentar.

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

1) Comparar a presença de neofobia alimentar em pacientes com PKU e em controles saudáveis;

Os resultados do presente trabalho evidenciaram alta prevalência de neofobia alimentar em pacientes com PKU quando foram comparados com controles saudáveis, que não possuíam nenhuma doença metabólica, pareados por sexo e idade.

2) Verificar os possíveis fatores associados à doença com a neofobia alimentar;

Vários possíveis fatores ligados ao aparecimento da neofobia alimentar foram investigados, como: o gênero, a introdução de alimentos sólidos antes dos seis meses de vida, o diagnóstico precoce, o estado nutricional, o controle metabólico, a idade, o tipo de PKU e os níveis de Phe ao diagnóstico. Acredita-se que essa investigação tenha sido bem completa. Contudo, somente a variável gênero apresentou associação significativa com os níveis de fobia alimentar, sendo este mais prevalente no sexo masculino.

Destaca-se ainda que o período de amamentação destes pacientes não foi o ideal. Além disso, a introdução de fórmula metabólica e a necessidade de dieta rígida e controlada de forma tão precoce podem ter contribuído para o surgimento de neofobia alimentar.

3) Comparar a percepção dos sentidos químicos (gustativa e olfativa) em pacientes com PKU e em controles saudáveis;

Observou-se significativa redução da percepção gustatória e olfatória dos pacientes com PKU quando comparado a controles saudáveis pareados por sexo e idade e que não possuíam nenhuma doença metabólica de tratamento dietético. Com o intuito de verificar qualquer defeito anatômico nestes pacientes, alguns foram encaminhados para o Serviço de Otorrinolaringologia do Hospital de Clínicas de Porto Alegre, que descartou qualquer problema que pudesse prejudicar as percepções olfatórias e gustatórias dos pacientes, nos levando a crer que estes achados são secundários ao tratamento rígido da PKU.

4) Verificar os possíveis fatores associados à doença com a percepção dos sentidos químicos (gustativa e olfativa).

Não foi observada correlação entre as variáveis hiposmia e hipogeusia com os níveis de Phe ao diagnóstico, níveis de Phe do ano anterior ao teste, controle metabólico e estado nutricional.

A única correlação encontrada foi os altos níveis de Phe ao diagnóstico com a menor percepção gustativa do sabor amargo, nos levando a crer que o rígido tratamento precoce aumenta a possibilidade de problemas sensórios.

Cabe ressaltar que dentre os Erros Inatos do Metabolismo, a PKU é uma doença relativamente frequente. Contudo, quando comparada à população geral, esta é considerada uma doença rara, de forma que o tamanho da amostra no presente estudo pode ter limitado o poder das análises estatísticas.

9. PERSPECTIVAS

A partir dos achados apresentados nesta tese, percebe-se que os pacientes com PKU estão mais suscetíveis a distúrbios alimentares e problemas que envolvem os sentidos químicos. Além disso, vale ressaltar a dificuldade de encontrar estudos que abordam o tema, principalmente ligados a doenças genéticas com restrições alimentares tão graves.

Os trabalhos existentes até o momento mostram a realidade de pacientes com PKU tratados em centros diferenciados, providos de opções terapêuticas que visam minimizar sua rígida rotina.

Nesse contexto, sugere-se a continuidade de pesquisas com esse tema, que englobem de modo mais profundo as dificuldades alimentares, o estado nutricional, as dificuldades orofaciais, e até mesmo que avaliem o real prazer que os pacientes com PKU têm em relação à alimentação.

Para o futuro, pretende-se incluir um maior número de pacientes, através de parcerias com outros centros de tratamento, no intuito de aumentar o poder estatístico das análises e melhorar a caracterização dessas condições em pacientes brasileiros com PKU.

APÊNDICE 1 – Aprovação do projeto no Comitê de Ética em Pesquisa (HCPA)

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: 150072
Data da Versão do Projeto: 19/02/2015

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
FILIPPO PINTO VAIRO
CHENIA CALDEIRA MARTINEZ
TÁSSIA TONON
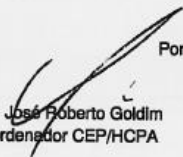CAROLINA FISCHINGER MOURA DE SOUZA
TATIELE NALIN

Título: Aspectos motores e sensoriais das estruturas e funções estomatognáticas de pacientes com Glicogenose Hepática

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, 27 de maio de 2015.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

APÊNDICE 2 – Termo de Consentimento Livre e esclarecido.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
(INDIVÍDUOS CONTROLES COM FENILCETONÚRIA)

PROJETO
Aspectos motores e sensoriais das estruturas e funções estomatognáticas de pacientes com Glicogenose Hepática

PESQUISADOR RESPONSÁVEL
Dra. Ida Vanessa Doederlein Schwartz. Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre (HCPA). Rua Ramiro Barcelos, 2350. Porto Alegre-RS. Telefone (51)3359.8011.

NOME DO PARTICIPANTE: _____

Prezado participante (ou responsável),

Gostaríamos de convidá-lo a participar do grupo controle de um estudo que avaliará o sistema orofacial de paciente com Glicogenose Hepática e de controles (pessoas com Fenilcetonúria e pessoas sem doença genética metabólica). A Glicogenose Hepática 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 pode provocar diversos sintomas, tais como: tremores, suor frio, cansaço, sonolência, convulsão. O tratamento da Glicogenose é a alimentação frequente com alimentos que mantém os níveis de açúcar no sangue elevados por mais tempo, como o amido de milho cru.

Devido a este tratamento com dieta rigorosa, os pacientes com Glicogenose Hepática podem ter alterações nas estruturas orofaciais (face, bochechas, lábios, dentes, boca, língua), nas funções de respirar, mastigar, deglutir e falar, bem como na percepção do cheiro e sabor dos alimentos. Por isso, estamos realizando este estudo com objetivo de avaliar as estruturas e funções relacionadas à boca e à face. Também iremos comparar os resultados das avaliações das pessoas com

Glicogenose Hepática com os resultados de pessoas sem doença e de pessoas com outra doença metabólica, que também necessite de tratamento dietético, com restrição de diversos alimentos (no caso, pessoas com Fenilcetonúria). Esta comparação irá ajudar na compreensão e na identificação de fatores que podem estar relacionados às dificuldades nas estruturas e funções da face e da boca.

No seu caso, serão coletados dados de prontuário, como por exemplo, idade, peso, altura, escolaridade e outras informações sobre sua saúde. Será realizada avaliação miofuncional orofacial, que consiste na visualização da face, bochechas, lábios, língua, dentes, boca, e da movimentação destas estruturas quando você estiver falando, respirando, mastigando ou engolindo. Este exame será gravado em vídeo com o objetivo de ser analisado por um pesquisador especialista nesta avaliação, que não tenha conhecimento sobre você, sua condição de saúde ou sobre qual grupo de estudo você pertence (indivíduos com Glicogenose Hepática, Fenilcetonúria ou Sem doença genética metabólica). Também será realizada a avaliação da face através de um aparelho que faz fotografia digital em 3D da face ou através de foto com câmera digital. Esta fotografia será analisada, em um segundo momento, por um pesquisador especialista na área, que não tenha conhecimento sobre você, sua condição de saúde ou sobre qual grupo de estudo você pertence (indivíduos com Glicogenose Hepática, Fenilcetonúria ou Sem doença genética metabólica). Realizaremos também uma avaliação do olfato (percepção do cheiro) através da inspiração do odor contido em fitas de papel que possuem diferentes aromas e avaliação do paladar (percepção do sabor) através da colocação de tiras de papel na língua, que possuem diferentes sabores. Ainda será feita avaliação da alimentação do participante. Neste procedimento, será perguntado a você (ou ao seu responsável), informações sobre sua alimentação, alimentos preferidos, dificuldades na alimentação.

Todos os exames citados são indolores e não invasivos. Estima-se que as avaliações terão duração de aproximadamente 2 horas, sendo que o participante poderá fazer estas avaliações em dois ou três encontros, se assim achar conveniente.

RISCOS E BENEFÍCIOS

Não são conhecidos riscos à sua saúde relacionados aos procedimentos do estudo, ficando apenas o desconforto mínimo por responder aos

testes e questionamentos, bem como o tempo despendido para fazer estes procedimentos. Os benefícios consistem na realização de uma avaliação detalhada da região orofacial (boca e face), que poderá ajudar na identificação de possíveis alterações destas estruturas (bochechas, lábios, língua, dentes, boca), das funções de respirar, falar, sugar, mastigar e engolir e de perceber os cheiros e sabores de alimentos. Você (participante ou responsável) poderá ter acesso aos resultados dos seus testes (ou dos testes do seu filho) e esclarecer dúvidas sobre os procedimentos ou sobre a pesquisa a qualquer momento.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa Doederlein Schwartz, telefone (51) 33598011, 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 do telefone (51) 33597640 ou localmente no segundo andar do HCPA, na Rua Ramiro Barcelos, 2350, Bairro Santa Cecilia, Porto Alegre-RS, horário de funcionamento das 8h às 17h de segunda à sexta-feira.

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 HCPA. A sua participação pode ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado e não haverá a necessidade de justificar a sua decisão. A concordância ou a não concordância em participar não irá alterar o tratamento já estabelecido para você ou para o seu familiar.

CONFIDENCIALIDADE DAS INFORMAÇÕES

As informações dessa pesquisa serão mantidas em sigilo, sendo apenas utilizadas de forma científica, e sem identificação de seu nome. Caso alguma informação derivada desse estudo for importante a você, todo esforço será realizado para informa-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 por _____. Declara ter sido esclarecido

que não receberá nenhuma remuneração financeira e que também não terá nenhum custo por participar do estudo. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que assinou duas vias deste consentimento, que uma delas ficou em seu poder.

Data: _____/_____/_____

_____   _____
Nome do participante   Assinatura

_____   _____
Nome do responsável (se aplicável)   Assinatura

Eu expliquei a _____ os objetivos e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para este (a) pessoa.

Data: _____/_____/_____

_____   _____
Nome do pesquisador   Assinatura

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(INDIVÍDUOS CONTROLES SEM DOENÇA GENÉTICA METABÓLICA)

PROJETO

Aspectos motores e sensoriais das estruturas e funções estomatognáticas de pacientes com Glicogenose Hepática

PESQUISADOR RESPONSÁVEL

Dra. Ida Vanessa Doederlein Schwartz. Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre (HCPA). Rua Ramiro Barcelos, 2350. Porto Alegre-RS. Telefone (51)3359.8011.

NOME DO PARTICIPANTE: _____

Prezado participante (ou responsável),

Gostaríamos de convidá-lo a participar do grupo controle de um estudo que avaliará o sistema orofacial de paciente com Glicogenose Hepática e de controles (pessoas com Fenilcetonúria e pessoas sem doença genética metabólica). A Glicogenose Hepática 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 pode provocar diversos sintomas, tais como: tremores, suor frio, cansaço, sonolência, convulsão. O tratamento da Glicogenose é a alimentação frequente com alimentos que mantém os níveis de açúcar no sangue elevados por mais tempo, como o amido de milho cru.

Devido a este tratamento com dieta rigorosa, os pacientes com Glicogenose Hepática podem ter alterações nas estruturas orofaciais (face, bochechas, lábios, dentes, boca, língua), nas funções de respirar, mastigar, deglutir e falar, bem como na percepção do cheiro e sabor dos alimentos. Por isso, estamos realizando este estudo com objetivo de avaliar as estruturas e funções relacionadas à boca e à face. Também iremos comparar os resultados das avaliações das pessoas com Glicogenose Hepática com os resultados de pessoas sem doença e de pessoas com outra doença metabólica, que também necessite de tratamento dietético, com

restrição de diversos alimentos (no caso, pessoas com Fenilcetonúria). Esta comparação irá ajudar na compreensão e na identificação de fatores que podem estar relacionados às dificuldades nas estruturas e funções da face e da boca.

No seu caso, serão coletados informações, através de perguntas dirigidas a você (ou seu responsável), sobre idade, peso, altura, escolaridade e outras informações sobre sua saúde. Será realizada avaliação miofuncional orofacial, que consiste na visualização da face, bochechas, lábios, língua, dentes, boca, e da movimentação destas estruturas quando você estiver falando, respirando, mastigando ou engolindo. Este exame será gravado em vídeo com o objetivo de ser analisado por um pesquisador especialista nesta avaliação e que não tenha conhecimento sobre você, sua condição de saúde ou sobre qual grupo de estudo você pertence (indivíduos com Glicogenose, Fenilcetonúria ou Sem doença genética metabólica). Também será realizada a avaliação da face através de um aparelho que faz fotografia digital em 3D da face ou através de foto com câmera digital. Esta fotografia será analisada, em um segundo momento, por um pesquisador especialista na área, que não tenha conhecimento sobre você, sua condição de saúde ou sobre qual grupo de estudo você pertence. Realizaremos também uma avaliação do olfato (percepção do cheiro) através da inspiração do odor contido em fitas de papel que possuem diferentes aromas e avaliação do paladar (percepção do sabor) através da colocação de tiras de papel na língua, que possuem diferentes sabores. Ainda será feita avaliação da sua alimentação. Neste procedimento, será perguntado a você (ou ao seu responsável), informações sobre sua alimentação, alimentos preferidos, dificuldades na alimentação.

Todos os exames citados são indolores e não invasivos. Estima-se que as avaliações terão duração de aproximadamente 2 horas, sendo que o participante poderá fazer estas avaliações em dois ou três encontros, se assim achar conveniente.

RISCOS E BENEFÍCIOS

Não são conhecidos riscos à sua saúde relacionados aos procedimentos do estudo, ficando apenas o desconforto mínimo por responder aos testes e questionamentos, bem como o tempo despendido para fazer estes procedimentos. Os benefícios consistem na realização de uma avaliação detalhada da região orofacial (boca e face), que poderá ajudar na identificação de possíveis

alterações destas estruturas (bochechas, lábios, língua, dentes, boca), das funções de respirar, falar, sugar, mastigar e engolir e de perceber os cheiros e sabores de alimentos. Você (participante ou responsável) poderá ter acesso aos resultados dos seus testes (ou dos testes do seu filho) e esclarecer dúvidas sobre os procedimentos ou sobre a pesquisa a qualquer momento.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa Doederlein Schwartz, telefone (51) 33598011, 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 do telefone (51) 33597640 ou localmente no segundo andar do HCPA, na Rua Ramiro Barcelos, 2350, Bairro Santa Cecilia, Porto Alegre-RS, horário de funcionamento das 8h às 17h de segunda à sexta-feira.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará qualquer tratamento de saúde que você faça ou precise fazer no HCPA. A sua participação pode ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado e não haverá a necessidade de justificar a sua decisão. A concordância ou a não concordância em participar do estudo não irá alterar qualquer tratamento já estabelecido para você ou para o seu familiar.

CONFIDENCIALIDADE DAS INFORMAÇÕES

As informações dessa pesquisa serão mantidas em sigilo, sendo apenas utilizadas de forma científica, e sem identificação de seu nome. Caso alguma informação derivada desse estudo for importante a você, todo esforço será realizado para informa-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 por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira e que também não terá nenhum custo por participar do estudo. Declara que foi informado da garantia de receber

resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que assinou duas vias deste consentimento, que uma delas ficou em seu poder.

Data: _____/_____/_____

_____   _____

Nome do participante   Assinatura

_____   _____

Nome do responsável (se aplicável)   Assinatura

Eu expliquei a _____ os objetivos e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para este (a) pessoa.

Data: _____/_____/_____

_____   _____

Nome do pesquisador   Assinatura

APÊNDICE 3 – Artigos Publicados durante o doutorado.

1) Artigo **"ASSESMENT OF NEWBORN SCREENING IN THE PUBLIC HEALTH SYSTEM OF A MUNICIPALITY IN NORTHEEN RIO GRANDE DO SUL" (2018).**

Revista: Clinical and Biochemical Research.

Autores: **Tássia Tonon**, Elisa Sisti, Tatiéle Nalin, Ida Vanessa Doederlein Schwartz.

2) Artigo **"FEEDING DIFFICULTIES AND OROFACIAL MYOFUNCTIONAL DISORDER IN PATIENTS WITH HEPATIC GLYCOGEN STORAGE DISEASES" (2018).**

Revista: JIMD Reports.

Autores: Chenia Caldeira Martinez, **Tássia Tonon**, Tatiéle Nalin, Lilia Farret Refosco, Carolina Fischinger de Moura Souza, Ida Vanessa Doederlein Schwartz.

3) Artigo **"DOMINO LIVER TRANSPLANT IN MAPLE SYRUP URINE DISEASE: TECHNICAL DETAILS OF CASES IN WICH THE FIRST SURGERY INVOLVED A LIVING DONOR" (2018).**

Revista: Transplantation.

Autores: Karina Roda, Rodrigo Vincenzi, Eduardo Fonseca, Marcel Benavides, Plínio Turine, Rogério Afonso, **Tássia Tonon**, Ida Schwartz, Irene Miura, Renata Pugliese, Gilda Porta, Paulo Chapchap, João Seda Neto.

4) Artigo **"EVALUATION OF PLASMA BIOMARKERS OF INFAMMATION IN PATIENTS WITH MAPLE SYRUP URINE DISEASE" (2018).**

Revista: Jounal of Inherited Metabolic Disease.

Autores: Giselli Scaini, **Tássia Tonon**, Carolina F Moura de Souza, Patricia Schuck, Gustavo Ferreira, João Quevedo, João Seda Neto, Tatiana Amorim, Jose Camelo, Ana Vitoria Barban Margutti, Rafael Hencke Tresbach, Fernanda Sperb-Ludwig, Raquel Boy, Paula de Medeiros, Ida Vanessa Schwartz, Emilio Luiz Streck.

5) Artigo **"QUALITY OF LIFE AND ADHERENCE TO TREATMENT IN EARLY-TREATED BRAZILIAN PHENYLKETONURIA PEDIATRIC PATIENTS" (2017).**

Revista: Brazilian Journal of Medical and Biological Research.

Autores: E. Vieira Neto, H.S. Maia Filho, C.B Monteiro, L.M. Carvalho, **T. Tonon**, A.P. Vanz, I.V.D. Schwartz, M.G. Ribeiro.

6) Artigo **"SERUM MARKERS OF NEURODEGENERATION IN MAPLE URINE DISEASE" (2016)**

Revista: Molecular Neurobiology.

Autores: Giselli Scaini, **Tássia Tonon**, Carolina F. Moura de Souza, Patrícia F. Schuck, Gustavo C. Ferreira, João Seda Neto, Tatiana Amorim, Ida Vanessa D Schwartz, Emilio Streck.

7) Artigo **"ACESS TO TREATMENT FOR PHENYLKETONURIA BY JUDICIAL MEANS IN RIO GRANDE DO SUL, BRAZIL" (2015).**

Revista: Ciência e Saúde Coletiva.

Autores: Luciano Mangueira Trevisan, Tatiéle Nalin, **Tássia Tonon**, Lauren Monteiro Veiga, Paula Vargas, Bárbara Correa Krug, Paulo Gilberto Cogo Leivas, Ida Vanessa Doederlein Schwartz

Assessment of newborn screening in the public health system of a municipality in northern Rio Grande do Sul

Tássia Tonon[1,2], Elisa Sisti[3], Tatiéle Nalin[1,2],
Ida Vanessa Doederlein Schwartz[2,4]

ABSTRACT

Introduction: Newborn screening allows the screening of diseases that are still in the asymptomatic period and whose early diagnosis and treatment are associated with reduced infant morbidity and mortality. The aim of this study was to evaluate the public National Newborn Screening Program in the municipality of Carazinho, state of Rio Grande do Sul (RS), Brazil.

Methods: This was a population-based, retrospective, descriptive study. We collected and transcribed data from a database of the Carazinho municipal laboratory, which is affiliated with the referral center for newborn screening in RS. The records of all individuals undergoing newborn screening from 2005 to 2010 were reviewed, and information was collected on the program coverage, time elapsed between birth and screening (first collection), and test results.

Results: The program had a coverage of 75.5%. One suspected case of phenylketonuria, three suspected cases of congenital hypothyroidism and no suspected cases of hemoglobinopathy were identified. In addition, there were 18 positive results for hemoglobin S heterozygosity, five for hemoglobin D heterozygosity, two for hemoglobin C heterozygosity, and one for a rare variant hemoglobin. When analyzing the newborn's age at the time of blood collection, it was observed that 63.1% were within the recommended age range.

Conclusions: Our findings suggest the need for optimization of public newborn screening in the evaluated municipality. The strategies to be adopted should include education of the population and especially of managers and health professionals about the importance of newborn screening.

Keywords: Newborn screening; public health; mass screening

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Newborn screening, also known as the heel-prick test because blood is collected from the newborn's heel, includes a series of laboratory tests that aim to identify certain genetic diseases in the asymptomatic stage. The first blood sample should preferably be collected between the third and fifth day of life[1]. This is an important strategy to reduce infant morbidity and mortality through early detection of treatable diseases that may lead to severe clinical presentations[2,3]. Therefore, the adoption of preventive actions, as early diagnosis and treatment, can change the natural history of diseases detected by newborn screening[4]. The selection of diseases for inclusion in a newborn screening program is mainly based on three criteria: a) disease detection should be feasible in the pre-symptomatic period; b) the disease should be treatable; and c) there should be the possibility of starting treatment at early disease stages[5].

Over the past two decades, the introduction of tandem mass spectrometry (MS/MS) has substantially expanded the number of disorders that can be detected in dried blood spot on filter paper. This test allows the simultaneous detection of more than 30 diseases, including organic acidemias and disorders of fatty acid metabolism[5]. Congenital hypothyroidism (CH) is the most widely screened disease in newborn screening programs worldwide[6].

In Brazil, a newborn screening program was initiated in the city of São Paulo in the 1970s by the pediatrician Benjamim Schmidt, with screening tests for phenylketonuria in the Association of Parents and Friends of Special Need Individuals (Associação de Pais e Amigos dos Excepcionais, APAE). In 2001, the Brazilian Ministry of Health implemented the National Newborn Screening Program (Programa Nacional de Triagem Neonatal, PNTN) through Regulation No. 822 of June 06, 2001. This regulation established a series of tests for early disease screening, diagnostic confirmation, treatment, follow-up, and database recording, with an intended coverage of 100% live births. The availability of tests was defined for each Brazilian state based on the preexisting coverage and infrastructure of health care services. The program was divided into three screening phases, as follows: Phase I – phenylketonuria and CH; Phase II – phenylketonuria, CH, and hemoglobinopathies; and Phase III – phenylketonuria, CH, hemoglobinopathies, and cystic fibrosis[7].

Later, Regulation No. 2,829 of December 14, 2012, included newborn screening for congenital adrenal hyperplasia and biotinidase deficiency within the scope of the program, encompassing all states in a single category[8]. Currently, there are 30 referral centers for newborn screening (Serviços de Referência em Triagem Neonatal, SRTN) in the 27 Brazilian federative units, which serve 17,854 collection stations[9]. Expanded screening, which enables the detection of up to 30 diseases, is provided by the public health system only in the Federal District and is available as a pilot program in the state of Minas Gerais[6].

Newborn screening in the state of Rio Grande do Sul (RS) began in the late 1980s as isolated initiatives in public hospital laboratories. It was only in 1994 that the state government began to offer screening tests in partnership with Universidade Federal do Rio Grande do Sul (UFRGS) and UFRGS School of Pharmacy. Currently, the referral institution for newborn screening in the state of RS is Hospital Materno Infantil Presidente Vargas, where the SRTN operates. Like most centers in other states, this SRTN is equipped with its own laboratory, which performs more than 60,000 laboratory tests per month. In 2015, the SRTN served 1,307 collection stations throughout the 497 municipalities that provide newborn screening as a public health service in the state[1].

First introduced in the United States in 1963, newborn screening is celebrating its 55th anniversary in 2018, coinciding with the 17th anniversary of the PNTN in Brazil. Therefore, an update on results is warranted to reflect the current state of newborn screening practice at the municipal level. The present study aimed to describe and evaluate newborn screening provided as a public health service in a municipality of the state of RS from 2005 to 2010.

METHODS

This population-based, observational, descriptive study with retrospective data collection was conducted in Carazinho, a municipality located in central-northern RS. In 2010, the population was 59,317 inhabitants[10], and the municipality was served by the 6th Regional Health District of Rio Grande do Sul.

The study was approved and data collection was authorized by the Municipal Health Department in Carazinho. Data were collected from the database of the Carazinho municipal laboratory, which contains all the results of newborn screening tests performed by the SRTN in the municipality.

The records of all individuals undergoing newborn screening from 2005 to 2010 were reviewed for information on the time elapsed between birth and screening and on the results of screening tests. During the study period, RS was in the Phase II of PNTN, i.e., screening for phenylketonuria, CH, and hemoglobinopathies.

The coverage of the screening program in Carazinho was determined based on data from the IT Department of the Brazilian Unified Health System (DATASUS), considering all live births occurring in the municipality from 2005 to 2010. Coverage was calculated as the number of tests performed multiplied by 100 and divided by the number of live births.

For age at collection of biological samples, we considered the period recommended before the Regulation No. 2,829 of 2012 became effective, i.e., blood collection up to the seventh day of life. After 2012, the ideal collection period was shortened to the fifth day of life, and delayed collection was defined as that performed after the sixth day of life.

Of note, regarding the screened diseases, we describe the results of screening tests, and not of confirmatory tests, as the latter were not made available to the researchers.

RESULTS

From 2005 to 2010, 4,647 children were born in Carazinho. Of these, 3,417 (75.5%) were screened for phenylketonuria and CH in the public health system. However, 3,410 (73.4%) children were screened for hemoglobinopathies because they had undergone transfusion procedures before collection. The results of the seven children screened for hemoglobinopathies 30 days after transfusion were not available at the time of data collection.

One suspected case of phenylketonuria and three suspected cases of CH were identified by laboratory analysis. There was no suspected case of hemoglobinopathy. In 18 cases, the results were positive for hemoglobin S heterozygosity, in five cases

Table 1: Newborn's age at blood collection for newborn screening (2005-2010).

Newborn's age	%	n
< 7 days of life*	63.1	2.157
7-30 days of life*	34.9	1.192
> 30 days of life*	2.0	68

*Collection period recommended by the Brazilian Ministry of Health before Regulation no. 2.829 of 2012.

for hemoglobin D heterozygosity, in two cases for hemoglobin C heterozygosity, and in one case for a rare variant hemoglobin.

Table 1 shows the newborn's age at the time of blood collection stratified according to the collection period recommended by the Brazilian Ministry of Health.

DISCUSSION

Newborn screening, which was consolidated in Brazil through the PNTN, aims to improve the quality of life of individuals affected by several diseases through early diagnosis. The introduction of the PNTN has been associated with a marked reduction in infant mortality over the years[4]. However, the proper implementation of this program still faces difficulties, such as the lack of financial resources, mobilization of the target audience and awareness of health managers, and inappropriate time of collection or transportation. This becomes evident from the wide variation and discrepancy across Brazilian states in the results reported in the "Situation Diagnosis of the PNTN", a survey conducted together with the Brazilian Ministry of Health in 2013[11].

Estimating the coverage of newborn screening is extremely important because it enables to analyze the percentage of children who were not screened, thereby allowing a partial analysis of the situation of the program under study. After the PNTN was established in 2001, there was an increase in the coverage of newborn screening in Brazil, reaching 80% in 2005, although it was heterogeneously distributed among states[2]. Currently, all Brazilian states and the Federal District have SRTNs and collection stations, the latter being usually located in primary health care units. In 2013, the exact number of tests performed and patients receiving follow-up was unknown by state managers and by the Brazilian Ministry of Health[11]. In 2016, according to the Brazilian Ministry of Health, the coverage of public newborn screening was 83.6% in Brazil[12]. In 2015, the coverage reached 83% in RS[1,2]. In the municipality of Carazinho, according to data from a master's degree thesis, the coverage was 72.2% in 2006[13].

A newborn screening program is successful when there is government priority and funding, population education and acceptance, engagement of health care professionals, and government participation in the implementation of the program[11]. The municipality under study did not achieve the ideal coverage of public screening recommended by the Brazilian Ministry of Health. However, it is important to point out that coverage rates may have been underestimated, since tests may have been performed in private health facilities and, therefore, were not available for analysis.

The low coverage of newborn screening in Brazil is justified by various reasons, the most important of which are the tests performed in the private health system, poor dissemination of the PNTN, and cultural traits that neglect the importance of newborn screening. The extent of this coverage is known to be directly influenced by cultural and socioeconomic factors, as demonstrated in more developed countries like Spain, France, Italy, Austria, Russia, New Zealand, Scotland, and Australia, where coverage is close to or reaches 100%[2].

According to some authors, it is essential to collect blood samples for newborn screening before hospital discharge in order to achieve a satisfactory coverage and to meet the ideal collection period[14]. However, it would be necessary, in the case of phenylketonuria, for example, to reduce cutoff points for normal and abnormal phenylalanine levels in order to identify children who had low protein intake through breastfeeding in their first hours of life. The unfavorable aspect of this strategy is that it would increase the number of false positive cases, which may increase costs because of the need for additional tests, also increasing the family's anxiety. Also worthy of note is that blood collection through the Brazilian Unified Health System (Sistema Único de Saúde) and the PNTN should be performed in an outpatient setting, except in special cases such as preterm infants and those with prolonged stay in the neonatal intensive care unit.

The PNTN has some guidelines and standards for the period of blood sample collection for newborn screening. The ideal collection period was up to the seventh day of life before Regulation No. 2,829 of 2012, which was shortened to the fifth day of life after the publication of this regulation. In 2015, 2.5 million newborns were screened in the PNTN. Of these,

only 53% were tested before the fifth day of life[11]. In Carazinho, most children were screened within the ideal period recommended by the guidelines in effect at the time of the study. It is worth noting that this period may vary among different countries, since it depends of the sensitivity of diagnostic technologies[4] and needs inherent in the diseases included within the scope of the screening program.

Regarding the incidence of the screened diseases, the reported numbers vary worldwide. For example, the worldwide incidence of phenylketonuria, a rare genetic disease in which a metabolic defect leads to the accumulation of blood phenylalanine, is estimated at 1 in approximately 10,000 newborns[5], and varies among countries and regions because of differences in rates of consanguinity[15]. In Brazil, the incidence of phenylketonuria was estimated at 1:15,839 newborns in 2001 and 1:24,780 newborns in 2002[16]. In 1996, Jardim et al. estimated that 1 in every 12,500 live births had phenylketonuria in RS[17]. In this state, screening (first sample) is performed through quantitative dosing of blood phenylalanine using filter paper, and abnormal results are confirmed by more specific tests (second sample) performed using serum, whole blood or urine samples[1].

Similar findings have been reported for CH, the most common congenital endocrine disorder, whose incidence has been found to vary among ethnic groups, with lower prevalence rates in African Americans than in Hispanics. Moreover, women are known to be more affected than men (at a 2:1 ratio), as well as children with Down syndrome. CH is caused by failure of the thyroid gland to produce adequate amounts of thyroid hormones, resulting in a generalized reduction in metabolic processes[4]. Currently, CH has a worldwide incidence of 1 in approximately 3,500 live births[4]. A similar incidence has been observed in Brazil, where the estimated rates range from 1:2,595 to 1:4,795 live births[1,18]. In the PNTN, CH is screened by dosing thyroid-stimulating hormone (TSH) with filter paper. CH is confirmed if serum TSH levels are above 20 mUI/L. Reduced levels of total T4, free T3 and free T4 confirm the diagnosis of primary defect in the thyroid gland[1].

As for hemoglobinopathies, these are the most prevalent genetic diseases in humans, and the most frequent and clinically significant variants are those affecting hemoglobin S and C. This disease is characterized by a structural defect in the beta chain of hemoglobin leading to the formation of sickle-shaped red blood cells. Data from the literature show that sickle cell anemia is 10 to 30 times more common than phenylketonuria, especially in regions where the population is of mixed race, like the Brazilian state of Bahia, where the incidence of sickle cell anemia is 1:650 live births[19,20].

The heel-prick test is economically feasible for the government, leading to a reduction in the number of disabled individuals and reducing expenditures on benefits to people with disabilities[21]. This study highlights the complexity of the PNTN, resulting from the vastness of the Brazilian territory and cultural diversity in the country, with an urgent need to improve the program. In conclusion, future strategies should focus on improving indicators and enabling the proper development of the program, including the following aspects: population awareness; education of managers from the three government spheres and of health professionals about the importance of the program; and consequent enhancement of actions and results for the benefit of the entire Brazilian population.

This study was performed at Universidade Luterana do Brasil (Carazinho, southern Brazil).

Conflicts of Interest

The authors declare no conflicts of interest.

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Feeding Difficulties and Orofacial Myofunctional Disorder in Patients with Hepatic Glycogen Storage Diseases

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Abstract Hepatic glycogen storage diseases (GSDs) are inborn errors of metabolism whose dietary treatment involves uncooked cornstarch administration and restriction of simple carbohydrate intake. The prevalence of feeding difficulties (FDs) and orofacial myofunctional disorders (OMDs) in these patients is unknown. *Objective*: To ascertain the prevalence of FDs and OMDs in GSD. *Methods*: This was a cross-sectional, prospective study of 36 patients (19 males; median age, 12.0 years; range, 8.0–18.7 years) with confirmed diagnoses of GSD (type Ia = 22; Ib = 8; III = 2; IXa = 3; IXc = 1). All patients were being treated by medical geneticists and dietitians. Evaluation included a questionnaire for evaluation of feeding behavior, the orofacial myofunctional evaluation (AMIOFE), olfactory and taste performance (Sniffin' Sticks and Taste Strips tests), and facial anthropometry. *Results*: Nine (25%) patients had decreased olfactory perception, and four (11%) had decreased taste perception for all flavours. Eight patients (22.2%) had decreased perception for sour taste. Twenty-six patients (72.2%) had FD, and 18 (50%) had OMD. OMD was significantly associated with FD, tube feeding, selective intake, preference for fluid and semisolid foods, and mealtime stress ($p < 0.05$). Thirteen patients (36.1%) exhibited mouth or oronasal breathing, which was significantly associated with selective intake ($p = 0.011$) and not eating together with the rest of the family ($p = 0.041$). Lower swallowing and chewing scores were associated with FD and with specific issues related to eating behavior ($p < 0.05$). *Conclusion*: There is a high prevalence of FDs and OMDs in patients with GSD. Eating behavior, decreased taste and smell perception, and orofacial myofunctional issues are associated with GSD.

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Introduction

Hepatic glycogen storage diseases (GSDs) are inborn errors of glycogen metabolism. These conditions are divided into subtypes, depending on the enzyme defect involved (Wolfsdorf and Weinstein 2003; Walter et al. 2016). Phenotype depends on the disease subtype and extent of metabolic control, but major features include growth retardation, short stature, a doll-like face, hepatomegaly, hypoglycemia, hyperlactatemia, hypercholesterolemia, and hypertriglyceridemia (Chen and Kishnani 2012; Kishnani et al. 2014; Derks and Smit 2015).

Treatment can include restricted intake of simple carbohydrates (fructose, maltose, glucose, lactose, galactose), administration of uncooked cornstarch several times a day (including overnight, as some patients do not tolerate fasting for more than 3 h), and management of clinical and laboratory parameters (Weinstein and Wolfsdorf 2002; Chen and Kishnani 2012; Derks and Smit 2015). Sometimes, due to the dietary restrictions and continuous feeding

required, tube feeding is indicated to maintain normoglycemia and proper metabolic control (Rake et al. 2002; Weinstein and Wolfsdorf 2002; Flanagan et al. 2015).

Feeding difficulties (FDs) are common in childhood, affecting up to 50% of children regardless of sex or socioeconomic status. Causes include a variety of organic and behavioral issues, as well as the feeding style of the caregivers; specific features and severity vary widely (Carruth et al. 2004; Wright et al. 2007; Dunitz-Scheer et al. 2009; Mascola et al. 2010; Benjasuwantep et al. 2013; Edwards et al. 2015; Kerzner et al. 2015).

The main organic conditions associated with FDs are dysphagia; gastrointestinal, metabolic, and cardiorespiratory abnormalities; structural/mechanical abnormalities; orofacial myofunctional disorders (OMDs); growth failure; and tube feeding. Other issues that are also directly related include prolonged mealtimes, food refusal, mealtime stress, lack of autonomy to self-feed, lack of distractions to increase intake, difficulty in eating foods with different textures, and picky eating. FDs can cause significant nutritional and emotional problems in children and in their caregivers (Carruth et al. 2004; Wright et al. 2007; Dunitz-Scheer et al. 2009; Mascola et al. 2010; Benjasuwantep et al. 2013; Edwards et al. 2015; Kerzner et al. 2015).

Few studies have assessed the issue of OMD and FDs in patients with GSDs. This population is particularly susceptible to FDs, as both the disease and its treatment are associated with risk factors for the development of feeding disorders. Poor acceptance of dietary plans by patients and families is also a concern (Correia et al. 2008; Santos et al. 2014; Flanagan et al. 2015). Within this context, the present study aimed to investigate FDs, OMDs, and olfactory and gustatory perception in Brazilian patients with hepatic GSDs.

Materials and Methods

Sample

This was a cross-sectional, prospective study of 36 patients (19 males; median age, 12.0 years; range, 8.0–18.7 years) with confirmed diagnoses of GSD (type Ia, $n = 22$; Ib, $n = 8$; III, $n = 2$; IXa, $n = 3$; IXc, $n = 1$) who were being treated at Hospital de Clínicas de Porto Alegre, a referral center in Southern Brazil. Fifteen patients (41.7%) were being tube-fed, 12 (36.1%) through a gastrostomy. Three patients (8.3%) were on tube feeding due to severe food refusal, and five (13.8%) were fed either orally or by tube. Data were collected from 2015 to 2017. A convenience sampling strategy was used.

Due to similarities in clinical characteristics and treatment, GSD subtypes were pooled as "subtype I" (Ia and Ib) and "other subtypes" (III and IX). Treatment included follow-up by an interdisciplinary team (a medical geneticist specializing in inborn errors of metabolism, a specialized dietitian, nurse, and clinical psychologist), with visits every 3 months; control of clinical and laboratory parameters; dietary management; and group therapy. Individuals under the age of 5 were excluded by recommendation of the tests. The study was approved by the Institutional Review Board of the hospital where it was carried out (protocol no. 150072), and written informed consent was obtained from all individuals before participation.

Procedures

Clinical information was collected from medical records and through a structured interview with the patient about FDs. To investigate dietary habits and feeding difficulties, a questionnaire of relevant items was constructed according to Edwards et al. (2015) and Kerzner et al. (2015); caregivers answered the questionnaire when patients were unable to understand the questions. Relevant behavioral signs and issues included selective intake, fear/aversion of feeding, prolonged mealtimes, mealtime stress (e.g., parents' and/or caregivers' insistence on offering food, constant resistance and/or refusal to feed, especially in childhood), preference for fluid/semisolid foods, and family eating habits (e.g., not eating together). Subjects were classified as having a "feeding difficulty" on the basis of the following three aspects: (1) self-report by patients/caregivers, (2) clinical evaluation by the researcher, and (3) presence of one or more of the aforementioned behaviors.

Clinical evaluations were performed on the same day by a trained speech–language pathologist with experience in administration of the study instruments, namely, (a) a validated protocol for investigation of OMD, the orofacial myofunctional evaluation with scores (AMIOFE) (De Felício and Ferreira 2008); (b) analysis of olfactory perception by the *Sniffin' Sticks* test (Hummel et al. 1997); and (c) Analysis of taste perception by the *Taste Strips* test (Mueller et al. 2003). This test evaluates four tastes (sweet, sour, salty, bitter), being possible to obtain the total score or the score of each flavor separately. For the present study, it were analyzed the total score and the sour taste, since sour taste is often present in foods restricted in the diet of patients with GSDs.

Statistical Analysis

The chi-square, Fisher's exact, Mann–Whitney U, and Spearman correlation tests were used for nonparametric variables, and Poisson regression with robust variance to analyze risk factors by prevalence ratio (PR). The

Kolmogorov–Smirnov test was used to evaluate the assumption of normality. The significance level was set at 5% ($p \leq 0.05$).

Results

Assessment of smell and taste perception was performed in 22 patients (61.1% of the sample), all aged ≥11 years, in accordance with the test recommendations. Regarding olfactory perception, the median score was 10.0 (8.8–11.2) points. Nine patients (40.9%) had median scores below the cutoff point, indicating hyposmia. For taste perception, the median score was 11.5 (10.0–14.0) points. Four patients (18.2%) had scores below the cutoff point for all flavors, suggesting hypogeusia. The scale for sourness alone ranges from 0 to 4 points. The sample median was 3.0 (0.8–3.0) points, with eight patients (36.4%) having a score indicative of decreased sour taste perception.

Variables related to the reduction of olfactory, gustatory, and sourness perception were compared to behavioral issues regarding food. Decreased olfactory perception was associated with selective intake ($p = 0.027$), while decreased sourness perception was associated with preference for fluid and/or semisolid foods ($p = 0.006$).

The prevalence of feeding issues (feeding behaviors or conditions that may impact on the child's feeding) and a comparison with the presence of FD are presented in Table 1. The overall prevalence of FD in this sample was 72.2% ($n = 26$). Since GSD I requires a more restrictive diet than other subtypes, the potential association between this subtype and FD needs to be investigated. In this study, we could not conduct a statistical comparison due to the discrepancy in sample size (30 participants with GSD I vs. 6 patients with other GSDs).

Findings related to feeding behavior were analyzed and compared to median scores in the orofacial myofunctional scale, specifically total, deglutition, and mastication scores (Table 2). The total orofacial myofunctional score correlated positively with age ($r = 0.493$, $p = 0.002$), suggesting that younger individuals had lower test scores.

Variables were analyzed by Poisson regression with robust variances to investigate risk factors for OMD, using prevalence ratios controlled by age. Preference for fluid/semisolid foods (PR = 10.29, 95% CI 1.4–75.1, $p = 0.021$) and selective intake (PR = 7.94, 95% CI 1.1–56.6, $p = 0.038$) were significant. This suggests that, even after controlling for age, these feeding issues are risk factors for OMD.

Table 3 presents an analysis of posture/appearance, mobility of orofacial structures, and orofacial functioning, stratified by age range (Table 3). Regarding stomatognathic functions, 1 patient presented with mouth breathing and 12 (33.33%) with oronasal-type breathing. Both breathing patterns were associated with selective intake ($p = 0.011$) and nonparticipation in family meals ($p = 0.014$). Mastication could not be assessed in three children due to lack of oral feeding secondary to complete food refusal. In these cases, the minimum score of one point was assigned, in accordance with test recommendations.

Table 1 Hepatic glycogen storage disorders: feeding aspects and feeding difficulty

| | Sample prevalence ($n = 36$) | Feeding difficulty[a] | | |
| | | Presence of feeding difficulty ($n = 26$) | Absence of feeding difficulty ($n = 10$) | |
	n (%)			*p-value
Tube feeding >1 year	15 (41.7%)	13 (50.0%)	2 (20.0%)	0.142
Exclusive breastfeeding <6 months	25 (69.4%)	21 (80.8%)	4 (40.0%)	0.039*
Feeding behaviors and conditions				
Selective intake	23 (63.9%)	22 (84.6%)	1 (10.0%)	<0.001*
Fear of feeding (or food aversion)	11 (30.6%)	11 (42.3%)	0 (0.0%)	0.016*
Preference for fluid/semisolid foods	21 (58.3%)	19 (73.1%)	2 (20.0%)	0.007*
Prolonged mealtimes	18 (50.0%)	14 (53.8%)	4 (40.0%)	0.360
Not eating together with the family	14 (38.8%)	14 (100%)	0 (0.0%)	0.003*
Mealtime stress	19 (52.8%)	16 (61.5%)	3 (30.0%)	0.139
Gastrointestinal conditions	13 (36.1%)	12 (46.2%)	1 (10.0%)	0.060

*Statistical significance by Fisher's Exact test to "feeding difficulty" ($p \leq 0.05$). Data presented by frequency (percentage)

[a] "Feeding difficulties" were determined on the basis of tree of these aspects: (1) self-reports by patients/family members, (2) clinical evaluation by the researcher, and (3) presence of one or more of the aforementioned behaviors

Table 2 Hepatic glycogen storage disorders: comparison between orofacial myofunctional evaluation scores with feeding aspects ($n = 36$)

	Scores of orofacial myofunctional evaluation					
	Total score	p-valor	Deglutition score	p-valor	Mastication score	p-valor
Tube feeding >1 year	84.0 (76.0–88.0)	0.012*	11.0 (9.0–13.0)	0.008*	5.0 (2.0–6.9)	0.077
Exclusive breastfeeding ≥6 months	87.0 (82.0–91.5)	0.256	12.0 (11.0–14.0)	0.728	5.0 (3.5–7.0)	0.446
Feeding difficulty	84.5 (81.2–90.0)	0.001*	12.0 (10.5–13.0)	0.001*	4.5 (3.0–6.0)	0.009*
Selective intake	84.0 (79.0–89.0)	<0.001*	12.0 (9.0–13.0)	0.004*	4.0 (2.0–6.0)	0.001*
Fear of feeding (or food aversion)	83.0 (69.0–90.0)	0.010*	11.0 (8.0–12.0)	0.015*	5.0 (1.0–6.0)	0.282
Preference for fluid/semisolid foods	84.0 (77.5–87.0)	<0.001*	11.0 (8.5–13.0)	<0.001*	5.0 (3.0–6.0)	0.051*
Prolonged mealtimes	84.0 (80.5–90.2)	0.038*	12.5 (11.0–14.0)	0.532	5.0 (2.7–7.0)	0.322
Not eating together with the family	84.6 (76.5–89.2)	0.019*	12.0 (8.0–12.2)	0.008*	5.0 (1.7–6.0)	0.088*
Mealtime stress	84.0 (79.0–90.0)	0.010*	12.0 (9.0–13.0)	0.058*	5.0 (3.0–6.0)	0.030*
Gastrointestinal conditions	85.0 (84.0–98.2)	0.339	12.0 (8.5–13.5)	0.152	5.0 (4.0–6.5)	0.690

*Statistical significance by Mann–Whitney test to "scores of orofacial myofunctional evaluation" ($p \leq 0.05$). Data presented by median (interquartile range)

Table 3 Hepatic glycogen storage disorders: children and adults in the specific abilities of orofacial myofunctional test

		Data by age range	
		Child ≤12 years old ($n = 22$)	Teenagers and adults >12 years old ($n = 14$)
	Reference score	Median (IQR)	Median (IQR)
Lips		3.0 (2.0–3.0)	3.0 (3.0–3.0)
Mandible/maxilla	3	2.0 (2.0–3.0)	3.0 (2.0–3.0)
Cheeks	3	2.0 (2.0–3.0)	3.0 (2.0–3.0)
Face	3	2.0 (2.0–3.0)	2.5 (2.0–3.0)
Tongue	3	3.0 (2.0–3.0)	3.0 (2.0–3.0)
Palate	3	3.0 (2.0–3.0)	3.0 (2.8–3.0)
Movements			
Lips	12	10.0 (9.8–11.3)	11.0 (9.5–12.0)
Tongue	18	17.0 (15.8–18.0)	18.0 (16.8–18.0)
Jaw	15	14.0 (12.0–15.0)	15.0 (14.8–15.0)
Cheeks	12	12.0 (10.8–12.0)	12.0 (11.0–12.0)
Functions			
Breathing	3	2.0 (2.0–3.0)	3.0 (3.0–3.0)
Deglutition	15	12.0 (11.0–13.0)	14.5 (11.8–15.0)
Mastication	10	5.0 (2.8–6.3)	7.0 (4.0–7.0)
Total	≥88	85.0 (81.3–90.3)	91.5 (84.8–99.0)

Data presented by median (IQR interquartile range)

Discussion

This was the first study in the literature to include a speech–language pathology viewpoint in the investigation of orofacial myofunctional issues and feeding behavior, as well as evaluate the possible association of these issues with the senses of smell and taste, in a sample of patients with hepatic glycogen storage diseases. Our findings indicate that feeding difficulties and orofacial myofunctional disorders are prevalent in this population, which may be particularly susceptible to the development of stomatognathic abnormalities.

GSD Ia was the most prevalent subtype in our sample, which is consistent with the literature (Janecke et al. 2001; Chou et al. 2002). Alternative feeding routes were used in a substantial portion of patients, which is consistent with the

need for uninterrupted dietary treatment to prevent fatal hypoglycemia. It is also worth noting that three patients presented with complete refusal of oral feeding secondary to progressive food refusal. Although alternative feeding routes are a necessary resource for some patients with GSD (Rake et al. 2002), tube feeding is known to cause adverse events, including negative impact on the stomatognathic system, and hinder swallowing and feeding behavior (Dunitz-Scheer et al. 2009; Gomes et al. 2015).

The study participants exhibited reduced olfactory and taste perception, and we identified an association between this reduced perception and feeding issues. These findings are consistent with the literature on FDs (Dunitz-Scheer et al. 2009; Edwards et al. 2015; Evans et al. 2017). It is well known that varied sensory experiences in childhood feeding (olfactory, gustatory, and others) play an important role in promoting proper and pleasant eating habits. It is understood that, in GSDs, olfactory and taste perception may be limited by the lack of stimuli caused by the highly restrictive diet, particularly regarding fruits and some vegetables.

We also found a high prevalence of FDs in the sample, which suggests that individuals with GSD have a higher frequency of selective intake and fear of feeding when compared to children without these diseases. Benjasuwantep et al. (2013) reported a 15.4% prevalence of selective intake and 0.25% prevalence of fear of feeding in the general population. Kerzner et al. (2015) and Edwards et al. (2015) note that children with chronic diseases or behavioral issues tend to develop feeding difficulties. In their study of phenylketonuria, an inborn error of metabolism which also requires a restrictive diet for proper management, Evans et al. (2017) showed that neophobia is mainly caused by fear of eating foods that may be forbidden in the patient's diet.

The high frequency of negative eating situations and behaviors identified in this sample corroborates previous studies showing that gastrointestinal abnormalities, orofacial myofunctional disorders, and the use of alternative feeding routes are mechanical and structural aspects that frequently cause feeding difficulty. Family habits and unfavorable and stressful environments have also been described as behavioral factors that predispose to food refusal and selective intake (Dunitz-Scheer et al. 2009; Kerzner et al. 2015; Edwards et al. 2015). Benjasuwantep et al. (2013) reported that children with eating problems tend to eat at the table with their families less often and have prolonged feeding times.

Within this context, we identified that several participants in our sample did not eat meals as a family and found an association between GSD type I and feeding difficulty. These findings may be related to the high overall prevalence of feeding difficulty in the sample, as individuals with feeding problems often do not eat at the family table (Dunitz-Scheer et al. 2009; Benjasuwantep et al. 2013), as well as to the disease itself and its treatment, since patients with hepatic GSD need to eat at prescribed times, which may diverge from family mealtimes (Rake et al. 2002; Weinstein and Wolfsdorf 2002; Flanagan et al. 2015). In the case of GSD type I, dietary control is associated with even greater restrictions and need for even more frequent intake of uncooked cornstarch to maintain normoglycemia and prevent secondary metabolic disorders than in other GSD subtypes (Rake et al. 2002; Flanagan et al. 2015).

In the present sample, the youngest patients and those with feeding difficulties performed worse on the orofacial myofunctional test. This finding is consistent with previous studies describing that structural and mechanical abnormalities, such as OMDs, can cause feeding difficulties (mainly selective intake and food aversion). Refusal of solid or difficult-to-chew foods is usually due to changes in breathing, swallowing, and mastication patterns, as well as aversive behaviors due to gagging, odynophagia, and increased protective oral reflexes (Dunitz-Scheer et al. 2009; Kerzner et al. 2015; Edwards et al. 2015).

We conclude that there is a high prevalence of feeding difficulties and orofacial myofunctional disorders in Brazilian patients with hepatic GSD. Our results suggest that individuals with GSD I subtypes may be at higher risk of feeding disorders and orofacial myofunctional disorders compared to those with other GSD subtypes also requiring strict dietary management. This warrants further evaluation. Likewise, our suspicion of decreased olfactory and taste perception in these patients was confirmed, especially for sourness. Weaknesses of this study include the fact that no validated protocol was used to assess feeding behavior, the small sample size, and the single-center design, which will have influenced dietary treatment practices and the eating habits of patients.

Our results also indicate that individuals with hepatic GSD may be inordinately susceptible to orofacial myofunctional disorders and feeding difficulties, due to factors related to the disease itself, to its treatment, and to eating habits. We suggest that clinicians involved in the management of GSDs need to be alert for selective intake, food refusal, and difficulties in chewing and swallowing in childhood and adulthood, especially in children during the period of food introduction, and should refer patients with these issues to specialist professionals for evaluation and follow-up. Further research on this topic be conducted to confirm whether olfactory and taste perception are reduced in these patients and investigate possible causes for

these sensory impairments, as well as to support early identification of eating disorders and feeding difficulties and development of therapeutic interventions to address these issues.

Concise One Sentence About Manuscript

Prevalence of feeding difficulties and orofacial myofunctional disorders in patients with hepatic glycogen storage disease.

General Rules

- *Details of the Contributions of Individual Authors*

  Chenia Caldeira Martinez: author took the lead in design project; acquisition, interpretation, and analysis of data; was responsible for the writing of manuscript.

  Tássia Tonon: contributed to the design of study; acquisition, interpretation, and analysis of data; revised the manuscript in order to approve the final version.

  Tatiéle Nalin and Lilia Farret Refosco: both coauthors contributed to the conception and design of study; interpretation and analysis of data; revised critically the article in order to approve the final version of this manuscript.

  Carolina Fischinger Moura de Souza and Ida Vanessa Doederlein Schwartz: both coauthors contributed to the idealization; conception and design of study; interpretation and analysis of data; revised critically the article in order to approve the final version of this manuscript.

- *A Competing Interest Statement*

  Chenia Caldeira Martinez, Tatiéle Nalin, Tássia Tonon, Lilia Farret Refosco, Carolina Fischinger Moura de Souza, and Ida Vanessa Doederlein Schwartz declare that they have no conflict of interest.

- *Details of Funding*

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- *Details of Ethics Approval*

  The study was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (protocol no. 150072).

- *A Patient Consent Statement*

  According to the project no. 150072 of Ethics Committee of Hospital de Clínicas de Porto Alegre, a written informed consent was obtained from all individuals before participation in the study.

- *Documentation of Approval from the Institutional Committee for Care and Use of Laboratory Animals (or Comparable Committee)*

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Domino Liver Transplant in Maple Syrup Urine Disease: Technical Details of Cases in which the First Surgery Involved a Living Donor

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Abbreviations

BCAAs: branched-chain amino acids

BCKDH: branched-chain ketoacid dehydrogenase

BMI: body mass index

CIT: cold ischemia time

CMV: cytomegalovirus

DLT: domino liver transplantation

GDA: gastroduodenal artery

GRWR: graft-to-recipient weight ratio

HA: hepatic artery

HV: hepatic veins

LD-DLT: living donor domino liver transplantation

LD: living donor

LDLT: living donor liver transplantation

LHA: left hepatic artery

LLS: left lateral segment

LT: liver transplantation

LL: left lobe

MHV: middle hepatic vein

MSUD: maple syrup urine disease

OT: operation time

PELD: pediatric end-stage liver disease

PHA: proper hepatic artery

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POD: postoperative day

PV: portal vein

RHV: right hepatic vein

RRHA: replaced right hepatic artery

US: ultrasound

VG: vascular graft

WIT: warm ischemia time

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Abstract

Background: Data describing the technical aspects of living donor domino liver transplantation (LD-DLT) in maple syrup urine disease (MSUD) are limited. The largest published series includes only 3 cases. One great challenge of this procedure is to ensure adequate vascular stumps for the living donor, the MSUD patient, and the recipient of the domino graft. Here, we describe our experience in 11 cases of LD-DLT in MSUD, highlighting the technical aspects of LD-DLT.

Methods: From September 2012 to September 2017, 11 patients with MSUD underwent LDLT at our institution, and MSUD livers were used as domino grafts in 11 children.

Results:1- MSUD patients: Ten patients received a left lateral segment. The donor's left hepatic vein was anastomosed to the confluence of the recipient's 3 hepatic veins (HV). No venous grafts (VG) were required for portal vein (PV) anastomosis. Single arterial anastomosis was performed with microsurgery in 10 out of 11 patients.

2- MSUD graft recipients: In 8 cases, HV reconstruction was performed between the graft's HV confluence and the recipient's HV confluence, and in 3 cases, a vena cava triangulation was necessary; 6 MSUD grafts required HV venoplasty. No VG were needed for HV reconstruction. VG were used for PV reconstruction in 3 cases due to sclerotic PV. In 2 cases, double arterial anastomoses were performed in the MSUD liver. All patients remain alive and well.

Conclusion: LDLT followed by DLT for MSUD is a complex procedure and demands technical refinement. Special attention must be paid to vascular reconstruction.

Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive disease caused by mutations of the branched-chain ketoacid dehydrogenase (BCKDH) complex that abrogates the function of this enzyme complex and leads to the accumulation of the branched-chain amino acids (BCAAs) leucine, isoleucine and valine. MSUD can cause serious neurologic disability and even death by brain swelling.[1] Despite progress in nutritional and medical treatment, very few patients are responsive to treatment, and neurological impairment can still occur.[2]

Liver transplantation (LT) is a therapeutic option for MSUD because the liver is responsible for 9-13% of the body's total BCKDH production. LT can restore BCAA homeostasis and prevent long-term neurologic consequences.[3,4] Living donor liver transplantation (LDLT) is an option that is especially useful in the pediatric population, where the shortage of size-matched donors is greater.

The MSUD liver is structurally and functionally normal and may be used as a graft for domino liver transplantation (DLT), as previously published.[5] The recipient of an MSUD domino graft will not develop the disease because BCAA metabolism will occur in tissues other than the liver.[5] DLT for MSUD has been performed mainly with deceased donors, and reports describing the use of a living donor in this situation are scarce.

In 2014, our group was the first to report a successful DLT in MSUD where the first surgery involved a related living donor (LD).[6] The available data describing the technical aspects of these cases is limited. Only 7 cases have been reported to date, and the largest series included 3 cases.

The great challenge of this procedure is to ensure adequate vascular stumps for both the LD and the MSUD patient as well as the recipient of the domino graft. The aim of this study is to

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describe our center's experience with 11 cases of living donor domino liver transplantation (LD-DLT) in MSUD, highlighting the technical aspects of this procedure.

Materials and Methods

From April 1991 to October 2017, our group performed a total of 1000 LTs in patients aged under 18 years. From September 2012 to September 2017, 11 instances of LD-DLT in MSUD were performed in patients aged under 18 years at Hospital Sírio-Libanês, Sao Paulo, Brazil. Data on the transplant recipients were collected through a retrospective examination of medical records and a prospectively collected database. This study was approved by the hospital's ethics committee. The patients were divided into 3 groups: live donors, MSUD patients and MSUD graft recipients, to achieve a better understanding of the results.

Organ Allocation for DLT

When DLT was scheduled, the graft from the MSUD patient was offered to patients in the waiting list, containing ABO compatible adults and children from all liver transplant centers in our region; position on the waiting list was determined by MELD score (12 years old and older) or the modified PELD score (additional score for patients aging less than 12 years old).[7]

Live Donors

The voluntary intent of the donor was first assessed, and informed consent was mandatory. The donors' preoperative evaluations and the surgical techniques used have been reported in previous publications and followed the principles described by Yamaoka et al.[8] A preoperative aminoacidogram was performed in all donors. The related donors (mother or

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father), who were assumed to be heterozygous with a normal phenotype, were only accepted if the aminoacidogram was normal.

MSUD Recipients and MSUD Graft Recipients

The preoperative image evaluations of the MSUD patient and the MSUD graft recipient were similar, and all were subjected to doppler US examination, especially for portal vein (PV) diameter analysis, to predict the use of vascular grafts (VG) during PV reconstruction (usually in patients with a PV diameter <5 mm).

MSUD grafts were flushed with histidine tryptophan ketoglutarate solution at 4°C and prepared for implantation during the bench procedure. A venoplasty was performed if the hepatic veins (HV) were distant from each other using a 6-0 polypropylene suture to unite the veins, thereby creating a single ostium.

MSUD patients received either an LLS or an LL from the LD, and the whole liver was used in the MSUD graft recipient. For the safety of both recipients, the surgery of the second recipient started only after the bench procedure with the MSUD graft was finished, and the graft from the LD successfully reperfused in the MSUD patient.

The grafts were implanted using a "piggyback technique." In both groups, the graft's HV were anastomosed to the recipient's HV or through a triangulation technique with the inferior vena cava, depending on the diameter of the veins. The graft's PV was anastomosed in an end-to-end fashion, either to the recipient's PV trunk or using an interposition VG. The hepatic artery (HA) was always reconstructed using microvascular techniques with 9-0 or 10-0 nylon sutures (Ethicon, Edinburgh, UK). The surgical microscope used was an OPMI PENTERO 900 (Carl Zeiss, Germany). Double arterial anastomosis in the presence of 2 arteries to the graft was

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mandatory, especially when a back flow was not obtained after the completion of the first anastomosis. Biliary anastomosis was performed either as a duct-to-duct anastomosis or as a Roux-en-Y bilioenteric reconstruction.

Doppler US scans were routinely performed on postoperative day (POD) 1 to evaluate vascular patency. Tacrolimus and steroids were used for immunosuppression. Preoperative and post-LT metabolic control was assessed by measuring o-phthaldialdehyde-derivatized amino acid concentrations from plasma or dried filter paper blood spots using high-performance liquid chromatography (Agilent Technologies). Aminoacidogram was collected 24 hours before LT, on POD 1, POD 3, POD 7, and then monthly within the first year of LT. The analysis presented in this manuscript compares the pretransplant values collected 24h before the procedure and the values collected 2-months posttransplant. Alloisoleucine was analysed only in patients 1, 2, 3 pre and postoperatively.

Statistical analysis

Continuous variables were presented as mean $\pm$ standard deviation. For statistical analysis, the means and standard deviations were evaluated by the paired Student's t test, using GraphPad Prism version 7.0 (GraphPad Software, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Demographics

There were 11 LD-DLTs for MSUD in the study period. The live donors were all related (mother or father) except for 2 who were unrelated; the donors' ages ranged from 25 years to 42

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years, and their median body mass index (BMI) was 23.5. The MSUD patients were children (10 females and 1 male) with ages ranging from 9 months to 96 months (median 38 months; IR 24-79 months). The MSUD patients' weights ranged from 5.9 kg to 22 kg (mean 13 kg ± 5.2 kg). Pediatric end-stage liver disease (PELD) scores ranged from 1 to 4, and mean Weight Z-scores was -1.9 ± 1.83 (ranging from -4.99 to 0.95). Only 1 patient had previously undergone a gastrostomy operation. In this case series, all but 1 MSUD patient presented with developmental delay, cognitive impairment and previous episodes of seizures, many of them with multiple hospital admissions prior to liver transplantation (Table 1). The MSUD graft recipients were children (7 females and 4 males) with ages ranging from 6 months to 68 months (median 18 months; IR 8-33 months). The patients' weights ranged from 5.3 kg to 18.6 kg (mean 10 kg ± 4 kg). The mean PELD score and Weight Z-scores were 18.5 ± 7.4 and -1.4 ± 1.6, respectively. The following indications were used for liver replacement: biliary atresia (n=10, 91%) and α1-antitrypsin deficiency (n=1, 9%). Five patients (45.5%) previously underwent a Kasai operation. Nine (82%) patients presented ascites prior to transplant (Table 2).

Technical Aspects- MSUD Patients

Ten patients (91%) received an LLS graft. In 1 case, an LL graft was used. The mean graft weight was 300 g ± 36 g (ranging from 250 g to 350 g), and the mean graft-to-recipient weight ratio (GRWR) was 2.7% ± 1.2% (ranging from 1.4% to 4.8%) (Table 3). The graft's HV were anastomosed to the confluence of the recipient's 3 HV in all cases; in 2 cases, the LD graft had a venoplasty between the LHV and an accessory vein. The median diameters of the graft veins and recipient veins were 2 cm (ranging from 2 cm to 3.2 cm) and 3 cm (ranging from 2 cm to 3.5 cm), respectively. Portal vein anastomosis was performed between the left PV of the graft

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and the PV trunk of the recipient in all patients, and no VG was required. In 10 patients (91%), there was only 1 anastomosis between the graft's left hepatic artery (LHA) and the recipient's proper hepatic artery (PHA). One patient required 2 arterial anastomoses; one between the graft's LHA and the recipient's PHA and the other between the graft's segment IV artery and the recipient's replaced right hepatic artery. Biliary reconstruction was performed with bilioenteric anastomosis in 5 (45.5%) patients and with duct-to-duct biliary anastomosis in 6 (54.5%) patients. The median operation time (OT), cold ischemia time (CIT) and warm ischemia time (WIT) were 360 minutes (ranging from 255 to 420 min), 60 minutes (ranging from 24 to 83 min) and 26 minutes (ranging from 22 to 28 min), respectively. The mean packed red blood cell transfusion volume was 7 ml/kg (ranging from 0 to 30 ml/kg).

Technical Aspects- MSUD Graft Recipients

The mean graft weight was 437 g ± 105.5 g (ranging from 275 g to 585 g), and the mean GRWR was 4.8% ± 1.8% (ranging from 2.2% to 7.8%). The technical aspects related to the MSUD graft recipients are summarized in Table 4.

In 8 (73%) patients, HV reconstruction was performed between the graft's HV confluence and the recipient's HV confluence. In 3 (27%) patients, the graft's HV confluence was anastomosed to the recipient's vena cava in a triangulated fashion. Venoplasty was performed in 6 (54.5%) MSUD grafts; 4 of these were between the middle hepatic vein (MHV) and right hepatic vein (RHV), and 2 used 2 venoplasties (one between the MHV and the RHV and the other between the LHV and the MHV). The mean diameters of the MSUD graft veins and the recipient's veins were 3.5 cm ± 0.5 cm (ranging from 3 cm to 4.5 cm) and 3 cm ± 0.4 cm (ranging from 2.5 cm to 4 cm), respectively. Three (27%) patients required the use of VG during

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PV anastomosis (a cryopreserved iliac vein from a deceased donor). In 9 (82%) patients, the arterial anastomosis was single and was performed between the MSUD graft's PHA and the recipient's LHA or RHA. Double arterial anastomosis was only performed in 2 patients. Biliary reconstruction was performed with bilioenteric anastomosis in all 11 patients. It was necessary to use a surgical mesh (Proceed mesh, Ethicon, USA) for abdominal closure in 3 (27%) patients. The median values of OT, CIT and WIT were 350 minutes (ranging from 255 to 540 min), 240 minutes (ranging from 98 to 328 min) and 27 minutes (ranging from 24 to 30 min), respectively. The mean intraoperative packed red blood cell transfusion volume was 14 ml/kg.

Outcomes- Live Donors

There were no surgical or clinical complications for the live donors, and the mean hospital stay was 4.9 ±1.3 days.

Outcomes- MSUD Patients

Patient and graft survival were 100% during a median follow-up of 8 months (ranging from 2 months to 60 months). Following transplantation on POD 1, all patients were switched to an unrestricted natural protein intake (dietary leucine tolerance ≥100 mg/kg/day) and stopped consuming the BCAA-free formula. Mean Pre-LT leucine levels (normal values: 49-216 μM) decreased from 330.5 ± 221.6 μM to 184.4 ± 48 μM (p=0.055); mean pre-LT isoleucine levels (normal values: 22-107 μM) decreased from 254.5 ± 163.6 μM to 126 ± 31.4 μM (p=0.037) and mean pre-LT valine levels (normal values: 74-321 μM) remained stable from 267 ± 160 μM to 272 ± 77.7 μM (p=0.941) (Figure 1). For patients 1, 2 and 3, the pretransplant alloisoleucine

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levels were 355 μM, 639 μM, 346 μM, respectively. One-month posttransplant the levels were undetectable.

Two (18%) patients required reoperation. One patient in POD 1 required reoperation due to an intra-abdominal clot and coagulopathy, and the other patient, also in POD 1, required reoperation due to bile leakage from the duct-to-duct biliary anastomosis and was converted to a bilioenteric anastomosis.

One patient developed septic shock with a positive blood culture for *Enterococcus faecalis* on POD 8, and this infection was successfully treated with antibiotics. There were no episodes of acute metabolic intoxication during follow-up, even during the septic shock episode in the patient mentioned above. Posttransplant hospitalizations of MSUD patients are summarized in table 5.

Outcomes- MSUD Graft Recipients

Patient and graft survival were 100% during the same median follow-up. Three patients required reoperation. One patient in POD 1 required reoperation due to intra-abdominal bleeding; one in POD 1 required reoperation due to unconfirmed arterial thrombosis, and the other required reoperation due to multiple perforations along the transverse colon secondary to multiple adhesions from a previous Kasai surgery.

One patient developed intraoperative HA and PV thrombosis. The HA was redone, and the portal vein was also re-anastomosed with the interposition of a VG; fifteen months after LT, this patient presented with splenomegaly and thrombocytopenia and was diagnosed with chronic portal vein thrombosis, which was successfully treated by percutaneous angioplasty and stent placement.

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Discussion

LT is an established treatment for classical MSUD; unfortunately, the availability of organs from deceased donors is limited in the pediatric population. In this scenario, LDLT plays an important role in overcoming this organ shortage.

DLT is another important tool to mitigate the shortage of grafts and was first performed in 1995. Patients with familial amyloid polyneuropathy are the main donors. Even though recipients of these grafts require several years to manifest the adverse effects of the disease, there are still benefits in cases where the survival of the recipient is questionable at the time of transplantation.[9] Domino liver grafts are typically of good quality; they originate from young, stable donors with a short cold ischemia time.[5]

Regarding MSUD patients, care must be taken in the selection of the live donor to guarantee sufficient GRWR, especially when using heterozygous donors, because the transplanted liver will be the only source of BCKDH activity. The liver from an obligate heterozygous parent expresses only 50% of BCKDH activity and, in theory, can restore approximately 7% of enzyme activity in the recipient. Previously, we reported the safe use of heterozygous donors in LDLT in MSUD, where effective BCAA homeostasis was obtained on an unrestricted diet, and no metabolic intoxication was observed.[10] In this series, GRWR ranged from 1.4% to 4.8%, and following LT, recipients didn't present signs of metabolic syndrome or brain injury, despite unrestricted natural protein intake. Moreover, amino acid concentrations remained stable in the postoperative period.

In this study, except for 1 patient, all MSUD patients had a previous degree of neurological impairment. Brain damage secondary to metabolic crisis is the leading cause of neurological insufficiency in MSUD patients.[11] The problem lies in the fact that even with a

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strict diet and a close amino acid monitoring, MSUD patients are at risk for metabolic crisis during viral illnesses, infections or any situation of organic stress.[12] Therefore, due to the unpredictability of a metabolic crisis, it is impossible to completely prevent severe brain injury. Moreover, dietary restriction and oxidative stress may exert a negative effect during the period of rapid brain growth of infancy and early childhood, contributing to neurological impairment.[12] Considering these deleterious effects, liver transplantation may be considered early in life in order to improve neurocognitive outcome in MSUD patients. However, further studies are required to clarify this issue because this case series is limited.

MSUD grafts are allocated to a unified waiting list containing patients from all liver transplant centers in our area, according to their MELD score or modified PELD criteria.[7] In addition to ABO compatibility, candidate selection for the domino MSUD graft considers the weight similarity between the children. Mohan et al[13] described a series of 2 cases in India, in which they accepted recipients for the domino graft with a weight within 20% of the weight of the domino donor. Weight match is an important parameter but is not the only one to be considered. Other parameters, such as the size of the abdominal cavity and presence of ascites, are also important when selecting a good candidate match for the MSUD graft. In this cohort, 3 MSUD graft recipients had GRWR>6%, and these patients had uneventful recoveries. This was possible, in part, due to the size of the abdominal cavity and the presence of ascites at the time of LT (Table 2). Whenever possible, adequate physical matching for all potential liver recipients is favored. However, when the domino graft is offered to 1 of our patients in the waiting list, the list priority dictates the allocation despite the size mismatch.

When size mismatch is inevitable, some intraoperative strategies are used in order to prevent complications secondary to large-for-size syndrome, such as using prosthetic mesh for

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abdominal closure and examining the graft's vascular flows (Doppler US) before and after abdominal closure. If the discrepancy in size is critical, graft reduction may also be employed (not required in this case series). Three cases with GRWR 5.5%, 5.6% and 6.7% required the use of ventral mesh (Proceed mesh, Ethicon, Somerville, NJ, USA). In other 2 patients with GRWR greater than 6%, primary abdominal closure was performed without a mesh; cases #2 and #11 (GRWR 7.8%% and 6.3%) were children with biliary atresia and large volumes of ascites.

The decision regarding where to divide the vascular structures in the living donor DLT is crucial to the success of the procedure. It is paramount to have a sufficient length of the hepatic veins, hepatic arteries and portal vein for both the LD and the MSUD patients as well as the recipient of the domino graft. The safety of the LD and the MSUD patients is crucial for the procedure.

The dissection of the HV in the MSUD patient goes as high as possible in the diaphragm to achieve good exposure of the 3 HV. In 5 MSUD grafts, no venoplasty was necessary between the 3 HV, and the veins could be anastomosed in the recipient as a single cuff. The remaining 6 cases required venoplasty to unite the HV, but no additional difficulties were observed during graft implantation despite the vascular reconstruction (Figure 2). None of the MSUD grafts required the use of a VG for HV reconstruction. Overall, our incidence of hepatic venous outflow obstruction with LDLT using LLS and LL, using the same technique, is 1.73%.[14] Matsunami et al[15] described a different technique when dissecting the HV; they used an ultrasonic surgical aspirator to exteriorize the HV as far as possible to facilitate reconstruction, and this may be another feasible option to assure safe vascular reconstruction.

Both surgical procedures (for the LD and MSUD patients) occurs simultaneously; thus, the communication between the surgical teams when deciding where to divide the artery is

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paramount to try to recover a single artery in the MSUD graft with safety. The aim of the dissection is to leave the HA as long as possible in the MSUD patient and to use a single artery for the MSUD graft. For this purpose, the gastroduodenal artery (GDA) is usually ligated to increase the length and mobility in the MSUD patient and MSUD graft recipient.

In 9 cases, the transection line was just above the GDA in the proper HA (Figure 3), and the anastomoses were single; in these cases, all grafts obtained from the LD had a single artery. It is important to have alternative HA reconstructions in mind in case of the eventual necessity to perform 2 arterial anastomoses. In this cohort, only 1 of the LD grafts had more than 1 hepatic artery (1 LHA and 1 segment IV artery); in this case, as the MSUD patient had a replaced right hepatic artery from the superior mesenteric artery and a LHA from the celiac trunk, 2 arterial anastomoses could be performed without technical difficulty. Since 2012, our group standardly uses 2 arterial anastomoses whenever possible. After we analyzed our own data on 654 primary LDLTs, a statistically significant trend for a decreased incidence of hepatic arterial thrombosis and an increased use of 2-arterial anastomosis over time was revealed.[16] Another strategy to achieve this goal when the LD has 2 arteries is to dissect high above the bifurcation of the HA to guarantee the availability of 2 arteries for reconstruction. This strategy necessarily recovers a graft from the MSUD patient with 2 arteries that must be reconstructed in the MSUD graft recipient.

It is important not to sacrifice the hepatic artery of the MSUD patient in order to provide a MSUD graft with a single hepatic artery. For instance, in circumstances where the hepatic artery of the LD is short, or when the MSUD patient has a low bifurcation of the right and left hepatic arteries, no attempts to obtain a single hepatic artery in the MSUD graft should be

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performed, as this may result in an artery with insufficient length in the MSUD patient and consequently jeopardize the arterial anastomosis.

Reports addressing the subject of arterial anatomy assessment preoperatively are scarce. The Tokyo group performed a 3D-CT in both the living donor and the MSUD patient to evaluate the anatomy of their HAs and to select the dividing point according to these findings.[15]

For PV reconstruction, there was no need to use a VG in the MSUD patients. In the MSUD graft recipients, all 3 cases that required VG were biliary atresia with sclerotic PVs. In the groups from Tokyo, Aichi (Japan) and India, the PV reconstructions were all fashioned end to end with no need to use VG.[13,15,17]

Roux-en-Y anastomosis was employed for biliary reconstruction in most of the cases (for both the MSUD patients and the MSUD graft recipients). However, no contra-indication exists for performing a duct-to-duct anastomosis in MSUD patients; nevertheless, careful arterial dissection is necessary to avoid ischemia of the remaining hepatic duct.

In conclusion, LDLT followed by DLT for MSUD is an effective and complex procedure. Short vascular stumps, venoplasty, the use of vascular grafts and the eventual necessity to perform 2 arterial anastomoses are some of the difficulties that must be overcome in these procedures.

Acknowledgments

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Figure Legends

Figure 1. 24 hours before LT and 2 months post LT plasma branched chain amino acid concentrations.

Figure 2. Venous outflow reconstruction in the MSUD graft recipient. Anastomosis between the graft's HV confluence with venoplasty and the recipient's HV confluence (aspect: posterior wall).

Figure 3. Proper hepatic artery and portal vein. Arrows demonstrate the transection point in the MSUD patient.

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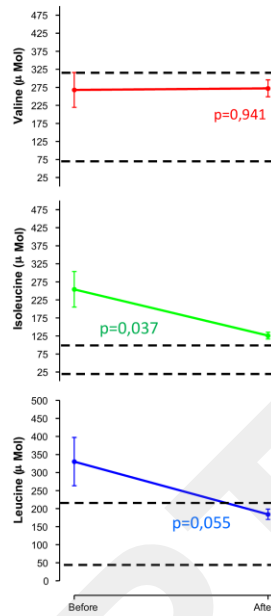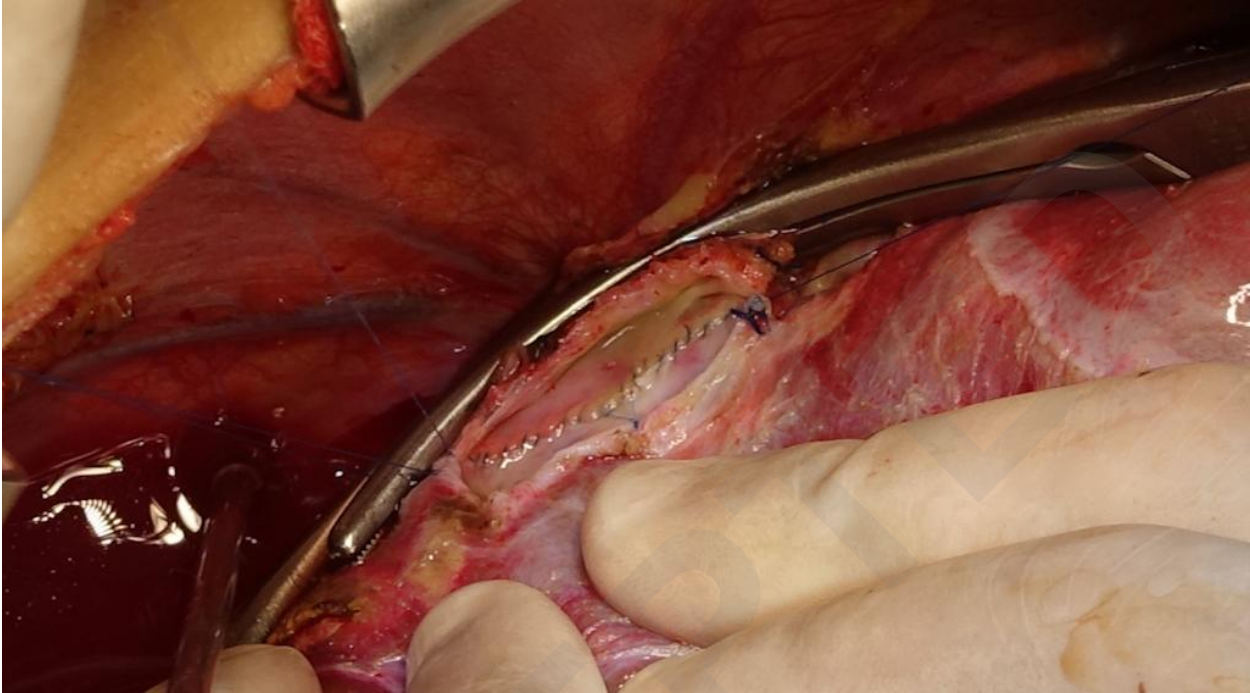**Figure 1**

Figure 2

Figure 3
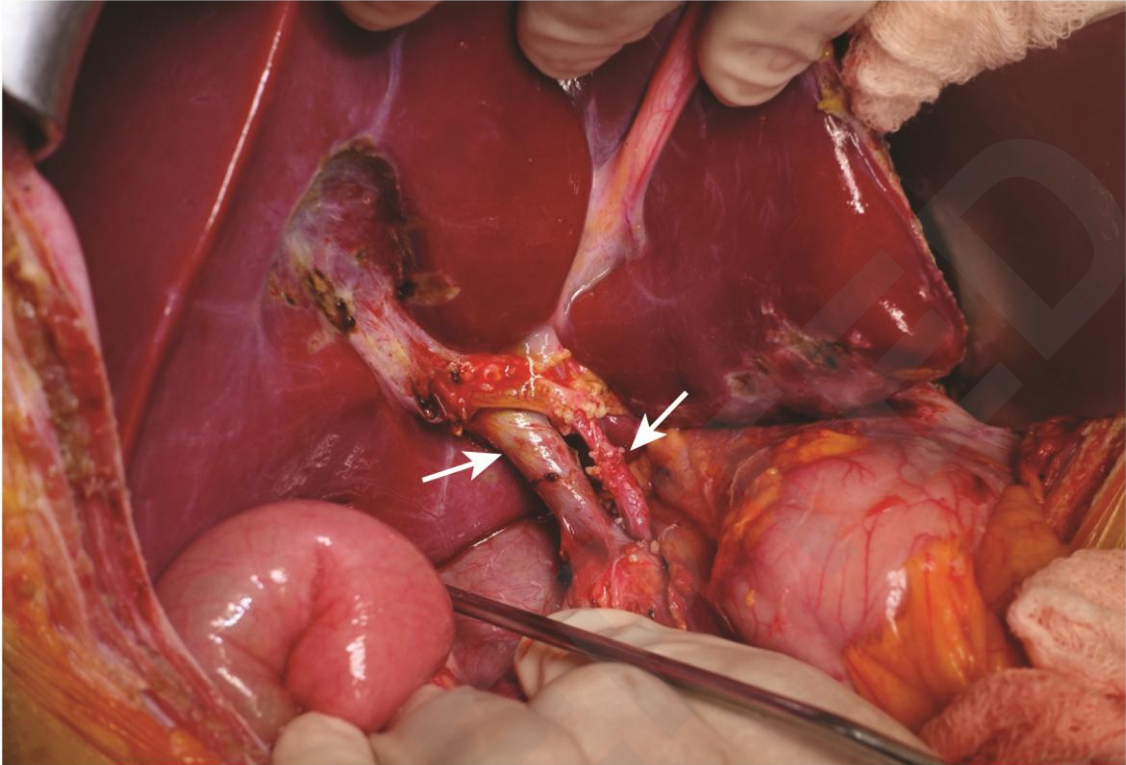
Table 1: Neurological clinical data on MSUD patients

Case	Age at MSUD diagnosis (days)	Developmental/motor delays and/or cognitive impairments	Seizures	Hospitalizations prior to LT	Age at LT (months)	Perception of neurologic improvement post LT	Hospitalizations due to metabolic crises post LT
1	7	Yes	Yes	5	24	Yes	No
2	30	Yes	Yes	>10	38	Yes	No
3	35	Yes	Yes	2	19	Yes	No
4	17	Yes	Yes	>10	94	Yes	No
5	38	Yes	Yes	2	44	Yes	No
6	5	No	No	0	9	Yes	No
7	425	Yes	Yes	3	79	Yes	No
8	575	Yes	Yes	3	96	Yes	No
9	90	Yes	Yes	>10	24	Yes	No
10	90	Yes	Yes	2	24	Yes	No
11	455	Yes	Yes	2	40	Yes	No

MSUD: maple syrup urine disease; LT: liver transplantation

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Table 2. Demographic data of MSUD patients and MSUD graft recipients

Case	MSUD Patient			MSUD Graft Recipient				
	Age (months)	Weight (kg)	Liver Weight (g)	Age (months)	Weight (kg)	Diagnosis	PELD	Ascites
1	24	10.77	465	33	13.15	BA	9	no
2	38	8.44	540	24	6.94	BA	29	yes
3	19	5.95	275	17	10.8	BA	25	yes
4	94	18	425	18	7.5	BA	13	yes
5	44	11.6	425	11	7.7	BA	18	yes
6	9	7.7	320	6	6.6	BA	25	yes
7	79	20	585	68	18.6	BA	10	yes
8	96	22	550	24	9.8	A1	19	no
9	24	12	360	8	5.3	BA	28	yes
10	24	11.4	335	36	15	BA	10	yes
11	40	16.6	530	8	8.3	BA	17	yes

MSUD: maple syrup urine disease; PELD: pediatric end-stage liver disease; BA: biliary atresia; A1: alpha 1-antitrypsin deficiency

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Table 3. Clinical data and technical aspects of the MSUD patients

Ca se	Age/Se x (month s)	Graf t/ GR WR	Cold ischemia time (min)	Warm ischemia time (min)	Venous outflow reconstru ction	Portal vein anastom osis	Arterial anastom osis	PO Surgical Complicat ions	Outcome s/ Follow-up
1	24/Male	LLS/ 2.6%	110	40	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/5 years
2	38/Female	LLS/ 4.1%	24	31	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/3 years
3	19/Female	LLS/ 4.8%	30	26	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/3 years
4	94/Female	LLS/ 1.47 %	58	26	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/1 year
5	44/Female	LLS/ 2.3%	27	25	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/1 year
6	9/Female	LLS/ 4.4%	82	22	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/8months
7	79/Female	LL/ 1.4%	107	23	Graft's LHV + MHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/7months
8	96/Female	LLS/ 1.5%	83	27	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient' s PHA	None	Alive/4months
9	24/Female	LLS/ 2.8%	60	28	Graft's LHV + acessory HV - recipient's HVC (venoplast y)	PV trunk	Graft's LHA - recipient' s PHA	Reop intra-abdominal clot	Alive/3months

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10	24/Female	LLS/2.63%	26	24	Graft's LHV - recipient's HVC	PV trunk	Graft's LHA - recipient's PHA + Graft's seg IV - recipient's RRHA	Reop bile leakage	Alive/2months
11	40/Female	LLS/1.6%	73	27	Graft's LHV + acessory HV - recipient's HVC (venoplasty)	PV trunk	Graft's LHA - recipient's PHA	None	Alive/2months

GRWR: graft-to-recipient weight ratio; PO: postoperative; LLS: left lateral segment; PV: portal vein; LHV: left hepatic vein; HVC: hepatic veins confluence; LHA: left hepatic artery; PHA: proper hepatic artery; LL: left lobe; MHV: middle hepatic vein; Reop: reoperation

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Table 4. Clinical data and technical aspects of the MSUD graft recipients

Case	Age/Sex (months)	GRWR	Cold ischemia time (min)	Warm ischemia time (min)	Venous outflow reconstruction	Portal vein anastomosis	Arterial anastomosis	PO Surgical Complications	Outcomes/ Follow-up
1	33/Male	3.5%	285	27	Graft's 3 HV - recipient 's HVC	PV - PV	Graft's PHA - recipient's RHA	None	Alive/5 years
2	24/Female	7.8%	259	30	Graft's 3 HV - triangulation IVC	PV - VG	Graft's PHA - recipient's RHA	Reop-bleeding	Alive/3 years
3	17/Male	2.5%	227	24	Graft's 3 HV - recipient 's HVC	PV - PV	Graft's PHA - recipient's RHA	None	Alive/3 years
4	18/Female	5.5%	142	30	Graft's 3 HV (venoplasty) - triangulation IVC	PV - VG	Graft's RHA - recipient's RHA + graft's LHA - recipient's LHA	None	Alive/ 1 year
5	11/Female	5.5%	168	27	Graft's 3 HV - recipient 's HVC	PV - PV	Graft's PHA - recipient's RHA	None	Alive/ 1 year
6	6/Female	4.8%	300	30	Graft's 3 HV (venoplasty) - recipient's HVC	PV - VG	Graft's PHA - recipient's RHA	Reop-intra-abdominal clot	Alive/8 months
7	68/Female	3.14%	328	25	Graft's 3 HV (venoplasty) - recipient's HVC	PV - PV	Graft's PHA - recipient's RHA	None	Alive/7 months
8	24/Female	5.6%	98	27	Graft's 3 HV (venoplasty) - recipient's HVC	PV - PV	Graft's PHA - recipient's LHA	None	Alive/4 months

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9	8/Female	6.7%	270	27	Graft's 3 HV (venoplasty) - recipient's HVC	PV - PV	Graft's PHA - recipient's RHA	None	Alive/3 months
10	36/Male	2.2%	420	25	Graft's 3 HV (venoplasty) - triangulation IVC	PV - PV	graft's RHA - recipient's RHA + graft's LHA - recipient's LHA	Reop- multiple perforations in the colon	Alive/2 months
11	8/Male	6.3%	154	24	Graft's 3 HV - recipient 's HVC	PV - PV	graft's PHA - recipient's RHA	None	Alive/2 months

GRWR: graft-to-recipient weight ratio; PO: postoperative; HV: hepatic vein; HVC: hepatic veins confluence; PV: portal vein; PHA: proper hepatic artery; RHA: right hepatic artery; VG: venous graft; Reop: reoperation; IVC: inferior vena cava; LHA: left hepatic artery

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Table 5: Post transplant hospitalizations - MSUD patients

Case	Hospitalizations post LT	Causes	Acute metabolic decompensation
1	3	CMV viremia, Gastroenteritis	No
2	2	Gastroenteritis, otitis media and seizures	No
3	1	CMV viremia	No
4	1	CMV viremia	No
5	2	CMV viremia, urinary tract infection	No
6	0	None	No
7	3	CMV viremia, Gastroenteritis, Pneumonia	No
8	1	CMV viremia	No
9	2	Pneumonia, urinary tract infection	No
10	2	Intra abdominal collection, Gastroenteritis	No
11	0	None	No

LT: liver transplantation; CMV: cytomegalovirus

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ORIGINAL ARTICLE

CrossMark

Evaluation of plasma biomarkers of inflammation in patients with maple syrup urine disease

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Abstract
Maple syrup urine disease (MSUD) is an autosomal recessive inherited disorder that affects branched-chain amino acid (BCAA) catabolism and is associated with acute and chronic brain dysfunction. Recent studies have shown that inflammation may be involved in the neuropathology of MSUD. However, these studies have mainly focused on single or small subsets of proteins or molecules. Here we performed a case-control study, including 12 treated-MSUD patients, in order to investigate the plasmatic biomarkers of inflammation, to help to establish a possible relationship between these biomarkers and the disease. Our results showed that MSUD patients in treatment with restricted protein diets have high levels of pro-inflammatory cytokines [IFN-γ, TNF-α, IL-1β and IL-6] and cell adhesion molecules [sICAM-1 and sVCAM-1] compared to the control group. However, no significant alterations were found in the levels of IL-2, IL-4, IL-5, IL-7, IL-8, and IL-10 between healthy controls and MSUD patients. Moreover, we found a positive correlation between number of metabolic crisis and IL-1β levels and sICAM-1 in MSUD patients. In conclusion, our findings in plasma of patients with MSUD suggest that inflammation may play an important role in the pathogenesis of MSUD, although this process is not directly associated with BCAA blood levels. Overall, data reported here are consistent with the working hypothesis that inflammation may be involved in the pathophysiological mechanism underlying the brain damage observed in MSUD patients.

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Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive amino acid disorder caused by mutations in genes that encode the components of the branched-chain α-ketoacid dehydrogenase (BCKDH) complex, which catalyzes the first irreversible step in branched-chain amino acid (BCAA) catabolism (Menkes 1959; Dancis et al 1977). The resulting metabolic block leads to elevated plasma concentrations of BCAA (leucine, isoleucine and valine) and the corresponding branched-chain α-ketoacids (α-ketoisocaproic acid, α-keto-β-methylvaleric acid and α-ketoisovaleric acid) (Treacy et al 1992; Chuang and Shih 2001). If untreated, affected individuals accumulate substantial BCAA and their corresponding ketoacids—mostly leucine and α-ketoisocaproic acid—and suffer encephalopathy within the first week of life as well as cerebral oedema and dysmyelination with chronic brain injury (Crome et al 1961; Silberman et al 1961; Chuang and Shih 2001). The global frequency is approximately one in 185,000 live newborns (Mackenzie and Woolf 1959; Chuang and Shih 2001; Zinnanti et al 2009), although data retrieved from newborn screenings suggest this rate can be higher (Simon et al 2006; Fingerhut 2009; Quental et al 2010).

Although numerous factors and mechanisms have been proposed to understand the pathogenesis of MSUD, its aetiopathology remains unknown. However, several studies have proposed that leucine and/or its ketoacid are the main neurotoxic metabolites in MSUD since the appearance of neurological symptoms is related to increased plasma concentrations of these compounds. Indeed, the elevated BCAA level may saturate the LAT-1 transporter and block the uptake of other large neutral amino acids, leading to decreased synthesis of neurotransmitters such as dopamine, serotonin, norepinephrine, and histamine (Wajner and Vargas 1999; Tavares et al 2000; Wajner et al 2000; Araujo et al 2001; Zielke et al 2002). Moreover, it has been demonstrated that metabolite accumulation in MSUD causes mitochondrial bioenergetics dysfunction (Sgaravatti et al 2003; Funchal et al 2006a, b; Wajner et al 2007; Ribeiro et al 2008; Amaral et al 2010), oxidative stress (Bridi et al 2003, 2005; Funchal et al 2006a, b; Barschak et al 2008, 2009; Mescka et al 2013, 2015a, b; Sitta et al 2014), apoptosis of neural cells (Jouvet et al 1998, 2000a, b), and alterations in the cholinergic system (Scaini et al 2012), in neurotrophin levels (Scaini et al 2013a, b, 2015) and in lysosomal proteases (Scaini et al 2016).

Inflammation has been recognized as a risk factor for age-related neurodegenerative diseases (Amor et al 2010, 2014; Fakhoury 2015), and high inflammatory profiles at baseline assessment may increase risk of conversion to dementia at long-term follow-up (Schmidt et al 2002; Cunningham and Hennessy 2015). A growing body of evidence demonstrates that higher levels of circulating inflammatory markers, especially interleukin-6 (IL-6) and C-reactive protein, are associated with greater cognitive decline (Yaffe et al 2003; Singh-Manoux et al 2014; Palta et al 2015). Moreover, studies have shown that modulators of the peripheral immune system can induce psychiatric symptoms in animal models and humans (Dantzer et al 2008; Laske et al 2008; Harrison et al 2009; Eisenberger et al 2010; Raison and Miller 2011). For example, healthy participants exposed to low-dose endotoxin (*Escherichia coli*) showed greater increases in self-reported and observer-rated depressed mood, suggesting that inflammation alters reward-related neural responses in humans and these in turn mediate the effects of inflammation on depressed mood (Eisenberger et al 2010). In this line, previous studies have shown that inflammatory processes play an integral role in the pathophysiology of certain inborn errors of metabolism, including phenylketonuria (Deon et al 2015), glutaric acidemia type I (Seminotti et al 2016), and methylmalonic acidemia (Ribeiro et al 2013). It has been shown that patients with MSUD treated with restricted protein diets have high levels of pro-inflammatory cytokines (Mescka et al 2015a, b). Moreover, studies using animal models and cell cultures have also demonstrated that BCAAs alter the balance between pro-inflammatory and anti-inflammatory cytokines (De Simone et al 2013; Rosa et al 2016).

Considering the hypothesis that BCAAs themselves and/or one of their metabolites could be responsible for MSUD's central nervous system (CNS) dysfunction and that there are few studies about inflammation in this disease, the aim of this study was to investigate plasma biomarkers of inflammation from patients with MSUD in treatment to establish a possible relationship between these biomarkers and the disease.

Material and methods

Sample and subjects

Plasma specimens from 12 treated patients with MSUD with the classic form and nine healthy individuals (control group) were used to evaluate the inflammatory markers and concentrations of amino acids. The diagnosis of MSUD was based on the high concentration of leucine and alloisoleucine in plasma, or by DNA analysis. The patients were between 15 days and 2 months old at diagnosis and followed a treatment that

consisted of a natural, protein-restricted diet with low BCAAs and supplemented with a semi-synthetic formula of essential amino acids containing small amounts of vitamins and minerals. In addition, the patients with MSUD were supplemented with isoleucine and valine capsules (Table 1). Table 2 displays the age at diagnosis, age at testing, length of treatment, genotypes, and clinical profiles of the patients with MSUD at presentation and under treatment. In addition, this study included a control group, comprising nine subjects, age- and gender-controlled. The present study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre, RS, Brazil. All parents of the patients included in the present study provided informed consent according to the guidelines of our committee.

Plasma preparation

Human blood samples were collected in heparin-coated collection tubes from fasting patients with MSUD (2 h) or nine healthy individuals (control group) by venous puncture during the baseline visits. Whole blood was centrifuged at $1000 \times g$, and plasma was removed by aspiration and frozen at −80 °C until analysis.

Amino acid determination

The free amino acids in plasma were determined by HPLC according to Joseph and Marsden (1986), with slight modifications (Wajner et al 2000). Amino acids were quantified by comparing their chromatographic peak area to those obtained from a known standard mixture and to that of an internal standard peak area (homocysteic acid).

Gene sequencing

A DNA sample was extracted from the peripheral blood of each proband and their biological parents. *BCKDHA*, *BCKDHB*, and *DBT* were amplified by polymerase chain reaction (PCR) with specific oligonucleotides, including exons and exon-intron boundaries. The amplification products were purified and sequenced by Sanger method in an ABI 3130 Genetic Analyzer (Applied Biosystems). Genetic variants were identified by comparison with reference sequences NG_013004.1 (*BCKDHA*), NG_009775.1 (*BCKDHB*), and NG_011852.2 (*DBT*) (GenBank/NCBI). Most pathogenic variations occur within coding regions or consensus splice and branch sites, and thus our approach can detect most but not all mutations. Moreover, this method does not detect large genomic deletions or deletions involving oligonucleotide primer sequences.

Table 1 Doses of valine and isoleucine supplementation (mg/day)

Patients	Valine (mg/kg)	Isoleucine (mg/kg)
1	2.824	2.824
2	7.299	9.124
3	4.016	4.016
4	2.777	2.777
5	3.521	0
6	1.766	6.183
7	NA	NA
8	16.949	16.949
9	NA	NA
10	3.500	16.336
11	NA	NA
12	1.020	2.040

NA not available

Inflammatory markers

Samples were randomized based on diagnostic group and plasma inflammatory markers (GM-CSF, RANTES, sICAM1, sVCAM1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, INF-ɣ, and TNF-α) were assayed in duplicate with Luminex xMAP Technology (Millipore, USA & Canada). The xMAP platform used here was based on the Rules-Based Medicine (RBM) fluorescent beads and antibody pairs. These are sensitive, specific, and widely used reagents sourced by numerous manufacturers, and data collected using xMAP multiplex beads have been widely reported in the literature in studies with simultaneous assay of multiple proteins.

The assays were conducted in 96-well polystyrene, round-bottom microplates. Initially, 200 μL of wash buffer was added to each well of the plate, and the plate was sealed and mixed on a plate shaker for 10 min at room temperature. Subsequently, 50 μL of the standard and 25 μL of the sample were added to the appropriate wells. A 25-μL aliquot of the working bead mixture was transferred into the wells. The plate was incubated with agitation on a plate shaker overnight (16–18 h) at 4 °C. The plate was then placed into the magnetic separator and left to separate for 60 s. The supernatant was carefully removed from each well by manual inversion. Beads were washed three times by adding 100 μL of assay buffer into each well to ensure absence of any undesirable or non-specifically bound antibodies. Following the protocol, 50 μL of a detection antibody was added to each well. Incubation was again performed in darkness and at RT on a plate shaker (850 rpm) for 60 min. Finally, 50 μL of streptavidin-PE was added to each well. The plate was incubated on a plate shaker (850 rpm) in the dark at RT for 30 min. The supernatant was carefully removed after magnetic separation of the beads by manual inversion, and washing was performed as previously

Table 2 Age at diagnosis, age at testing, length of treatment and clinical findings of MSUD patients

Patients	Sex	Age at diagnosis	Age at testing	Length of treatment	Genotypes	Clinical Features	
						At diagnosis	At inclusion
1	F	2 months	8 years	7.10 years	NA	Poor feeding, poor sucking, changes in respiratory pattern, hypertonia, encephalopathy, psychomotor developmental delay	Cognitive impairment
2	M	17 days	11.5 years	11.4 years	NA	Seizures, vomiting, psychomotor and neurodevelopmental delay	Cognitive impairment
3	F	17 days	6.7 years	6.6 years	*BCKDHB* c.[498G>C];[595_596delAG] p.[(Lys166Asn)];[(Pro200Ter)]	Vomiting, irritability	Normal development
4	M	15 days	11.8 years	11.7 years	*DBT* c.[346G>A];[346G>A] p.[(Gly116Arg)];[(Gly116Arg)]	Poor feeding, poor sucking, changes in respiratory pattern, hypotonia, vomiting, hypoactivity, psychomotor and neurodevelopmental delay	Cognitive impairment
5	F	15 days	3.8 years	3.7 years	NA	Irritability, sleep disturbances	Neuropsychomotor development delay
6	M	24 days	2.2 years	2.1 years	NA	Irritability, poor sucking, growth failure, pallor, nystagmus	Neuropsychomotor development delay
7	M	4 days	0.3 years	0.2 years	NA	Asymptomatic	Neuropsychomotor development delay
8	F	1 month and 16 days	3 years	2.11 years	*BCKDHA* c.[109-3575_288+ 4del];[859C>T] p.[?];[(Arg242Ter)]	Poor feeding, poor sucking, characteristic odor, metabolic acidosis, seizures, changes in respiratory pattern, apnea, upper respiratory tract infection	Neuropsychomotor development delay
9	M	27 days	1.5 year	1.4 year	*BCKDHB* c. [502C>T];[595_596delAG] p.[(Arg168Cys)];[(Pro200Ter)]	Poor feeding, poor sucking, characteristic odor, hypotonia, hypertonia, lethargy, metabolic acidosis, seizures, coma, changes in respiratory pattern, skin lesions, psychomotor developmental delay	Neuropsychomotor development delay
10	F	16 days	1.11 year	1.10 year	*BCKDHB* c.[743delC;c.764_ 840+22delinsGTAG;c.932+ 9_+22del]; [743delC;c.764_ 840+22delinsGTAG;c.932+ 9_+22del] p.[?];[?]	Asymptomatic	Normal development
11	M	1 month	0.2 years	0.1 years	NA	Refusal of food, poor sucking, characteristic odor, lethargy, changes in respiratory pattern, seizures, coma	Neuropsychomotor development delay
12	M	26 days	15.2 years	15.1 years	NA	Refusal of food, characteristic odor, lethargy, seizures, cerebral edema	Cognitive impairment

described. Sheath fluid (150 μL) was added into each well, and the plate was placed onto a plate shaker for approximately 5 min to achieve gentle agitation of the beads. Samples were run in duplicate using a Luminex 200™ system (Millipore, USA & Canada), and data analysis was conducted in MILLIPLEX® Analyst 5.1 software using a 5-parameter logistic regression model. Inflammatory markers were expressed as pg/mL.

Statistical analysis

The association between dichotomous variables was assessed with the Fisher exact test. All variables were tested for Gaussian distribution by the Kolmogorov-Smirnov normality test. The initial comparisons between the two groups (patients vs. controls) were determined using Mann-Whitney or Student's t tests when non-normally or normally distributed,

respectively. Linear regression was used to adjust for possible confounding variables. We considered all variables associated with diagnostic group and biomarkers with $p < 0.20$ as possible confounding factors (Victora et al 1997). Spearman's Rho(r) correlation analyses were performed to examine the relationship between clinical variables and plasma levels of inflammatory markers. Data are presented as the means and 95% confidence intervals. All statistical tests were two-tailed and were performed using a significance level of $\alpha = 0.05$. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA) as well as GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The variants involving premature stop codons and deletions as observed in patients 3, 8, and 10 usually cause a substantial impact on the enzyme activity, with significant or total loss of function. These findings are consistent with the phenotype observed in "age of diagnosis" of the patients, with severe symptoms (Table 2). However, the p.Pro200Ter mutation in the BCKDHB gene has already been associated with a mild phenotype (Henneke et al 2003). The p.Lys166Asn and p.Arg168Cys mutations in the BCKDHB gene have already been associated with the classical phenotype of the disease (Imtiaz et al 2017). Functional or expression studies of alleles have not been performed. Maintaining good metabolic control and early diagnosis does not provide knowledge on the natural history of the disease and the phenotypic impact of the mutations found. Flaschker et al (2007) suggest that changes in the BCKDHA gene are more severe than in the BCKDHB and DTB genes; however, this observation is directly related to the mutations found, and many patients with alterations in the last two genes present the severe phenotype. The compound heterozygotes, (as observed in many of our patients) present the combination of the two different altered alleles, affecting individual predictions about alleles.

Notably, no significant differences in age or gender emerged between groups (Table 3). The levels of leucine in the MSUD group were 166.43 μmol/l ± 27.85, and their mean isoleucine and valine levels were 180.00 μmol/l ± 27.72 and 207.08 μmol/l ± 48.92, respectively. At the time of the study, all patients with MSUD were in treatment with a protein-restricted diet with isoleucine and valine supplementation as shown in Table 1. In this context, no significant alterations in leucine and valine levels were found between the healthy controls and the patients with MSUD, while isoleucine levels were significantly higher in the patients with MSUD. Moreover, phenylalanine, serine, ornithine, glutamate, and aspartate levels were significantly reduced, and glycine/threonine/arginine levels were increased in the patients with MSUD compared with the healthy controls (Table 3).

The role of inflammatory markers in MSUD was investigated by analyzing the protein levels of GM-CSF, RANTES, sICAM1, sVCAM1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, INF-γ, and TNF-α using a Luminex xMAP kit. As seen in Table 4, a significant increase in pro-inflammatory cytokines IL-1β and IL-6 were found in the patients with MSUD compared to the control group in the adjusted analyses. However, no significant alterations in the levels of IL-2, IL-4, IL-5, IL-7, IL-8, and IL-10 were found between the healthy controls and the patients with MSUD. On the other hand, we found increased levels of TNF-α and INF-γ as well as an increase in cell adhesion molecules, sICAM1 and sVCAM1 in the patients with MSUD when compared to the controls. In addition, IL-1β, IL-6, TNF-α, INF-γ, sICAM1, and sVCAM1 remained significantly increased in the patients with MUSD in comparison with the control group after controlling for age (linear regression model). To further investigate the cytokine profile in MSUD, we also compared cytokine ratios. The patients with MSUD showed higher IFN-γ/IL-4, IL-2/IL-4, and IFN-γ/IL-10 ratios than the controls, suggesting a bias toward a Th1 profile. There was no significant difference regarding the IL-6/IL-10 ratio and TNF/IL-10 ratio.

Finally, to explore the potential clinical correlates of our findings, we tested possible correlations between the number of metabolic crises and LNAA levels with inflammatory markers. Our results showed a positive correlation between the number of metabolic crises and IL-1β levels (rho = 0.788, $p = 0.0052$, Fig. 1a) and sICAM-1 (rho = 0.816, $p = 0.0030$, Fig. 1b). However, no associations were found between protein levels of IL-6, TNF-α, INF-γ, and sVCAM1 and metabolic crisis (Suppl. Table 1). Moreover, the results of Spearman's rank correlation showed no correlations between LNAA blood levels and IL-1β, IL-6, TNF-α, INF-γ, sICAM1, and sVCAM1 levels (Suppl. Table 2).

Discussion

MSUD treatment involves long-term dietary management by restricting BCAA intake along with optimal nutritional supply to keep the plasma branched-chain metabolites continually close to normal range, protecting the brain from functional disturbances and structural damage (Strauss et al 2010). Abnormalities of immune function and cytokine levels are implicated in the pathophysiology of MSUD. The abnormalities of immune function that have been suggested to occur in MSUD are based on both direct and indirect evidence. For example, De Simone et al (2013) showed that H-BCAA microglial cells exhibit a peculiar phenotype characterized by a partial skewing toward the anti-inflammatory state, with enhanced IL-10 expression and phagocytic activity and increased free radical generation and decreased neuroprotective function. Consistent with this result, we have recently

Table 3 Plasma concentrations of amino acids (μmol/L) in MSUD patients and controls

	MSUD patients	Controls	P value	Plasma amino acid reference values (μmol/l)
Age	5.459 ± 5.097	9.10 ± 4.86	0.104	
Gender (female/male)	5/7	4/5	0.623	
Alloisoleucine (μmol/L)	137.33 ± 109.42	NA		<2 nmol/mL
Amino acids levels				
Alanine	373.20 ± 59.81	513.54 ± 72.11	0.166	152–547
Aspartate	10.60 ± 9.24.67*	48.18 ± 4.47	0.001	1–24
Glutamate	75.72 ± 15.61*	154.18 ± 24.75	0.016	5–150
Glutamine	558.34 ± 37.87	590.12 ± 69.61	0.669	254–823
Glycine/threonine/arginine	533.90 ± 40.04*	287.36 ± 33.51	0.001	172–707
Histidine	76.68 ± 4.94*	139.00 ± 30.23	0.010	41–125
Isoleucine	180.00 ± 27.72*	83.60 ± 17.78	0.011	22–107
Leucine	166.43 ± 27.85	205.54 ± 35.86	0.412	49–216
Lysine	183.40 ± 19.13	198.56 ± 33.27	0.680	48–284
Methionine	27.72 ± 2.62	28.14 ± 3.56	0.929	7–47
Ornithine	60.52 ± 5.54*	108.30 ± 17.35	0.004	10–163
Phenylalanine	63.63 ± 4.62*	130.90 ± 11.85	0.001	26–91
Serine	140.25 ± 10.17*	202.60 ± 25.73	0.015	69–187
Tryptophan	83.30 ± 8.98	78.32 ± 11.31	0.751	0–79
Tyrosine	91.47 ± 9.89	83.40 ± 5.50	0.608	24–115
Valine	207.08 ± 48.92	307.46 ± 33.55	0.214	74–321

All values are expressed as mean ± SD. P values stated have been calculated by Student's t test. Different from control, $*p < 0.05$

verified that chronic administration of H-BCAA decreases pro-inflammatory cytokine levels in the brains of rats; however, acute administration of H-BCAA increases IL-1β, IL-6, and TNF-α levels and decreases IL-10 levels in the brains of infant rats but not in those of young rats (Rosa et al 2016).

To extend this investigation and better understand the involvement of inflammation in the pathophysiology of MSUD, in the present study, we measured inflammatory markers in plasma from treated patients with the classic form of MSUD. We also investigated whether alterations of those parameters were correlated with plasma leucine, isoleucine, and valine concentrations. We demonstrated that patients with MSUD in treatment with restricted protein diets have elevated levels of pro-inflammatory cytokines [IFN-γ, TNF-α, IL-1β, and IL-6] and cell adhesion molecules [sICAM-1 and sVCAM-1] compared to controls. Our study corroborates previous work published by Mescka et al (2015a, b), which demonstrated that patients with MSUD in treatment with restricted protein diets and without L-carnitine supplementation have increased levels of pro-inflammatory cytokines IL-1β, IL-6, and IFN-γ in comparison to controls, and L-carnitine supplementation improved cellular defense against inflammation and oxidative stress. Moreover, we observed that patients with MSUD showed increased ratios of IFN-γ/IL-4, IL-2/IL-4, and IFN-γ/IL-10 in comparison to controls, suggesting a bias toward a Th1 profile in

MSUD. When activated, Th cells can differentiate into Th1, Th2, and Th17 cells. These have different immune responses via different patterns of cytokine production; Th1 cells act mainly in macrophage and T cytotoxic cell activation, Th2 cells contribute to B lymphocyte activation, while Th17 cells are responsible for tissue inflammation induction and protection against extracellular pathogens (Striz et al 2014). The bias toward a Th1 profile seen in MSUD reinforces the hypothesis that MSUD is associated with immunological imbalance or dysfunction. The combinations of IFN-γ with TNF-α or IL-1 can strikingly upregulate the expression of two Ig family adhesion molecules: ICAM-1 and VCAM-1. To the best of our knowledge, this is the first report to describe elevated levels of cell adhesion molecules [sICAM-1 and sVCAM-1] in patients with MSUD compared to controls. Thus, we could consider that altered levels of sICAM-1 and sVCAM-1 could be a result of a chronic low-grade inflammation mediated by immune markers that were previously reported to promote Th1 dominance and influence cellular adhesion in MSUD.

Cell adhesion molecules such as sICAM-1 and sVCAM-1 increase T cell infiltration and have been shown to be involved in the development of demyelinating diseases (Zameer and Hoffman 2003; Deem and Cook-Mills 2004; Bullard et al 2007). Ivkovic et al (2017) showed an involvement of sICAM-1 in state and sVCAM-1 in trait vulnerability to mood

Table 4 Plasma levels of pro-inflammatory [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, TNF-α, and IFN-γ], anti-inflammatory [IL-10] cytokines, and cell adhesion molecules [sICAM-1 and sVCAM-1] in controls and treated MSUD patients

| Inflammatory markers [pg/mg of protein]	MSUD patients	Controls	P value	Multiple linear regression
IL-1β	0.445 ± 0.061*	0.284 ± 0.047	0.0139	0.020
IL-2	0.482 ± 0.103	0.491 ± 0.095	0.4399	
IL-4	1.913 ± 0.180	1.802 ± 0.244	0.4470	
IL-5	0.481 ± 0.055	0.504 ± 0.042	0.9028	
IL-6	0.399 ± 0.026*	0.307 ± 0.024	0.0142	0.023
IL-7	2.086 ± 0.252	2.650 ± 0.192	0.2595	
IL-8	2.185 ± 0.658	3.754 ± 0.980	0.1917	
IL-10	1.004 ± 0.134	0.741 ± 0.095	0.0570	
TNF-α	1.435 ± 0.195*	0.938 ± 0.078	0.0302	0.013
INF-γ	2.427 ± 0.182*	1.876 ± 0.199	0.0307	0.005
sICAM-1	249,552 ± 10192*	206,625 ± 13,644	0.0133	0.007
sVCAM-1	1,161,703 ± 102430*	710,939 ± 35,184	0.0028	0.001
INF-γ/IL-4 ratio	1.582 ± 0.182*	1.162 ± 0.146	0.0319	
INF-γ/IL-10 ratio	2.504 ± 0.248*	1.820 ± 0.293	0.0354	
TNF-α/IL-10 ratio	1.178 ± 0.274	1.327 ± 0.207	0.9787	
IL-6/IL-10 ratio	0.394 ± 0.084	0.476 ± 0.079	0.8249	
IL-2/IL-4	0.3977 ± 0.034*	0.263 ± 0.043	0.0076	

Samples were randomized based on diagnostic group and assayed in duplicate on a Luminex 200™ system. Data represent the mean ± standard error of the mean (SEM). Student's t test was used to compare means between controls and MSUD patients, and multiple linear regression was used to adjust for possible confounders. *$p < 0.05$ compared to the control group

IFN interferon, *IL* interleukin, *TNF* tumor necrosis factor, *sICAM-1* soluble intercellular adhesion molecule-1, *sVCAM-1* soluble vascular cell adhesion molecule-1

symptoms. Similarly, another study reported a vulnerability of sICAM-1 to state symptoms in patients with schizophrenia (Schwarz et al 2000). The association of low-grade inflammation and endothelial dysfunction with depression was also demonstrated in a study published by van Dooren et al (2016). In this study, they showed that the levels of TNF-α, CRP, sICAM-1, and low-grade inflammation were associated with depressive disorder. Moreover, studies have shown that the plasma concentrations of sVCAM-1 and sICAM-1 are increased in AD (Rentzos et al 2005; Zuliani et al 2008; Popp et al 2017), suggesting that cell adhesion molecules play an essential role in Ab aggregate-stimulated endothelial-monocyte adhesion (Gonzalez-Velasquez et al 2010). Notably, generalized oedema in the CNS, atrophy of the cerebral hemispheres, spongy degeneration of white matter, and delayed myelinisation, followed by neurocognitive deficits, have been observed in patients with MSUD (Treacy et al 1992; Jan et al 2003; Strauss et al 2010; Shellmer et al 2011; Klee et al 2013; Muelly et al 2013). Taking these findings and the present results together, we suggest that the generalized spongy degeneration of white matter, delayed myelinisation, cognitive impairment, and neuropsychiatric illness observed in patients with MSUD may be mediated by inflammatory responses and endothelial dysfunction.

In an attempt to explore the potential clinical correlates of our findings we tested possible correlations between clinical characteristics and inflammatory markers. We found a positive correlation between IL-1β and sICAM-1 levels and metabolic crisis, suggesting that a higher number of metabolic crises may be involved in the immunological imbalance by Th1 profile. Corroborating these results, a study with 35 patients with neonatal-onset MSUD showed that the need for psychological follow-up was significantly associated with the number of lifetime metabolic decompensations (Abi-Warde et al 2017). Although correlation does not necessarily imply causation since we have confounding factors such as recurrent infections, correlation could be evidence for causation, and more work will be needed to explore these possibilities. On the other hand, we observed no correlations between blood amino acid levels and interleukin levels or cell adhesion molecules in this study. Taken together, it may be presumed that leucine and the other BCAAs are not directly associated with pro-inflammatory cytokine production in MSUD. In this line, Mescka et al (2015a, b) suggested that oxidative stress observed in patients with MSUD may be related, at least in part, to the increase in pro-inflammatory cytokine plasma levels, since a positive correlation was found between pro-inflammatory cytokines and MDA values, the main marker of lipid peroxidation, in the plasma of patients with MSUD. Moreover, studies have shown evidence of long-term deficits in neurocognition despite acceptable metabolic control
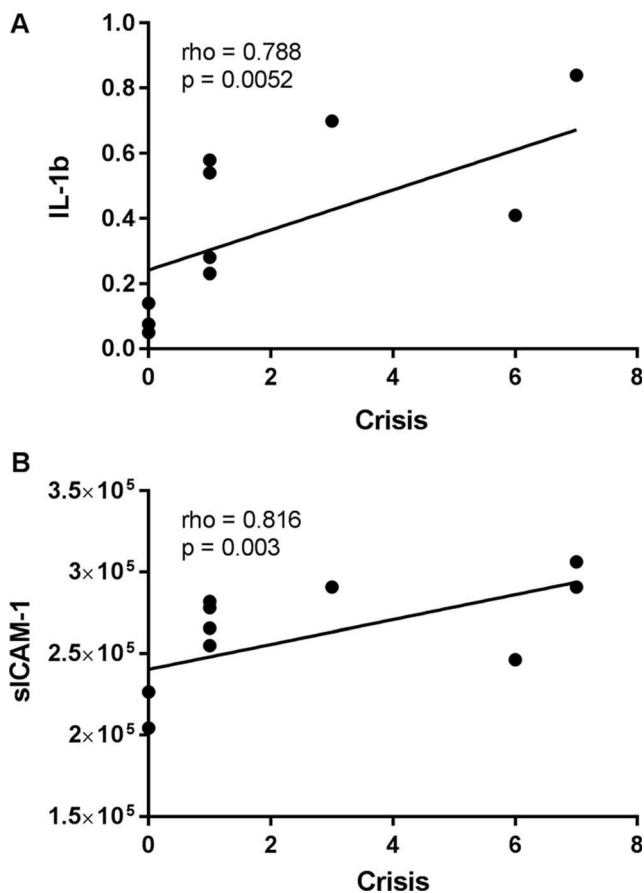
Fig. 1 Correlation coefficient of IL-1β (**a**) and sICAM-1 (**b**) with the number of metabolic crises in controls and treated patients with MSUDs. The results were assessed using Spearman's Rho(r) correlation analyses

immunological mechanisms are involved in the pathogenesis and psychopathology of MSUD, but this process is not directly associated with the BCAA blood levels. A better knowledge of these events may contribute to a more comprehensive understanding of the biological processes in this disease and provide new targets for future treatment strategies to improve the quality of life of MSUD patients.

Compliance with ethical standards

Conflict of interest G. Scaini, T. Tonon, C. F. Moura de Souza, P. F. Schuck, G. C. Ferreira, J. Quevedo, J. S. Neto, T. Amorim, J. S. Camelo Jr, A. V. B. Margutti, R. Tresbach, F. Sperb-Ludwig, R. Boy, P. F. V. de Medeiros, I. V. D. Schwartz, E. L. Streck declare that they have no conflict of interest.

(McLaughlin et al 2013; Vogel et al 2014), suggesting that early lesions to the brain may have long-term consequences (Muelly et al 2013). Additionally, a recent study showed that lower levels of BDNF and PDGF-AA, and higher levels of NCAM and cathepsin D, are not correlated with leucine levels in patients with MSUD under treatment (Scaini et al 2016).

Our results should be interpreted considering their limitations. The small number of patients and healthy controls may be a factor preventing us from finding significant differences in the biomarkers evaluated. Thus, a validation study comprising a larger sample size is needed to confirm the current findings. Moreover, we did not evaluate the impact of inflammatory markers and their relation with cognitive impairment and neuropsychiatric illness in patients with MSUD. Therefore, future longitudinal studies involving inflammatory markers levels related to the Th1 profile may help clarify the role of inflammation in cognitive impairment and psychiatric symptoms among individuals with MSUD.

In conclusion, our study corroborates and expands evidence from previous studies by showing significant changes in inflammatory markers in patients with MSUD. Our data together with previous studies corroborate the hypothesis that

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Quality of life and adherence to treatment in early-treated Brazilian phenylketonuria pediatric patients

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Abstract

Early dietary treatment of phenylketonuria (PKU), an inborn error of phenylalanine (Phe) metabolism, results in normal cognitive development. Although health-related quality of life (HRQoL) of PKU patients has been reported as unaffected in high-income countries, there are scarce data concerning HRQoL and adherence to treatment of PKU children and adolescents from Brazil. The present study compared HRQoL scores in core dimensions of Brazilian early-treated PKU pediatric patients with those of a reference population, and explored possible relationships between adherence to treatment and HRQoL. Early-treated PKU pediatric patient HRQoL was evaluated by self- and parent-proxy reports of the Pediatric Quality of Life Inventory (PedsQL) core scales. Adherence to treatment was evaluated by median Phe levels and percentage of results within the therapeutic target range in two periods. Means for total and core scales scores of PedsQL self- and parent proxy-reports of PKU patients were significantly lower than their respective means for controls. Adequacy of median Phe concentrations and the mean percentage of values in the target range fell substantially from the first year of life to the last year of this study. There was no significant difference in mean total and core scale scores for self- and parent proxy-reports between patients with adequate and those with inadequate median Phe concentrations. The harmful consequences for intellectual capacity caused by poor adherence to dietary treatment could explain the observed decrease in all HRQoL scales, especially in school functioning. Healthcare system and financial difficulties may also have influenced negatively all HRQoL dimensions.

Key words: Phenylketonuria; Quality of life; Questionnaires; Patient compliance; Diet therapy

Introduction

Phenylketonuria (PKU, OMIM 261600) is an autosomal recessive inborn error of phenylalanine metabolism, caused by a deficient activity of phenylalanine hydroxylase (PAH, EC 1.14.16.1) – an enzyme that coverts the essential amino acid phenylalanine (Phe) to tyrosine, leading to an accumulation of Phe in blood and other tissues (1). When untreated, most individuals with PKU develop severe intellectual disability, microcephaly, neurological disorders, eczema and decreased hair and skin pigmentation (2). In Latin America, it was estimated an incidence of PKU of about 1:21,000 live births (1:12,000 to 1:52,000) (3). In Brazil, the incidence of PKU was reported as approximately 1 in 25,000 live births (4), although there are important variations among different Brazilian states, from 1:9,000 to 1:33,000 (5–9).

The establishment of mass newborn screening programs allowed the detection of PKU in the neonatal period and the early establishment of dietary treatment, resulting in a normal cognitive development (10). In 2001, the National Neonatal Screening Program was established within the Brazilian National Health System, ensuring access of all newborns to screening, confirmatory tests, and treatment for PKU, including the provision of metabolic phenylalanine-free formula (4).

The need to adhere to a strict diet, frequent blood sampling to monitor the levels of Phe and regular visits to health services, can affect daily life and therefore have a negative impact on the health-related quality of life (HRQoL) of individuals affected by PKU (11–13). In addition, there is evidence that adherence to diet treatment in early-treated

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PKU patients deteriorates with increasing age, being unsatisfactory in older children and adolescents (14). Low adherence to treatment, besides its negative consequences for the psychomotor development of pediatric patients, could also reduce their HRQoL, as has been suggested for adults (15,16).

The present study intends to contribute to the knowledge of HRQoL of early-treated pediatric patients in Brazil, investigating differences in core dimensions of this outcome measure between them and a reference healthy schoolchildren population, and exploring possible relationships between adherence to treatment and HRQoL.

Material and Methods

Study design

The study was conducted from March 2012 to July 2014. Parents of patients with early-treated PKU, ranging in age from 6 to 18 years, followed in two centers in Brazil – State Institute of Diabetes and Endocrinology Luiz Capriglione (IEDE), Rio de Janeiro, RJ, and Medical Genetics Service, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, RS, were invited to participate in the study. The affected individuals should have had a diagnosis of classic, moderate or mild PKU in the first four months of life, and since then been submitted to a protein-restricted diet supplemented with a Phe-free amino acid formula. Classic PKU was defined as the finding of pre-treatment blood Phe levels repeatedly >1200 μmol/L (>20 mg/dL), whereas moderate and mild PKU were characterized by pre-treatment blood Phe levels in the ranges of 900–1200 μmol/L (15–20 mg/dL), and 600–900 μmol/L (10–15 mg/dL), respectively (17). They might have presented varying degrees of treatment adherence and metabolic control, but all participants had their blood Phe levels regularly monitored.

The following exclusion criteria were adopted: 1) inability of children and parents in understanding the study questionnaires; 2) presence of severe chronic or disabling diseases unrelated to PKU.

Participants' demographics

A total of 105 affected pediatric patients fulfilled the eligibility criteria, of which 63 were followed at IEDE and 42 at HCPA. Fifty-one of the 105 eligible children and adolescents with PKU were recruited and accepted to participate in the study (response rate 49%), including 38 followed at IEDE and 13 at HCPA. The sample included 33 classic PKU and 17 mild or moderate PKU patients (for 1 patient this information could not be obtained). The mean age at diagnosis was 45 ± 22 days, and the mean age at the start of treatment with a Phe-restricted diet was 48 ± 20 days.

Thirteen of 38 children and adolescents from Rio de Janeiro evaluated by RCPM and RSPM had their intellectual capacity classified as Grade IV (definitely below average in intellectual capacity) or Grade V (intellectually defective). Intellectual capacity of 1 of 13 patients from Rio Grande do Sul was evaluated clinically as defective with no formal psychometric test being applied.

Twenty-nine patients lived in Metropolitan areas, while 22 lived in the countryside of both states. Twenty-two females and 29 males were enrolled in the study. Twenty-five children and adolescents belonged to middle-income families (class C), 20 to high-income families (classes A and B), and 2 to low-income families (class D) – four families did not fill the socioeconomic status evaluation form.

The gender distribution of our sample of patients did not differ from that of the normative sample of healthy school children and adolescents (21) (χ^2=1.2240, P=0.269). The socioeconomic status distribution of our sample of patients differed from the normative sample (χ^2=6.7518, P=0.034), due to the higher percentage of high-income families in our sample – 42.6 *vs* 31.1%. Nevertheless, the socioeconomic status distribution of our PKU patient sample did not differ significantly from those of the populations of Rio de Janeiro (χ^2=7.6612, P=0.176) and Porto Alegre (χ^2=2.5856, P=0.764) metropolitan areas (22).

Ethical issues

This study was approved by the Research Ethics Review Committee of IEDE and HCPA. At all times, the study was conducted under ethical principles and in line with the Guidelines and Standards for Research in Human Beings, established by Resolution No. 466/2012 of the Brazilian National Health Council (18). Informed consent was obtained from the parents or guardians of all pediatric patients engaged in this study. Active consent was then obtained from each individual child and adolescent whose parents agreed to his/her participation.

Quality of life and socioeconomic status questionnaires, and intellectual capacity tests

Pediatric Quality of Life Inventory (PedsQL) Version 4.0 is a generic questionnaire for assessing HRQoL of children and adolescents aged 2 to 18 years, originally developed by Varni et al. (19). Its core scales are designed to evaluate four dimensions – physical health, emotional functioning, social functioning, and school functioning. The aggregation of emotional, social and school dimensions is the psychosocial health summary score (19). Items of the questionnaire are Likert scales that are reverse-scored and linearly transformed to a 0–100 scale, where higher scores represent better quality of life. Self-assessment and parent proxy forms of the questionnaire were employed according to the child or adolescent's age.

Normative data from healthy children and adolescents were collected in the city of São Paulo, SP, Brazil, by the authors of the translation and validation to Brazilian Portuguese of the original English version of PedsQL 4.0, which results have been published elsewhere (20,21). This reference population included 240 healthy children

and adolescents from 2 to 18 years of age living in urban areas of the outskirts of Greater São Paulo, and their respective parents. Enrolment locations were public schools for children aged 5 to 18 and primary healthcare units for children aged 2 to 4. Inclusion criteria included ability to answer the HRQoL questionnaire, absence of chronic or acute illness in the last 30 days before the interview, and presence of at least 1 parent on the day the questionnaire was applied.

Socioeconomic status evaluation of the families of children and adolescents with PKU was carried out according to the criteria of the Brazilian Association of Research Companies (22). For comparison purposes, these criteria were simplified to three classes: low-income (classes D and E), middle-income (classes C1 and C2), and high-income classes (classes A1, A2, B1, and B2).

Intellectual capacity of children aged 6 to 11 years was evaluated by Raven's Colored Progressive Matrices - RCPM (23), and of adolescents aged 12 to 18 years by Raven's Standard Progressive Matrices - RSPM (24). These are non-verbal intelligence tests developed by John C. Raven in 1936 in the United Kingdom, which employ abstract geometrical figures to directly measure general intelligence, and avoid influences of cultural elements and training. In the present study, we used the Brazilian standardizations of RCPM and of RSPM. The Brazilian standardization of RCPM is based on the results of 1547 kindergarten and elementary school children, aged 5 to 11.5 years, from the city of São Paulo (25), and of RSPM is based on 366 individuals with educational level ranging from elementary to postgraduate school, from the city of Rio de Janeiro (24).

Adherence to treatment

Adherence to treatment was measured indirectly by the median Phe level in the first year of life and in the last year of treatment evaluated by this study (2010–2012). We also evaluated, for both periods, the percentage of blood Phe results in the treatment target range according to the Brazilian PKU Guidelines (26). Adherent children and adolescents were defined as those with median Phe levels $\leqslant 6$ mg/dL in the first year of life, or $\leqslant 10$ mg/dL for those $\geqslant 13$ years of age and $\leqslant 6$ mg/dL for those < 13, in the last year of the study.

Statistical analyses

The t-test for two independent samples was conducted between the normative pediatric data and the results of the self-assessment and parent proxy reports for the PedsQL core scales of PKU children and adolescents in this study. This same test was employed to evaluate potential significant differences in HRQoL between states or area of residence (Rio de Janeiro and Porto Alegre Metropolitan areas or countryside), gender, and severity of disease (classic or mild PKU). One-way ANOVA was used to assess differences in HRQoL scores due to

socioeconomic status. A multi-way ANOVA taking into account state or area of residence, gender, disease severity, and socioeconomic status was also performed.

Associations between scales of the PedsQL dimensions for self-assessment and parent proxy reports and the adherence to treatment variables were examined by linear regression analysis. Two independent samples t-test was conducted between the results of the self-assessment and parent proxy reports of the PedsQL scales for adherent and non-adherent PKU children and adolescents. A multi-way ANOVA taking into account adherence to treatment in the first year of life and in the last year of the study and the other previously mentioned independent variables was also carried out.

All statistical analyses were conducted with Stata/SE 12.1 for Mac software package (StataCorp, USA).

Minimum sample size

The minimum sample size was calculated from the previously published mean scores and standard deviations for the PedsQL core scales results of the self-assessment and parent proxy reports of Brazilian healthy children and children with rheumatic diseases (20). A minimum sample size of 35 children in each group was considered in order to detect a difference of ten points on a hypothetical scale, the variance of which was the mean of the weighted variances of the physical, emotional, social and school scales of the self-assessment and parent proxy reports (219.5), accepting an alpha error of 5% and a beta error of 20%.

Results

HRQoL

PedsQL self-reports were administered to 49 early-treated PKU patients aged 6 to 17 years – self-reports could not be administered to 2 patients (1 refused to answer the questionnaire and the other was not able to fill it out owing to intellectual disability). Means for total score and for the scores from physical health, emotional functioning, social functioning, school functioning, and psychosocial health scales for PedsQL self-reports were significantly lower than the respective means from the normative sample of healthy schoolchildren (Table 1).

In addition, PedsQL parent proxy-reports were administered to 34 parents and caretakers of early-treated PKU children and adolescents. Most of these parents and caretakers (33) were from Rio de Janeiro, which corresponds to 86.8% of this state sample. With the exception of emotional functioning scale, mean scores for the generic core scales for parent proxy-reports were significantly lower than the respective means for parent proxy-reports from the normative sample (Table 2).

The mean total score for PedsQL self-reports of children and adolescents from Rio de Janeiro (n=37) was not significantly different from the mean of their peers from

Table 1. Self-reported scores for PedsQL 4.0 generic core scales of Brazilian early-treated phenylketonuria (PKU) pediatric patients.

Aspect	Items	PKU (n=49)	Controls (n=180)	P
Total score	23	75.31 ± 12.04	88.90 ± 7.35	<0.0001
Physical health	8	82.54 ± 14.67	95.94 ± 5.83	<0.0001
Emotional functioning	5	66.84 ± 18.31	73.03 ± 16.52	0.0241
Social functioning	5	79.29 ± 22.52	93.14 ± 10.54	0.0001
School functioning	5	68.27 ± 17.00	89.31 ± 11.80[a]	<0.0001
Psychosocial health	15	71.44 ± 13.73	85.03 ± 9.66	<0.0001

Data are reported as means ± SD. The *t*-test for two independent samples considering as controls a group of healthy school children of the city of São Paulo, Brazil was used. [a] n=173.

Table 2. Parent-proxy scores for PedsQL 4.0 generic core scales of Brazilian early-treated phenylketonuria (PKU) pediatric patients.

Aspect	Items	PKU (n=34)	Controls n=240)	P
Total score	23	79.98 ± 16.26	92.32 ± 6.01	0.0001
Physical health	8	89.62 ± 16.71	97.86 ± 4.31	0.0072
Emotional functioning	5	73.97 ± 20.59	80.52 ± 12.59	0.0789[a]
Social functioning	5	84.85 ± 21.93	96.38 ± 8.89	0.0046
School functioning	5	65.74 ± 24.25	90.93 ± 11.85[b]	<0.0001
Psychosocial health	15	74.85 ± 18.24	89.18 ± 8.19	0.0001

Data are reported as means ± SD. The *t*-test for two independent samples considering as controls a group of healthy school children of the city of São Paulo, Brazil was used. [a] Non-significant difference. [b] n=207.

Rio Grande do Sul (n=12, t=0.5911, P=0.5573). We did not find significant differences between pediatric patients from the two states with regard to emotional functioning (t=0.4868, P=0.6287), social functioning (t=–0.7870, P=0.4352), school functioning (t=–0.6960, P=0.4899), and psychosocial health (t=–0.4910, P=0.6257) scores. Unexpectedly, the patients from Rio de Janeiro evaluated their physical health more positively than their peers from Rio Grande do Sul (t=2.3615, P=0.0224). This difference was upheld even when other independent variables such as region, gender, socioeconomic class, and disease severity were taken into account in a multi-way ANOVA model (F=11.85, P=0.0014). The number of parent proxy-reports from Rio Grande do Sul (n=1) was too small to allow any statistical inference.

In addition, children and adolescents from the interior of both states (n=22) evaluated their global quality of life similar to those living in the metropolitan areas (n=29) (t=–0.8086, P=0.4228). Mean scores for all the generic self-report core scales did not differ significantly between countryside and metropolitan area pediatric patients.

Parent proxy-reports from Rio de Janeiro pediatric patients also did not show any significant difference between metropolitan area (n=23) and countryside (n=10) concerning PedsQL total score (t=–0.6348, P=0.5302). Mean scores for all the generic parent proxy-report core

scales did not differ significantly between patients from countryside and metropolitan areas of Rio de Janeiro.

We did not find any significant gender difference concerning the self-perception (t=–1.3172, P=0.1942) and the parents' perception (t=–0.8625, P=0.3948) of quality of life assessed by PedsQL self-report and parent proxy-report total scores. But girls self-evaluated their emotional functioning more negatively than boys (t=–2.8485, P=0.0065). This was the only PedsQL core scale that showed any gender difference for self-reports, but this difference tended to disappear when other independent variables were included in a multi-way ANOVA model (F=3.06, P=0.0884). Considering exclusively parent proxy-reports, there was no gender difference in any PedsQL core scale.

Mean total score for PedsQL self-reports of children and adolescents with mild or moderate PKU was not significantly different compared to pediatric patients affected by classic PKU (t=–0.1901, P=0.8501). Physical health (t=–0.2400, P=0.8114), emotional functioning (t=0.6868, P=0.4957), social functioning (t=–0.5163, P=0.6081), school functioning (t=–0.3594, P=0.7210), and psychosocial health (t=–0.1240, P=0.9019) self-report scores did not show any significant difference between mild or moderate and classic PKU pediatric patients either.

Nevertheless, the mean total score for PedsQL parent-reports of children and adolescents with mild or moderate

PKU was significantly higher than that of parent-reports of pediatric patients affected by classic PKU (t=–2.5012, P=0.0189). This difference tended to disappear when other independent variables (region, socioeconomic class, and disease severity) were included in a multi-way ANOVA model (F=3.97, P=0.0561). Similar differences were also found concerning physical health (t=–2.2383, P=0.0323), and school functioning (t=–2.0841, P=0.0452) in parent-report scores. However, only mean score for school functioning in parent-reports was persistently lower in classic PKU (62.2) than mild or moderate PKU (80.6) in a multi-way ANOVA model (F=4.42, P=0.0446). No significant differences were found between the two groups with regard to emotional functioning (t=–1.2524, P=0.2195), social functioning (t=–1.0938, P=0.2838), and psychosocial health (t=–1.6988, P=0.0991) scores.

The number of low-income class families of PKU patients (classes D and E, n=2) was too small for making any significant conclusions. Thus, the assertions regarding socioeconomic classes refer to differences between middle-income – C (n=24) and high-income – A and B (n=20) classes. There was no significant difference in mean total score for PedsQL self-reports of children and adolescents among different socioeconomic classes (F=0.68, P=0.5113). Physical health (F=0.20, P=0.8199), social functioning (F=0.18, P=0.8367), school functioning (F=0.93, P=0.4014), and psychosocial health (F=0.79, P=0.4596) self-report scores also did not show any significant difference among socioeconomic classes, but surprisingly children and adolescents from middle-income class evaluated their emotional functioning more positively than their high-income class peers (F=6.60, P=0.0032). This difference was retained even when other independent variables were considered in a multi-way ANOVA model (F=11.29, P=0.0018).

With reference to the parent proxy-reports, we also did not find a significant difference in mean total score among different socioeconomic classes (t=0.5448, P=0.5898). Physical health (t=0.6749, P=0.5070), emotional functioning (t=–0.0050, P=0.9960), social functioning (t=0.3839, P=0.7037), school functioning (t=0.6228, P=0.5379), and psychosocial health (t=0.4210, P=0.6767) parent proxy-report scores also did not show any significant difference among socioeconomic classes.

Adherence to treatment

The mean value for the median Phe concentration in the first year of life was 5.3 ± 4.1 mg/dL. Thirty-five children (70.0%) had adequate median Phe concentrations at this age, i.e., below the upper limit of the target range for children 0 to 12 months of age – 2 to 6 mg/dL, according to the Brazilian PKU Guidelines (26). The distribution of the median Phe concentrations in the first year of life is depicted in Figure 1. The mean percentage of values in the target range was $61.3 \pm 28.9\%$ in this period. There was no significant difference in median Phe concentrations in the first year of life among pediatric patients of the two states (t=–0.6663, P=0.5087) or of the two regions – countryside and metropolitan areas (t=1.4452, P=0.1549) included in the present study. Gender (t=0.0888, P=0.9296) and severity of disease (t=1.0046, P=0.3203) also did not influence this adherence parameter. The median Phe concentrations in the first year of life of middle-income class patients did not differ significantly from those of high-income class patients (t=–0.3114, P=0.7571). Similar results were found for the other parameter of adherence in the first year of life (percentage of values in the target range).

In the last year of treatment evaluated by this study (2010–2012) the mean value for the median Phe
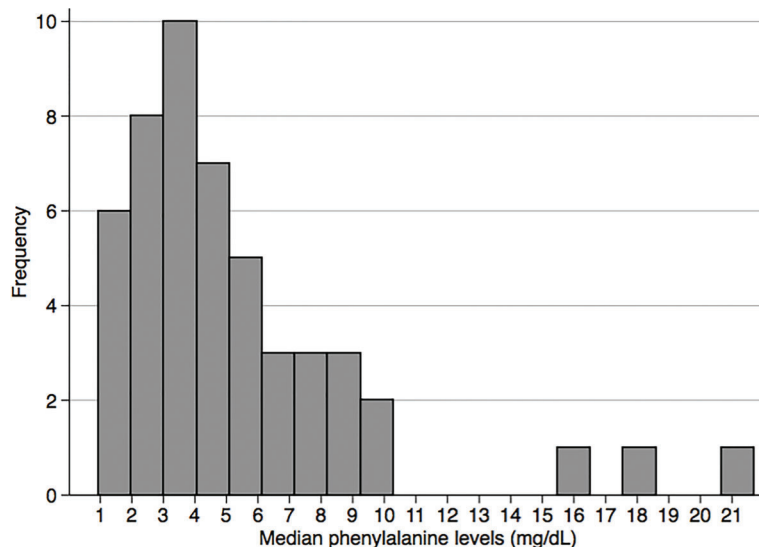
Figure 1. Median phenylalanine levels in the first year of life of Brazilian early-treated PKU pediatric patients. Thirty percent of the patients had median phenylalanine levels in the first year of life above the upper limit of the recommended target range for this age (6 mg/dL). PKU: phenylketonuria.

concentration increased to 9.2 ± 4.3 mg/dL, and the percentage of children with adequate median Phe concentrations fell to 31.4% (16/51). The mean percentage of values in the target range also dropped to 30.9 ± 36.7% in the last year of treatment. There was no significant difference in median Phe concentrations in the last year of treatment among pediatric patients of the two states (t=0.4737, P=0.6378) or of the countryside and metropolitan areas (t=–0.8735, P=0.3867) included in the present study. Gender (t=0.4941, P=0.6235), disease severity (t=1.9745, P=0.0541), and socioeconomic status (t=0.4676, P=0.6424) also did not influence this adherence parameter. Similar results were found for percentage of values in the target range.

No statistically significant association was found between scores for self-report PedsQL generic core scales and the four 'adherence to treatment' variables by linear regression analysis (Table 3).

There was a significant inverse relationship between parent proxy-report school functioning score and median Phe levels in the first year of life (t=–2.64, P=0.013; Figure 2). No other statistically significant association was found between scores for parent proxy-report PedsQL generic core scales and the four 'adherence to treatment' variables by linear regression analysis (Table 4)

Means for all self-report and parent proxy-report scores of PedsQL generic core scales of adherent PKU children and adolescents were not different from the respective means of their non-adherent peers, both in the first year of life and in the last year of the study (Supplementary Table S1). A multi-way ANOVA taking into account adherence to treatment in these two periods and the other previously mentioned independent variables did not show any significant difference attributable to adherence for the self-report and parent proxy-report PedsQL generic core scales score of adherent and non-adherent PKU pediatric patients (Supplementary Table S2).

Discussion

A generic instrument of pediatric HRQoL, PedsQL, was chosen to ascertain the impact of PKU and its treatment on the daily life of affected individuals, in their physical, emotional, social, and school dimensions, allowing the cross-comparison to a normal healthy population of school-age children. Generic instruments of HRQoL permit comparisons between different populations (27), and consequently are indispensable for exploratory studies as ours. Disease-specific instruments of HRQoL are generally more sensitive than generic versions for assessing the impact of particular interventions, and are essential to demonstrate small changes over time in HRQoL, for example after the start of BH4 treatment (27,28). Nonetheless, Brazilian and European Portuguese versions of the PKU-specific HRQoL questionnaire, PKUQOL (28,29), were not available during the execution of this study.

Lower scores were obtained for almost all the generic core scales for PedsQL self-reports and parent-proxy reports of PKU children and adolescents when compared to a normative sample of healthy Brazilian schoolchildren. This definitely is in contrast to the findings of most studies done in high-income countries that demonstrated that HRQoL of patients with PKU is similar to that of the general population (11,12,28,30,31). PedsQL was employed only in one of these studies (28), a recent multicenter European study that showed that HRQoL scores of children, adolescents, and adults with PKU evaluated by generic instruments were in the same range as those established as reference values for the US population.

This striking difference between our findings and those of the studies from high-income countries could be attributed, in the case of the European multicenter study (28), to the access to pharmacological treatment with BH4, as increased Phe tolerance and reduced metabolic formula requirement in BH4-responsive patients can improve HRQoL (32).

Table 3. Linear regression analysis of the self-report scores for PedsQL 4.0 generic core scales and adherence to treatment variables of Brazilian early-treated phenylketonuria (PKU) pediatric patients.

| Aspect (dependent variable) | Independent adherence to treatment variables | | | | | | | |
| | Median Phe 1st year | | % Phe results in the target range 1st year | | Median Phe last year | | % Phe results in the target range last year | |
	t^a	P	t	P	t	P	t	P
Total score	–0.93	0.356	–0.14	0.891	–0.70	0.488	0.33	0.747
Physical health	–1.28	0.208	1.67	0.102	–0.45	0.653	0.30	0.766
Emotional functioning	0.34	0.733	–1.45	0.154	0.21	0.831	–0.21	0.832
Social functioning	–0.41	0.685	–0.60	0.550	–0.91	0.365	0.29	0.776
School functioning	–1.19	0.242	–0.42	0.678	–0.66	0.512	0.49	0.630
Psychosocial health	–0.53	0.599	–1.13	0.264	–0.68	0.501	0.27	0.791

Phe: phenylalanine. [a] t-statistic.
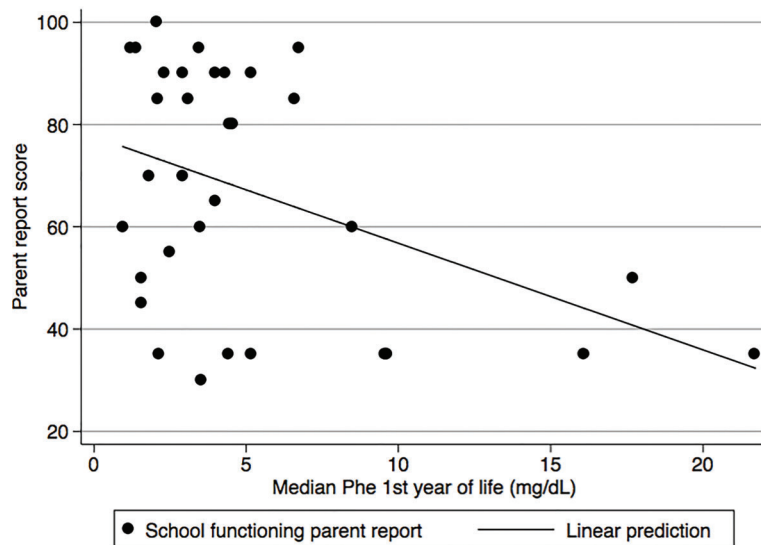
Figure 2. Parent proxy-report school functioning score and median phenylalanine (Phe) levels in the first year of life. A significant inverse relationship between these two variables was found by linear regression analysis (t=–2.64, P=0.013).

Table 4. Linear regression analysis of the parent proxy-report scores for PedsQL 4.0 generic core scales and adherence to treatment variables of Brazilian early-treated phenylketonuria (PKU) pediatric patients.

Aspect (dependent variable)	Independent adherence to treatment variables							
	Median Phe 1st year		% Phe results in the target range 1st year		Median Phe last year		% Phe results in the target range last year	
	t^a	P	t	P	t	P	t	P
Total score	–1.31	0.200	0.49	0.630	–0.42	0.679	–0.48	0.632
Physical health	–0.83	0.415	0.50	0.617	–0.53	0.598	–0.40	0.689
Emotional functioning	–0.71	0.484	–0.53	0.603	0.20	0.844	–0.51	0.616
Social functioning	–0.17	0.869	–0.15	0.878	0.56	0.579	–1.55	0.130
School functioning	–2.64	0.013*	1.64	0.111	–1.41	0.169	0.74	0.465
Psychosocial health	–1.39	0.175	0.42	0.676	–0.31	0.760	–0.47	0.643

Phe: phenylalanine. ^{a} t-statistic. * $P \leqslant 0.05$.

Nevertheless, this cannot be sustained as the other four studies were conducted before BH4 became available. Difficulties faced by the Brazilian National Health System in ensuring a regular supply of PKU metabolic formula to patients, and the financial burden of out-of-pocket acquisition of low protein food by families may have a negative impact on HRQoL of Brazilian PKU patients (33).

The relatively low response rate of the present study - 49%, compared to the studies of PKU patients in European countries, ranging from 59 to 91% (11,12,28,30,31), could not explain the lower HRQoL of Brazilian PKU children and adolescents, since the included patients were those that regularly attended the scheduled metabolic and nutritional consultations, and therefore had presumably a higher HRQoL than their counterparts that were dropping out of follow-up.

Although our sample size was larger (self-assessment form) or at the limit (parent proxy form) of the calculated minimum number, the low response rate may have affected the clinical strength of our results. Therefore, extrapolation of our results to a national perspective ought to be made with caution. A Brazilian multi-center study with a larger cohort is necessary to achieve a stronger statistical and especially clinical significance.

Two studies showed results similar to ours. One study conducted in the Russian Federation, using the same questionnaire as in the present study, showed statistically significant lower scores for PKU children when compared to healthy peers in all PedsQL core scales (34). Lower HRQoL scores, assessed by the Child Health Questionnaire (CHQ), were also demonstrated in a group of children and adolescents in Italy (35).

An increasingly low adherence to treatment over time as PKU patients reached school age and particularly adolescence was found in this study. This deterioration in treatment adherence is expected in PKU patients as well as in other chronic conditions that require diet therapy for many reasons. Adolescents and adults perceive dietary therapy as a constraint to their activities of daily living, find it hard to prepare protein substitutes, are more prone to social and peer group pressures, and may perceive their condition as stable (14,31,36).

This non-adherence could have influenced negatively HRQoL by reducing the intellectual capacity of PKU children and adolescents. We could not demonstrate, on the other hand, a correlation between adherence to treatment and quality of life in these patients. The lower scores obtained for almost all HRQoL scales by PKU children and adolescents in comparison to a normative population could not be attributed to non-adherence in the present study. This absence of correlation between adherence to treatment and HRQoL in PKU was also found by Cotugno et al. (35) in Italy. Cazzorla et al. (37), also from Italy, reached opposing results, finding an association between Phe levels and HRQoL scores in PKU. However, they included patients on BH4 treatment, and consequently their results are not comparable to our and Cotugno et al. (35) studies.

There is a scarcity of studies addressing the correlation between adherence to treatment and HRQoL in PKU children and adolescents. These two Italian studies are the few that directly concentrated on the subject.

Even though the correlation between compliance and HRQoL in PKU is debatable, there is solid evidence that PKU diet is hard to comply with (14,38), and, as a result, patients and their families consider it as a limitation to their daily life activities. This may have obscured any potential difference attributable to adherence, and may possibly have negatively influenced all HRQoL dimensions independently of achieving recommended phenylalanine levels.

Nevertheless, the importance of an adequate adherence to treatment at an early age on cognitive development and school performance cannot be overemphasized. The lower performance of Brazilian PKU children in executive functions (39), and intellectual capacity (40)

are most probably due to the highly unsatisfactory adherence to diet treatment found in the reference centers around the country. The significant inverse relationship between parent proxy-report school functioning score and median Phe levels in the first year of life found in the present study (Figure 2) is a strong evidence of the impact of non-adherence in cognitive performance.

Lower scores were obtained for almost all HRQoL scales for self-reports and parent-proxy reports of PKU patients when compared to a normative sample of healthy Brazilian school children. Poor adherence to dietary treatment, ascertained by phenylalanine blood levels, was also found not to be optimal in the first year of life and tended to worsen, as patients grew older. Nonetheless, no significant differences in HRQoL scores were found between adherent and non-adherent children and adolescents. Failure of the healthcare system to ensure a regular supply of PKU metabolic formula to patients and the financial burden of acquisition of low protein food by families can be compounded by the well-known barriers to dietary compliance in PKU children and adolescents from Brazil affecting negatively all HRQoL dimensions irrespective of maintaining a satisfactory phenylalanine blood level. Few articles have addressed the impact of adherence to dietary treatment on HRQoL of patients with PKU and consequently further studies are urgently needed to clarify if adherence to diet therapy leads in fact to a better HRQoL, especially among children and adolescents.

Supplementary material

Click here to view [pdf].

Acknowledgments

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Serum Markers of Neurodegeneration in Maple Syrup Urine Disease

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Abstract Maple syrup urine disease (MSUD) is an inherited disorder caused by deficient activity of the branched-chain α-keto acid dehydrogenase complex involved in the degradation pathway of branched-chain amino acids (BCAAs) and their respective α-keto-acids. Patients affected by MSUD present severe neurological symptoms and brain abnormalities, whose pathophysiology is poorly known. However, preclinical studies have suggested alterations in markers involved with neurodegeneration. Because there are no studies in the literature that report the neurodegenerative markers in MSUD patients, the present study evaluated neurodegenerative markers (brain-derived neurotrophic factor (BDNF), cathepsin D, neural cell adhesion molecule (NCAM), plasminogen activator inhibitor-1 total (PAI-1 (total)), platelet-derived growth factor AA (PDGF-AA), PDGF-AB/BB) in plasma from 10 MSUD patients during dietary treatment. Our results showed a significant decrease in BDNF and PDGF-AA levels in MSUD patients. On the other hand, NCAM and cathepsin D levels were significantly greater in MSUD patients compared to the control group, while no significant changes were observed in the levels of PAI-1 (total) and PDGF-AB/BB between the control and MSUD groups. Our data show that MSUD patients present alterations in proteins involved in the neurodegenerative process. Thus, the present findings corroborate previous studies that demonstrated that neurotrophic factors and lysosomal proteases may contribute, along with other mechanisms, to the intellectual deficit and neurodegeneration observed in MSUD.

Keywords Maple syrup urine disease · Neurodegeneration · Brain-derived neurotrophic factor (BDNF) · Platelet-derived growth factor AA (PDGF-AA) · Neural cell adhesion molecule (NCAM) · Cathepsin D

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Introduction

Maple syrup urine disease (MSUD; branched-chain keto aciduria; OMIM 248600) is an autosomal recessive inborn error of metabolism, with an estimated world frequency of approximately 1 in 185,000 newborns. It is caused by deficiency of the activity of the mitochondrial enzyme complex branched-chain l-2-keto acid dehydrogenase (BCKAD, E.C. 1.2.4.4), which leads to the accumulation of the branched-chain amino acids (BCAAs) leucine, isoleucine and valine and the corresponding branched-chain α-keto acids (BCKAs) α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV) [1]. MSUD manifests itself with heterogeneous clinical and molecular phenotypes, characterized by ketoacidosis,

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hypoglycemia, poor feeding, apnea, ataxia, convulsions, coma, psychomotor delay, and mental retardation, as well as generalized edema in the central nervous system (CNS), atrophy of the cerebral hemispheres, spongy degeneration of white matter, and delayed myelinization [1, 2].

Although neurological sequelae are common in untreated and late-treated MSUD patients, the mechanisms of brain damage are still poorly understood. However, leucine and/or its keto acid are considered to be the main neurotoxic metabolites in MSUD, as their increased plasma concentrations (approximately 5.0 mM) are associated with the appearance of neurological symptoms [1, 3]. The neurotoxicity of leucine stems in part from its ability to interfere with the transport of other large neutral amino acids across the blood-brain barrier, reducing the supply of tryptophan, methionine, tyrosine, phenylalanine, histidine, valine, and threonine to the brain and leading to brain injury as a result of the decreased synthesis of neurotransmitters, such as dopamine, serotonin, norepinephrine, and histamine [4–7]. Additionally, it has been reported that metabolite accumulation in MSUD produces dysfunction in mitochondria bioenergetics [8–11], apoptosis of neural cells [12, 13], oxidative stress [14–21], and alteration of neurotrophin levels [22–24].

Several studies have advanced the search for a greater understanding of neurodegeneration in MSUD patients. Previous studies from our laboratory have shown that BCAAs decreased the levels of nerve growth factor (NGF) in the hippocampus and increased acetylcholinesterase (AChE) activity in the cerebral cortex, striatum, and hippocampus of rats [23, 25]. Cholinergic deficits and alterations in neurotrophic factors are the leading hypothesized mechanisms to explain MSUD's pathophysiology and are associated with memory loss and other cognitive dysfunctions, a finding that overlaps the mechanisms of neurodegenerative diseases [26–32]. Many of the neurodegenerative diseases share common mechanisms associated with the pathological aggregation of misfolded proteins that suggests that their pathology may be directly comparable. For example, alterations in AChE expression and distribution have been reported in Alzheimer's disease (AD) brains [33, 34], while the cholinesterase inhibitor induces a favorable response in Parkinson's disease (PD) patients with dementia (Mori 2002). Previous studies have also established that disequilibrium in neurotrophic signaling and/or processing have been associated with a number of neurodegenerative conditions, including AD [26, 35–38], PD [39], and Huntington's disease (HD) [40, 41]. Another important molecule involved in neurodegeneration and abnormal neurodevelopment is the neural cell adhesion molecule (NCAM) [42], since it has been shown that disrupting NCAM function impairs synaptic plasticity in the hippocampus, which can lead to several memory and learning deficits [42–45].

Patients affected by MSUD present severe neurological symptoms and brain abnormalities whose pathophysiology is poorly known. Preclinical studies have suggested that alterations in markers involved with neurodegeneration may be involved, as the increase in AChE and inflammation and the decrease in NGF levels, present in MSUD brains, can play a role in the neurodegeneration observed in MSUD. However, no studies in the literature have reported the neurodegenerative markers in MSUD patients; thus, the present study evaluated neurodegenerative markers [BDNF; platelet-derived growth factor AA and AB/BB (PDGF-AA and PDGF-AB/BB), NCAM, cathepsin D, and plasminogen activator inhibitor-1 total (PAI-1 total)] in plasma from MSUD patients during dietary treatment.

Material and Methods

Sample and Subjects

Plasma specimens from ten treated MSUD patients with the classic form (mean age 6.48 ± 4.97 years) and nine age- and sex-matched healthy children (mean age 10.70 ± 3.35 years) were used to evaluate the neurodegenerative markers and the concentrations of amino acids. The diagnosis of MSUD was based on the high concentration of leucine plus alloisoleucine in plasma, or plus DNA analysis. The patients were aged between 15 days and 2 months at diagnosis and followed a treatment that consisted of a natural protein-restricted diet with low BCAAs and supplemented with a semi-synthetic formula of essential amino acids containing small amounts of vitamins and minerals. In addition, MSUD patients were supplemented with isoleucine and valine capsules (Table 1). Table 2 displays the age at diagnosis, age at testing, length of treatment, and clinical profile of MSUD patients at presentation and under treatment. The present study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil. All parents of the patients included in the present study provided informed consent according to the guidelines of our committee.

Table 1 Doses of valine and isoleucine supplementation (mg/day)

Patients	Valine (mg/day)	Isoleucine (mg/day)
1	50	50
2	200	250
3	100	100
4	150	150
5	50	0
6	20	70
7	NA	NA
8	150	150
9	30	10
10	50	100

NA not available

Table 2 Age at diagnosis, age at testing, length of treatment, and clinical findings of MSUD patients

Patients	Sex	Age at diagnosis	Age at testing	Length of treatment	Clinical features	
					At diagnosis	At inclusion
1	F	2 months	8 years	7.10 years	Poor feeding, poor sucking, changes in respiratory pattern, hypertonia, encephalopathy, psychomotor developmental delay	Cognitive impairment
2	M	17 days	11.5 years	11.4 years	Seizures, vomiting, psychomotor and neurodevelopmental delay	Cognitive impairment
3	F	17 days	6.7 years	6.6 years	Vomiting, irritability	Normal development
4	M	15 days	11.8 years	11.7 years	Poor feeding, poor sucking, changes in respiratory pattern, hypotonia, vomiting, hypoactivity, psychomotor and neurodevelopmental delay	Cognitive impairment
5	F	15 days	3.8 years	3.7 years	Irritability, sleep disturbances	Neuropsychomotor development delay
6	M	24 days	2.2 years	2.1 years	Irritability, poor sucking, growth failure, pallor, nystagmus	Neuropsychomotor development delay
7	F	1 month and 16 days	3 years	2.11 years	Poor feeding, poor sucking, characteristic odor, metabolic acidosis, seizures, changes in respiratory pattern, apnea, upper respiratory tract infection	Neuropsychomotor development delay
8	M	27 days	1.5 year	1.4 years	Poor feeding, poor sucking, characteristic odor, hypotonia, hypertonia, lethargy, metabolic acidosis, seizures, coma, changes in respiratory pattern, skin lesions, psychomotor developmental delay	Neuropsychomotor development delay
9	F	16 days	1.11 year	1.10 years	Asymptomatic	Normal development
10	M	26 days	15.2 years	15.1 years	Refusal of food, characteristic odor, lethargy, seizures, cerebral edema	Cognitive impairment

Plasma Preparation

Plasma was separated from whole blood samples obtained from fasting individuals (controls and MSUD patients) by venous puncture with heparinized vials. Whole blood was centrifuged at 1000×g, and plasma was removed by aspiration and frozen at −80 °C until analysis.

Amino Acid Determination

The free amino acids in plasma were determined by HPLC according to Joseph and Marsden [48] with slight modifications [6]. Amino acids were quantitatively determined by relating their chromatographic peak area to those obtained from a known standard mixture and to that of an internal standard peak area (homocysteic acid).

Neurodegenerative and Inflammatory Markers

Plasma neurodegenerative markers (BDNF, cathepsin D, NCAM, PAI-1 (total), PDGF-AA, and PDGF-AB/BB) were assayed with the Luminex xMap technology (Millipore, USA and Canada). Briefly, the antibody specific to the analyte of interest was immobilized within microspheres through covalent bonds. After the analytic sample bound to the capture antibodies located on the surface of microspheres, end detection was performed via a third fluorescent marker, phycoerythrin, connected to the detection antibody. The end result was a sandwich test through the microspheres. Neurodegenerative markers were expressed as pictograms per milliliter

Statistical Analysis

The results are presented as the mean ± standard error of the mean (SEM). All assays were performed in duplicate, and the mean was used for statistical analysis. Student's *t* test was used to compare means between controls and MSUD patients. Multiple linear regression was used to adjust for possible confounders. We considered possible confounding factors all variables associated with diagnostic group and biomarkers with $p < 0.20$ [49]. Correlations were carried out using Spearman's rho (*r*). Differences between the groups were considered to be

significant at $p < 0.05$. All analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences software (Armonk, New York, USA).

Results

Table 3 shows the results of the quantitative analysis of amino acids in plasma specimens from MSUD patients and controls. It can be seen that the concentrations of leucine and valine were not significantly increased in MSUD patients compared to the control group. Furthermore, the concentrations of aspartate, glutamate, histidine, ornithine, and phenylalanine were significantly reduced relative to the control group, probably due in part to dietary restrictions. However, isoleucine, glycine, threonine, and arginine levels were significantly increased in MSUD patients compared to the control group.

We next investigated whether neurodegenerative markers were altered in plasma from MSUD patients. The results showed that in MSUD patients, BDNF levels were significantly reduced (B = −0.602 [CI 95 % −4359.92 to −473.44], $p = 0.019$), but no significant changes were observed in the PAI-1 (total) levels (Fig. 1a). As detailed in Fig. 1b, PDGF-AA levels were significantly decreased in MSUD patients (B = −0.614 [CI 95 % −5137.535 to −587.872], $p = 0.018$), while there were no significant differences in the levels of PDGF-AB/BB between the control and MSUD groups. However, post hoc analysis showed significantly greater

levels of NCAM (B = 0.376 [CI 95 % 12,257.425 to 215,463.279], $p = 0.031$) and cathepsin D (B = 0.359 [CI 95 % 6256.995 to 149,435.926], $p = 0.035$) in MSUD patients compared to the control group (Fig. 1c). Finally, we found no correlation between amino acid blood levels and NCAM, BDNF, PDGF-AA, and cathepsin D measurements (Table 4).

Discussion

MSUD patients usually present a variable degree of neurological dysfunction as well as atrophy of the cerebral hemispheres, spongy degeneration of white matter, and delayed myelinization. Although dietary intervention prevents the severe developmental delays associated with chronic hyperleucinemia, it nonetheless presents challenges that include maintenance and adherence to diet. There is increasing evidence of long-term deficits in neurocognition despite acceptable metabolic control because the longitudinal control of circulating BCAAs and BCKAs is linked to neurological outcome [2, 50–56]. Moreover, Muelly et al. [51] suggested that early insults to the brain may have long-term consequences, which highlights the importance of carrier testing and screening programs that allow for early identification and intervention.

Chronic neurological sequelae of MSUD are likely caused by several interacting mechanisms also thought to be responsible for acute neurotoxicity, including unbalanced cerebral
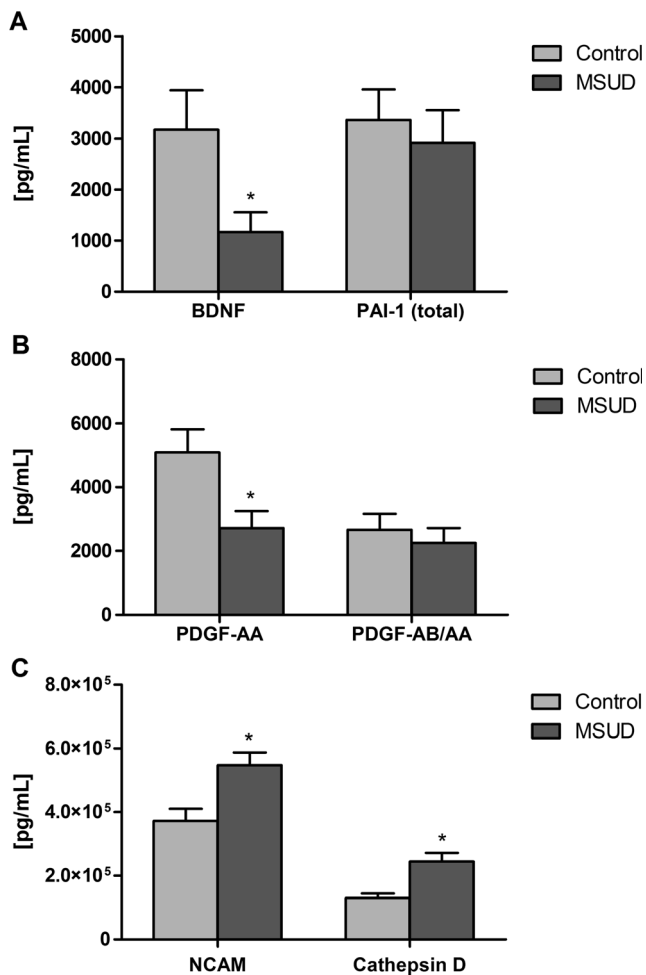
Table 3 Plasma concentrations of amino acids (μmol/L) in MSUD patients and controls

Amino acids	MSUD patients	Controls	p value	Plasma amino acid reference values
Alanine	373.20 ± 59.81	513.54 ± 72.11	0.166	152–547
Aspartate	8.85 ± 1.67*	48.18 ± 4.47	0.001	1–24
Glutamate	75.72 ± 15.61*	154.18 ± 24.75	0.016	5–150
Glutamine	558.34 ± 37.87	590.12 ± 69.61	0.669	254–823
Glycine/threonine/arginine	533.90 ± 40.04*	287.36 ± 33.51	0.001	172–707
Histidine	76.68 ± 4.94*	139.00 ± 30.23	0.010	41–125
Isoleucine	180.00 ± 27.72*	83.60 ± 17.78	0.011	22–107
Leucine	166.43 ± 27.85	205.54 ± 35.86	0.412	49–216
Lysine	183.40 ± 19.13	198.56 ± 33.27	0.680	48–284
Methionine	27.72 ± 2.62	28.14 ± 3.56	0.929	7–47
Ornithine	60.52 ± 5.54*	108.30 ± 17.35	0.004	10–163
Phenylalanine	63.63 ± 4.62*	130.90 ± 11.85	0.001	26–91
Serine	140.25 ± 10.17*	202.60 ± 25.73	0.015	69–187
Tryptophan	83.30 ± 8.98	78.32 ± 11.31	0.751	0–79
Tyrosine	91.47 ± 9.89	83.40 ± 5.50	0.608	24–115
Valine	207.08 ± 48.92	307.46 ± 33.55	0.214	74–321

All values are expressed as mean ± SD. p Values stated have been calculated by Student's t test. Different from control

*$P < 0.05$

Fig. 1 Plasma levels of brain-derived neurotrophic factor and plasminogen activator inhibitor-1 total (a), platelet-derived growth factor AA and AB/BB (b), and neural cell adhesion molecule and cathepsin D (c) in controls and MSUD patients. Data represent the mean ± standard error of the mean (SEM). Student's *t* test was used to compare means between controls and MSUD patients, and multiple linear regression was used to adjust for possible confounders. *$p < 0.05$ compared to the control group. (*BDNF* brain-derived neurotrophic factor; *PAI-1 total* plasminogen activator inhibitor-1 total; *PDGF-AA* platelet-derived growth factor AA; *PDGF-AB/BB* platelet-derived growth factor AB/BB; *NCAM* neural cell adhesion molecule]

essential amino acid uptake, neurotransmitter deficiencies, energy deprivation, alterations in levels of neurotrophic factors, and osmotic dysregulation. However, the molecular triggers that induce the progression of disease and neurodegeneration and that contribute to cognitive dysfunctions are currently unknown. Thus, the aim of the present study was to determinate whether there is any difference in the plasma values of markers involved in plasticity, synaptogenesis, neurogenesis, myelination, and autophagy between MSUD and control subjects.

One class of molecules that may contribute to plasticity in the central nervous system are glycoproteins involved in the adhesion of neural cells, such as NCAM. This glycoprotein is involved in signal transduction, neurite outgrowth and fasciculation, neural crest cell migration, synaptogenesis, and adult neurogenesis [57–61] and is considered to be a reliable marker of synaptic plasticity, with its concentration reflecting the numbers of recently generated synapses [62]. In this study, we showed that NCAM levels are significantly higher in MSUD patients compared to the control group. This increase has been related to increased neurogenesis and may be indicative of an attempt by the brain to restore structure and function or to compensate for the damage caused by the disease. However, evidence suggests that NCAM levels in the blood plasma of patients may be used for the differential diagnosis of AD [63, 64], as the disturbance of NCAM expression is involved in the pathogenesis of AD and there is a strong tendency for an increase in the soluble fragments of NCAM in the cerebrospinal fluid and in the occipital cortex and hippocampus of Alzheimer's patients [65–68]. Moreover, levels of low-molecular-weight forms of NCAM in the serum samples are correlated with the severity of dementia [65].

In addition, for comparative purposes, we studied BDNF, another classically used marker of plasticity. Our results showed low plasma levels of BDNF in MSUD patients. There is accumulating evidence for the involvement of BDNF, a key mediator of neuronal proliferation, differentiation, survival, and response to stress [69, 70] in the pathogenesis of MSUD, as preclinical investigations in animals indicate that BCAAs and KIC decrease BDNF levels in the brain of rats [24, 71]. BDNF is also important for learning and memory processes by inducing long-term potentiation in the hippocampus with structural changes in synapses [72–74]. There is evidence linking changes in BDNF to core psychopathological features of neurodegenerative diseases, including AD, HD, and PD [75–80]. Indeed, BDNF messenger RNA (mRNA) and protein are decreased in postmortem brain tissue from subjects with AD [38, 81–83], and changes in BDNF serum concentrations depend on the severity of AD [84, 85]. Recent data suggest that plasma BDNF is a biomarker of impaired memory and general cognitive function in aging women [86].

Additionally, studies have shown that BDNF is capable of stimulating neural stem/progenitor cell differentiation and myelination. Silva et al. [87] showed that BDNF increased the number of differentiated neurons and decreased the number of neuronal precursors compared to NGF or RA-KCl, and the exposure to BDNF resulted in an increase in differentiated neurons, which acquired a GABAergic neuronal phenotype. These results are consistent with the study by Soltys et al. [88] that demonstrated that BDNF does not stimulate the proliferation of neural stem/progenitor cells or induce apoptosis but is capable of inducing the differentiation of neural stem/progenitor cells into the oligodendrocyte lineage. Cellerino et al. [89] showed that BDNF-deficient mice exhibit hypomyelination of the optic nerve, which suggests that BDNF influences the axonal signals via the direct myelination

Table 4 Spearman's rho correlation coefficients and *p* values between neurodegenerative markers and amino acids

		NCAM	BDNF	PDGF-AA	Cathepsin-D
Leucine	rho *p* value	−0.110	0.099	0.258	0.000
		0.721	0.748	0.394	1.000
Isoleucine	rho	0.268	−0.375	−0.346	0.168
	p value	0.335	0.168	0.206	0.550
Valine	rho	−0.204	0.235	0.367	0.029
	p value	0.483	0.418	0.197	0.923
Alanine	rho	0.139	−0.036	0.212	−0.391
	p value	0.701	0.915	0.556	0.235
Aspartate	rho	0.280	0.387	0.547	0.296
	p value	0.434	0.239	0.102	0.377
Glutamate	rho	0.091	0.100	0.285	0.227
	p value	0.803	0.770	0.425	0.507
Glutamine	rho	0.273	−0.450	0.127	0.200
	p value	0.446	0.984	0.726	0.606
Glycine/threonine/arginine	rho	−0.018	0.036	0.042	−0.545
	p value	0.960	0.915	0.907	0.083
Histidine	rho	0.200	0.091	0.467	0.152
	p value	0.606	0.803	0.174	0.676
Lysine	rho	0.083	0.127	0.100	−0.564
	p value	0.831	0.726	0.798	0.090
Methionine	rho	0.588	0.464	0.079	0.055
	p value	0.074	0.151	0.829	0.873
Ornithine	rho	−0.018	0.064	0.139	−0.200
	p value	0.960	0.853	0.701	0.555
Phenylalanine	rho	−0.491	−0.355	0.018	−0.345
	p value	0.145	0.285	0.960	0.298
Serine	rho	−0.224	0.227	0.248	−0.573
	p value	0.532	0.502	0.489	0.066
Tryptophan	rho	−0.091	−0.491	−0.030	0.136
	p value	0.803	0.125	0.934	0.689
Tyrosine	rho	−0.224	−0.336	−0.358	−0.255
	p value	0.533	0.312	0.310	0.450

of TrkB-expressing retinal ganglion cell axons. These authors also showed that the analysis of the hippocampus and cortex showed significant reductions in myelin basic protein (MBP) and proteolipid protein (PLP) mRNA levels.

Another factor required for the development of CNS connections is the PDGF-AA. In this work, we demonstrate that PDGF-AA levels are decreased in MSUD patients. This growth factor is a key signaling molecule that specifically regulates the differentiation of neural stem/progenitor cells to oligodendrocytes [90–92], and the oligodendrocytes, through their ability to myelinate axons, play an essential role in the mature CNS. Vana et al. [93] showed that PDGF-AA supports oligodendrocyte proliferation and survival and helps with the remyelination of chronic lesions. Furthermore, PDGF-AA levels in the CSF decrease with the progression of multiple sclerosis [94]. Murtie et al. [95] showed that PDGFa receptor

(PDGFaR) signaling played a key role in maintaining the density of oligodendrocytes in the normal adult CNS, and PDGFaR+/− mice showed insufficient proliferation of oligodendrocytes and impaired compensatory remyelination. Hypomyelination has been reported in association with MSUD, and studies in iMSUD mice and patients with MSUD maintained on a low branched-chain amino acid diet continue to have increased T2 signals on MRI, suggesting a lingering myelin disruption [2, 7]. Previous characterizations of MSUD patients have also described cognitive levels consistent with mental retardation, including nonverbal reasoning and visuospatial impairments [1, 56, 96]. Taking together these findings and the present results, we suggest that hypomyelination and cognitive impairment observed in MSUD patients may be mediated by the reduced trophic support of BDNF and PDGF-AA.

Autophagy may also be involved in neurodegenerative diseases [97]. In this study, we showed that levels of cathepsin D, the major lysosomal aspartic protease, are increased in MSUD patients. Cathepsin D upregulation has already been shown in experimental models of neurodegeneration [98–102]. Yelamanchili et al. [103] showed that cathepsin D can been involved in the long-term neuronal damage in PD in regions remote from the site of primary insult and underscores the need to study brain regions outside the sites of initial damage in neurodegeneration to help understand the basis of disease progression. Moreover, using a mouse model of Niemann–Pick disease type C, German et al. [100] showed a strong increase in cathepsin D-positive microglia cells, while only a mild increase in cathepsin D content in nerve cells was detected.

Although the major function of cathepsin D is related to intracellular catabolism in lysosomal compartments [104], this protease seems to play an important role in the processes that regulate apoptosis [105–108], as the released cathepsin D then cleaves the Bcl-2 family member Bid and leads to the subsequent release of cytochrome c from mitochondria as well as the activation of caspase-9 and caspase-3 [109]. In addition, a number of studies have demonstrated that cathepsin D is induced by several stimuli, such as IFN-γ, TNF-α, Fas/CD95/APO-1, staurosporine, etoposide, 5-fluorouracil, cisplatin [105, 110, 111], and oxidative stress [112, 113]. However, this relationship between inflammation and cathepsin D may be bidirectional, wherein procathepsin D initiates the secretion of inflammatory cytokines, including IL-4, IL-8, IL-10, and IL-13 [114], and the acidification of cytokine-treated media converts procathepsin D into cathepsin D [115]. A recent analysis of activated microglia revealed that cathepsin D mediates microglial neurotoxicity and might represent a potential biomarker for neurodegenerative diseases associated with extensive microglial activation and neurotoxic inflammation [116]. In this context, several other studies also support the hypothesis that there may be increased apoptosis, oxidative stress, and inflammation in MSUD [12, 13, 46, 47]. Based on these findings, it is tempting to speculate that the increased cathepsin D levels and apoptosis in MSUD patients could be induced by elevated levels of cytokines and oxidative stress and that cathepsin D may be involved in the mediation of apoptosis induced by the cytokines.

Although all of these data strongly suggest the occurrence of alterations in neurodegenerative markers in patients with MSUD and that leucine together with KIC can be considered the main neurotoxic agents in this disease, our results from patients with MSUD have not shown a clear association between these metabolites and markers of neurodegeneration. Therefore, it is possible that other factors may be related to the neurodegeneration observed in MSUD, which needs to be better investigated.

In conclusion, this is the first study to examine BDNF, cathepsin D, NCAM, PAI-1 (total), PDGF-AA, and PDGF-AB/BB in plasma of MSUD patients, and the results showed that cathepsin D and NCAM levels were increased, while BDNF and PDGF-AA levels were decreased in the plasma of MSUD subjects compared with controls. The present findings corroborate previous studies that demonstrate that neurotrophic factors and lysosomal protease may contribute, along with other mechanisms, to the intellectual deficit and neurodegeneration observed in MSUD. A better knowledge of these events may be useful to develop new therapeutic strategies aimed to normalize, or at least ameliorate, the pathological consequences of the negative impact of neurodegeneration on brain structure and function in this disease.

Compliance with Ethical Standards The present study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil. All parents of the patients included in the present study provided informed consent according to the guidelines of our committee.

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ERRATUM

Erratum to: Serum Markers of Neurodegeneration in Maple Syrup Urine Disease

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The original version of this article unfortunately contained mistakes.

   The affiliation of the co-author "Tássia Tonon" should be changed to 2) BRAIN Laboratory (Basic Research and Advanced Investigations in Neurosciences), Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil AND 3) Post Graduation Program in Medicine: Medical Sciences, Universidade Federal do Rio Grande do Sul, Brazil.

   The name of the co-author published as "Tatiana Amorin" should also be changed to the correct form which is Tatiana Amorim.

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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*

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/

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 re-

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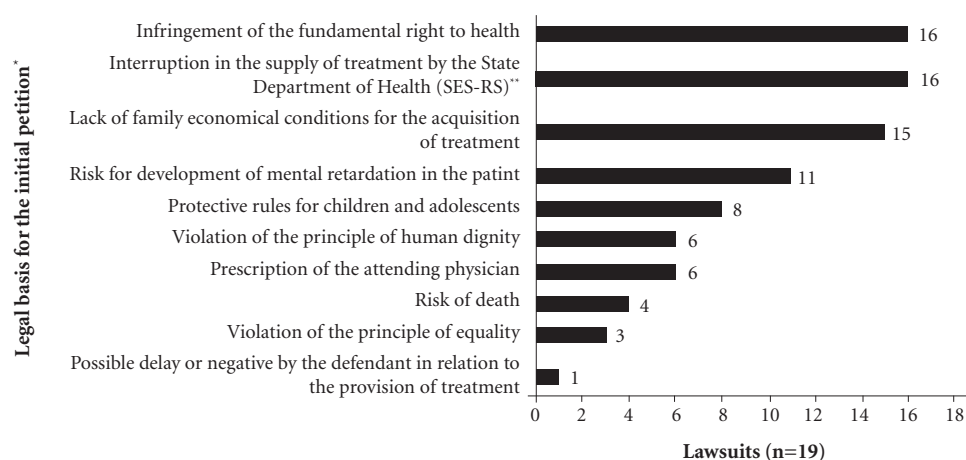
Figure 1. Access to PKU treatment by judicial means in Rio Grande do Sul, Brazil: legal basis for the initial petition.

[*] 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 hos-

pitals, 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-

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 regard-

ing 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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