UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

HOMOCISTINÚRIA E METABOLISMO DA HOMOCISTEÍNA: TRATAMENTO COM CREATINA E FENILCETONÚRIA COMO MODELOS

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

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Maio de 2019

Este trabalho foi desenvolvido no Hospital de Clínicas de Porto Alegre (Serviço de Genética Médica e Laboratório BRAIN/Centro de Pesquisa Experimental) e na Universidade de Freiburg/Alemanha (Laboratory for Clinical Biochemistry and Metabolism, University Medical Center Freiburg).

Esta tese é resultado três projetos aprovados pela Comissão Científica do Hospital de Clínicas de Porto Alegre (HCPA) com base nos pareceres do Comitê de Ética em Pesquisa e Serviço de Gestão em Pesquisa do HCPA. O projeto foi financiado com recursos do Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (projetos 13-0525, 15-0536, 16-0140) e do PPGBM-UFRGS (tradução de artigos). Além disso, a aluna recebeu bolsa de doutorado do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). E bolsa de doutorado sanduíche da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES.

AGRADECIMENTOS

É muito bom ter a quem agradecer ao final deste trabalho, que não reflete apenas anos de estudo e pesquisa, mas sim uma formação e jornada ao aprimoramento acadêmico.

Primeiramente, agradeço a Deus que é Senhor, Luz e Direção em cada dia. Agradeço pela calma, força, tranquilidade e confiança de que tudo está correndo de acordo com o planejado. Agradeço pelas oportunidades que encontrei e experiências que tive e que hoje formam a profissional que sou.

Agradeço a meus pais Tarcisio e Cléris Weber, pelo apoio incondicional aos meus sonhos e anseios, além de orgulho que sempre demonstraram, e auxílio em cada pequena necessidade que tive.

Agradeço a minha orientadora Dra. Ida Schwartz, mentora que transmitiu muito de seu conhecimento teórico e prático. Ela que cuidou de detalhes, esperou por resultados e explicações, argumentos para fortalecer nossas teorias. Trabalhar com a Ida também abriu muitas portas para mim, tive várias ótimas oportunidades, sempre fui incentivada e assim acumulei diversas experiências em diferentes áreas. Da mesma forma, agradeço à minha co-orientador Dra. Fernanda Sperb-Ludwig por sua instrução, direcionamento, participação no trabalho prático e teórico.

Agradeço a meu orientador internacional Henk Blom, grande mentor, grande pesquisador, referência no estudo da homocisteína e homocistinúrias. Ele foi muito presente durante todo meu doutorado, participou ativamente em todos os projetos e artigos, e além disso, também sempre se mostrou interessado em meu bem estar, realização e satisfação no trabalho a ser feito. Obrigada por seu cuidado e encorajamento.

Agradeço aos meus professores, colegas no pós, no laboratório, amigos e companheiros no dia a dia. Em especial às colegas, Soraia Poloni, Taciane Borsatto, Suélen Basgallup, Karina Kolonetti e Franciéli Cabral, pela companhia diária, suporte nos experimentos, discussões e amizade construída.

Também ao meu querido marido, Cristiano, que sempre me ouviu e tentou ajudar na medida do possível, me aconselhando e incentivando em tudo que precisei. Muito obrigada Cris, é muito bom ter e certeza de que sempre posso contar contigo pra me apoiar e consolar.

Ao Hospital de Clínicas de Porto Alegre pela estrutura fornecida no Centro de Pesquisa Experimental, bem como a todos os seus funcionários pela competência.

Ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, pelo constante aprendizado, toda estrutura disponível e auxílio dispensados nesta jornada.

Agradeço às instituições alemãs, Universitätsklinikum Hamburg-Eppendorf e Universitätsklinikum Freiburg, que me receberam, permitiram o desenvolvimento de meus projetos, com a convívio e aprendizado com ótimos pesquisadores.

Ao Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo aporte financeiro para que esse estudo fosse concluído.

LISTA DE ABREVIATURAS

AdoCbl: Adenosilcobalamina

AdoHcy: S-adenosil-homocísteina

AdoMet: S-adenosilmetionina

AGAT: L-arginina:glicina amidinotransferase

B12: Vitamina B12 (cobalamina)

B2: Riboflavina

B6: Piridoxina

B9: Ácido fólico

BH4: tetra-hidrobiopterina

BHMT: betaína-homocísteina metiltransferase

CAT: Catalase

Cbl: Cobalamina

cblC: Acidemia metilmalônica com homocistinúria tipo C

cDNA: DNA complementar

Cre: Creatina

CreaT: Transportador da creatina

CβS: Cistationina beta-sintase

CγL: Cistationina gama-liase

DCFH: Ensaio oxidativo 2′7′Diclorofluorescente

DNA: Ácido desoxirribonucleico

EIM: Erro inato do metabolismo

GAA: Guanidinoacetato

GAMT: Guanidilacetato metiltransferase

gnomAD: Genome Aggregation Database

GPx: Glutationa peroxidase

GSH: Glutationa

H_2O_2: Peróxido de hidrogênio

HCPA: Hospital de Clínicas de Porto Alegre

HCU: Homocistinúria clássica

Hcy: Homocisteína

HMG-CoA redutase: 3-hidroxi-3-methyl-glutaril-CoA redutase

IL: Interleucina

LAT1: Aminoácido neutros do tipo L

LCR: Líquido cefalorraquidiano

LNAAs: Grandes aminoácidos neutros

MAT: Metionina adenosiltransferase

MCM: Metilmalonil-CoA mutase

MeCbl: Metilcobalamina

Met: Metionina

MMA: Ácido metilmalônico

MMACHC: Proteína da acidemia metilmalônica com homocístinuria tipo C

MTHFR: 5,10-metileno-THF redutase

MTR: Metionina sintase

MUT: Gene da Metilmalonil-CoA mutase

O2-: Oxigênio molecular

OHCbl: Hidroxicobalamina

PAH: Fenilalanina hidroxilase

Phe: Fenilalanina

PKU: Fenilcetonúria

RN: Recém-nascido

RNA: Ácido ribonucleico

SAHH: S-adenosil-L-homocisteína hidrolase

SH: Conteúdo total de sulfidrilas

SHMT: serina-hidroximetiltransferase

SNC: Sistema Nervoso Central

SOD: Enzima superóxido dismutase

TBARS: Substâncias reativas ao ácido tiobarbitúrico

tHcy: Homocisteína total

THF: Tetra-hidrofolato

SUMÁRIO

1. RESUMO

Introdução: Homocistinúria é um grupo de erros inatos do metabolismo (EIM) que resultam em aumento marcante dos níveis de homocisteína (Hcy). As causas mais comuns são homocistinúria clássica (HCU), com incidência estimada em ao menos 0.29 a 1 a cada 100,000 indivíduos, e as deficiências de cblC e MTHFR. Já o termo hiperhomocisteinemia refere-se ao aumento de Hcy total (tHcy) que pode ser de origem genética, ambiental, ou multifatorial. Uma importante porção da Hcy é formada em consequência da síntese endógena de creatina (Cre), que requer a incorporação de um grupo metil doado no processo de metabolismo de metionina (Met). Além disso, a Cre é descrita com potencial antioxidante. Pacientes com fenilcetonúria (PKU) tem seu tratamento baseado em restrição dietética de fenilalanina (Phe) e uso de fórmula metabólica. Com base na dieta pobre em proteínas esses pacientes estão em risco de desenvolver deficiência de Met e vitamina B12, aumentando consequentemente níveis de Hcy e ácido metilmalônico (MMA). **Objetivos:** 1) Caracterizar aspectos epidemiológicos, clínicos e bioquímicos de homocistinúrias; 2) Avaliar o papel da Cre como novo tratamento das homocistinúrias; 3) Avaliar o metabolismo da Hcy em PKU, de forma a propor novos mecanismos fisiopatológicos para a doença. **Métodos:** Etapa 1: revisão de literatura acerca das formas mais comuns de homocistinúria, comparando suas apresentações clínicas e bioquímicas. Etapa 2: análise de dados clínicos de 72 pacientes brasileiros (60 famílias) com HCU provenientes das cinco regiões do país. Etapa 3: estimativa da incidência mundial de HCU com base no número de heterozigotos, aplicando regras de equilíbrio de Hardy-Weinberg, para as 25 mutações mais comuns no gene *CBS*, presentes em bancos de dados genômicos. Etapa 4: análise de marcadores inflamatórios e de estresse oxidativo em cultivo celular de células comerciais imortalizadas, controle (n=3) e células com defeito de remetilação (n=6) tratadas com Cre. Etapa 5: análise dos níveis de Phe, Hcy, MMA, vitamina B12, Met, tirosina, leucina, isoleucina e valina em soro de 25 pacientes com PKU. **Resultados:** Etapa 1: a manifestação bioquímica comum à HCU e às deficiências de cblC e MTHFR é o acúmulo de tHcy. Os outros aminoácidos sulfurados apresentam perfis diversos e até inversos. Alguns achados clínicos comuns dentre as homocistinúrias, são atraso no desenvolvimento e convulsões, bem como eventos tromboembólicos. No entanto, as complicações do sistema nervoso central (SNC) diferem em uma ampla variedade de apresentações e

severidades e são aparentemente menos pronunciadas na HCU. Etapa 2: nos pacientes brasileiros com HCU, manifestações oculares foram as mais precoces e prevalentes, bem como a principal suspeita diagnóstica. Observou-se maior prevalência de pacientes não responsivos à piridoxina, e uma pequena porção dos pacientes não responsivos manteve os níveis de Hcy indicados (<100umol/L). Os tratamentos mais comuns foram respectivamente: piridoxina, ácido fólico, betaína, vitamina B12 e dieta restrita em Met associada ao uso de fórmula metabólica. Etapa 3: conforme a frequência de heterozigotos para *CBS* no banco de dados gnomAD, a incidência mundial de HCU foi calculada em aproximadamente 0,38:100.000 indivíduos. Etapa 4: observamos que o conteúdo intracelular de Cre aumentou nos grupos tratados, e no grupo de células saudáveis após o tratamento houve uma redução de CAT, SOD, SH e DCFH, mas não houve diferença estatística, provavelmente devido ao pequeno número amostral. Etapa 5: o tratamento de pacientes PKU com fórmula metabólica reduz os níveis de tHcy. Nenhum dos pacientes com PKU apresentou valores de tHcy acima da faixa normal, indicando que não há deficiências evidentes de folato ou vitamina B12 entre os pacientes em tratamento, provavelmente devido ao enriquecimento das fórmulas metabólicas com estes compostos. Nas pacientes grávidas, os níveis de Phe e Hcy foram menores durante a gestação indicando melhor aderência ao tratamento. **Conclusões:** Há pouca sobreposição nas apresentações bioquímicas e clínicas das três formas mais comuns de homocistinúria, o que nos faz questionar se a própria Hcy é o principal metabólito patogênico. Apresentamos o panorama clínico mais abrangente da HCU já realizado no Brasil. Observa-se que a maioria dos pacientes apresenta fenótipo grave, o que sugere subdiagnóstico das formas de HCU atenuadas/responsivas à piridoxina. O grande número de indivíduos analisados e a cobertura populacional do banco de dados utilizado nos dão grande precisão na estimativa de incidência da HCU, que é um distúrbio tratável. Nossos resultados apoiam a implementação da triagem neonatal na Europa (~0,72:100.000) e na América Latina (~0,45:100.000). A Cre tem o potencial de reduzir marcadores de estresse oxidativo. Em pacientes com PKU o acompanhamento clínico e adesão ao tratamento dietético são muito importantes. O acompanhamento dos pacientes deve abordar a adequação da ingestão de proteínas, além de monitorar os níveis de Phe e os aminoácidos plasmáticos, Hcy e MMA, que devem ser avaliados para detectar a deficiência de vitamina B12, especialmente antes da concepção, para minimizar os riscos para o feto.

2. ABSTRACT

Introduction: Homocystinuria is a group of inborn errors of metabolism (EIM) that result in marked increase in homocysteine (Hcy) levels. The most common causes are classical homocystinuria (HCU), with an estimated incidence of to be between 0.29 and 1:100,000 individuals, and deficiencies of cblC and MTHFR. The term hyperhomocysteinemia refers to the increase in total Hcy (tHcy) levels that may be due to genetic, environmental, or multifactorial. An important portion of Hcy is formed as a consequence of endogenous creatine (Cre) synthesis, which requires methyl groups donated in the methionine (Met) metabolism process. In addition, Cre is described with antioxidant potential. Patients with phenylketonuria (PKU) have their treatment based on dietary restriction of phenylalanine (Phe) and use of metabolic formula. Because of the low protein diet these patients are at risk of developing Met and vitamin B12 deficiency, thereby increasing levels of Hcy and methylmalonic acid (MMA).

Objectives: 1) To characterize epidemiological, clinical and biochemical aspects of homocystinuria; 2) To evaluate the role of Cre as a new treatment for homocystinuria; 3) To evaluate the metabolism of Hcy in PKU, in order to propose new pathophysiological mechanisms for the disease.

Methods: Phase 1: literature review on the most common forms of homocystinuria, comparing its clinical and biochemical presentations. Phase 2: clinical data analysis of 72 Brazilian patients (60 families) with HCU from the five regions of the country. Phase 3: estimation of the worldwide incidence of HCU based on the number of heterozygotes, applying Hardy-Weinberg equilibrium rules, for the 25 most common mutations in the *CBS* gene, present in genomic databases. Phase 4: analysis of inflammatory and oxidative stress markers in cell culture of immortalized commercial cells, control (n = 3) and cells with remethylation defect (n = 6) treated with Cre. Phase 5: analysis of Phe, tHcy, MMA, vitamin B12, Met, tyrosine, leucine, isoleucine and valine levels in plasma of 25 PKU patients.

Results: Phase 1: the common biochemical manifestation to HCU and deficiencies of cblC and MTHFR is the accumulation of tHcy. The other sulfur amino acids have different and even inverse profiles. Some common clinical findings among homocystinurias are development delay and seizures, as well as thromboembolic events. However, central

nervous system complications differ in a wide variety of presentations and severities, and are apparently less pronounced in HCU. Phase 2: In Brazilian HCU patients, ocular manifestations were the earliest and most prevalent, as well as the main diagnostic suspicion. A higher prevalence of patients non responsive to pyridoxine was observed, and a small portion of nonresponsive patients maintained the indicated tHcy levels (<100umol/L). The most common treatments were: pyridoxine, folic acid, betaine, vitamin B12 and restricted diet in Met associated with the use of metabolic formula. Phase 3: according to the frequency of heterozygotes for *CBS* in the gnomAD database, the worldwide incidence of HCU was estimated at approximately 0.38:100,000 individuals. Phase 4: we observed that the intracellular content of Cre increased in the treated groups, and in the group of healthy cells after the treatment there was a reduction of CAT, SOD, SH and DCFH, but there was no statistical difference, probably due to the small sample. Phase 5: Treatment of PKU patients with metabolic formula reduces tHcy levels. None of the patients with PKU presented tHcy values above the normal range, indicating that there are no evident deficiencies of folate or vitamin B12 among the patients under treatment, probably due to the enrichment of metabolic formulas. In pregnant patients, Phe and tHcy levels were lower during gestation indicating better adherence to the treatment.

Conclusions: There is little overlap in the biochemical and clinical presentations of the three most common forms of homocystinuria, which makes us question whether Hcy itself is the main pathological metabolite. We present the most extensive clinical picture of the HCU already performed in Brazil. It is observed that the majority of the patients present severe phenotype, which suggests underdiagnosis of HCU forms attenuated / responsive to pyridoxine. The large number of individuals analyzed and the population coverage of the database used give us great accuracy in estimating the incidence of HCU, which is a treatable disorder. Our results support the implementation of neonatal screening in Europe (~0.72:100,000) and in Latin America (~0.45:100,000). Cre has the potential to reduce oxidative stress markers. In patients with PKU, clinical follow-up and adherence to dietary treatment are very important. Patient follow-up should address adequacy of protein intake, as well as monitoring Phe levels and plasma amino acids, Hcy and MMA, which should be evaluated for vitamin B12 deficiency, especially prior to conception, to minimize risks to the fetus.

3. INTRODUÇÃO

3.1 HOMOCISTEÍNA

A Hcy é um aminoácido não essencial, contem enxofre, não participa da sínteses de proteínas, é formado exclusivamente a partir da desmetilação da Met, e é considerado tóxico em níveis aumentados. Hcy é um metabólito chave nos processos de a) metilação e ressíntese de S-adenosil-homocísteina (AdoHcy) através da atividade reversa da S-adenosil-L-homocisteína hidrolase (SAHH; EC:3.3.1.1); b) remetilação para Met por duas vias, dependente de folato e vitamina B12 ou ainda pela via dependente de betaína e c) via da transulfuração para cistationina (Figura 1) (Škovierová *et al.*, 2016).

Hiper-homocisteinemia refere-se ao aumento de tHcy no plasma ou soro que pode ser de origem genética, ambiental, ou multifatorial (Mudd *et al.*, 2000). Em indivíduos adultos saudáveis, os níveis plasmáticos de Hcy mantém-se abaixo de 15μmol/L, com variações de acordo com idade e gênero. A hiperhomocisteinemia pode ser classificada como leve (15–30 μmol/L), moderada (31–100 μmol/L) ou grave (>100 μmol/L), embora não haja consenso na literatura sobre os pontos de corte desta classificação (Mudd *et al.*, 2000; Weiss *et al.*, 2002; Brustolin *et al.*, 2010).

A hiperhomocisteinemia grave resulta em homocistinúria, um grupo de EIM dos aminoácidos sulfurados, que apresentam aumento marcante dos níveis de Hcy no plasma ou soro, e foi assim denominada devido à eliminação excessiva de homocistina (homocisteína dissulfeto) na urina observada nos pacientes com a doença. Os principais tipos de homocistinúria serão descritos em mais detalhes a seguir. Os principais aminoácidos sulfurados em humanos são a Met, cisteína (Cys), Hcy e taurina. Eles desempenham papel essencial em diversas rotas metabólicas, como a síntese de glutationa, síntese proteica e metilação de DNA, RNA, proteínas e lipídeos (Blom & Smulders, 2011; Castro *et al.*, 2006). O metabolismo dos aminoácidos sulfurados é resumido na Figura 1.

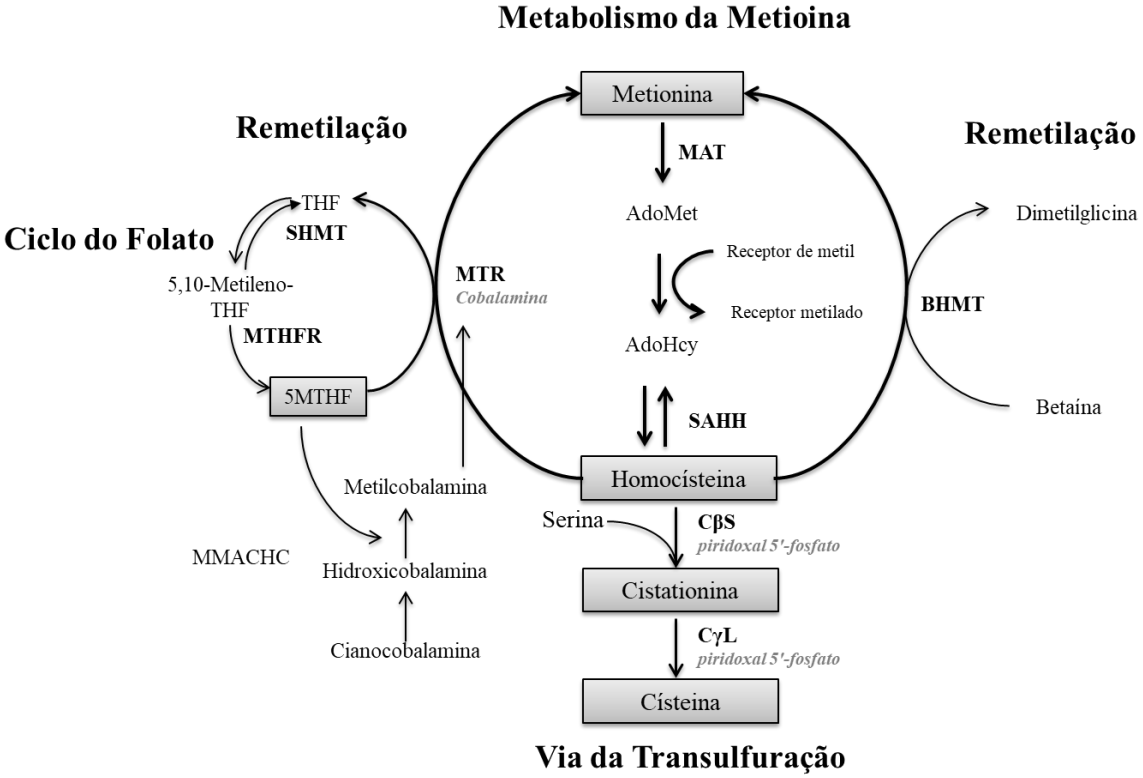


Figura 1. Visão geral do metabolismo dos aminoácidos sulfurados. Homocisteína (Hcy) é formado a partir da desmetilação da metionina por metionina adenosiltransferase (MAT), com intermediários S-adenosilmetionina (AdoMet) e S-adenosilhomocísteina (AdoHcy). Hcy pode ser degradada pela via de transsulfuração, em que se transforma em cistationina através da ação da enzima cistationina beta-sintase (CBS) e em seguida cisteína pela ação da enzima cistationina gama-liase (CγL). Hcy pode voltar a AdoHcy pela ação de S-Adenosil-homocísteina hidrolase (SAHH). Hcy também pode ser remetilada à Met por metionina sintase (MTR), uma enzima dependente de vitamina B12 e folato, ou ainda, pela rota da homocisteína metiltransferase (BHMT) que usa betaína, formada pelo catabolismo da colina, como doador do grupo metil. THF: tetrahidrofolato; MTHFR: 5,10-metileno-THF redutase; SHMT: serina-hidroximetiltransferase; MMACHC: proteína da acidemia metilmalônica com homocístinuria tipo C. Enzimas estão representados em letras maíusculas e seus cofators em itálico. **Fonte:** autor.

3.1.1 Metabolismo da Homocisteína

Depois de formada, a Hcy pode seguir duas rotas metabólicas: a transsulfuração, onde é degradada; ou a remetilação, onde é reconvertida a Met. Pela via de transsulfuração, a Hcy se transforma em cistationina através da ação da enzima cistationina beta sintase (CBS; EC 4.2.1.23), que utiliza como cofator o piridoxal fosfato, forma ativa da vitamina B6. A cistationina é então convertida à Cys, molécula precursora da glutationa e da taurina (Pico & Bermudez, 2006). A Hcy também pode ser remetilada à Met por duas rotas alternativas. A mais importante é catalisada pela metionina sintase (MTR; EC 1.16.1.8), uma enzima dependente de vitamina B12 e folato, ou ainda, pela rota da homocisteína

metiltransferase (BHMT; EC 2.1.1.5) que também pode catalisar a remetilação da Hcy, usando betaína, formada pelo catabolismo da colina, como doador do grupo metil (Fowler, 1997).

De forma detalhada, a Hcy é formada como um produto de reações de transferência de metil no metabolismo da Met. Neste processo, a Met é ativada por ATP em S-adenosilmetionina (AdoMet), que é o doador universal de grupos metil, resultando na formação de AdoHcy. A AdoHcy é convertida em Hcy e adenosina através de SAHH. O equilíbrio da reação favorece a formação da AdoHcy. Como consequência, o aumento da Hcy deve resultar em um acúmulo pronunciado de AdoHcy, que é um potente inibidor de muitas reações da metiltransferase. Como a metilação é essencial para funções celulares em todos os órgãos, a Hcy é formada em todos os tecidos.

Já a eliminação de Hcy via CBS está confinada ao fígado e rins. Assim, em todos os outros tecidos, como o sistema vascular e o cérebro, as únicas formas disponíveis para eliminar a Hcy são a remetilação pela MTR ou a exportação para fora da célula (Hannibal & Blom, 2017).

No processo de remetilação, a Hcy recebe um grupo metil do 5-metiltetrahidrofolato (5MTHF), que é formado a partir de 5,10-metilenotetrahidrofolato pela metilenotetrahidrofolato redutase (MTHFR; EC 1.5.1.20) (Figura 1). Notavelmente, 5MTHF é a forma circulante de folato no sangue e líquor. O fígado e o rim contêm também metiltransferase de betaína-homocisteína (BHMT; EC 2.1.1.5), que é uma via alternativa de remetilação de Hcy (Figura 1).

A vitamina B12, na forma de metilcobalamina (MeCbl), está envolvida como cofator da MTR, que catalisa a remetilação da Hcy em Met no citosol. Já a adenosilcobalamina (AdoCbl) é o cofator da metilmalonil-CoA mutase (MCM; EC:5.4.99.2), que converte metilmalonil-CoA em succinil-CoA na mitocôndria. Assim, a deficiência de vitamina B12 leva ao aumento dos níveis de Hcy e MMA.

Os níveis de AdoMet regulam se a Hcy será degradada via CBS ou remetilada de volta para Met. No fígado, o excesso de Met aumenta os níveis de AdoMet, que ativa CBS e inibe MTHFR, fazendo com que a Hcy seja primária e irreversivelmente convertida em cistationina (Castro *et al.*, 2006; Blom & Smulders, 2011). Se os níveis de Met estiverem baixos, CBS não é ativado e MTHFR não é inibido por AdoMet, fazendo com que a Hcy seja principalmente remetilada (Thomas & Rosenblatt, 2005; Burda *et al.*, 2015).

3.1.2 Patogenicidade da Homocisteína

O significado e o impacto patológico dos altos níveis de Hcy tem sido assunto de intenso debate entre pesquisadores e médicos durante as últimas décadas. Há três ramos principais de discussão quanto à toxicidade da Hcy: a) modificações na estrutura proteica conhecida como homocisteinilação; b) indução de estresse oxidativo; e c) excitotoxicidade. Seja a Hcy causa, mediador, biomarcador ou apenas espectador, a compreensão da homeostase da Hcy continua a ser uma matéria de pesquisa ativa em todo o mundo.

Homocisteinilação é consequência da ligação covalente de Hcy a proteínas, levando à modificação de suas funções, e é considerada uma modificação pós-traducional de proteínas. O grau de homocisteinilação de proteínas é proporcional ao aumento do nível plasmático de Hcy (Perła-Kaján *et al.*, 2007). Vários estudos indicaram uma associação entre níveis elevados de Hcy e a formação de espécies reativas de oxigênio, formadas em reações redox envolvendo o grupo tiol da Hcy, especialmente o ânion superóxido (O_2-) e H_2O_2 (Huang *et al.*, 2001; Weiss *et al.*, 2002; Weiss, 2005).

O efeito tóxico da Hcy no tecido cerebral pode ser influenciado pela ausência das duas principais rotas para a eliminação de Hcy: remetilação de Hcy para Met mediada por betaína e transulfuração de Hcy para Cys (Škovierová *et al.*, 2016). A relação entre Hcy e problemas neurológicos, como depressão, mal de Parkinson e Alzheimer são amplamente descritas (Soni *et al.*, 2019). Suplementação com vitaminas B2, B6, B9 e B12 diminuem de forma eficaz os níveis plasmáticos de Hcy e também reduzem sintomas depressivos (Gariballa, 2011).

A Hcy também foi descrita como patogênica à atividade de osteoclastos. Estudo i*n vitro* de células da medula óssea com meios enriquecidos com Hcy mostrou que ela regula positivamente a formação de osteoclastos e suprime a apoptose nessas células devido à maior produção de espécies reativas de oxigênio. Assim, em pacientes com hiperhomocisteinemia, a atividade elevada de osteoclastos levará a um aumento na reabsorção óssea seguido por maior risco de fraturas e diminuição da densidade mineral óssea (Schalinske & Smazal, 2012). Hiperhomocisteinemia também já foi descrita em uma variedade de doenças gastrintestinais, incluindo constipação, doença de Crohn, doença inflamatória intestinal e câncer colorretal (Givvimani *et al.*, 2012; Casella *et al.*, 2013; Liu *et al.*, 2018).

De modo mais marcante, a Hcy tem sido reconhecida como um fator de risco independente para doenças cardiovasculares; primeiramente descrita por McCully (1969) em dois pacientes com HCU e outro pacientes com um defeito no metabolismo da vitamina B12. Em seguida, inúmeros estudos mostraram que hiperhomocisteinemia associa-se a risco aumentado para várias formas de doença vascular (Lühmann *et al.*, 2007; McCully, 2015). No entanto uma recente metanálise mostrou que a redução dos níveis de tHcy por folato e vitamina B12 não reduziu o risco de eventos vasculares (Martí-Carvajal *et al.*, 2017).

3.2 ÁCIDO METILMALÔNICO

As acidurias metilmalônicas são um grupo de EIM que são bioquimicamente caracterizados por aumento de MMA. Elas são causadas por uma deficiência hereditária de MCM ou por defeitos na síntese da desoxiadenosilcobalamina, o cofator da MCM. O MMA é uma molécula de quatro carbonos relacionada ao catabolismo da valina, isoleucina e ácido propiônico. As concentrações de MMA geralmente aumentam nos estágios iniciais da deficiência de vitamina B12 antes mesmo de reduções mensuráveis na vitamina B12 sérica (Klee, 2000). O MMA já foi descrito como metabólito neurotóxico, *in vitro* e *in vivo* em estudos com animais, uma vez que compromete o metabolismo energético cerebral (Brusque *et al.*, 2002; Pettenuzzo *et al.*, 2006; Mirandola *et al.*, 2008). Outro estudo mostrou que MMA provoca danos ao DNA em leucócitos de sangue periférico humano (Ribas *et al.*, 2010). Andrade *et al.* (2014) relatam que o MMA aumentou os danos ao DNA de córtex cerebral e rim de ratos.

3.3 VITAMINA B12 e FOLATO

O termo vitamina B12 refere-se a uma família de substâncias compostas por anéis tetrapirrólicos em torno de um átomo central de cobalto com cadeias laterais de nucleotídeos ligadas ao cobalto. O nome geral do grupo é cobalamina, com cada um dos diferentes ligantes axiais superiores ligados ao cobalto conferindo um nome diferente: metil (metilcobalamina/MeCbl), hidroxila (hidroxicobalamina/OhCbl), adenosil (adenosilcobalamina/AdoCbl), água (aquacobalamina) e cianeto (cianocobalamina).

Quimicamente, o termo vitamina B12 refere-se à hidroxocobalamina ou cianocobalamina, embora em uso geral este termo se aplique a todas as formas de cobalamina. A forma predominante no soro é a MeCbl e a forma predominante no citosol é a AdoCbl (Klee, 2000).

A vitamina B12 é hidrossolúvel, sintetizada exclusivamente por microrganismos, encontrada em praticamente todos os tecidos animais e estocada primariamente no fígado na forma de AdoCbl (Paniz *et al.*, 2005). A fonte natural de vitamina B12 na dieta humana restringe-se a alimentos de origem animal, especialmente leite, carne e ovos (Herrmann *et al.*, 2003; Vugteveen *et al.*, 2011).

A vitamina B12 e o folato (B9) são duas vitaminas que têm papéis interdependentes na síntese de ácidos nucleicos. Na ausência de vitamina B12, o folato é "preso" e não pode ser reciclado de volta para o pool de folato. Eventualmente, isso leva a uma redução na síntese de timidina monofosfato que leva à anemia megaloblástica. O consumo inadequado de folato durante o início da gravidez pode causar defeitos do tubo neural no feto em desenvolvimento. Além disso, a deficiência de folato e vitamina B12 e o consequente aumento de Hcy são considerados um fator de risco significativo para doenças cardiovasculares.

Tanto a deficiência de vitamina B12 como a de folato estão associadas a distúrbios neuropsiquiátricos. Os mecanismos destas perturbações não são conhecidos. Essas deficiências podem causar depressão, demência, neuropatia desmielinizante, autismo e esquizofrenia (Bottiglieri, 1996; Coşar *et al.*, 2014; Zhang *et al.*, 2016). Outros estudos sugeriram que pacientes idosos com alterações cognitivas e depressivas podem se beneficiar da suplementação com folato (Abalan, 1999). Os índices hematológicos para muitos desses pacientes com distúrbios neuropsiquiátricos estão dentro dos valores de referência, portanto, não se deve descartar a possibilidade de deficiência de vitamina B12 com base apenas em testes hematológicos normais (Brett & Roberts, 1994).

3.4 CREATINA

A Cre desempenha um papel energético fundamental na célula, particularmente nos tecidos muscular e cerebral (Wyss & Kaddurah-Daouk, 2000). A deficiência de Cre está associada a manifestações neurológicas que podem estar relacionadas a possíveis efeitos

antioxidantes (Lawler *et al.*, 2002; Schulze, 2003; Kolling *et al.*, 2014) ou a um possível papel da Cre como um neuromodulador (Almeida *et al.*, 2006).

A síntese de Cre requer três aminoácidos glicina, arginina e Met - bem como a ação de duas enzimas. A primeira enzima, a L-arginina:glicina amidinotransferase (AGAT; EC:2.1.4.1) catalisa a formação de guanidinoacetato e ornitina a partir de arginina e glicina, ativa principalmente no rim e pâncreas. A segunda enzima, ativa no fígado, guanidilacetato metiltransferase (GAMT; EC:2.1.1.2) utiliza AdoMet para metilação de guanidinoacetato, produzindo Cre e AdoHcy (Figura 2) (Brosnan *et al.*, 2007). Cre tanto de origem alimentar ou sintetizada endogenamente, é retirada do plasma através de um transportador específico (CreaT). O CreaT é encontrado em vários tecidos, incluindo o músculo esquelético, rim, coração, cérebro, cerebelo e fígado (Snow & Murphy, 2001).

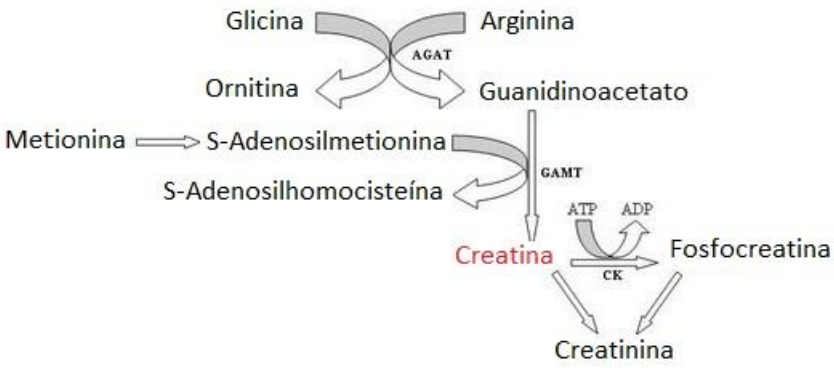


Figura 2. Síntese de creatina. AGAT: L-arginina:glicina amidinotransferase; GAMT: guanidilacetato metiltransferase.

A regulação da síntese de Cre ocorre principalmente através de mudanças nos níveis de guanidinoacetato (GAA). A atividade renal de AGAT é regulada positivamente pelo hormônio do crescimento e negativamente pela Cre da dieta (Guthmiller *et al.*, 1994). A síntese de Cre também pode ser regulada pela disponibilidade de substrato, particularmente arginina (Edison *et al.*, 2007).

Teoricamente, a Met pode ser poupada e o acúmulo de Hcy evitado se menos grupos metil forem doados por reações que utilizam AdoMet como substrato para metiltransferase (Finkelstein, 2006). Em particular, a síntese de Cre e fosfatidilcolina consomem muitos grupos metil. Aproximadamente 40% dos grupos metil fornecidos por AdoMet são consumidos na síntese de Cre, portanto ela é responsável por 40% da

produção corporal de Hcy (Stead *et al.*, 2006). Como consequência, a administração oral de Cre poderia levar a uma formação reduzida de Hcy e assim reduzir seu acúmulo em homocistinúrias (Schiff & Blom, 2012).

Vários estudos tem investigado o potencial da Cre em diminuir níveis de Hcy. Petr *et al.* (2013), por exemplo, mostraram diminuição de 50% nos níveis séricos de Hcy em paciente homozigoto para mutação c.677C>T no gene da *metilenotetrahidrofolato redutase* (*MTHFR*) após 30 dias com suplemento diário de 5g de Cre monohidratada. Já Moraes *et al.* (2014) não observaram alteração sérica de Hcy ao avaliar 40 homens jovens e saudáveis submetidos, durante sete dias, à suplementação de Cre monohidratada na dose de 20g/dia. Já em cultivo celular de cardiomiócitos utilizando suplementação de 5mM de Cre, observou-se uma diminuição de citotoxicidade, apoptose e produção de espécies reativas de oxigênio, pelas células quando expostas a tratamento quimioterápico (Santacruz *et al.*, 2015).

3.5 HOMOCISTINÚRIA CLÁSSICA

A HCU (OMIM # 236200) é um EIM associado à deficiência da CBS, responsável pela degradação da Hcy, que resulta em aumento nos níveis plasmáticos de Hcy, Met e AdoHcy, bem como diminuição dos níveis de Cys, conforme rota do metabolismo da Hcy e Met (Figura 1) (Mudd. *et al.*, 2001; Wilcken, 2006).

O primeiro relato de HCU foi descrito na Irlanda, em 1963, por Nina Carson e Desmond Neill. Enquanto realizavam testes em urina de indivíduos com retardo mental a fim de detectar distúrbios metabólicos, eles verificaram que duas irmãs excretavam grande quantidade de homocistina. Além do retardo mental, as irmãs apresentavam alterações clínicas como luxação de cristalino, deformidades esqueléticas e alterações na pele e cabelos. Os pesquisadores denominaram este novo distúrbio metabólico de homocistinúria (Carson *et al.*, 1963). Dois anos depois, descobriu-se que a doença era causada por um defeito na enzima CBS (Mudd *et al.*, 1964).

O diagnóstico da HCU é dado através da combinação de sinais clínicos e das avaliações bioquímica e molecular. O teste de triagem para HCU mensura a concentração de homocistina na urina do paciente (Pico & Bermudez, 2006). Como esse teste não é

conclusivo, também são mensuradas as concentrações plasmáticas de Met, Hcy e seus metabólitos. A hipermetioninemia é um achado importante, já que outras homocistinúrias, como deficiência de MTHFR e defeitos de cobalamina, apresentam concentrações normais ou baixas de Met (Picker & Levy, 2014). A confirmação do diagnóstico de HCU é dada, através do padrão-ouro, com ensaios diretos da atividade da enzima CBS. Ela pode ser avaliada em cultura de fibroblastos, biópsias hepáticas ou linfócitos estimulados por fitohemaglutinina (Yap, 2005). A atividade enzimática em indivíduos com HCU varia de 0 a 1,8 U/mg de proteína, enquanto que em controles fica na faixa de 3,7-60 U/mg de proteína. A enzima CBS utiliza como cofator o piridoxal fosfato, forma ativa da vitamina B6, assim após a confirmação do diagnóstico de HCU é importante determinar a responsividade do paciente ao tratamento com piridoxina (Walter *et al.*, 1998; Mudd. *et al.*, 2001; Picker & Levy, 2014).

3.5.1 Sintomas e Apresentação Clínica

Bioquimicamente, a HCU é caracterizada pela elevação do nível plasmático de Hcy, acompanhado pela elevação de seu precursor, o aminoácido essencial Met, bem como a redução dos produtos da via de transulfuração da Hcy, cistationina e Cys. Também ocorre a eliminação de homocistina, metabólito formado por duas moléculas de Hcy ligadas por uma ponte dissulfeto na urina, característica que resultou na denominação da doença. As concentrações plasmáticas normais de Hcy variam de 5-15 µmol/L, podendo atingir 500 µmol/L em pacientes homocistinúricos não tratados (Mudd. *et al.*, 2001).

A HCU é uma doença multissistêmica, de início lento e caráter progressivo e expressividade variável dentre os pacientes. Os sinais clínicos da doença envolvem principalmente quatro sistemas: ocular, vascular, nervoso central e ósseo (Mudd *et al.*, 1985). Dentre os sinais e sintomas mais frequentes estão: luxação do cristalino (*ectopia lentis*), retardo mental, distúrbios psiquiátricos, crises convulsivas, aterosclerose, tromboembolismo, miopia, osteoporose, tromboembolismo pulmonar e uma variedade de deformações ósseas (Mudd. *et al.*, 2001; Jiang *et al.*, 2005; Picker & Levy, 2014).

3.5.2 Tratamento dos Pacientes com Homocistinúria Clássica

O principal objetivo do tratamento da HCU é a redução nos níveis de Hcy. Quando uma boa adesão terapêutica e um bom controle metabólico são mantidos ao longo da vida, e o tratamento iniciado no período neonatal, é possível prevenir todas as complicações da HCU (Mudd *et al.*, 1985; Yap & Naughten, 1998). No Brasil, ainda não há pesquisa de HCU pelo teste do pezinho oferecido pelo SUS, portanto o diagnóstico geralmente é tardio e a adesão ao tratamento, principalmente à dieta que é muito restrita, é de difícil aceitação pelos pacientes.

Atualmente, o plano de ação terapêutica está focado em manter os níveis de tHcy <50 μmol/L para pacientes responsivos ao tratamento com piridoxina e <100 μmol/L para pacientes não responsivos, e a Cys em níveis normais e assim, garantir o crescimento e desenvolvimento adequados (Morris *et al.*, 2017). Para isto, três estratégias podem ser utilizadas: a estimulação da atividade residual da CBS (com uso de piridoxina), a redução da sobrecarga de Met, inclusive com dieta restrita, e o aumento da remetilação de Hcy à Met (com uso de betaína). Entretanto, ainda assim, vários pacientes não alcançam os níveis almejados dos metabólitos.

A piridoxina atua aumentando a atividade residual da enzima, o que ocasiona redução da concentração de Hcy e Met e aumento de Cys (Mudd *et al.*, 1970). Aproximadamente 50% dos pacientes com HCU apresentam algum nível de responsividade à piridoxina (Mudd *et al.*, 2001). Em geral, pacientes não responsivos desenvolvem a forma mais grave da doença, apresentando complicações mais precocemente e maior mortalidade (Mudd *et al.*, 1985).

Recomenda-se que o ácido fólico e a vitamina B12 sejam adicionados ao tratamento, a fim de otimizar a conversão de Hcy em Met, porque a resposta à piridoxina é influenciada pela depleção de folato, que pode ocorrer pela própria administração da piridoxina (Pico & Bermudez, 2006). Além disso, acredita-se que os requerimentos de folato sejam maiores nos pacientes com deficiência de CBS devido a um aumento do fluxo pela rota de remetilação.

A betaína fornece um caminho alternativo de remetilação atuando como doador de grupos metil para converter o excesso de Hcy em Met através da enzima BHMT, podendo assim ajudar a prevenir as complicações, principalmente a trombose (Lawson-Yuen &

Levy, 2006). A ingestão de 6-9g de betaína ao dia pode reduzir em mais de 70% os níveis de Hcy, e é acompanhada por um aumento importante da concentração de Met (Walter *et al.*, 1998; Wilcken, 2006). Em alguns casos também se indica uma dieta restrita em Met, através da limitação do aporte de proteínas naturais. No entanto, para atingir as necessidades proteicas diárias utiliza-se um suplemento (fórmula metabólica) de aminoácidos isento de Met e suplementado com Cys, vitaminas, minerais e elementos-traço (Frangipani *et al.*, 2006).

3.5.3 Incidência de Homocistinúria Clássica

A incidência de HCU varia dramaticamente entre as regiões, de 416:100.000 indivíduos entre uma tribo Tao em uma ilha em Taiwan, para menos de um em um milhão na população Han de Taiwan (Lu *et al.*, 2012). A prevalência mundial é estimada em 0.29 a 1 a cada 100.000 indivíduos (Mudd. *et al.*, 2001; Moorthie *et al.*, 2014). O país com maior incidência de HCU no mundo é o Catar. Provavelmente a alta incidência da doença seja influenciada por um efeito fundador, uma vez que a doença é mais comum em três tribos da população, que também tem uma alta incidência de casamento consanguíneo. Após a implementação da triagem neonatal medindo tHcy e Met, combinada à triagem genética, a incidência estimada de HCU no Catar chega a 55:100.000 recém-nascidos (RN) (Gan-Schreier *et al.*, 2010).

Baseado em dados de triagem neonatal (ainda disponível em poucos países), com mensuração de Met, Naughten *et al.* (1998) relataram uma incidência de HCU na Alemanha de 0,77:100.000 RN, na Inglaterra 0,8:100.000 RN, nos EUA 0,34:100.000 RN e mais frequentes como 1,5:100.000 RN na Irlanda e 1,8:100.000 RN na Itália. A triagem bioquímica neonatal de mais de 800 mil indivíduos na Austrália por volta da década de 1960, revelou 14 casos de HCU, resultando em uma incidência de 1,7:100.000 RN (Wilcken & Turner, 1978). Uma incidência de HCU muito menor é observada na Ásia. No Japão, por exemplo, uma incidência extremamente baixa de 0,1:100.000 RN foi observada apesar de um programa de triagem bioquímica eficaz (Naughten *et al.*, 1998). Em Taiwan, 5 milhões de indivíduos foram incluídos na triagem bioquímica neonatal para HCU e apenas 3 foram diagnosticados (Lu *et al.*, 2012). No Brasil o número de pacientes em

acompanhamento estimado pela Associação Brasileira de Homocistinúria é de 80-100 pacientes (relato verbal).

Outra estratégia para calcular a incidência de HCU é determinar a frequência de heterozigotos para mutações em *CBS* e assim, através da equação de equilíbrio Hardy-Weinberg, estimar o número de pacientes com HCU. Os primeiros a utilizar essa abordagem foram Gaustadnes *et al.* (1999), esse grupo seleciounou 500 RNs dinamarqueses consecutivos e avaliaram a mutação c.833 T>C, estimando uma incidência de HCU de ao menos 4,8:100.000. Na Noruega, Refsum *et al.* (2004) determinaram a prevalência de seis mutações do gene *CBS* em 1.133 amostras selecionadas aleatoriamente dentre ~12.000 amostras de screening neonatal e calcularam uma prevalência de HCU de ~15,6:100.000 RN.

3.6 DEFICIÊNCIA DE CblC

A acidemia metilmalônica com homocistinúria é uma doença hereditária do metabolismo da vitamina B12 (cobalamina/cbl) caracterizada por anemia megaloblástica, letargia, atraso de crescimento, atraso no desenvolvimento, déficit intelectual e convulsões. Existem três defeitos da cobalamina - cblC, cblD e cblF - que são responsáveis pela acidemia metilmalônica associada à homocistinúria. A acidúria metilmalônica e homocistinúria tipo cblC (OMIM # 277400) é o EIM mais comum do metabolismo da cobalamina (Lerner-Ellis *et al.*, 2009). CblC é causada por mutações no gene *MMACHC* (GenBank ID: 25974). O diagnóstico e tratamento precoces são importantes, os primeiros sintomas podem surgir entre a primeira infância e/ou a idade adulta. Os doentes que se tornam sintomáticos depois da infância apresentam melhor prognóstico, mas podem apresentar ataxia, demência ou psicose (Carrillo-Carrasco *et al.*, 2012).

Em 1969, o primeiro caso de deficiência de cblC foi relatado por Mudd et al. em um lactente com homocistinúria, acidúria metilmalônica, cistinemia e hipometioninemia. A criança morreu com 7,5 semanas de idade. A análise *in vitro* identificou um defeito nas duas reações em que os derivados da vitamina B12 funcionam como coenzimas: 1) AdoCbl na formação de Met a partir Hcy por MTR, e 2) MeClb para isomerização de metilmalonil-CoA para succinil-CoA por MCM. Como a vitamina B12 estava presente em

concentrações normais no fígado, os autores concluíram que o gene responsável por esse defeito envolvia a conversão de vitamina B12 em suas coenzimas ativas (Mudd *et al.*, 1969).

3.6.1 Sintomas e Apresentação Clínica

Pacientes com deficiência de cblC geralmente sofrem de um amplo espectro de complicações clínicas, incluindo aspectos de desenvolvimento, metabólicos, hematológicos, neurológicos, oftalmológicos e dermatológicos (Rosenblatt *et al.*, 1997). Embora seja considerada uma doença da infância, os pacientes podem apresentar sintomas a qualquer momento desde o período neonatal até a idade adulta, e podem até mesmo ser afetados intra-útero. A doença é classificada como: forma de início precoce e de início tardio (Thauvin-Robinet *et al.*, 2008).

Pacientes com doença de início precoce, definida pelo início dos sintomas antes do primeiro ano de vida, geralmente apresentam comprometimento sistêmico grave. Os sintomas incluem dificuldades de alimentação, hipotonia, deficit de crescimento, convulsões, microcefalia e atraso no desenvolvimento. Acidose foi observada em alguns pacientes. Nistagmo, retinopatia pigmentar e diminuição da acuidade visual são comuns. Os achados hematológicos incluíram trombocitopenia, anemia macrocítica megaloblástica e/ou neutrófilos polimorfonucleares hipersegmentados e leucopenia, provavelmente todos relacionados à deficiência celular de folato. Também ocorrem complicações renais, que não são induzidas pelo aumento de MMA, mas sim por microangiopatia (que pode ocorrer em qualquer órgão), resultando em síndrome hemolítico-urêmica (Rosenblatt *et al.*, 1997).

O início tardio da doença parece ser mais raro do que a forma de início precoce. Além de anormalidades hematológicas leves, o curso clínico é caracterizado por distúrbios comportamentais, psiquiátricos, rápida deterioração mental com confusão e desorientação, demência, delirium e psicose (Martinelli *et al.*, 2011). Embora sua ocorrência seja rara, a deficiência de cblC de início tardio deve ser considerada em diagnóstico diferencial de pacientes que apresentam sintomas neurológicos que não são consistentes com doenças neurológicas comuns, especialmente quando a cognição, o trato piramidal e nervos periféricos estão envolvidos (Wang *et al.*, 2012). É importante ressaltar que facilmente os

pacientes podem ser diagnosticados erroneamente ou até mesmo permanecerem sem diagnóstico.

3.6.2 Tratamento dos Pacientes com deficiência de cblC

O tratamento para deficiência de cblC consiste numa abordagem combinada que utiliza uma alta dose de vitamina B12 intramuscular, de preferência sob a forma de hidroxicobalamina (OHCbl). Em uma diretriz para o diagnóstico e tratamento de distúrbios da remetilação, Huemer *et al.* (2017) recomendam uma dose inicial de 1 mg de OHCbl por dia, administrada por via parenteral. A betaína oral é usada para melhorar a remetilação de Hcy pela via alternativa, e o ácido fólico oral melhora o "aprisionamento" de folato (Martinelli *et al.*, 2011; Fischer *et al.*, 2014). Restrição proteica para reduzir MMA é contraindicada porque resulta em uma grande redução de Met (Manoli *et al.*, 2016). Bom controle metabólico e correção de problemas hematológicos podem por vezes ser alcançados com tratamento, mas a maioria dos doentes continua a ter sinais de atraso motor e da linguagem, déficit intelectual e achados oftalmológicos anormais (Rosenblatt *et al.*, 1997).

3.7 DEFICIÊNCIA DE METILENOTETRAHIDROFOLATO REDUTASE (MTHFR)

A homocistinúria devido à deficiência de MTHFR (OMIM # 236250) resulta no metabolismo intracelular anormal do ácido fólico e impede a redução de 5-10 metilenotetrahidrofolato a 5-metilenotetrahidrofolato, doador do grupo metil para a remetilação da Hcy em Met. Como resultado, a doença leva à deficiência de metiletrahidrofolato e, consequentemente, a homocistinúria com hipometioninemia (Huemer *et al.*, 2015).

O gene que codifica a enzima MTHFR, também chamado *MTHFR*, localiza-se no braço longo do cromossomo 1 (GenBank ID: 4524), e é composto por 11 éxons, de tamanhos que variam de 102 a 432 pb. A sequência codifica um proteína de aproximadamente 70 kDa (Goyette *et al.*, 1998). Em humanos, quatro transcritos diferentes podem ser produzidos, diferindo entre si no primeiro éxon (Homberger *et al.*, 2000). Mais

de 130 mutações causadoras de homocistinúria já foram identificadas no gene, sendo a maioria delas privada e de troca de sentido. Exceções são as mutações c.1141C>T, frequente em populações Amish, e c.1542G>A, detectada em mais de 20 alelos não relacionados (Burda *et al.*, 2015).

Muito mais frequentes que as mutações causadoras de homocistinúria por deficiência de MTHFR são os polimorfismos que resultam em hiperhomocisteinemia leve a moderada. O polimorfismo c.677C>T é o mais frequente, resultando na redução da atividade enzimática em 70% em homozigotos. Está associado com baixos níveis de folato no soro, plasma e eritrócitos e hiperhomocisteinemia (Jacques *et al.*, 1996; Brustolin *et al.*, 2010). No Brasil, o estudo de Arruda *et al.* (1998) encontrou alta prevalência de homozigoze para este polimorfismo entre descendentes de caucasianos (10%) e baixa entre negros (1,45%) e indígenas (1,2%).

3.7.1 Sintomas e Apresentação Clínica

Deficiência de MTHFR é uma doença grave que afeta principalmente o SNC, provavelmente devido à reduzida disponibilidade de metilenotetrahidrofolato e Met, causando redução da metilação cerebral, como sugerido pela diminuição dos níveis de AdoMet no líquor (Surtees *et al.*, 1991; Strauss *et al.*, 2007). Assim, está associada a um lento desenvolvimento do cérebro, à incapacidade neurológica grave e à morte prematura (Rosenblatt & Erbe, 2001).

Foi descrita pela primeira vez em 1972 por Mudd et al. em três pacientes: 1) menino de 16 anos com fraqueza muscular, convulsões e sinais encefálicos anormais, 2) menina de 17 anos com degradação mental e esquizofrenia e 3) irmã de 15 anos de idade do paciente 2. Esses três pacientes apresentavam homocistinúria, mas níveis normais de Met, atividade normal de CBS em fibroblastos e baixa atividade de MTHFR (Mudd *et al.*, 1972). A deficiência grave de MTHFR é caracterizada bioquimicamente por hiper-homocisteinemia, homocistinúria, cistationina aumentada e Met baixa ou normal, em contraste com a deficiência de CBS, que apresenta Met elevada. Em cultura de fibroblastos, a atividade enzimática residual é inferior a 20% do valor médio de controle (Rosenblatt & Erbe, 2001; Watkins & Rosenblatt, 2012; Huemer *et al.*, 2017).

Uma apresentação clássica sugestiva de deficiência de MTHFR é um recém-nascido com hipotonia generalizada, dificuldade de alimentação, déficit de crescimento, letargia, apneia e, eventualmente, microcefalia. Os pacientes também podem apresentar sintomas durante a infância e até na idade adulta, na forma tardia da doença. Os pacientes com a forma tardia têm um quadro clínico variável, incluindo marcos tardios de desenvolvimento, comprometimento cognitivo e/ou anormalidades de marcha, bem como degradação mental e encefalopatia progressiva, compatíveis com mielopatia ou ataxia, problemas comportamentais de espasticidade, um espectro inespecífico de sintomas psiquiátricos e ocasionalmente trombose. A anemia megaloblástica e/ou macrocítica é tipicamente ausente (Thomas & Rosenblatt, 2005; Saudubray *et al.*, 2012; Diekman *et al.*, 2014).

3.7.2 Tratamento dos Pacientes com deficiência de MTHFR

Pacientes não tratados com a forma precoce da doença apresentam retardo progressivo do desenvolvimento e retardo mental, alguns pacientes também podem apresentar epilepsia e doença neurológica (marcha anormal, espasticidade). No entanto, um grande número de pacientes não tratados vem a óbito (Thomas & Rosenblatt, 2005; Diekman *et al.*, 2014).

O tratamento envolve a administração (doses sugestivas) de betaína (100 a 250 mg/kg/dia em crianças e 5 a 20 g/dia em adultos) para fornecer uma via alternativa para a remetilação de Hcy. Além disso, OHcbl (1-2 g/dia) e ácido fólico (400 mg/d) são indicados. Piridoxina, como cofator para CBS, pode ser administrada para maximizar a via de transulfuração e a riboflavina como cofator da enzima MTHFR (Huemer *et al.*, 2017).

Met pode ser suplementada se seu nível permanecer baixo, apesar do tratamento. Em geral, o tratamento melhora o curso da doença em casos de início precoce (Rosenblatt & Erbe, 2001; Sibani *et al.*, 2003; Thomas & Rosenblatt, 2005). Em uma revisão sistemática, incluindo 36 pacientes, o impacto do tratamento com betaína em pacientes com a forma precoce da doença mostrou que todos os cinco pacientes tratados precocemente sobreviveram com desenvolvimento psicomotor normal. Enquanto nove dos dez pacientes não tratados e dois dos 21 tratados tardiamente morreram. O desenvolvimento psicomotor foi prejudicado em todas as crianças com atraso no início do

tratamento, apesar da estabilização clínica e bioquímica observada desde a introdução do tratamento com betaína (Diekman *et al.*, 2014).

3.8 FENILCETONÚRIA

PKU e as hiperfenilalaninemias são doenças hereditárias do metabolismo do aminoácido Phe, caracterizadas pela deficiência de fenilalanina hidroxilase (PAH; EC:1.14.16.1). PKU é uma doença metabólica autossômica recessiva em que o aminoácido essencial Phe não pode ser convertido em tirosina, levando ao acúmulo de metabólitos tóxicos (Dobbelaere *et al.*, 2003). PKU é uma hiperfenilalaninemia caracterizada por mutações no gene *PAH* (GenBank ID: 5053) que sintetiza enzima hepática com mesmo nome. PAH é responsável por converter a Phe no aminoácido tirosina na presença do cofator tetrahidrobiopterina (BH4), oxigênio molecular e ferro (figura 3) (Blau *et al.*, 2010; Flydal & Martinez, 2013).

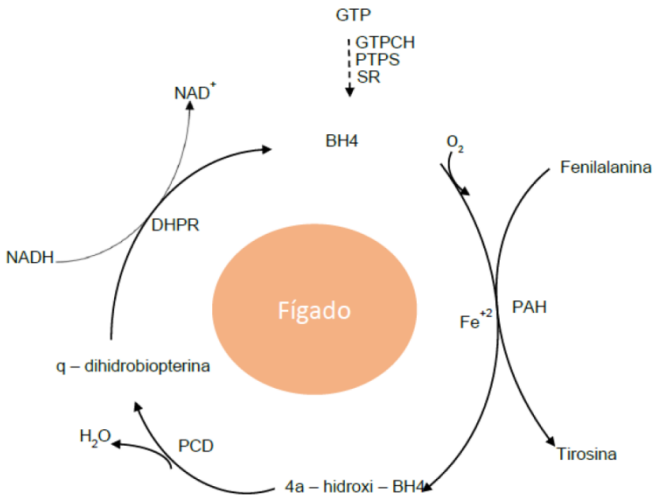


Figura 3. Sistema de hidroxilação de fenilalanina. Durante a hidroxilação de Phe pela PAH, na presença de oxigênio molecular (O_2) e ferro (Fe^{+2}), tetra-hidrobiopterina (BH4) é oxidada para 4a-hidroxi-BH4 intermediário, que é subsequentemente regenerado em BH4 via quinonoide (q) de dihidrobiopterina pela enzima carbinolamina-4adehidratase (PCD) e pela dihidropteridina redutase dependente de NADH (DHPR). BH4 é sintetizada a partir da guanosina trifosfatada (GTP) por três enzimas adicionais: ciclohidrolase I GTP (GTPCH), 6-piruvoil-tetrahidropterina sintase (PTPS), e sepiapterina redutase (SR). **Fonte:** Adaptado e traduzido de Blau *et al.*, 2010.

O tratamento para PKU é principalmente dietético e consiste em: (1) fornecer uma quantidade rigorosa e restrita de Phe, tipicamente inferior a 10g/dia (2) uso de um substituto proteico livre de Phe, constituído de todos os outros aminoácidos, vitaminas e traços minerais (fórmula metabólica) e (3) alimentos naturalmente pobres em Phe que são fornecidos livremente (MacDonald *et al.*, 2011). O início precoce, logo após o nascimento, de uma dieta restrita em Phe evita complicações neuropsicológicas (Blau *et al.*, 2010). Cada indivíduo apresenta uma "tolerância" distinta de Phe, por isso, a quantidade de Phe que será consumida na dieta deve se adequar às necessidades do paciente (Casey, 2013). A dieta restrita em Phe mostrou ter efeito positivo para o desenvolvimento mental, mas um efeito negativo no crescimento físico (Dobbelaere *et al.*, 2003; Blau *et al.*, 2010).

Durante a infância, o aleitamento materno é recomendado para fornecer proteínas naturais de acordo com a tolerância de Phe individual, e/ou se necessário uma fórmula metabólica infantil livre de Phe é usada. Com a introdução de alimentos sólidos, as crianças com PKU têm de evitar alimentos ricos em proteínas (carne, peixe, ovos, produtos lácteos, pão padrão, nozes e sementes) (Verduci *et al.*, 2016).

Uma vez que a Phe faz parte da proteína natural, pacientes com PKU consomem quantidades limitadas de proteína natural para manter as concentrações de Phe no sangue dentro da faixa alvo para a idade. A demanda pelos demais aminoácidos é preenchida com uma fórmula substituta de proteína isenta de Phe. Esta fórmula é enriquecida com micronutrientes, normalmente fornecidos com a ingestão de proteínas e com tirosina extra sendo um aminoácido especialmente deficiente na PKU (Vugteveen *et al.*, 2011).

3.8.1 Sintomas, Apresentação Clínica e Mecanismos Fisiopatológicos

A PKU foi descrita pela primeira vez em 1934 por Følling quando ele detectou fenilcetonas na urina de indivíduos afetados e em 1953, Bickel et al. relataram pela primeira vez a eficácia de uma dieta pobre em Phe em uma criança com PKU. Os fenótipos podem variar de um aumento médio de Phe no soro para um fenótipo clássico grave com hiperfenilalaninemia pronunciada que, se não tratada, resulta em incapacidade mental profunda e irreversível. PKU não tratada está associada com comprometimento intelectual progressivo, acompanhado por uma série de sintomas adicionais como erupção eczematosa, autismo, convulsões, e déficits motores. Problemas de desenvolvimento,

comportamento aberrante e sintomas psiquiátricos frequentemente se tornam aparentes à medida que a criança cresce (Blau *et al.*, 2010; van Wegberg *et al.*, 2017). Estudos de imagem descrevem lesões na substância branca do cérebro associadas à redução da formação de mielina, embora ainda não haja ligação causal entre a desmielinização e dano neuropsicológico (Pearsen *et al.*, 1990).

As mulheres com PKU com controle inadequado de Phe durante a gestação estão sob alto risco de ter um filho com retardo mental, defeitos cardíacos congênitos, retardo de crescimento intra-uterino e outros defeitos uma vez que a Phe é teratogênica. Portanto, os níveis de Phe inferiores a 360 umol/L antes da concepção e durante toda a gravidez são indicados (Rouse & Azen, 2004; Prick *et al.*, 2012).

Os níveis elevados de Phe no sangue são neurotóxicos principalmente devido ao seu efeito inibitório sobre o transportador de aminoácido neutros do tipo L (LAT1), que é a o principal meio de entrada de Phe no SNC. LAT1 também é a única rota de entrada para outros grandes aminoácidos neutros (LNAAs) ao SNC – triptofano, histidina, tirosina, isoleucina, leucina, valina, Met e lisina (Pratt, 1980). Destacando que a tirosina é um precursor de dopamina e norepinefrina; e triptofano é um precursor da serotonina. Altas doses de Phe no sangue podem inibir LAT1, e assim inibir o transporte de outros grandes aminoácidos neutros para o SNC, aumentando o potencial para disfunção de neurotransmissores pela menor disponibilidade de substrato para a sua síntese (Hoeksma *et al.*, 2009).

Outros mecanismos possíveis para indução de danos ao cérebro pela hiperfenilalaninemia incluem atividade reduzida de piruvato quinase (Hörster *et al.*, 2006), neurotransmissão glutamatérgica perturbada (Martynyuk *et al.*, 2005), atividade reduzida da HMG-CoA redutase (ou 3-hidroxi-3-methyl-glutaril-CoA redutase) (Shefer *et al.*, 2000), e a função da monoamina oxidase B, que degrada feniletilamina (metabólito tóxico da Phe) (Ghozlan *et al.*, 2004).

4. JUSTIFICATIVA

No Brasil, há poucos estudos descrevendo o perfil genético e clínico de pacientes com HCU. O serviço de Genética Médica do HCPA é referência no diagnóstico e tratamento de pacientes com EIMs, incluindo homocistinúrias, e a amostra analisada no presente estudo inclui pacientes das diversas regiões do Brasil. A incidência de HCU é estimada em ao menos 0.29 a 1 a cada 100,000 indivíduos pessoas em todo o mundo. Parece ser mais comum em alguns países, como Alemanha (0,7:100.000), Irlanda (1,5:100.000) e Qatar (55:100.000). Por outro lado a descrição de pacientes é muito rara na Ásia, com 0,1:100.000 RN no Japão. As formas mais brandas da doença, que geralmente inclui pacientes responsivos ao tratamento com piridoxina, podem não ser percebidos e diagnosticados, e a incidência de HCU pode ser muito mais comum do que a descrita. Assim, a estimativa de incidência a partir de heterozigotos descritos em grandes bancos de dados genômicos pode ser de grande valia, pelo alto número de indivíduos analisados e sua diversidade genética.

Pacientes com HCU são tratados com piridoxina e grande parte deles tem que ser tratada por uma dieta restrita em Met, a qual é muito difícil de seguir, em particular depois da infância. Pacientes com defeitos de remetilação são tratados com betaína, no entanto, estratégias alternativas de tratamento são urgentemente necessárias. A síntese endógena de Cre requer uma considerável porção de grupos metil no fígado, e é responsável pela formação de uma importante parcela de Hcy. Além disso, a Cre é descrita com potencial antioxidante, assim poderia ser um adjuvante no tratamento dos pacientes com homocistinúria.

Já os pacientes com PKU tem seu tratamento baseado em restrição dietética de Phe e uso de fórmula metabólica. Com base na dieta pobre em proteínas esses pacientes estão em risco de desenvolver deficiência de Met e vitamina B12, e consequentemente aumento nos níveis de Hcy e MMA. Hiperhomocisteinemia e deficiência de vitamina B12 foram relatadas em pacientes com PKU, mas há estudos discordantes. A análise de Hcy, MMA e vitamina B12 em pacientes com PKU no pré-tratamento e pós-tratamento; pacientes em momentos aderentes à dieta hipoproteica, comparados a períodos não aderentes; e mulheres nos estágios pré, pós e durante a gravidez, pode auxiliar no entendimento das

apresentações clínicas e tratamento de pacientes com PKU, já que o aumento dos níveis de Hcy pode elevar o risco de eventos tromboembólicos e cardiovasculares.

5. OBJETIVOS

5. 1 OBJETIVOS GERAIS:

1) Caracterizar aspectos epidemiológicos, clínicos e bioquímicos da homocistínúria clássica;
2) Avaliar o papel da creatina como novo tratamento das homocistinúrias;
3) Avaliar o metabolismo da homocisteína na fenilcetonúria, de forma a propor novos mecanismos fisiopatológicos para a doença;

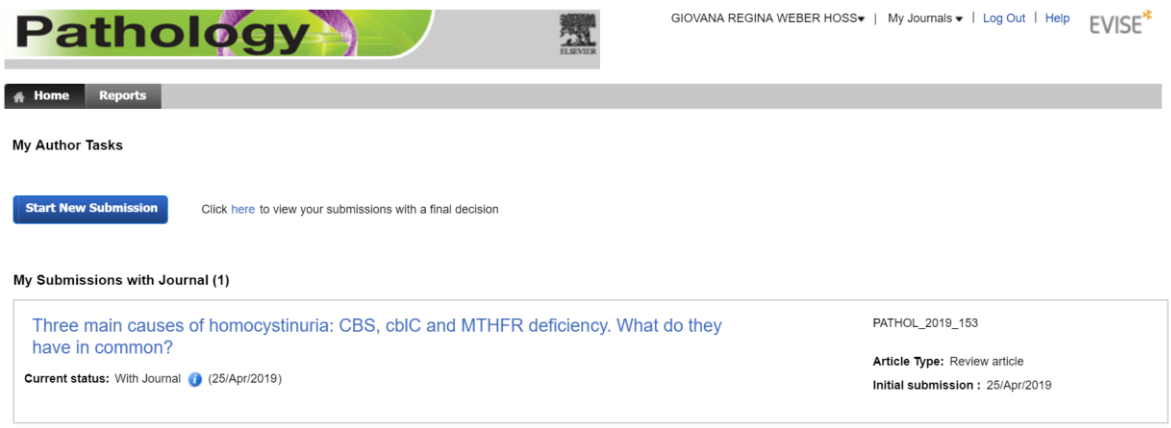
5.2 OBJETIVOS ESPECÍFICOS:

- Caracterizar e comparar as principais causas de homocistinúria: homocistinúria por deficiência de CBS, cblC e MTHFR;
- Discutir o papel da homocisteína na patogenicidade das homocistinúrias;
- Caracterizar o perfil clínico da homocistinúria clássica no Brasil;
- Estimar a incidência de homocistinúria clássica com base no número de heterozigotos em bancos de dados genômicos;
- Avaliar a ação antioxidante da creatina em cultura celular de fibroblastos com defeito de remetilação da homocisteína e controles;
- Avaliar os níveis de homocisteína, ácido metilmalônico e vitamina B12 em pacientes com fenilcetonúria.

6. CAPÍTULOS

6.1 CAPÍTULO 1: "*Three main causes of homocystinuria: CBS, cblC and MTHFR deficiency. What do they have in common?"*

O capítulo 1 consiste em uma revisão da literatura que descreve e compara as principais causas de homocistinúria, discutindo a patogenicidade da Hcy. Este capítulo será apresentado na forma de artigo, o qual está submetido à publicação na revista Pathology, cujo fator de impacto é 3,068 e o Qualis CAPES da área de genética (Biotecnologia) é A2. O formato obedece às normas de submissão da revista.



Three main causes of homocystinuria: CBS, cblC and MTHFR deficiency. What do they have in common?

What do homocystinuria have in common?

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SUMMARY

In this review, we aim to highlight the clinical and biochemical similarities and differences between the most frequent causes of homocystinuria. The discussion addresses the controversial involvement of homocysteine (Hcy) as a main disease-causing metabolite. Genetic homocystinurias are a group of inborn errors of metabolism that result in the massive excretion of Hcy in the urine due to Hcy accumulation in the body. The three most frequent causes are classical homocystinuria (deficiency of cystathionine beta-synthase (CBS)], methylmalonic aciduria with homocystinuria, cblC type (cblC deficiency) and severe methylenetetrahydrofolate reductase (MTHFR) deficiency. Their joint manifestation is the accumulation of tHcy and treatment focuses mainly on lowering tHcy. Besides the severely elevated tHcy, we observed very little overlap in the biochemical and clinical presentations of the three most common forms of homocystinuria. The clear difference in clinical presentations makes us questioning if Hcy itself is the main disease-causing metabolite in homocystinurias. Our findings challenge the paradigm that elevated Hcy levels are causally related to the clinical presentations. Plasma tHcy is an inadequate biomarker for disease outcome and evaluation of treatment. As a consequence, treatment strategies for homocystinurias needs to be reformulated.

Keywords: Classical homocystinuria; Methylmalonic aciduria with homocystinuria, cblC type; Severe MTHFR deficiency; Homocysteine.

1. BACKGROUND

Hyperhomocysteinemia (tHcy >15 μM)[1] can be caused by environmental (such as nutritional deficiency of vitamins B12 or folate) or genetic factors, including inborn errors of metabolism (Table 1). Mild or moderate forms of hyperhomocysteinemia are present in 5–10% of the population[2]. The severe form of hyperhomocysteinemia (tHcy>50 μM) is rare, and typically associated with severe vitamin B12 or folate deficiency or inherited defects in Hcy metabolism[3].

Hcy is a sulfur-containing amino acid not used in protein synthesis and is considered toxic at increased levels. Hcy is formed as a product of methyl-transfer reactions in methionine (Met) metabolism. In this process, Met is activated by ATP into S-adenosylmethionine (SAM), which is the universal methyl group donor, resulting in the formation of S-adenosylhomocysteine (SAH). SAH is converted to Hcy and adenosine through S-adenosyl-L-homocysteine hydrolase (SAHH). The equilibrium of the reaction favors the formation of SAH. As a consequence, increased Hcy should result in a pronounced accumulation of SAH, which is a potent inhibitor of many methyltransferase reactions. Because methylation is essential for cellular functions in all organs, Hcy is formed in all tissues. Its elimination via CBS (EC 4.2.1.23), is confined to the liver and kidney. Thus, in all other tissues, such as the vascular system and the brain, the only available ways to eliminate Hcy are remethylation by methionine synthase (MTR - EC 1.16.1.8) or exportation out of the cell[4]. In remethylation, Hcy receives a methyl group from 5-methyltetrahydrofolate (5MTHF), which is formed from 5,10-methylenetetrahydrofolate by MTHFR (methylenetetrahydrofolate reductase - EC 1.5.1.20)

(Figure 1). Notably, 5MTHF is the circulating form of folate in blood and cerebrospinal fluid (CSF).

SAM regulates whether Hcy is degraded via CBS or remethylated back to Met. In the liver excess of Met increases SAM, which activates CBS and inhibits MTHFR, causing Hcy to be primarily and irreversibly converted to cystathionine[5, 6]. If Met is low then CBS is not activated and MTHFR is not inhibited by SAM, causing Hcy to be mainly remethylated back to Met[7, 8]. Vitamin B12, as methylcobalamin (Mecbl), is involved as a cofactor for MTR, which catalyzes the remethylation of Hcy into Met in the cytosol. Adenosylcobalamin (Adocbl) is the cofactor for methylmalonyl-CoA mutase, which converts methylmalonyl-CoA into succinyl-CoA in mitochondria (Figure 2). The liver and kidney also contain betaine-homocysteine methyltransferase (BHMT - EC 2.1.1.5), which is an alternative pathway of Hcy remethylation (Figure 1).

This paper reviews the clinical and biochemical findings and management of the three most frequent genetic causes of homocystinurias: CBS deficiency or classical homocystinuria (HCU), methylmalonic aciduria with homocystinuria cblC type, and severe MTHFR deficiency. These three disorders all present with severe hyperhomocysteinemia, while Met levels are increased in HCU and decreased or normal in cblC and MTHFR, and methylmalonic acid (MMA) is increased only in cblC[9] (Table 1). Because folate and Hcy metabolism are intertwined, defects in Hcy metabolism influence folate homeostasis. In CBS deficient patients at diagnosis, folate is often deficient likely due to the inhibition of MTHFR by the elevated SAM, resulting in decreases of 5MTHF, the circulation form of folate[10]. In MTHFR deficiency folate is severely deficient, whereas in cblC defect all folates accumulate as 5MTHF resulting in functional cellular folate deficiency.

2. Classical Homocystinuria

2.1 Clinical presentation

CBS deficiency or classical homocystinuria (HCU; OMIM +236200) is the most common type of homocystinuria. The prevalence of HCU dramatically varies between regions, from 1:240 among a Tao tribe of the Orchid Island, to less than one in one million in the Taiwanese Han population[11]. The worldwide prevalence is estimated at 1:100,000 to 1:344,000 individuals[12, 13].

Four organ systems are primarily affected in HCU: ocular, vascular, central nervous (CNS) and skeletal[14] (Table 2). The hallmark study of Mudd et al. in 1985, concerning a cohort of over 600 HCU patients, described that eye disease, particularly lens dislocation (ectopia lentis), was the main reason for HCU investigation (85% of the cases) and commonly the first symptom, manifesting after the age of two years old and affecting more than 50% of non-treated patients at the age of 10 years old[15]. Other ocular abnormalities that might occur in HCU include high myopia, iridodonesis, glaucoma, optic atrophy, retinal degeneration, retinal detachment, cataracts and corneal abnormalities[12, 16-18].

Another common feature of HCU is vascular disease. Thromboembolic events can occur at any age and at any vein[15, 19-22]. Although it is less common than eye disease, 50% of non-treated patients presented a thromboembolic event at age 29, and the importance of this disease is demonstrated by the high mortality rate from vascular complications. In Mudd's survey[15], over 70% of the deceased patients died because of thromboembolism. Special circumstances, such as pregnancy, surgery and association with mutations in Factor V, seem to increase the risk of vascular events[23-25]. Interestingly, recent studies showed that vascular manifestations can be the sole symptom and can appear even after the

second or third decade of life, especially in patients homozygous for the *CBS* p. Ile278Thr mutation[19, 26].

Cognitive impairment is also considered a common CNS manifestation of HCU. In Mudd's survey, a wide range of IQ's from 10 to 138 was reported, with a median of 78[15]. In addition to the direct impact of Hcy accumulation on CNS, recurrent strokes can also impact cognitive skills[12, 23]. Seizures occur in nearly 20% of the patients[15]. Psychiatric disorders are also highly prevalent, affecting up to 50% of patients[27]. Schizophrenia, anxiety and depression are well documented in HCU[12, 27-30]. The most common symptoms observed by Abbott et al., (1987) in 63 patients include episodic depression (10%), chronic behavior disorders (17%), chronic obsessive-compulsive disorder (5%), and personality disorders (19%)[27]. Additionally, psychiatric disorder as an isolated symptom of HCU has been reported[31, 32].

The skeletal features of HCU include osteoporosis and osteopenia, scoliosis, dolichostenomelia, tall stature, *genu valgum*, sternal deformities and arachnodactyly[15, 33]. The most frequent finding is osteoporosis, which affects approximately half of non-treated patients in their second decade of life[15]. In addition to eye abnormalities (ectopia lentis), the bone deformities (with the exception of osteoporosis) resemble those observed in Marfan Syndrome, what has led to misdiagnosis in the past[34]. Liver injury has been reported in HCU patients, but it is rather rare[35]. Other clinical findings reported in HCU include hypopigmentation of the hair and skin and malar flush, and there is a case report on acute pancreatitis and chronic diarrhea[36-39].

2.2 Diagnosis

Markedly high total Hcy (tHcy >50 µmol/L in children; tHcy >100 µmol/L in adults) together with increased Met and low cysteine in plasma are the classical biochemical features of HCU (Table 2). However, these biochemical abnormalities may be less pronounced in patients with milder forms of HCU or those taking vitamin supplements[23, 40, 41].

Confirmation can be made via the measurement of CBS activity (typically in fibroblasts). Since this method is not broadly available, molecular genetic analysis is most often used for confirmation of the diagnosis. DNA analysis can also be performed, especially in high-risk populations and families with known *CBS* mutations. The human *CBS* gene is located at chromosome 21q22.3[42], and more than 200 disease-causing mutations have been identified[43]. The most frequent mutations (p.Ile278Thr, p.Thr191Met and p.Gly307Ser) account for nearly half of the HCU alleles worldwide (http://cbs.lf1.cuni.cz/index.php). Prenatal diagnosis can be achieved by molecular genetic analyses or the extraction and culture of cells from amniotic fluid to measure CBS activity[12, 44, 45].

Newborn screening has been performed, especially in countries with high incidences of HCU, such as Ireland and Qatar[46, 47]. The most common method is the measurement of Met in dried blood spots (DBS), but a high proportion of false negatives have been obtained by using this method[48-50]. The accuracy of Met to detect HCU is low since, in particular, the pyridoxine-responsive forms of HCU do not develop hypermethioninemia in the first days of life. In addition, other diseases can also lead to increased Met concentrations[51]. To increase sensitivity, reducing the cutoff for Met has

been suggested[51]. In high-risk populations, the direct measurement of tHcy in DBS or analyses of mutations should be performed[52-54].

2.3 Management

CBS deficiency results in markedly increased plasma levels of tHcy and Met and low cysteine[12, 23]. The main goal in HCU treatment is to reduce tHcy (<50 μmol/L for pyridoxine responsive and <100 μmol/L for non-responsive patients)[14]. The first strategy in the treatment is to test whether the patient is pyridoxine responsive. Pyridoxal phosphate, the active form of pyridoxine, is a co-factor of CBS and high dose of oral administration of pyridoxine markedly decreases tHcy in approximately 50% of HCU patients[15, 55]. Dosages from 100 to 500 mg/day in adults can be used[9, 40]. Typically, patients who are responsive to pyridoxine present delayed and less severe clinical symptoms and these individuals may not even require any additional treatment[14, 15]. In addition to pyridoxine, oral folinic acid supplementation (1 to 5 mg/day) should be administered because many patients are folate deficient at the time of diagnosis. Pyridoxine responsiveness should only be tested under normal folate levels[9, 41, 56, 57].

If pyridoxine plus folate is not able to reduce tHcy levels to the target values, then additional therapies should be used. A very efficient strategy to lower tHcy is dietary Met restriction. Met is an essential amino acid and a diet low in Met will result in a significant decline of tHcy. To meet protein and micronutrient requirements, a free-Met amino acid-based formula should be taken daily. The amount of Met tolerated per patient varies from 15 to 60 mg/kg/day[58]. Poor compliance to diet is common, especially in adults and late diagnosed patients.

Betaine acts in the remethylation route through a pathway independent of folate[5]. Oral betaine supplementation (6 - 9 g/day or 150 - 250 mg/kg/day) can reduce tHcy more than 70%, but this treatment results in an even more pronounced increase in Met. High levels of Met are acceptable, as long as this molecule does not exceed 1000 µmol/L, as levels higher may cause cerebral edema[41, 54, 56].

Cobalamin (cbl) or vitamin B12 participates in the remethylation route together with folate, and its deficiency is common in HCU; thus, vitamin B12 should be monitored and supplemented when deficient[9, 41]. N-acetylcysteine may be administered to increase cysteine levels[14]. For patients with high thrombosis risk (previous thromboembolic event or mutations in factor V), salicylic acid is recommended[41].

Good metabolic control starting in the neonatal period prevents the clinical complications of HCU[15, 59, 60]. In late diagnosed patients, treatment can also significantly prevents morbidity and mortality[46]. Even when tHcy remained much higher than reference values, a major reduction in vascular disease risk is observed in HCU treated patients[21].

3. Methylmalonic Aciduria and Homocystinuria, cblC Type

3.1 Clinical Picture

Methylmalonic aciduria and homocystinuria cblC type (MIM# 277400) is rare but remains the most common inborn error of cbl metabolism[61]. Newborn screening studies suggest that the incidence of cblC deficiency is higher than the previous estimate of 1/200,000 births[62]. A neonatal screening program in New York state estimates the incidence of cblC as approximately 1:100,000 live births[63], and according to Han et al. (2015), the incidence of cblC was approximately 1:3,920 in Shandong Province, China[64].

In 1969, the first case was reported by Mudd et al., concerning an infant with homocystinuria, methylmalonic aciduria, cystathioninemia, and hypomethioninemia. The infant died at 7.5 weeks of age. In vitro analysis identified a defect in the two reactions in which vitamin B12 derivatives function as coenzymes: 1) Met formation from 5MTHF and Hcy by MTR, and 2) the isomerization of methylmalonyl-CoA to succinyl-CoA (MUT) (Figure 2). Since vitamin B12 was present at normal concentrations in the liver, these authors concluded that the gene-determined defect involved the conversion of B12 to its active coenzymes[65]. It is nowadays known that CblC deficiency is caused by homozygous or compound heterozygous mutations in the *MMACHC* gene on chromosome 1p34.

Individuals with cblC deficiency often suffer from a wide range of clinical complications, including developmental, metabolic, hematologic, neurologic, ophthalmologic and dermatologic findings[66]. Although considered a disease of infancy or childhood, patients can present at any time from the neonatal period to adulthood and can even be intrauterine affected. The disease has been classified into early-onset (infantile) and late-onset (noninfantile) forms[67]. Patients with early-onset disease, defined by the onset of symptoms before the age of one year, in general have severe systemic involvement. Symptoms include feeding difficulties, hypotonia, failure to thrive, seizures, microcephaly and developmental delay. Acidosis was observed in some patients. Progressive CNS findings were prominent and included hydrocephalus and neuroimaging evidence of cortical atrophy. Other systems became progressively involved. Nystagmus, pigmentary retinopathy, and decreased visual acuity were common. Hematological findings included thrombocytopenia, macrocytic anemia, megaloblastic marrow and/or hypersegmented polymorphonuclear neutrophils, leukopenia and neutropenia, probably all related to cellular folate deficiency. Also renal complications occur, which are not induced

by increased MMA but are rather due to microangiopathy, resulting in hemolytic-uremic syndrome[66]. Microangiopathy may occur in any organ. Mild facial anomalies have also been described in cblC patients; features included a long face, high forehead, large, floppy, and low-set ears, and flat philtrum. The morphologic characteristics became more evident after three years of age[68].

The late-onset of the disease seems rarer than the early-onset form. In addition to mild or even no hematological abnormalities, the clinical course is characterized by behavioral and psychiatric disturbances and rapid mental deterioration with confusion and disorientation, dementia, delirium, and psychosis[69]. Although its occurrence is rare, late-onset combined methylmalonic aciduria and homocystinuria, cblC type, should be considered when making a differential diagnosis in patients who present with neurological symptoms that are not consistent with common neurological diseases, especially when cognition, the pyramidal tract and peripheral nerves are involved[70]. Importantly, patients can be easily misdiagnosed or even missed.

Rosenblatt et al. (1997) reviewed 50 cblC patients who could be classified into the two broad phenotypes: 44 patients had early-onset, and six patients had later-onset diseases. The 44 patients presented in the first year of life with feeding difficulties, hypotonia, developmental delay, seizures, pigmentary retinopathy, and anemia. The outcome is often poor, as approximately one-fourth of the patients died, and those who survived suffered in general from severe neurological impairment[66].

3.2 Diagnosis

CblC should be suspected when both tHcy and MMA are markedly elevated. CblC is a disorder of intracellular cbl metabolism causing impaired delivery of intracellular cbl

to its two metabolically active forms, Mecbl and Adocbl. This results in obstruction of the activity of the enzymes MTR and methylmalonyl-CoA mutase causing severe elevations of tHcy and MMA as well as low-normal or reduced Met[65, 66]. The overflow of Hcy into the transsulfuration pathway explains the increases of cystathionine. In addition to overt B12 deficiency, other genetic defects in cbl metabolism, such as deficiency of cblD, cblF and cblJ, also result in homocystinuria and MMA. The differential diagnosis is mainly based on gene analyses but may also be performed with functional complementation studies in cultured fibroblasts. We focus on cblC because it is the most common inborn error of cbl metabolism and so it clinical presentation is relative well known.

Because biochemical abnormalities are present in neonates, the diagnosis of cblC could be made by newborn screening, enabling the initiation of treatment prior to the development of notable pathology[71]. Key investigations for the diagnosis include measurements of plasma tHcy, MMA and Met and urinary organic acids MMA, propionic acid and methylcitrate. In acylcarnitine profiling, cblC patients often show marked increases of propionylcarnitine (C3) and to a lesser extend of methylmalonylcarnitine (C4DC). Newborn screening can detect affected infants through decreased Met and elevated C3 or C3/C0 and C3/C2 ratios measured by tandem mass spectrometry (MS/MS). MMA and/or tHcy by MS/MS can be applied in second-tier analyses[63, 72], although in theory, these metabolites could be used directly in newborn screening.

Some common mutations are found in the *MMACHC* gene: c.271dupA, c.331C>T (p.Arg111Ter) and c.394C>T (p.Arg132Ter)[73, 74]. The c.271dupA and c.331C>T mutations were associated with early-onset disease, while the c.394C>T mutation is primarily associated with late-onset disease[75, 76]. Wang et al. (2010) reported that the c.609 G>A mutation (p.Trp203Ter), which results in a premature termination codon at amino acid

residue 203 located in the C-terminal region of *MMACHC*, was detected in 39 of 46 patients, or 85% of alleles, making this mutation the most frequent in Chinese cblC patients[72]. Recently, a cblC patient was reported to be heterozygous for the c.270_271insA, at the *MMACHC* gene, and the c.515-1G>T, in the *PRDX1* gene[77]. These mutations were *in trans*. The *PRDX1* gene is located at the same locus than *MMACHC*, but transcribed in the opposite strand. The c.515-1G>T is considered an epimutation, since it leads to a hypermethylated sequence encompassing the promoter and first exon of *MMACHC* gene.

3.3. Management

Treatment of the cblC defect typically consists of a combined approach that utilizes mega-dose vitamin B12 IM., preferably in the form of hydroxycobalamin (OHcbl). In a guideline for the diagnosis and management of remethylation disorders, Huemer et al. (2017) recommended a starting dose of 1 mg of OHcbl daily and administered parenterally[78]. Oral betaine is provided to enhances the remethylation via an alternative pathway and oral folinic acid ameliorates folate trapping[69, 73, 79]. Protein restriction to reduce MMA is contraindicated because it results in a great reduction of Met[80].

In a retrospective analysis of 50 patients with cblC disease, Rosenblatt et al. (1997) described a shorter interval between the onset of symptoms and the diagnosis in patients who died, likely because these individuals were sicker and progressed more rapidly than those who survived. The overall mortality rate was 30% (13/44) in early-onset cblC, and the six patients with late-onset cblC survived with good neurological outcomes. In general treatment has a positive effect on symptoms like feeding difficulties, failure to thrive and haematological abnormalities. However neurological and ocular impairments are therapy resistent[66].

In a review of published case reports and case series, Weisfeld-Adams et al. (2015) reported that maculopathy and nystagmus with abnormal vision affect a majority of children with early-onset cblC, and strabismus and optic atrophy present at relatively high frequency. Treatment fails to prevent ocular disease, despite apparently adequate plasma Met levels and moderately elevated plasma tHcy. Even if treatment is started in the prenatal period through OHcbl administration to the mother[81].

Andersson, Marble and Shapira (1999) described the clinical and biochemical features of eight cblC patients who were treated for an average of 5.7 years. The age at diagnosis was between 1 week and 11 months, and treatment consisted of OHcbl IM. and daily oral carnitine supplementation. The earliest treated patient had one of the most severe developmental delay, showing that early treatment is no guarantee for better outcome. All patients presented with poor growth, feeding problems, and/or seizures[82].

More recently, in a retrospective study, Fischer et al. (2014) described 76 cblC patients with early-onset and 12 patients with late-onset diseases. The number of males affected was almost twice as high as the number of affected females. In early-onset group, 43 of the 76 patients presented symptoms in the first month of life. Parenteral OHcbl was prescribed to approximately 90% of the patients, but even after treatment, neurological and ophthalmological problems remained, such as developmental delay, seizures, failure to thrive, microcephaly and optic atrophy. Ten patients (11.4%) died, and these individuals were primarily non-treated with OHcbl[73].

4. Severe 5, 10-methylenetetrahydrofolate reductase (MTHFR) deficiency

4.1 Clinical Picture

Severe methylenetetrahydrofolate reductase (MTHFR) deficiency is inherited as an autosomal recessive metabolic disorder of folate metabolism caused by mutations in the *MTHFR* gene on chromosome 1p36.3[83]. Homozygous or compound heterozygous loss-of-function mutations in *MTHFR* result in systemic 5MTHF deficiency and so hampered Hcy remethylation (Figure 1). This rare disorder is associated with slow brain growth, severe neurological disability, and untimely death[84].

Homocystinuria due to MTHFR deficiency (OMIM ID: 236250) was first described in 1972 by Mudd et al. in three patients: 1) 16-year-old boy with muscle weakness, seizures and abnormal encephalographic signs, 2) 17-year-old girl with mental degradation and schizophrenia and 3) 15-year-old sister of patient 2. These three patients had homocystinuria but normal levels of Met, normal CBS activity in fibroblasts and low MTHFR activity[85]. Severe MTHFR deficiency is biochemically characterized by hyperhomocysteinemia, homocystinuria, increased cystathionine, and low or low-normal Met, in contrast with CBS deficiency, which presents with elevated Met. In cultured fibroblasts, residual activity is less than 20% of the mean control value[78, 84, 86] (Table 2).

A classical presentation suggestive of MTHFR deficiency is a neonate with acute neurological distress, including generalized muscular hypotonia, feeding problems, failure to thrive, lethargy, apnea, and eventually microcephaly[7, 87]. Patients may also present with late-onset disease during childhood and even in adulthood. The latter patients have a more variable picture, encompassing delayed developmental milestones, cognitive impairment and/or gait abnormalities, as well as mental degradation and progressive encephalopathy,

compatible with myelopathy or ataxia, spasticity behavioral problems, an unspecific spectrum of psychiatric symptoms, and occasionally thrombosis. Megaloblastic and/or macrocytic anemia is typically absent[7, 87, 88].

In contrast to the defects blocking MTR function, the block in the conversion of methylene-THF to methyl-THF does not result in the trapping of folates, as methyl-THF, and so does not interfere with the availability of reduced folates for purine and pyrimidine synthesis. This finding explains why patients do not have megaloblastic anemia and do not suffer from microangiopathy[7]. The product of MTHFR is methyl-THF, which is the circulating form of folate. As a consequence MTHFR deficiency results in reduced folate levels, especially in the brain. Cerebral folate deficiency is a common finding in this inborn disorder[89]. Another interesting finding in the brain of patients with remethylation defects, compared to controls, is that choline, a precursor of betaine and so a source of methyl groups, seems deficient. The decrease in choline levels is possibly a side effect of methyl groups depletion due to a reduced function of the transmethylation pathway[90].

Age of presentation and clinical pattern correlate with residual enzyme activity[7]. In a review of 33 patients, Huemer et al. (2016) showed the median age at onset of symptoms was 1.25 months (mean 21; range 0.1 to 216 months). In 14 patients, the first symptoms were observed within the first month of life; and in another 11 patients, the symptoms were observed by the 6th month of life. The remaining five patients became symptomatic at the ages of 2, 5, 11, 13 and 18 years[91].

In summary, MTHFR deficiency is a severe disease primarily affecting the CNS, likely to due to the reduced availability of methyl-THF and Met, causing reduced cerebral methylation as suggested by decreased SAM levels in CSF[92, 93]. MRI imaging of the brain often reveals white matter disease and brain atrophy[91].

4.2 Diagnosis

Froese et al. (2016) reported more than 100 different mutations in over 170 patients with severe MTHFR deficiency. Most mutations in the *MTHFR* gene are restricted to one or two families[94]. The p.Ala222Val change is a polymorphism leading to a thermolabile MTHFR variant with a propensity for monomer dissociation and flavin adenine dinucleotide binding loss, showing a 70% and 35% reduction of enzyme activity in lymphocytes in homozygotes and heterozygotes, respectively, when compared with wild-type controls[95]. Notably, this SNP does not cause severe MTHFR deficiency. However, this common variant is a fascinating gene-environment example because in homozygotes with low-normal folate levels, this variant hampers Hcy and folate homeostasis, causing moderate hyperhomocysteinemia. This variant has been associated with many common diseases, such as cardiovascular disease and neurodegenerative disorders, but so far only confirmed as a risk factor in neural tube defects[86, 96].

Neonatal screening for MTHFR deficiency is feasible by detecting a decreased Met and Met-to-phenylalanine ratio in DBS, followed by analysis of tHcy. To what extent patients are detected or missed remains obscure[51]. Direct measurement of MTHFR-specific activity can be performed in the liver tissue, leukocytes, lymphocytes and cultured fibroblasts. There is a rough inverse correlation between the specific activity of the reductase in cultured fibroblasts and clinical severity[97].

4.3 Management

Untreated patients show progressive developmental delay and mental retardation, whereas some patients may also present with epilepsy and neurological disease (abnormal gait, spasticity). A major number of untreated patients will even die[7, 87]. The goal of

treatment is to reduce plasma tHcy, normalize the levels of Met and folate, especially in CSF, and so presumably alleviate clinical symptoms. Treatment involves the administration (suggestive doses) of betaine (100– 250 mg/kg/day in children and 5–20 g/day in adults) to provide an alternative pathway for Hcy remethylation. In addition, OHcbl (1-2 g/d) and folinic acid (400 mg/d) are prescribed. Pyridoxine, as a cofactor for CBS, may be administered to maximize the transsulfuration pathway and riboflavin as cofactor of the MTHFR enzyme. Met may be supplemented if its level remains low, despite treatment. In general, treatment improves the disease course in early-onset cases[7, 84, 98, 99]. Some studies have shown that treatment with folinic acid, Met, pyridoxine, and different cbl preparations but without betaine has generally been considered unsuccessful[7]. In a systematic review, including 36 patients, the positive impact of early betaine treatment on the outcome in early-onset patients was shown: all five early treated patients survived with normal psychomotor development, while nine of ten non-treated and two of 21 late-treated patients died. In families with one or more deceased siblings, none of the treated but all of the untreated children died. Psychomotor development was impaired in all children with delayed treatment onset, despite the stabilization observed from the introduction of betaine treatment[87].

Regarding the infantile forms, the only patients who have done well are those who were treated from birth. Early treatment with betaine following prenatal diagnosis has resulted in the best outcome[100]. Without treatment, these early-onset forms may rapidly progress to coma and potentially death by central respiratory failure. Since MTHFR deficiency is a potentially treatable disease, early diagnosis is crucial and treatment, in particularly betaine should be administered as early as possible[94]. Although single reports

have described a benefit of treatment with folinic acid[101] or Met supplementation[98], the mainstay of treatment is betaine[93].

5. DISCUSSION

Homocystinurias are a group of inborn errors of sulfur amino acid metabolism. Their joint manifestation is the accumulation of tHcy and treatment has focused mainly on lowering tHcy. The other sulfur amino acids show various and even invers profiles. CBS deficiency shows high Met with low cystathionine and cysteine. Whereas in the remethylation defects, Met is low-normal or decreased and cystathionine increased. Total cysteine in plasma is reduced in all homocystinurias because the elevated tHcy displaces cysteine from albumin, which binds the major fraction of tHcy and cysteine in plasma. At diagnosis, folate is typically low or (functionally) deficient in all homocystinurias. MTHFR deficiency blocks the production of methyl-THF, which is the circulation form of folate. In CBS deficiency, folate is often deficient at diagnosis probably due to the inhibition of MTHFR by the increased concentration of SAM. In cblC deficiency, a different mechanism kicks in: a dysfunctional MTR results in the accumulation of all folates as methyl-THF, which cannot be converted back to methylene-THF because MTHFR is physiologically none-reversible, a phenomenon called folate trap. This results in a functional folate deficiency despite that plasma folate may be normal or even increased because of the leakage of methyl-THF out of the cell. Intracellularly, folate is not available for the folate-dependent pathways. In particular, the synthesis of thymidylate and purine will be compromised, which will hamper essential cellular functions, especially in cells that rapidly divide, such as those in bone marrow. This functional folate deficiency

occurs in any cell, which may explain why so many different organ systems can be affected in cblC patients, particularly when compared to the other remethylation defect, MTHFR deficiency, which mainly affects the CNS.

Some clinical findings were found in all homocystinurias, like developmental delay and seizures. However, the complications of CNS differ in a wide variety of presentations and severities among the different forms of homocystinuria and are apparently less pronounced in CBS. Moreover, patients with remethylation defects typically do not present ectopia lentis and bone disturbances, tall stature and osteoporosis. Whereas hematological alterations, such as megaloblastic anemia, thrombocytopenia neutropenia and life-threatening microangiopathy, are specific findings of cblC deficiency. The only remaining common clinical finding in all forms of homocystinuria is thromboembolic event. Taken together, there is only little overlap in the biochemical and clinical presentations of the three most common forms of homocystinuria. The clear difference in clinical presentations makes us questioning if Hcy itself is the main disease-causing metabolite in homocystinurias.

The main aim of treatment in all forms of homocystinurias is to lower plasma tHcy. In CBS deficiency this results in a striking improved clinical outcome[14], including the reduction of vascular events[20, 21], and prevention of mental retardation[59] and bone anomalies[60]. Remarkably, on treatment, the levels of plasma Hcy in general remained clearly elevated[20, 102], in particular in B6-nonresponders. Treated cblC patients have in general a marked reduction of tHcy below the levels found in CBS deficient patients who are B6-nonresponders. However, neurological and ophthalmological problems usually remain in cblC[73]. These different outcomes adds to our doubt if tHcy itself is directly causing the clinical manifestations in HCU.

The meaning of elevated plasma Hcy is also compromised by numerous studies on tHcy and risk for cardiovascular diseases. Although elevated tHcy is related to an increased risk for atherosclerosis and venous thrombosis, lowering of tHcy by folate and B12 failed to reduce risk in large randomized controlled trials[103]. The observations in homocystinurias and elevated tHcy as risk factor for vascular disease causes strong doubt if elevated tHcy levels are directly and causally related to the clinical presentations.

6. CONCLUSIONS

Plasma Hcy is an inadequate biomarker for disease outcome and evaluation of treatment. This conclusion is not only of academic relevance because current treatment strategies in homocystinurias mainly focuses on lowering tHcy. In particularly in cblC deficiency treatment fall short to halt the disease process. Accepting that Hcy is not the main disease-causing metabolite, should pave the road for more research dissecting the underlying disease-causing mechanisms in homocystinurias like hampered methylation and cellular folate and betaine deficiency. In line, research should aim to discover more relevant biomarkers for the evaluation of treatment.

GLOSSARY

Homocysteine (Hcy): A sulfur containing amino acid, formed during the metabolism of methionine to cysteine.

Total Homocysteine (tHcy) in plasma: Consists of free homocysteine (reduced plus oxidized homocysteine in the non-protein fraction of plasma) and protein-bound homocysteine.

5- methyltetrahydrofolate (5MTHF): Circulating active form of folic acid, and an important cofactor for the remethylation of homocysteine to methionine.

LIST OF ABBREVIATIONS

ABCD4*:** gene ATP binding cassette subfamily D member 4 related to cblJ; **Adocbl:** Adenosylcobalamin; **AR:** Autosomal recessive; **ATP:** Adenosine triphosphate; **BHMT:** Betaine-homocysteine methyltransferase; **C0:** Free carnitine; **C2:** Acetylcarnitine; **C3:** Propionylcarnitine; **Cbl:** Cobalamin; **CBS:** Cystathionine beta-synthase; **CNS:** Central nervous; **CSF:** cerebrospinal fluid; **CγL:** Cystathionine γ-lyase; **DBS:** Dried blood spot; **DNA:** Deoxyribonucleic acid; ***HCFC1: gene host cell factor C1 related to cblX; **HCU:** Classical homocystinuria; **Hcy:** Homocysteine; **IM:** Intramuscular; **IQ:** Intelligence quotient; ***LMBRD1*:** gene that encodes a lysosomal membrane protein that may be involved in the transport and metabolism of cobalamin affected in cblF; **Mecbl:** Methylcobalamin; **Met:** Methionine; **MMA:** Methylmalonic acid; **MMAA:** Methylmalonic aciduria type A; **MMAB:** Methylmalonic Aciduria cblB Type; **MMACHC:** methylmalonic aciduria and homocystinuria type C protein; **MMADHC:** methylmalonic aciduria and homocystinuria type D; **MS/MS:** Tandem mass spectrometry; **5MTHF:** 5-methyltetrahydrofolate; **MTHFR:** Methylenetetrahydrofolate reductase; **MTR:** Methionine synthase; ***MTRR***: gene methionine synthase reductase related to cblE; **MUT:** Methylmalonyl CoA mutase; **OHcbl:** Hydroxycobalamin; **SAH:** S-adenosylhomocysteine; **SAHH:** S-adenosyl-L-homocysteine hydrolase; **SAM:** S-adenosylmethionine; **SHMT:** Serine-hydroxymethyltransferase; **SNP:** Single nucleotide polymorphisms; **TCN2**: transcobalamin 2; **tHcy:** Total homocysteine; **THF:** Tetrahydrofolate; **XLR:** X-linked recessive.

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TABLE 1 - Summary of genetic homocystinurias.

Disease (OMIM number)	Deficient Enzymes	Gene (Locus)
Classical homocystinuria (# 236200)	Cystathionine beta-synthase (CBS - EC 4.2.1.23)	*CBS* (21q22.3)
Severe methylenetetrahydrofolate reductase (MTHFR) deficiency (# 236250)	Methylenetetrahydrofolate reductase (MTHFR - EC 1.5.1.20)	*MTHFR* (1p36.22)
Methylmalonic aciduria and homocystinuria, cblC type (# 277400)	Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR - EC 1.16.1.8)	*MMACHC* (1p34.1)
Methylmalonic aciduria and homocystinuria, cblC type, digenic (epi-cblC #277400)	Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR - EC 1.16.1.8)	*PRDX1* (1p34.1) *MAHCC* (1p34.1)
Methylmalonic aciduria and homocystinuria, cblD type (# 277410)	Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR - EC 1.16.1.8)	*MMADHC* (2q23.2)
Homocystinuria-megaloblastic anemia, cblE type (# 236270)	Methionine synthase reductase (MTRR - EC 2.1.1.135)	*MTRR* (5p15.31)
Methylmalonic aciduria and homocystinuria, cblF type (# 277380)	Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and Methionine synthase (MTR - EC:2.1.1.13)	*LMBRD1* (6q13)
Homocystinuria-megaloblastic anemia, cblG type (# 250940)	Methionine synthase (MTR - EC:2.1.1.13)	*MTR* (1q43)
Methylmalonic aciduria and homocystinuria, cblJ type (# 614857)	Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and Methionine synthase (MTR - EC:2.1.1.13)	*ABCD4* (14q24.3)
Methylmalonic acidemia and homocystinuria, cblX type (# 309541)	HCF-1; Transcriptional regulation of *MMACHC.* Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR -EC 1.16.1.8)	*HCFC1* (Xq28)
Methylenetetrahydrofolate dehydrogenase (MTHFD) deficiency (# 617780)	5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 10-formyltetrahydrofolate synthetase (EC 6.3.4.3)	*MTHFD1* (14q23.3)
Transcobalamin II (TC) deficiency (# 275350)	Transcobalamin; Cellular delivery of cbl. Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR -EC 1.16.1.8)	*TCN2* (22q12.2)
Methylmalonic aciduria and homocystinuria, TcblR type (# 613646)	TCblR; Cellular receptor for TC. Methylmalonyl-CoA mutase (MUT - EC 5.4.99.2) and methionine synthase (MTR - EC 1.16.1.8)	*CD320* (19p13.2)

All diseases have an autosomal recessive inheritance pattern, excepted of Methylmalonic acidemia and homocystinuria, cblX type, that is X-linked recessive. cbl = cobalamin.

TABLE 2 – Biochemical presentation and clinical manifestation of non-treated homocystinuria disorders due to CBS, cblC and MTHFR defects

	CBS	cblC	MTHFR
Homocysteine	↑ - ↑↑	↑ - ↑↑	↑ - ↑↑
Methionine	Normal or ↑↑ *	Normal or ↓	Normal or ↓
Cystathionine	Normal or ↓ *	↑↑	↑↑
Methylmalonic acid	Normal	↑↑**	Normal
Cysteine	↓↓	↓	↓
Clinical findings in common			
Central nervous system	Seizures, psychiatric disorders, mental retardation.	**Early onset:** seizures, mental retardation **Late onset:** psychiatric disorder, mental retardation.	**Early onset:** seizures, mental retardation **Late onset:** psychiatric disorder, mental retardation.
Cardiovascular, Vessels and hematology	Thromboembolism	Thromboembolism	Thromboembolism
More specific clinical findings			
Central nervous system	Sequelae of thromboembolic events* Extrapyramidal signs (dystonia)	**Early onset:** Microcephaly, hydrocephalus, cortical atrophy**, hypotonia, lethargy, developmental delay. **Late onset:** Acute neurologic decompensation, extrapyramidal symptoms and tremor.	**Early onset:** Hypotonia, lethargy, apnea***, paresthesias***, feeding problems*** and eventually microcephaly. **Late onset:** progressive encephalopathy, ataxia, spasticity.
Eye	Ectopia lentis*, myopia* and glaucoma*	Pigmentary retinopathy**, nystagmus** and decreased visual acuity.	-
Bone	Normal to tall stature and generalized osteoporosis*	-	-
Cardiovascular, Vessels and hematology	-	Megaloblastic anemia**, thrombocytopenia** and neutropenia**	-
Treatment			
	Pyridoxine, folinic acid, dietary Met restriction, betaine, acetylsalicylic acid for patients with high thrombosis risk.	High dose of OHcbl, betaine, Met supplementation	Betaine, OHcbl, folinic acid, Met supplementation, riboflavin and pyridoxine.

*Specific of CBS deficiency; ** Specific of cblC deficiency; *** Specific of MTHFR deficiency. Met: methionine; OHcbl: hydroxycobalamin.
L: typical low limits of reference ranges; H: typical high limits of reference ranges. ↓: metabolite decreased; ↑ and ↑↑: metabolite increased and grossly elevated.

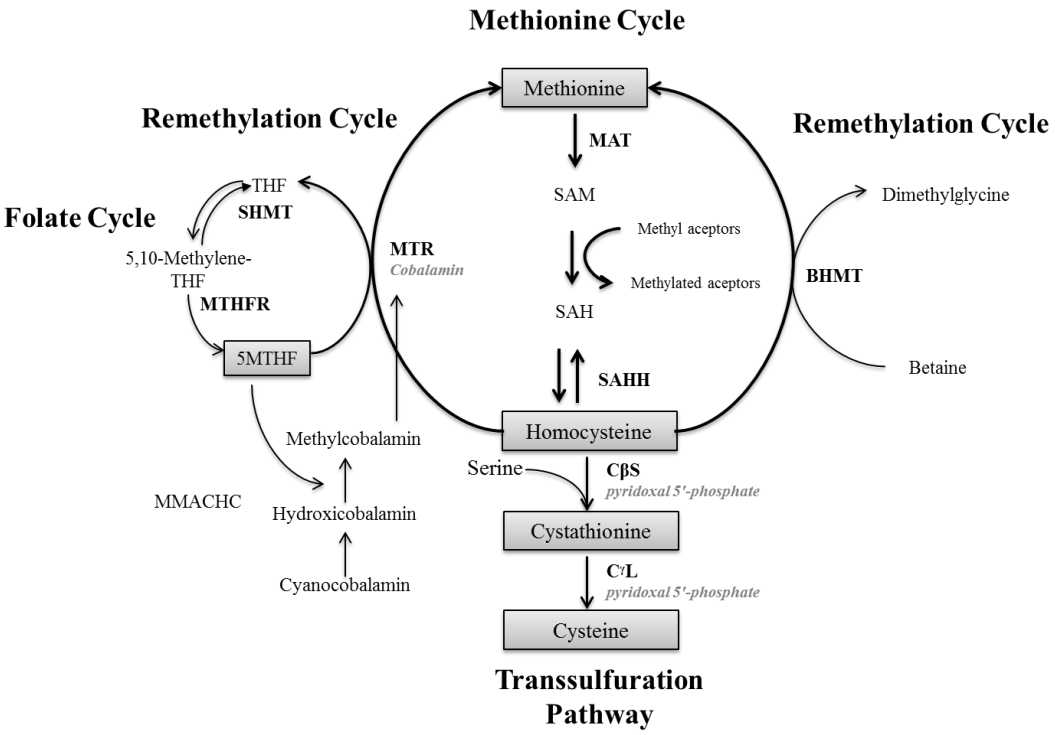

Figure 1. Overview of homocysteine metabolism. MAT: methionine adenosyltransferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SAHH: S-Adenosyl-homocysteine hydrolase; CβS: cystathionine β-synthase; CγL: cystathionine γ-lyase; MTR: methionine synthase; THF: tetrahydrofolate; MTHFR: 5,10-methylene-THF reductase; SHMT: serine-hydroxymethyltransferase; BHMT: betaine-homocysteine methyltransferase; MMACHC: methylmalonic aciduria and homocystinuria type C protein; Enzymes are shown in capitals, and their cofactors in italics.

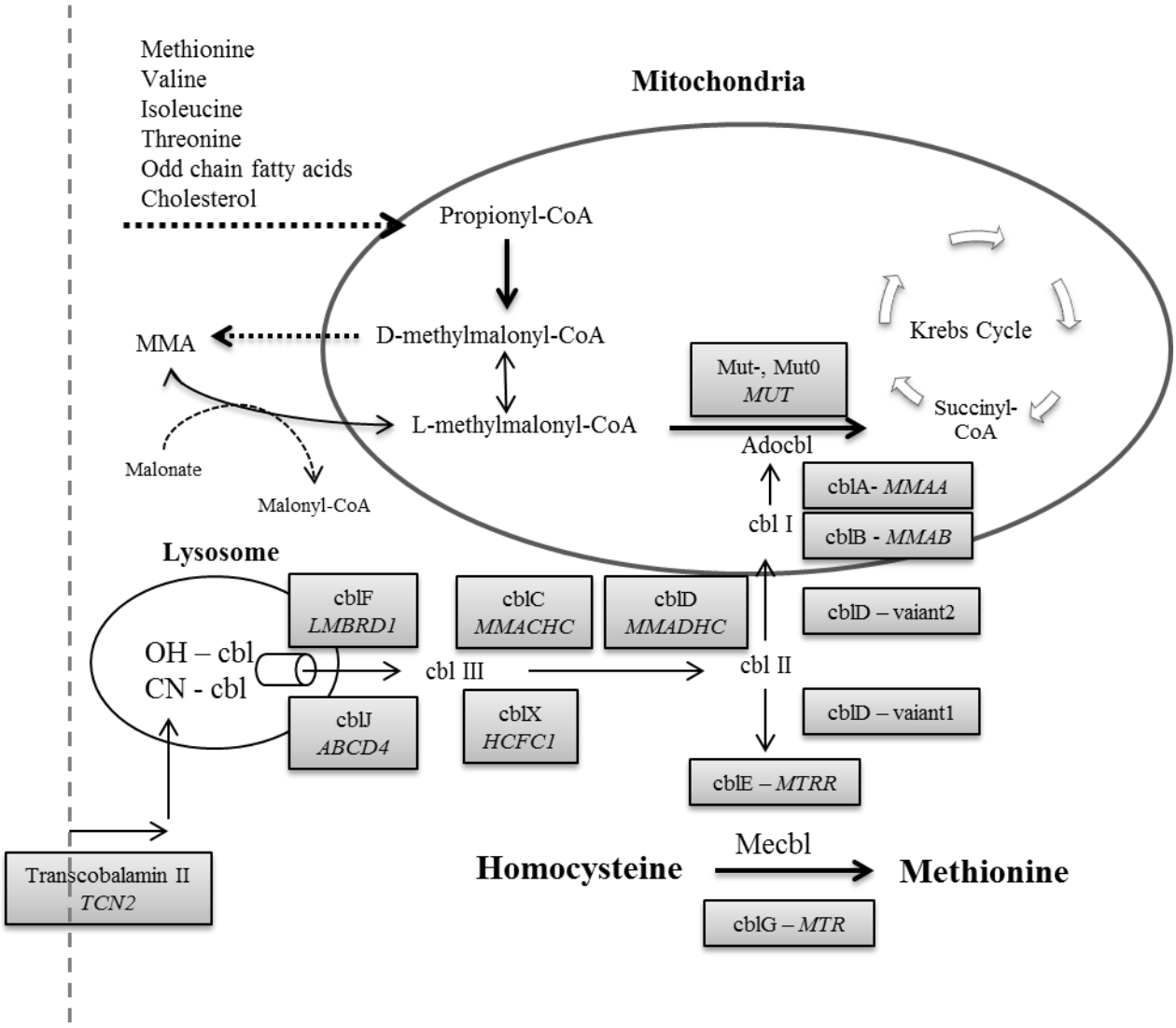

Figure 2. Overview of B12 metabolism. MMA: methylmalonic acid; *MUT*: methylmalonyl CoA mutase; Mut -: partial loss of MUT function; Mut 0: complete loss of MUT function; Adocbl: adenosylcobalamin; cbl: cobalamin; *MMAA*: Methylmalonic aciduria type A; *MMAB*: Methylmalonic Aciduria cblB Type; *MMADHC*: methylmalonic aciduria and homocystinuria type D; *MMACHC*: methylmalonic aciduria and homocystinuria type C protein; *LMBRD1*: gene that encodes a lysosomal membrane protein that may be involved in the transport and metabolism of cobalamin affected in cblF; *ABCD4*: gene ATP binding cassette subfamily D member 4 related to cblJ; *HCFC1*: gene host cell factor C1 related to cblX; OH-cbl: hydroxocobalamin; CN-cbl: cyanocobalamin; *TCN2*: transcobalamin 2; *MTRR*: gene methionine synthase reductase related to cblE; Mecbl: Methylcobalamin and *MTR*: methionine synthase related to cblG.

6.2 CAPÍTULO 2: *"Diagnosis and Management of Classical Homocystinuria in Brazil: A Summary of 72 Late-Diagnosed Patients"*

O capítulo 2 desta tese consiste na avaliação e caracterização clínica de 72 pacientes das diversas regiões do Brasil com HCU. O capítulo será apresentado na forma do artigo publicado na revista JIEMS em junho de 2018.

Original Article

Journal of Inborn Errors of Metabolism & Screening
2018, Volume 6: 1–6

DOI: 10.1177/2326409818788900
journals.sagepub.com/home/iem
SAGE

Diagnosis and Management of Classical Homocystinuria in Brazil: A Summary of 72 Late-Diagnosed Patients

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Abstract

This study described a broad clinical characterization of classical homocystinuria (HCU) in Brazil. This was a cross-sectional, observational study including clinical and biochemical data from 72 patients (60 families) from Brazil (South, n = 13; Southeast, n =

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Received April 16, 2018, and in revised form June 07, 2018. Accepted for publication June 12, 2018.

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37; Northeast, n = 8; North, n = 1; and Midwest, n = 1). Parental consanguinity was reported in 42% of families. Ocular manifestations were the earliest detected symptom (53% of cases), the main reason for diagnostic suspicion (63% of cases), and the most prevalent manifestation at diagnosis (67% of cases). Pyridoxine responsiveness was observed in 14% of patients. Only 22% of nonresponsive patients on treatment had total homocysteine levels <100 μmol/L. Most commonly used treatment strategies were pyridoxine (93% of patients), folic acid (90%), betaine (74%), vitamin B12 (27%), and low-methionine diet + metabolic formula (17%). Most patients diagnosed with HCU in Brazil are late diagnosed, express a severe phenotype, and poor metabolic control. Milder forms of HCU are likely underrepresented due to underdiagnosis.

Keywords
classical homocystinuria, CBS deficiency, homocysteine, pyridoxine responsiveness, diagnosis

Introduction

Classical homocystinuria (HCU; OMIM 236200) is an inborn error of methionine metabolism caused by deficient activity of cystathionine β-synthase (CβS; EC 4.2.1.22). Cystathionine β-synthase deficiency leads to massive accumulation of homocysteine and methionine and low levels of cysteine.[1,2] Homocystinuria was first described in 1962[3]; since then, many advances in treatment and early diagnosis, including newborn screening, have improved prognosis dramatically.[4–6] However, diagnosis and management of HCU is still a major challenge in developing countries, where newborn screening is unavailable and access to health-care services is often poor and unequal.[7]

From a clinical standpoint, the classic signs of HCU are lens dislocation, thromboembolism, mental retardation, psychiatric disorders, osteoporosis, and marfanoid features.[8] Established treatment strategies include supplementation of pyridoxine (CβS cofactor), folic/folinic acid, betaine, and a methionine-restricted diet supplemented with an essential amino acids admixture free of methionine.[9] Novel therapies with chaperones and enzyme replacement are currently under development.[10–12] Usually, patients who respond to pyridoxine supplementation exhibit a milder phenotype and have a better prognosis.[8]

The worldwide prevalence of HCU is estimated to be 1:100 000.[13] However, several mild and late presentation phenotypes have been described,[14–16] raising the issue of whether HCU is largely underdiagnosed. Newborn screening for HCU is performed in some countries with high incidence of the disease, such as Ireland and Qatar.[5,17] Usually, newborn screening is performed by the determination of methionine on filter paper. However, this method has a large percentage of false negatives (up to 50%), since pyridoxine-responsive HCU may not present with hypermethioninemia in the first days of life.[18,19] In Brazil, the prevalence of HCU is unknown, and the disease is not included in the National Neonatal Screening Program.

Brazil is a very large country with over 200 million inhabitants, and the country is characterized by intense admixture.[20,21] The country also has one of the world's largest publicly funded health-care systems, the Unified Health System (*Sistema Único de Saúde*), which was established to provide equitable and comprehensive care to all users. However, betaine and the methionine-free amino acid formulation are not available through Unified Health System, and few centers across the country offer biochemical testing for diagnosis and management of this condition. Furthermore, several factors make diagnosis and management of HCU within the Brazilian health system a major challenge; hence, a substantial number of patients with this treatable condition are believed to remain undiagnosed and thus untreated. Within this context, the present study sought to establish a broad clinical characterization of HCU in Brazil through a survey on diagnosis and management of a representative patient population that is being followed at several centers nationwide.

Materials and Methods

The present study was approved by the local research ethics committee. Collection procedures for the study were conducted only after participants or their caregivers had agreed to take part in the investigation and provided written informed consent.

Patients

The study sample comprised 72 Brazilian patients with a diagnosis of HCU, from 60 different families. Diagnosis was in general made on clinical symptoms followed by clear elevations in homocysteine and methionine. Mutation analyses was performed in 35 of the 72 patients.[22] Families from all 5 regions of Brazil were represented: South (n = 13), Southeast (n = 37), Northeast (n = 8), North (n = 1), and Midwest (n = 1). Patients were recruited through contact with physicians involved in care and/or research activities at medical genetics centers across the country. A structured questionnaire containing queries regarding diagnosis, consanguinity, treatment strategies, metabolic control, and current health condition of patients with HCU was sent to 15 medical centers that had agreed to participate in the study. Clinical data regarding diagnosis were available only for 28 patients.

All 72 patients had delayed diagnoses: 62 patients had been diagnosed after clinical suspicion and biochemical findings consistent with HCU (hyperhomocysteinemia and hypermethioninemia), and the remaining 10 had been diagnosed on family screening. Each of the participating centers used a different protocol for determination of pyridoxine responsiveness. For the purposes of this study, patients were classified as

Table 1. Classical Homocystinuria in Brazil: A Summary of Clinical Findings at Diagnosis.[a]

	Total (N = 72)	Pyridoxine Responsive		P
		Yes (n = 10)	No (n = 61)	
Current age, years	19 (5-45)	23 (14-35)	18 (5-45)	.120
Age at first symptom onset, years	5 (0-20)	2 (0.2-15)	5 (0.7-20)	.316
Age at diagnosis, years	10 (1-39)	11 (4-34)	9 (1-39)	.545
Systems affected at diagnosis, %				
Ocular	72	50	75	.100
CNS	60	70	59	.497
Skeletal	60	40	54	.401
Vascular	15	20	14	.625

Abbreviation: CNS, central nervous system.
[a]N = 72, data expressed as the median (range) or percentage. In 1 patient, pyridoxine responsiveness could not be determined, data from this patient were used alone in the whole-group analysis.

responsive if they achieved homocysteine levels <50 μmol/L on pyridoxine alone or pyridoxine + folic acid (regardless of the number of weeks since testing). All other patients were classified as nonresponsive to pyridoxine.

Target total homocysteine levels on treatment were set according HCU guidelines,[9] which were <50 μmol/L for pyridoxine-responsive patients and <100 μmol/L for nonresponsive patients. Treatment adherence was determined by the subjective impressions of the care team at each medical center.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows, version 18.0 (SPSS Inc, Chicago, Illinois). Asymmetrically distributed variables were expressed as the median (range). The Mann-Whitney *U* test (continuous variables) or χ^2 test (categorical variables) was used to assess between-group differences. Values of $P < .05$ were considered significant.

Results

Of the 72 patients included, 55% were male. Two patients were already deceased at the time of inclusion in the study (presumably due to thromboembolic events). The patients belonged to 60 families; parental consanguinity was reported in 25 (42%) families. Median age at assessment was 19 years. The youngest patient was aged 5, and the oldest was 45. Regarding pyridoxine responsiveness, 61 (85%) patients were classified as nonresponsive and 10 (13.8%) as responsive. In 1 patient, pyridoxine responsiveness was not reported/determined.

Journey to Diagnosis

The very first symptom noticed by families and/or physicians was visual impairment (mainly due to *ectopia lentis*) in 53% of the cases, followed by developmental delay (22% of cases), seizures (11% of cases), and isolated thromboembolic episodes (9.5% of cases). Table 1 reports clinical features at diagnosis for the sample as a whole and stratified by pyridoxine responsiveness.

The median time elapsed between symptom onset and diagnosis was 5 years (maximum, 34 years). One-third of the patients had 3 or more systems already affected at the time of diagnosis. The main clinical findings leading to investigation of HCU are represented in Figure 1. Eye disease, the most prevalent symptom at diagnosis (67% of cases), accounted for 63% of referrals for HCU investigation.

Management

Current clinical and biochemical data were available for 44 patients, of whom 7 were responsive and 37 were nonresponsive to pyridoxine. The median length of follow-up was 6 years (range, 0-27 years). Table 2 describes clinical manifestations and biochemical control in this group of patients. Ocular manifestations were more prevalent among pyridoxine-nonresponsive patients (71% vs 97%, $P = .01$). *Ectopia lentis* was the most common complication in our sample, affecting 91% of patients at the time of study inclusion.

Pyridoxine-responsive patients had significantly lower total homocysteine levels at study inclusion ($P < .001$). Only 22% of nonresponsive patients achieved target total homocysteine levels (<100 μmol/L) on treatment, while all responsive patients (n = 7) had total homocysteine <50 μmol/L. Treatment adherence was reported as appropriate in 44% of patients.

Regarding treatment strategies, 93% of patients were on pyridoxine supplementation, 90% on folic acid, 74% on betaine, 27% on vitamin B12, and only 17% on a low-methionine diet + metabolic formula.

Discussion

The present report provides the largest clinical profile of patients with HCU ever studied in Brazil to date. Clinical data of 72 patients (60 unrelated) from 15 medical genetics centers across Brazil were analyzed. Most families lived in the South and Southeast regions of the country. These regions are home to 57% of the country's population (http://www.ibge.gov.br/) and, compared to other regions of Brazil, have higher rates of access to health-care services and procedures. The fact that patients from other regions, particularly the North (n = 1) and Midwest (n = 1), were underrepresented suggests high rates of HCU underdiagnosis and/or limited access to care in these regions.[7]

A high proportion of pyridoxine-nonresponsive patients with HCU was found in our study (85%). Nonresponsive patients usually present a more severe phenotype, have more complications, and younger ages .[6,8,23] In our study, this proportion exceeded rates described worldwide of approximately 50%.[8] In countries where the proportion of nonresponsive patients is disproportionately high, such as Qatar and Ireland,

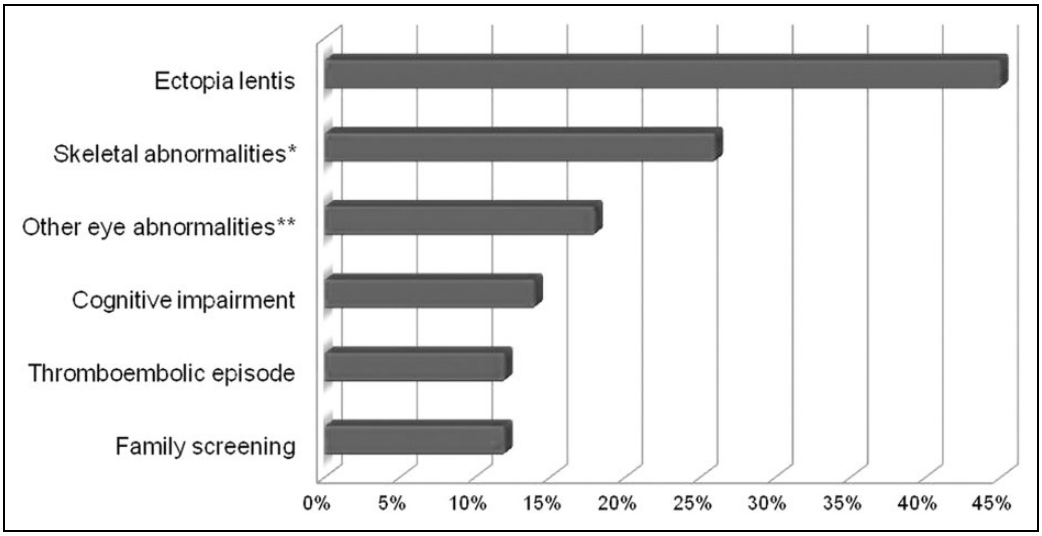

Figure 1. Main reasons for clinical suspicion of classical homocystinuria in our sample (N = 72). *Including marfanoid habitus, **Other than *ectopia lentis*.

Table 2. Classical Homocystinuria in Brazil: Clinical and Biochemical Profile of Patients on Treatment at the Time of Study Inclusion.[a]

		Pyridoxine Responsive		
	Total (n = 44)	Yes (n = 7)	No (n = 37)	*P*
Current age, years	16	19 (14-35)	16 (5-37)	.182
Homocysteine, μmol/L	168	19 (14-34)	212 (9-455)	<.001
Methionine, μmol/L	131	27 (23-650)	218 (6-881)	.065
Clinical manifestations, %				
Ocular	93	71	97	.013
CNS	70	86	68	.318
Skeletal	61	57	62	.803
Vascular	25	14	27	.475

Abbreviation: CNS, central nervous system.
[a]n = 44, data expressed as the median (range) or percentage; reference ranges: homocysteine, 5-15 μmol/L; methionine, 5-30 μmol/L.

specific genotypes are highly prevalent and account for these discrepancies .[24,25] In a previous study of our group, we explored in more depth the genotype of 35 patients with HCU who are also included in the current study.[22]

Our results show there is no single molecular basis for the high prevalence of HCU nonresponsive phenotypes in Brazil. In fact, the most prevalent mutation in that study (p.Ile278Thr) is a pyridoxine-responsive mutation. Another genetic study of 14 Brazilian patients with HCU showed similar results.[26] These findings rather suggest underdiagnosis of pyridoxine-responsive patients in Brazil who express milder phenotypes with fewer symptoms and later onset of clinical presentation.[6,8] In our study, no significant differences in age at symptom onset or age at diagnosis were found between pyridoxine-responsive and nonresponsive patients; however, we believe our analysis was underpowered because of the low number of pyridoxine-responsive patients (n = 10 vs n = 61, respectively).

The high prevalence of ocular manifestations at diagnosis and their predominant contribution to diagnostic suspicion reinforce the importance of eye disease in HCU. *Ectopia lentis* is usually the earliest manifestation of HCU, occurring in half of all untreated patients by age 10 years and in over 90% by age 24 years.[8] No significant difference in the prevalence of ocular manifestations at diagnosis was found between responsive and nonresponsive patients (75% vs 50%, $P = .10$), although the lack of significance could also be explained by the small sample size. However, this finding is consistent with previous reports in the literature.[8]

Skeletal and neurologic manifestations were also highly prevalent at diagnosis, affecting more than half of patients of our cohort. The lower prevalence of vascular events at diagnosis is consistent with the natural history of HCU, in which such manifestations usually occur in general at a later age.[8] However, vascular disease may have been underrepresented due to the high lethality of thromboembolic events. The relative large time gap between symptom onset to diagnosis (median, 5 years) and the presence of multiple clinical manifestations attest to the difficulty in establishing a definitive diagnosis in these patients.

The main strategies and goals of HCU treatment have been recently established in the first guidelines for the diagnosis and management of CβS deficiency.[9] Early diagnosis and early treatment are the key to prevent clinical manifestations and improve prognosis.[4,18]In the present study, current clinical and biochemical data were obtained from 44 patients in treatment. There was a clear difference in metabolic control between the groups: Responsive patients had low and even near-normal homocysteine and methionine levels, whereas most nonresponsive patients had persistently high homocysteine levels (>100 μmol/L) despite multiple treatment strategies. This difficulty in achieving metabolic control in pyridoxine-nonresponsive patients has been reported elsewhere.[18,27,28]

The high rate of betaine supplementation and comparatively low use of methionine-restricted diet in the nonresponsive patients may be attributed to several factors: (1) difficulties in obtaining the metabolic formula, which is expensive and not provided by the Unified Health System in Brazil;[29] (2) low adherence to dietary methionine restriction, particularly in patients with a late diagnosis; and (3) limited training of health-care professionals in dietary prescription. In a European survey of 181 patients with pyridoxine-nonresponsive HCU, 66% were on dietary treatment, that is, twice as many as in the present study.[30] Homocystinuria guidelines clearly state that betaine should not be considered a first-line treatment for HCU-nonresponsive patients but used as adjunct treatment in those who cannot achieve target levels of homocysteine by other means.[9] While dietary therapy dramatically reduces methionine and homocysteine and normalizes cysteine, betaine supplementation reduces homocysteine but increases methionine levels.[31,32] In animal models, betaine is less effective at preventing clinical manifestations,[33] and its efficacy declines over time.[34] In humans, there are no studies of the long-term efficacy of betaine supplementation alone in HCU.

In conclusion, this study provides the first broad clinical characterization of HCU in Brazil. All patients described here were late diagnosed, and most expressed a severe phenotype associated with nonresponsiveness to pyridoxine, early and multisystem clinical manifestations, and poor metabolic control. Limitations of this study include the underrepresented number of patients responsive to pyridoxine, and the number of patients coming from some regions of the country. We believe that our findings will contribute to the development of protocols and guidelines to improve diagnosis and management of HCU in Brazil.

Authors' Note

Soraia Poloni and Giovana Weber Hoss contributed equally to this article. Soraia Poloni, Giovana W. Hoss, Fernanda Sperb-Ludwig, Taciane Borsatto, and Ida V. D. Schwartz made substantial contributions to the conception and design, acquisition of data, analysis, and interpretation of data; Maria Juliana R. Doriqui, Emilia K.E.A Leão, Charles M. Lourenço, Chong A. Kim, Carolina F. M. Souza, Helio Rocha, Marcia Ribeiro, Carlos E. Steiner, Carolina A. Moreno, Pricila Bernardi, Eugenia Valadares, Osvaldo Artigalas, Gerson Carvalho, Hector Y. C. Wanderley, Ney Boa Sorte, and Luiz C. Santana made substantial contributions to the acquisition of data and were involved in revising the manuscript. Ida V. D. Schwartz and Henk J. Blom were involved in the analysis and interpretation of data and critically revising the manuscript for important intellectual content. All authors have given final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgments

The authors thank Prof. Roberto Giugliani, Rede EIM Brasil, Simone Allan Arede, and Associação Brasileira de Homocistinúria for their assistance with patient diagnosis and follow-up.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article. I. Schwartz and V. D'Almeida are National Council of Scientific and Technological Development (CNPq) research productivity fellows.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Financial support for this study was provided by CNPq, CAPES, DAAD, and FIPE-HCPA.

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6.3 CAPÍTULO 3: *"Classical homocystinuria: a common inborn error of metabolism? An epidemiological study based on genetic databases"*

O capítulo 3 estima a incidência mínima de HCU em diferentes populações a partir do número de heterozigotos para as 25 mutações mais comuns em CBS, descritos em grandes bancos de dados genômicos. Este capítulo será apresentado na forma de artigo, o qual será submetido para a revista Human Mutation, cujo fator de impacto é 5,359 e o Qualis CAPES da área de genética (Ciências biológicas I) é A1. O formato obedece às normas de submissão da revista.

Classical homocystinuria: a common inborn error of metabolism? An epidemiological study based on genetic databases

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Abstract

Classical homocystinuria (HCU) is the most common cause of homocystinuria. The worldwide prevalence of HCU is estimated to be 0.82:100,000 [95% CI, 1.73-0.39:100,000] according to clinical records and 1.09:100,000 [95% CI, 3.55-0.34:100,000] by neonatal screening. In this study, we aimed to estimate the minimal worldwide incidence of HCU. The 25 most common causative alleles of HCU were identified through a literature review. The minimal incidences of HCU in Europeans, Latin Americans, Africans and Asian were estimated based on the frequency of these common pathogenic alleles in a large genomic database (gnomAD). The minimum worldwide incidence of HCU was estimated to be ~ 0.38:100,000, and the incidence was higher in Europeans non-Finnish (~0.72:100,000) and Latin Americans (~0.45:100,000) and lower in Africans (~0.20:100,000) and Asian (~0.02:100,000). Our data regarding the minimum worldwide incidence are in accordance with the only published metanalysis on this topic. To our surprise, the observed incidence of HCU in Europeans was much lower than those described in articles exploring small populations from northern Europe but was similar to the incidence described on the basis of neonatal screening programs. In our opinion, the large dataset analyzed and its population coverage gave us greater precision in the estimation of incidence.

Key-words: Classical homocystinuria; Frequent mutations; Incidence; Genomic databases; Homocysteine; Methionine.

Introduction

The most common cause of homocystinuria is classical homocystinuria (HCU) or cystathionine beta-synthase (CBS) deficiency (OMIM #236200), which is an autosomal recessive disease biochemically characterized by the accumulation of homocysteine (Hcy) and methionine (Met) and by decreased cysteine levels. The main clinical complications in untreated HCU patients are found in the eyes, skeleton, central nervous system, and vascular system [Morris et al., 2017].

The *CBS* gene is located on chromosome 21q22.3. It spans 23 exons, with exons 1–16 constituting the coding region, which encodes a 551-amino acid polypeptide [Kraus et al., 1998]. More than 200 pathogenic variants have been described, and most of these are rare and private variants [Stenson et al., 2017]. However, the four most prevalent mutations (p.Ile278Thr, p.Thr191Met, p.Gly307Ser and p.Trp323Ter) represent half of all HCU alleles reported worldwide [Kraus, 2019].

The worldwide prevalence of HCU based on the number of known patients is estimated to be between 0.29 and 1:100,000 individuals [Mudd et al., 2001; Moorthie et al., 2014]. Moorthie et al., 2014 performed a systematic review and meta-analysis to estimate the prevalence of HCU and found a worldwide prevalence based on diagnosis of symptomatic individuals of 0.82:100,000 [95% CI, 0.39-1.73:100,000], while that based on neonatal screening by MS/MS was 1.01:100,000 [95% CI, 0.34-3.55:100,000] newborns (NBs). We should point out that studies involving the Qatari population were included in the study by Moorthie et al., 2014, and, so, the incidence found may be overestimated.

Another strategy is to determine the frequency of carrier for pathogenic alleles in the *CBS* gene and use it to calculate the expected number of patients with HCU via the Hardy-Weinberg equation. The first researchers to use this approach were Gaustadnes,

Ingerslev, & Rütiger, (1999), who screened 500 consecutive Danish NBs for the c.833 T>C mutation and estimated the incidence of HCU to be at least 4.8:100,000. Linnebank et al., (2001) also conducted screening for the c.833 T>C mutation in 200 healthy unrelated German controls and calculated the frequency of homozygosity for this mutation to be 5.6:100,000 individuals. In Norway, Refsum et al., (2004) determined the prevalence of six specific mutations of the *CBS* gene in 1,133 NB blood samples randomly selected from ~12,000 samples, and they calculated an HCU prevalence of ~15.6:100,000. Janosík et al., (2009) estimated the frequency of HCU in the Czech Republic via determining the presence of the c.1105C>T mutation in 600 NB blood spots, and they calculated the birth prevalence for HCU to be at least 2.5:100,000.

Thus, there is an approximately 6-fold discrepancy between the number of known patients with HCU and that calculated on the basis of the number of carriers detected via genetic analyses of relatively small populations. There is no clear explanation for this discrepancy, but it could be due to the low penetrance or expressivity of some genotypes or to underdiagnosis. Therefore, to obtain a more reliable estimate of the minimal worldwide incidence of HCU, we decided to explore the data available in relevant genomic databases.

Methods

We determined the 25 most common mutations in HCU patients via a literature review using the key terms “CBS mutation” and “Classical homocystinuria mutation” in PubMed (www.ncbi.nlm.nih.gov/pubmed) and by examining references cited in related papers. Forty papers were selected and used in the analysis, which resulted in the inclusion of 1026 independent alleles from 25 countries (Table S1). Since several of these studies described only a few patients, which could lead to overestimation of the frequency of a

specific allele, only data from those countries in which at least ten alleles (five non-related patients) were used in the analyses.

We conducted searches to determine the prevalence of these variants in the general population in two relevant genomic databases: gnomAD v2.1 [Lek et al., 2016, last accessed October 2018] and ABraOM [Naslavsky et al., 2017, last accessed October 2018]. The first database includes worldwide data from 141,456 unrelated individuals sequenced as part of various disease-specific and population genetic studies. The individuals are clustered according to their genetic determination of ancestry. For example, individuals residing in the USA or Brazil may be clustered as European, African or Asian according to their genetic background. The second database, ABraOM, uses data from 609 healthy elderly individuals who were selected by using a standardized sampling process from the city of São Paulo, Brazil; nearly 10% of the Brazilian population is located in this city, making it reasonably representative of the country.

The prevalence of HCU was calculated based on the assumption that Hardy-Weinberg equilibrium exists; thus, the frequencies are ‘‘p’’ for the wild-type allele and ‘‘q’’ for the mutant allele. The different allele frequencies for each mutation were summed.

Results

HCU patients from all selected studies were grouped according to their country of origin, and allelic frequencies were calculated for each variant in each country. The 25 most frequent variants of the *CBS* gene are described in Table 1.

Most Common Variants

The five most common pathogenic *CBS* variants identified in our research (46% of alleles) were p.Arg336Cys, p.Ile278Thr, p.Gly307Ser, p.Thr191Met, and p.Trp323Ter. The countries where these mutations are most common are highlighted in Figure 1 and Table 1.

p.Arg336Cys

In our study, p.Arg336Cys presented an overall allele frequency of 14% (149 alleles) among HCU patients, and it was by far the most common variant in Qatar (97% of alleles). p.Arg336Cys was found in 15% of HCU Saudi Arabian patients but in no more than 10% of cases in European and Asian patients.

In the gnomAD, this variant was found only in non-Finnish Europeans, and it was present in 0.004% of alleles in this population. Patients homozygous for p.Arg336Cys are usually unresponsive to treatment with pyridoxine, and untreated patients present a severe clinical phenotype with involvement of the eyes, bones and vascular and central nervous systems.

p.Ile278Thr

Our data showed an allele frequency of 13% (133 alleles) for p.Ile278Thr, which is the most widely dispersed variant in the world. Among HCU patients, p.Ile278Thr was the most common pathogenic variant in the USA, Brazil, France, Italy, Germany, the Netherlands, the Czech Republic, Slovakia, Poland, Denmark, England and Israel. Figure 2 illustrates the presence and frequency of this mutation around the world in HCU patients.

According to gnomAD, this variant was not present in Ashkenazi Jewish, Asian or Latin American individuals; however, it was present in almost 1% of alleles in the Brazilian sample studied in ABraOM. Patients homozygous for p.Ile278Thr are usually responsive to treatment with pyridoxine and present a mild to moderate phenotype.

p.Gly307Ser

An allele frequency of 9% (108 alleles) was found for p.Gly307Ser in patients from the USA, Europe, Israel, Australia and Qatar. p.Gly307Ser was the most common pathogenic variant in Ireland (66%) and Australia (22%).

According to the genomic database, the mutant allele was present in Europeans (0.03% of all alleles were mutated) and Africans (0.008%) (Table 2). Patients homozygous for p.Gly307Ser are usually non-responsive to treatment with pyridoxine and present a severe clinical phenotype.

p.Thr191Met

p.Thr191Met presented an allele frequency of 8% (82 alleles) in HCU patients and was the most common pathogenic variant in countries of the Iberian Peninsula and in their former colonies in Latin America. The highest frequencies of this variant among HCU patients were found in Spain (44% of the alleles), Portugal (23%), Colombia (73%) and Venezuela (20%).

Data from gnomAD indicated the presence of the variant in Latin Americans (0.038%), but it was not identified in ABraOM. Patients who are p.Thr191Met homozygous are usually non-responsive to pyridoxine and present a moderate to severe clinical phenotype.

p.Trp323Ter

The overall allele frequency of p.Trp323Ter among HCU patients was 2% (25 alleles). This variant was present in patients from Saudi Arabia (77% of alleles) and northeast Brazil (6% of alleles). Interestingly, according to gnomAD data (0.003% of alleles), this variant was found only in Asians. This variant was not observed in ABraOM, which analyzed persons from São Paulo, Brazil. Patients homozygous for p.Trp323Ter are usually non-responsive to treatment with pyridoxine and present a moderate to severe clinical phenotype.

HCU worldwide incidence

In the genetic database gnomAD, we found 303 individuals who were carriers for any of 21 of the 25 most frequent pathogenic alleles of the *CBS* gene, yielding an estimated HCU incidence (i.e., homozygosity or compound heterozygosity) of ~0.38:100,000 (95% CI, 0.39–0.29:100,000) individuals (Table 2). No homozygous individuals were found in this database.

Analyzing the data according to different ancestry, we calculated an HCU prevalence of ~0.72:100,000 individuals among Europeans (non-Finnish), ~0.45:100,000 individuals among Latin Americans, ~0.20:100,000 individuals among Africans and ~0.02:100,000 individuals in Asia (Table 2).

HCU incidence in southeastern Brazil

In the ABraOM database, we found only two of the 25 variants analyzed (p.Ile278Thr and p.Ala114Val). A total of 12 individuals carrying either of the mutations

were included in this database, yielding an estimated incidence of HCU of ~9.7:100,000 individuals. No homozygous individuals were found.

Discussion

Knowledge of the genetic background of HCU in different populations is generally poor and even contradictory. This omission hampers proper patient genetic counseling and appropriate genetic testing, since testing for the few most prevalent variants may be more efficient than testing for many rare variants. Knowledge of the prevalent mutations and their frequencies will support decision making within national screening programs. Furthermore, there is an approximately 6-fold discrepancy between the number of known patients with HCU and the estimate calculated on the basis of the number of heterozygotes detected via genetic analyses of relatively small populations.

In this study, we used the results of published articles to characterize the mutational profiles of HCU patients around the world, detailing the most common *CBS* gene pathogenic variants among these patients. Next, we used the 25 most common published mutations to determine the corresponding allele frequencies in genomic databases and to calculate the incidence of HCU in different ancestralities. Interestingly, the frequencies of these 25 most commonly reported mutations in various countries are in line with the data from genomic databases; for instance, p.Ile278Thr was very common in different ancestry, p.Thr191Met was found in Latin Americans, and variants such as p.Thr257Met and p.Ala114Val were described in patients with HCU in different continents and found in several ancestralities. Europeans seems to be the group with the greatest allelic diversity, which leads us to hypothesize that dispersion of these mutant alleles occurred during the colonization period of America and Africa. In addition to the much lower incidence of

HCU, we observed a distinct pattern of mutations in Asia and Russia, where 75% of alleles differ from the 10 most common pathogenic variants worldwide.

Based on neonatal screening data obtained by the measurement of Met in dried blood spots (DBSs), Naughten, Yap, & Mayne, (1998) reported HCU incidences of 0.77:100,000 NBs in Germany, 0.8:100,000 NBs in England and 0.34:100,000 NBs in the USA and higher incidences of 1.54:100,000 NBs in Ireland and 1.82:100,000 NBs in Italy. According to Mathias & Bickel, (1986), the incidence in Germany was 0.32:100,000 NBs based on biochemical neonatal screening data of almost 1 million individuals. Biochemical neonatal screening of 820,797 individuals in New South Wales, Australia, around the 1960s revealed 14 cases of HCU, resulting in an incidence of 1.72:100,000 NBs [Wilcken & Turner, 1978].

In Asian countries, a much lower HCU incidence was observed. In Japan, an extremely low incidence of 0.11:100,000 NBs was observed despite an effective biochemical screening program [Naughten et al., 1998]. National biochemical neonatal screening performed in the Philippines between 1996 and 2001 identified no HCU patients among 176,548 samples [Padilla, 2003]. In Taiwan, 5 million individuals were subjected to biochemical neonatal screening for HCU, and only 3 were diagnosed with the disease. In sharp contrast, an extremely high frequency of HCU of 416:100,000 individuals was found on an island inhabited by an Austronesian Taiwanese Tao tribe [Lu et al., 2012]. Kaur, Das, & Verma, (1994) investigated 2,560 high-risk patients with strong suspicion of an inborn error of metabolism in northern India, and the most commonly found disorder was HCU (0.6%).

The country with the highest incidence of HCU in the world is Qatar. This incidence may be influenced by a founder effect since the disease is more common in three

tribes of the Qatari population, which also exhibit a high incidence of consanguineous marriage. Based on the frequency of the most common mutation in the *CBS* gene (p.Arg336Cys), the incidence of the disease was initially estimated to be ~ 33:100,000 individuals [El-Said et al., 2006]. However, after the implementation of neonatal screening through the detection of tHcy and Met combined with genetic screening, the estimated incidence of HCU in the Qatari population increased to 55:100,000 NBs [Gan-Schreier et al., 2010].

Among *CBS* mutations, p.Ile278Thr is geographically the most widespread (Figure 2). Studying the emergence and dispersal of this mutation, Vyletal et al., (2007) reported that haplotype c. [833C; 844_845ins68] is very common in sub-Saharan Africa (up to 40% of control chromosomes), less frequent throughout Europe and America (5–10% of control chromosomes), and rare in Asia (0.16–2.5% of control chromosomes). It was concluded that the p.Ile278Thr mutation occurred repeatedly and independently in the recent history of the European population. Haplotype c. [833C; 844_845ins68] is a common variant present in ~12% of the general population according to gnomAD, but it is not pathogenic since 844_845ins68 creates an alternative splice site that rescues the wildtype CBS sequence from the mutated allele.

The prevalence of HCU varies dramatically between regions from 416:100,000 on Orchid Island and 55:100,000 in Qatar to less than one in one million in the Taiwanese Han population [Gan-Schreier et al., 2010; Lu et al., 2012]. In this study, we used the genetic database gnomAD to determine the frequency of *CBS* heterozygotes and then calculated the worldwide incidence of HCU, which was found to be approximately 0.38:100,000 individuals. Stratifying populations by ancestry, the highest incidence was found in Europeans and Latin Americans. A much lower incidence was found in Asians.

The incidences in these various regions were more or less in line with those found through neonatal biochemical screening. For instance, in Europeans, an incidence of 0.72:100,000 individuals was calculated on the basis of the number of heterozygotes in gnomAD versus 0.77:100,000 according to neonatal screening, and in Asian, the corresponding values were 0.02:100,000 versus 0.07: 100,000 individuals.

Another remarkable finding is that the incidence calculated in this study for Europeans of approximately ~0.72:100,000 individuals is much lower (approximately 7 times) than those described in other studies. Gaustadnes et al., (1999) estimated the incidence of HCU to be at least 4.8:100,000 live births in Denmark according to mutation screening among 500 newborns. Studying 200 unrelated German control subjects, Linnebank et al., (2001) calculated a frequency of HCU of 5.6:100,000 individuals. Janosík et al., (2009) calculated an incidence of 2.5:100,000 on the basis of 600 Czech NB blood spots. Refsum et al., (2004) calculated a prevalence of HCU of ~15.6:100,000 NBs based on 1133 Norwegian NB samples. We have no explanation for this discrepancy except that the numbers of studied individuals were relatively small and that publication bias may have played a role. The sample size of gnomAD is approximately 120 times larger than the sample sizes of these studies and should therefore provide a much more precise incidence rate.

We estimated the number of HCU patients by using Hardy-Weinberg (HW) equilibrium. The HW principle presents limitations because it analyzes allele frequencies and genotype counts in successive generations and predicts that in a random mating population of infinite size, allele and genotype frequencies should remain constant from one generation to the next. Some factors that can disrupt HW equilibrium are mutation, natural selection, migration, population structure (nonrandom marriage and/or

consanguinity) and nonrandom selection of the sample studied [Waples, 2015; Piel et al., 2016]. Overall, we assume that these limitations do not substantially affect the numbers we calculated.

The genomic database gnomAD includes data from consortia such as 1000 genomes, GO-ESP and TOPMed and provides sequence data from unrelated individuals from various disease-specific populations included in genetic studies. Therefore, our frequency analysis is based on diverse populations from various countries and ethnicities clustered according to their genetic determination of ancestry. Although the observed frequency of HCU is relatively low, we consider it to be closer to reality with greater precision in the estimation of incidence because of the large number of individuals and the different genetic backgrounds included.

Genetic data provided by databases present limitations because of the heterogeneous inclusion criteria of the original studies such as age or selection based on diseases. Although gnomAD is the largest public genetic database to our knowledge, it should be taken into account that individuals in this database are clustered according to their genetically determined ancestry and not according to the country or continent where they reside. Approximately 45% of individuals are classified as exhibiting European ancestry, so gnomAD only partially reflects global genetic diversity. To our knowledge, this is the first study to use this large genetic database to estimate the incidence of a monogenic disease.

Another possible limitation of this study was the method used to define the 25 most common mutations among HCU patients. An extensive review of the literature was performed to identify the most common mutations in HCU patients, but some relatively common variants may still have been missed. Each study presents its own methodology for

the inclusion and diagnosis of patients. In many countries, indicated in white in Figure 1, there are no reported HCU patients. No alleles were found in the genomic databases for five of the twenty-five mutations analyzed (p.Gln7Profs (c.19dupC), p.Leu101Pro, p.Cys165Tyr, p.Lys441Ter and p.Lys523Serfs). Our study included three mutations that are known to be responsive to pyridoxine (p.Arg266Lys, p.Ala226Thr and p.Ile278Thr), which accounted for an important percentage of the alleles present in this population (~20%). Pyridoxine-responsive patients are known to present with a mild clinical presentation later in life or may even have no symptoms.

The genetic database ABraOM consists of 1,218 alleles from individuals living in São Paulo, Brazil, and 12 mutant alleles were found in this database. This number resulted in a calculated incidence of HCU of ~9.7:100,000 individuals, which is in line with the incidence found in various small European studies. Among the *CBS* mutations reported in Brazilian HCU patients, the variant p.Ile278Thr was the most common (18%) [Poloni et al., 2018]. In our search in the genomic database ABraOM, p.Ile278Thr was the most common variant with a striking frequency of 0.00903. If ABraOM is representative of the Southeast Region of Brazil, this result shows that many patients are not being diagnosed. However, again, the relatively small sample size may affect the results.

Newborn screening is being carried out in countries with high incidences of HCU, such as Ireland and Qatar [Yap & Naughten, 1998; Zschocke et al., 2009]. For this purpose, tHcy is measured in DBSs with a dedicated method in Qatar. However, all other newborn screening programs measure Met in DBSs, which results in a high proportion of false negatives, particularly for pyridoxine-responsive forms of HCU, because these patients seem not to develop hypermethioninemia in the first days of life [Peterschmitt, Simmons, & Levy, 1999; Bowron, Barton, Scott, & Stansbie, 2005; McHugh et al., 2011].

Countries in Latin America do not have a neonatal screening program for HCU; however, we estimated HCU incidences for these populations of ~0.45:100,000. Other countries such as Japan and the USA have neonatal screening programs for HCU, even though the incidences in these countries are lower than (Japan) or similar to (USA) those estimated in Latin America.

Because HCU is a treatable disease and given the severe clinical complications of HCU, such as thromboembolic events, dislocation of the lens and neurological complications, the early recognition of HCU patients by health professionals is extremely relevant. The results presented in this study based on the number of heterozygotes for *CBS* mutations in a large genomic database reinforce efforts to recognize CBS patients and to implement effective newborn screening methods, particularly in regions with high incidences.

Acknowledgments

This project was supported by PPGBM, FIPE–HCPA, CAPES, CNPq (Brazil).

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Table 1. Frequency of the 25 most prevalent pathogenic *CBS* variants found among HCU published patients worldwide.

COUNTRY (N OF ALLELES)	ALLELIC FREQUENCY												
	p.Arg336Cys	p.Ile278Thr	p.Gly307Ser	p.Thr191Met	p.Trp323 Ter	IVS11 as A-C -2	p.Asp444Asn	p.Ala114Val	p.Arg125Gln	p.Thr353 Met	p.Arg266 Lys	c.1566delG	
CZECH REPUBLIC AND SLOVAKIA (60)		0.20				0.18		0.02					
DENMARK (10)		0.20					0.20						
ENGLAND (34)	0.03	0.29	0.23						0.03				
FRANCE (12)		0.17	0.08				0.08						
GERMANY (12)		0.33	0.17										
IRELAND (78)		0.01	0.66						0.02				
ITALY (62)		0.29						0.14	0.01				
NORWAY (32)		0.12	0.19								0.34		
POLAND (14)		0.36											
PORTUGAL (26)	0.08	0.04		0.23					0.11			0.23	
SPAIN (80)	0.01	0.02		0.44			0.10		0.01	0.05		0.05	
RUSSIA (22)		0.04				0.27				0.04			
THE NETHERLANDS (42)		0.55					0.05						
ARGENTINA (18)				0.11				0.05					
BRAZIL (82)		0.16		0.15	0.06					0.01			
COLOMBIA (34)				0.73									
USA (98)		0.19	0.07				0.01	0.01	0.02				
VENEZUELA (10)				0.20			0.20						
CHINA (20)	0.05	0.05							0.15				
JAPAN (24)		0.08						0.04					
KOREA (10)	0.10									0.10 (1/10)			
ISRAEL (16)		0.18	0.06										
QATAR (142)	0.97		0.01										
SAUDI ARABIA (26)	0.15				0.77								
ANGLO -CELTIC (AUSTRALIA) (50)	0.02	0.20	0.22							0.02			

COUNTRY (TOTAL Nº OF ALLELES)	ALLELIC FREQUENCY												
	p.Glu144Lys	p.Cys165Tyr	p.Thr257Met	p.Arg121His	p.Val320Ala	p.Gly85Arg	p.Gly151Arg	p.Leu101Pro	p.Thr262Met	c.19dupC	p.Ala226Thr	p.Lys441Ter	p.Gly148Arg
CZECH REPUBLIC/ SLOVAKIA (60)	0.03	0.03								0.05			
DENMARK (10)						0.10 (1/8)							
ENGLAND (34)	0.03												
FRANCE (12)													
GERMANY (12)	0.08												
IRELAND (78)								0.05	0.01				
ITALY (62)			0.01										
NORWAY (32)					0.12				0.06				
POLAND (14)													0.14
PORTUGAL (26)													
SPAIN (80)			0.025										
RUSSIA (22)													
THE NETHERLANDS (42)		0.09											
ARGENTINA (18)						0.05					0.22		0.11
BRAZIL (82)			0.01			0.02	0.06						
COLOMBIA (34)				0.09									
USA (98)					0.02			0.01	0.02				
VENEZUELA (10)						0.20							
CHINA (20)			0.05										
JAPAN (24)				0.16			0.04					0.16	
KOREA (10)			0.10										
ISRAEL (16)													
QATAR (142)													
SAUDI ARABIA (26)													
ANGLO -CELTIC (AUSTRALIA) (50)	0.10	0.02						0.02					

Country and the total number of independent alleles analyzed according to studies included in this research. Results for each pathogenic variant are presented as: frequency of allele (alleles described in each study in the country and the references used for this particular frequency). The most common pathogenic variant in each country is underlined.

Table 2. Incidence of carriers individuals for each pathogenic variant in the *CBS* gene in different populations according to the genomic database gnomAD.

Variant	Europeans non-Finnish Alleles (n): 126,706	Europeans Finnish Alleles (n): 25,744	Africans Alleles (n): 24,032	Latin Americans Alleles (n): 34,420	Asian Alleles (n): 49,650	TOTAL (allele frequency)
p.Arg336Cys (c.1006 C>T)	0.00004417	-	-	-	-	0.00001994
p.Ile278Thr (c.833 T>C)	0.00143200	0.00057600	0.00023230	-	-	0.00083270
p.Gly307Ser (c.919 G>A)	0.00031740	0.00003989	0.00008010	-	-	0.00016620
p.Thr191Met (c.572 C>T)	-	-	-	0.00037700	-	0.00006023
p.Trp323Term (c.969 G>A)	-	-	-	-	0.00003270	0.00000399
IVS11 as A-C -2 (c.1224-2 A>C)	0.00004088	-	-	-	-	0.00007850
p.Asp444Asn (c.1330 G>A)	0.00026390	0.00005424	0.00021710	0.00126600	-	0.00032120
p.Ala114Val (c.341 C>T)	0.00035680	-	0.00008024	0.00028230	0.00013296	0.00021600
p.Arg125Gln (c.374 G>A)	0.00002326	-	-	-	-	0.00001062
p.Thr353Met (c.1058 C>T)	-	-	0.00029160	0.00002840	-	0.00002973
p.Arg266Lys (c.797 G>A)	0.00001772	-	-	-	-	0.00000801
p.Lys523Serfs (c.1566delG)	-	-	-	-	-	-
p.Glu144Lys (c.430 G>A)	0.00003882	-	0.00004020	-	0.00009799	0.00003198
p.Cys165Tyr (c.494 G>A)	-	-	-	-	-	-
p.Thr257Met (c.770 C>T)	0.00001564	-	0.00004065	0.00011340	0.00013230	0.00004286
p.Arg121His (c.362 G>A)	0.00002325	-	0.00024090	-	-	0.00003187
p.Val320Ala (c.959T>C)	0.00003539	-	-	-	-	0.00001598
p.Gly85Arg (c.253 G>A)	0.00000879	-	-	-	-	0.00000398
p.Gly151Arg (c.451 G>A)	0.00002335	-	0.00008055	0.00002825	-	0.00002142
p.Leu101Pro (c.302 T>C)	-	-	-	-	-	-
p.Thr262Met (c.785C>T)	0.00001772	0.00014060	0.00006242	-	-	0.00002804
p.Gln7Profs (c.19dupC)	-	-	-	-	0.00005473	0.00000411
p.Ala226Thr (c.676G>A)	0.00000879	0.00004620	-	-	-	0.00000795
p.Lys441Ter (c.1321A>T)	-	-	-	-	-	-
p.Gly148Arg (c.442G>A)	0.000008825	-	0.00006176	0.00002893	-	0.00001202
TOTAL	0.00267672	0.00085693	0.00142782	0.00212428	0.00045068	0.00194734
PREVALENCE of HCU per 100,000 individuals	**~ 0.72**	**~ 0.07**	**~ 0.20**	**~ 0.45**	**~ 0.02**	**~ 0.38**

- = no alleles found. Total (allele frequency): contains all the data show in the table plus individuals classified as Ashkenazi Jewish and "others" in the gnomAD.

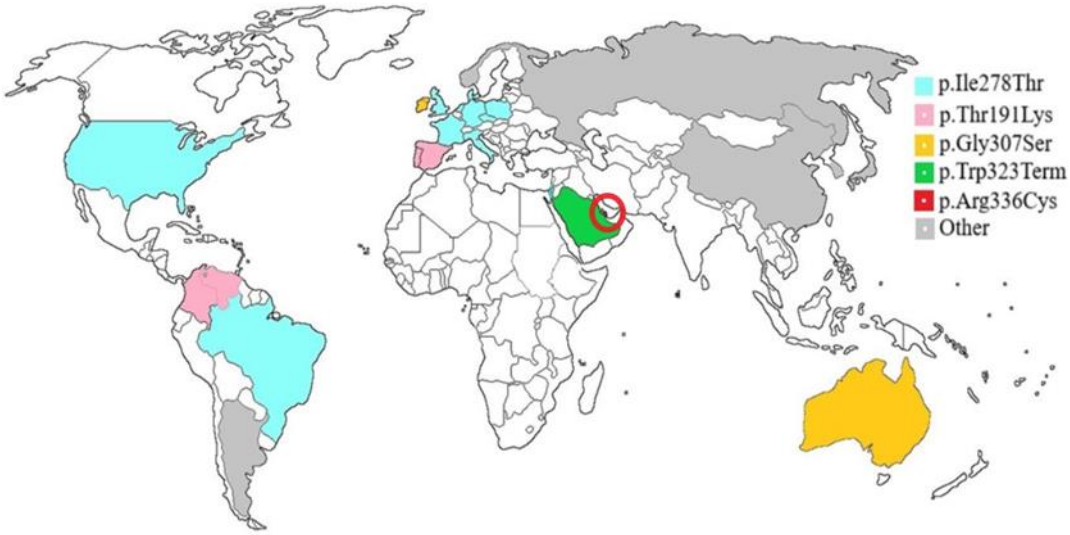

Figure 1. The most common pathogenic alleles of the *CBS* gene per country: p.Ile278Thr, p.Thr191Met, p.Gly307Ser, Trp323Term and p.Arg336Cys. The most prevalent variant in the world (p.Ile278Thr) is the most common in The Netherlands (allelic frequency: 55%), Poland (36%), Germany (33%), England (29%), Italy (29%), Denmark (20%), Czech Republic and Slovakia (20%), USA (19%), Israel (18%), France (17%) and Brazil (16%). The variant p.Thr191Met is the most common in Colombia (73%), Spain (44%), Portugal (23%) and Venezuela (20%). In Ireland (66%) and Australia (22%) the most common variant is p.Gly307Ser. The variant Trp323Term is the most common in Saudi Arabia (77%), and in Qatar (highlighted by the red circle) the most common variant is p.Arg336Cys (97%). Other prevalent mutations are c.700_702delGAC in Korea (20%), IVS11 as A-C -2 in Russia (27%), p.Arg121His and p.Lys441Term in Japan (16% each one), p.Arg125Gln in China (15%), in Argentina p.Ala226Thr (22%) and in Norway p.Arg266Lys (34%).

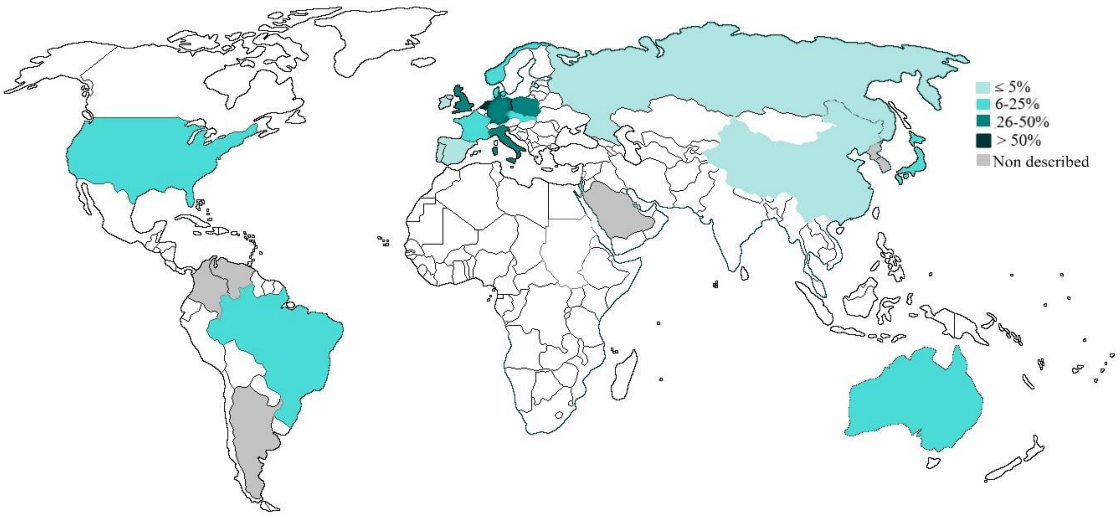

Figure 2. Distribution of the p.Ile278Thr variant among Classical Homocystinuria patients. Presence of the pathogenic variant in countries with at least 10 pathogenic alleles of HCU described in the literature. Countries without at least 10 published HCU alleles, or with no HCU patients genotyped, are presented in white. Gray indicates countries in which the variant p.Ile278Thr was not described among HCU patients.

Supplemental Table: Pathogenic variants reported in the literature in *CBS* gene.

	Location	Nº of Alleles	Codon	Protein Change	References	Countries
1	Exon 1	1	c.52C>T	p.Arg18Cys	(Lee et al., 2005)	Korea
2	Exon 1	1	c.129G>A	p.Trp43Ter	(Katsushima et al., 2006)	Japan
3	Exon 1	3	c.146C>T	p.Pro49Leu	(Cozar et al., 2011; Gaustadnes et al., 2002; Poloni et al., 2018)	Brazil; Spain; Australia
4	Exon 1	1	c.172C>T	p.Arg58Trp	(de Franchis, Kraus, Kozich, Sebastio, & Kraus, 1999)	Italy
5	Exon 1	1	c.194A>G	p.His65Arg	(Janosík et al., 2001)	Czech Republic
6	Exon 2	1	c.215A>T	p.Lys72Ile	(Li et al., 2018)	China
7	Exon 2	6	c.253G>A	p.Gly85Arg	(Cozar et al., 2011; De Lucca & Casique, 2004; Maclean et al., 2002; Poloni et al., 2018)	Venezuela; Argentina; Brazil; Dennmark
8	Exon 2	1	c.262C>T	p.Pro88Ser	(Sebastio et al., 1995)	Italy
9	Exon 2	2	c.284T>C	p.Ile95Thr	(Poloni et al., 2018)	Brazil
10	Exon 2	6	c.302T>C	p.Leu101Pro	(Gallagher et al., 1998; Gaustadnes et al., 2002; Kruger, Wang, Jhee, Singh, & Elsas, 2003)	EUA; Ireland; Australia
11	Exon 2	1	c.304A>C	Lys102Gln	(Gat-Yablonski, Mandel, Fowler, Taleb, & Sela, 2000)	Israel
12	Exon 3	2	c.325T>C	p. Cys109Arg	(Gaustadnes et al., 2002; Voskoboeva, Semyachkina, Yablonskaya, & Nikolaeva, 2018)	Australia; Russia
13	Exon 3	1	c.329A>T	p.Glu110Val	(Poloni et al., 2018)	Brazil
14	Exon 3	13	c.341C>T	p.Ala114Val	(de Franchis et al., 1999; Janosík et al., 2001; Katsushima et al., 2006; Moat et al., 2004; Sebastio et al., 1995; Sperandeo et al., 1995; Urreizti et al., 2006)	Argentina; EUA; Italy; Czech Republic and Slovakia; Japan
15	Exon 3	1	c.346G>A	p.Gly116Arg	(Li et al., 2018)	China
16	Exon 3	2	c.361C>T	p.Arg121Cys	(Cozar et al., 2011; Katsushima et al., 2006)	Spain; Japan
17	Exon 3	7	c.362G>A	p.Arg121His	(Katsushima et al., 2006; Urreizti et al., 2006)	Colombia; Japan
18	Exon 3	2	c.373C>T	p.Arg125Trp	(Kluijtmans et al., 1999; Urreizti et al., 2003)	The Netherlands; Spain
19	Exon 3	13	c.374G>A	p.Arg125Gln	(Castro et al., 2001; Cozar et al., 2011; Gaustadnes et al., 2002; Li et al., 2018; Marble, Geraghty, de Franchis, Kraus, & Valle, 1994; Moat et al., 2004; Sebastio et al., 1995)	EUA; Spain; Portugal; Italy; Ireland; England; China
20	Exon 3	1	c.384G>C	p.Glu128Asp	(Coudé, Aupetit, Zabot, Kamoun, & Chadefaux-Vekemans, 1998)	France
21	Exon 3	1	c.407T>C	p.Leu136Pro	(Gong et al., 2015)	China
22	Exon 3	1	c.415G>A	p.Gly139Arg	(Shih et al., 1995)	Italy
23	Exon 3	1	c.429C>G	p.Ile143Met	(Orendáè et al., 2004)	Poland

24	Exon 3	9	c.430G>A	p.Glu144Lys	(Gaustadnes et al., 2002; Janosík et al., 2001; Moat et al., 2004; Shih et al., 1995)	Germany; England; Australia; Czech Republic and Slovakia
25	Exon 3	2	c.434C>T	p.Pro145Leu	(Urreizti et al., 2006)	Spain
26	Exon 3	5	c.442G>A	p.Gly148Arg	(Katsushima et al., 2006; Orendáè et al., 2004; Urreizti et al., 2006)	Argentina; Poland; Japan
27	Exon 3	6	c.451G>A	p.Gly151Arg	(Katsushima et al., 2006; Poloni et al., 2018)	Brazil; Japan
28	Exon 4	3	c.456C>G	p.Ile152Met	(Kluijtmans et al., 1999)	The Netherlands
29	Exon 4	1	c.457G>A	p.Gly153Arg	(Zaidi et al., 2012)	Saudi Arabia
30	Exon 4	1	c.461T>A	p. Leu154Gln	(Lee et al., 2005)	Korea
31	Exon 4	1	c.464C>T	p.Ala155Val	(Lee et al., 2005)	Korea
32	Exon 4	2	c.463G>A	p.Ala155Thr	(Janosík et al., 2001)	Czech Republic
33	Exon 4	1	c.473C>T	p.Ala158Val	(Gong et al., 2015)	China
34	Exon 4	8	c.494G>A	p.Cys165Tyr	(Gaustadnes et al., 2002; Janosík et al., 2001; Kluijtmans et al., 1999)	The Netherlands; Australia; South Africa; Czech Republic and Slovakia
35	Exon 4	1	c.503T>C	p.Val168Ala	(Porto et al., 2005)	Brazil
36	Exon 4	1	c.517A>G	p.Met173Val	(Urreizti et al., 2006)	Spain
37	Exon 4	3	c.526G>A	p.Glu176Lys	(Janosík et al., 2001; Kozich et al., 1997)	Czech Republic and Slovakia
38	Exon 5	1	c.539T>C	Val180Ala	(Kluijtmans et al., 1999)	The Netherlands
39	Exon 5	82	c.572C>T	p.Thr191Met	(Bermúdez et al., 2006; Cozar et al., 2011; De Lucca & Casique, 2004; Poloni et al., 2018; Porto et al., 2005; Urreizti et al., 2006; Urreizti et al., 2003)	Venezuela; Colombia; Argentina; Brazil; Spain; Portugal
40	Exon 5	1	c.599C>T	p.Pro200Leu	(Cozar et al., 2011)	Spain
41	Exon 6	1	c.650C>T	p.Ser217Phe	(Katsushima et al., 2006)	Japan
42	Exon 6	5	c.676G>A	p.Ala226Thr	(Cozar et al., 2011; Kruger et al., 2003; Urreizti et al., 2006)	Argentina; EUA
43	Exon 6	2	c.683A>G	p. Asn228Ser	(Kruger et al., 2003)	EUA
44	Exon 6	3	c.684C>A	Asn228Lys	(Gaustadnes et al., 2002; Orendáè et al., 2004)	Australia; Poland
45	Exon 6	1	c.684C>G	p. Asn228Lys	(Gallagher et al., 1998)	Ireland
46	Exon 6	1	c.689T>A	p. Leu230Gln	(Li et al., 2018)	China
47	Exon 6	1	c.691G>C	p.Ala231Leu	(Poloni et al., 2018)	Brazil
48	Exon 6	1	c.691G>C	p. Ala231Pro	(Kruger et al., 2003)	EUA
49	Exon 6	2	c.694C>G	p. His232Asp	(Katsushima et al., 2006)	Japan
50	Exon 6	4	c.700G>A	p.Asp234Asn	(De Lucca & Casique, 2004; El-Said et al., 2006)	Venezuela; Qatar
51	Exon 6	2	c.707C>A	p.Thr236Asn	(Li et al., 2018)	China
52	Exon 6	1	c.727C>T	Gln243Ter	(De Lucca & Casique, 2004)	Venezuela
53	Exon 7	8	c.770C>T	p.Thr257Met	(Lee et al., 2005; Li et al., 2018; Poloni et al., 2018; Sebastio et al., 1995; Urreizti et al., 2006; Zaidi et al., 2012)	Brazil, Spain; Italy; Sudan; Korea; China
54	Exon 7	5	c.785C>T	p.Thr262Met	(Gallagher et al., 1998; Kim et	EUA; Ireland;

					al., 1997; Moat et al., 2004)	Norway
55	Exon 7	4	c.785C>G	p.Thr262Arg	(Gat-Yablonski et al., 2000; Guttormsen et al., 2001)	Norway; Israel
56	Exon 7	11	c.797G>A	p.Arg266Lys	(Guttormsen et al., 2001; Kim et al., 1997)	Norway
57	Exon 7	2	c.796A>G	p.Arg266Gly	(Katsushima et al., 2006)	Japan
58	Exon 7	2	c.824G>A	Cys275Tyr	(Cozar et al., 2011; Urreizti et al., 2003)	Spain
59	Exon 8	133	c.833T>G	p.Ile278Thr	(Cozar et al., 2011; Gallagher et al., 1998; Gallagher et al., 1995; Gat-Yablonski et al., 2000; Gaustadnes et al., 1998; Gaustadnes et al., 2002; Hu et al., 1993; Janosík et al., 2001; Katsushima et al., 2006; Kim et al., 1997; Kluijtmans et al., 1999; Kozich et al., 1997; Kruger et al., 2003; Kwok et al., 2011; Lefaucheur, Triquenot-Bagan, Quillard, Genevois, & Hannequin, 2008; Maclean et al., 2002; Moat et al., 2004; Orendáè et al., 2004; Poloni et al., 2018; Porto et al., 2005; Sebastio et al., 1995; Shih et al., 1995; Sperandeo et al., 1995; Voskoboeva et al., 2018)	Brazil; EUA; Spain; Portugal; France; Italy; Germany; The Netherlands; Ireland; Norway; England; Dennmark; Australia; Israel; Czech Republic and Slovakia; Poland; Russia; Japan; China
60	Exon 8	1	c.833T>G	Ile278Ser	(Cozar et al., 2011)	Spain
61	Exon 8	1	c.841G>A	Asp281Asn	(Cozar et al., 2011)	Spain
62	Exon 8	2	c.862G>A	p.Ala288Thr	(Bermúdez et al., 2006; Lee et al., 2005)	Colombia; Korea
63	Exon 8	1	c.869C>T	p.Pro290Leu	(Sperandeo et al., 1995)	Spain
64	Exon 8	2	c.904G>A	p. Glu302Lys	(Gaustadnes et al., 2002; Voskoboeva et al., 2018)	Australia; Russia
65	Exon 8	2	c.913G>A	p. Gly305Arg	(Voskoboeva et al., 2018)	Russia
66	Exon 8	108	c.919G>A	p.Gly307Ser	(de Franchis et al., 1999; Gallagher et al., 1998; Gaustadnes et al., 2002; Guttormsen et al., 2001; Hu et al., 1993; Kim et al., 1997; Kruger et al., 2003; Moat et al., 2004; Zschocke et al., 2009)	EUA; France; Germany; Ireland; Norway; Qatar; England; Australia; Israel
67	Exon 9	6	c.959T>C	p.Val320Ala	(Guttormsen et al., 2001; Kim et al., 1997; Kruger et al., 2003)	EUA; Norway
68	Exon 9	1	c.962A>T	p.Asp321Val	(Cozar et al., 2011)	Argentina
69	Exon 9	25	c.969G>A	p.Trp323Ter	(Poloni et al., 2018; Zaidi et al., 2012)	Brazil; Saudi Arabia
70	Exon 9	149	c.1006C>T	p.Arg336Cys	(de Franchis et al., 1999; El-Said et al., 2006; Gaustadnes et al., 2002; Kwok et al., 2011; Lee et al., 2005; Urreizti et al., 2003; Zaidi et al., 2012; Zschocke et al., 2009)	Spain; Portugal; Qatar; England; Australia; Saudi Arabia; Korea; China

71	Exon 9	2	c.1007G>A	p.Arg336His	(Coudé et al., 1998)	North Africa
72	Exon 9	4	c.1013T>C	p.Leu338Pro	(Urreizti et al., 2006; Urreizti et al., 2003)	Spain
73	Exon 9	3	c.1039G>A	p.Gly347Ser	(Katsushima et al., 2006; Lee et al., 2005)	Korea; Japan
74	Exon 10	3	c.1046G>A	p.Ser349Asn	(Urreizti et al., 2003)	Spain
75	Exon 10	12	c.1058C>T	p.Thr353Met	(Cozar et al., 2011; Gaustadnes et al., 2002; Kruger et al., 2003; Lee et al., 2005; Poloni et al., 2018; Urreizti et al., 2006; Voskoboeva et al., 2018)	Brazil; Spain; Australia; EUA; Russia; Korea
76	Exon 10	1	c.1060G>A	p.Val354Met	(Coudé et al., 1998)	Portugal
77	Exon 10	1	c.1063G>C	p. Ala355Pro	(Gallagher et al., 1998)	Ireland
78	Exon 10	2	c.1081G>A	p. Ala361Thr	(Castro et al., 2001)	Portugal
79	Exon 10	1	c.1102C>T	p.Gln368Ter	(Voskoboeva et al., 2018)	Russia
80	Exon 10	2	c.1105C>T	Arg369Cys	(Kim et al., 1997; Kluijtmans et al., 1999)	The Netherlands; Norway
81	Exon 10	3	c.1111G>A	p.Val371Met	(Gaustadnes et al., 2002; Kluijtmans et al., 1999)	The Netherlands; Australia
82	Exon 10	4	c.1126G>A	p. Asp376Asn	(Kruger et al., 2003; Poloni et al., 2018)	EUA; Brazil
83	Exon 10	4	c.1136G>A	p.Arg379Gln	(Cozar et al., 2011; Urreizti et al., 2006; Urreizti et al., 2003)	Spain
84	Exon 11	1	c.1152G>C	p.Lys384Asn	(Voskoboeva et al., 2018)	Russia
85	Exon 11	2	c.1150A>G	p.Lys384Glu	(Aral et al., 1997)	France
86	Exon 12	3	c.1226G>A	p.Trp409Ter	(Janosík et al., 2001; Kozich et al., 1997)	Czech Republic and Slovakia
87	Exon 12	1	c.1259C>G	p.Ser420Ter	(Coudé et al., 1998)	North Africa
88	Exon 12	1	c.1265C>T	p.Pro422Leu	(Maclean et al., 2002)	Dennmark
89	Exon 12	1	c.1301C>A	p.Thr434Asn	(Kluijtmans et al., 1999)	The Netherlands
90	Exon 12	1	c.1304T>C	p.Ile435Thr	(Maclean et al., 2002)	Dennmark
91	Exon 12	4	c.1321A>T	p.Lys441Ter	(Katsushima et al., 2006)	Japan
92	Exon 12	16	c.1330G>A	p.Asp444Asn	(Cozar et al., 2011; De Lucca & Casique, 2004; Kluijtmans et al., 1999; Lefaucheur et al., 2008; Maclean et al., 2002; Moat et al., 2004; Urreizti et al., 2006)	Venezuela; EUA; Spain; France; The Netherlands; Dennmark
93	Exon 12	2	c.1330G>T	p.Asp444Tyr	(Voskoboeva et al., 2018)	Russia
94	Exon 12	1	c.1336G>T	p. Ala446Ser	(Cozar et al., 2011)	Argentina
95	Exon 13	2	c.1367T>C	p.Leu456Pro	(Urreizti et al., 2003)	Spain
96	Exon 14	1	c.1471C>T	p.Arg491Cys	(Kluijtmans et al., 1999)	The Netherlands
97	Exon 13	1	c.1397C>T	p.Ser466Leu	(Maclean et al., 2002)	Dennmark
98	Exon 16	1	c.1576C>A	p. Gln526Lys	(Kruger et al., 2003)	EUA
99	Exon 16	2	c.1616T>C	p.Leu539Ser	(Aral et al., 1997)	France
100	Exon 1	1	c.2T>C	?	(Poloni et al., 2018)	Brazil
101	Exon1	5	c.19dupC	?	(Gaustadnes et al., 2002; Janosík et al., 2001)	Australia; Czech Republic and Slovakia
102	Exon1	2	c.28delG	p.Val10 fs	(Janosík et al., 2001; Poloni et al., 2018)	Brazil; Czech Republic and Slovakia
103	Intron 1	1	c.209+1delG	?	(Poloni et al., 2018)	Brazil
104	Intron 1	1	c.209+1G>A	?	(Poloni et al., 2018)	Brazil

105	Intron 1	1	IVS1+1insC	Del ex 1	(Gaustadnes et al., 2002)	Australia
106	Intron 1	2	IVS1+1G>A	Del ex 1	(Gaustadnes et al., 2002; Urreizti et al., 2003)	Australia; Spain
107	Intron 1	1	IVS1-1G>C	210del26, fs stop at aa 9	(Janosík et al., 2001)	Czech Republic
108	Intron 1	1	c.-541_532del10	?	(Urreizti et al., 2006)	Argentina
109	Exon 2	1	c.216-217delAT	?	(Voskoboeva et al., 2018)	Russia
110	Exon 3	1	c.442insG	N149fsX187	(Gaustadnes et al., 2002)	Australia
111	Exon 3	2	c.444delG	p.Asn149fs	(Poloni et al., 2018)	Brazil
112	Intron 3	2	c.IVS3+1G>A	?	(Li et al., 2018)	China
113	Exon 4	2	518delTGA	p.M173del	(Cozar et al., 2011)	India
114	Exon 4	1	493del22	fs,del aa 165-172	(Gaustadnes et al., 1998)	Dennmark
115	Exon 4	1	IVS4-29del129	Del ex 5	(Gat-Yablonski et al., 2000)	Israel
116	Exon 5	2	c.604_606delTA	?	(Li et al., 2018)	China
117	Intron 5	1	IVS5-14_-7del8	Y223GfsX23	(Cozar et al., 2011)	Argentina
118	Exon 6	2	c.689delT	L230RfsX39	(Cozar et al., 2011)	Argentina
119	Exon 6	2	c.700_702delGAC	234delD	(Lee et al., 2005)	Korea
120		3	c.536_533del18	del aa 179-184	(Gaustadnes et al., 2002)	Australia
121	Exon 6	2	c.689delT	L230RfsX39	(Cozar et al., 2011)	Argentina
122		1	532-37_736+438del794	?	(Cozar et al., 2011)	Portugal
123	Intron 6	1	c. IVS6-1 G>C	?	(Kruger et al., 2003)	EUA
124	Intron 7	1	c.IVS7+1 G>A	828ins104, 737del92	(Janosík et al., 2001)	Czech Republic
125	Exon 8	2	c.862_866del5	p.E289GfsX39	(Cozar et al., 2011)	Portugal
126	Exon 8	2	c.864_868delGAG	p.Glu289del	Poloni (1/60)	Brazil
127	Exon 8	1	c.892insC	Q298fsX329	Gaustadnes, 2002 (1/56)	Australia
128	Intron 8	9	c.828+1G>A	Del ex 8	Urreizti 2006 (0/54); Poloni (1/60); Maclean, 2002 (0/8); Gaustadnes, 2002 (1/56)	Argentina; Brazil; Dennmark; Australia
129	Exon 9	1	c.989_991delAGG	p.Glu330del	Poloni (1/60)	Brazil
130	Exon 9	1	c.1010_1011insT	?	Li, 2018 (0/16)	China
131	Intron 9	2	c.1039+1G>T	p.T318fsX11	Orendác, 2004 (0/10)	Poland
132	Exon 11	1	c.1221delC	W408fsX423	Gaustadnes, 2002 (1/56)	Australia
133	Intron 11	1	IVS11+1G>A	del aa382-407	Gaustadnes, 2002 (1/56)	Australia
134	Intron 11	18	c. IVS11-2 A>C	del ex 12	Moat (59/104); Kozich, 1997 (14/24); Janosik, 2001 (6/36); Voskoboeva, 2018 (3/22)	EUA; Czech Republic and Slovakia; Russia
135	Intron 11	1	c.IVS11+39del99	?	Janosik, 2001 (6/36)	Czech Republic

136	Intron 11	2	c.1223+5G>T	?	Poloni (1/60)	Brazil
137	Intron 11	2	c.1224-2A>C	p.W409_G453del	Orendác, 2004 (0/10)	Poland
138	Exon 12	1	c.1286_1288delTCA	p. I429del	Urreizti (0/54)	Argentina
139	Exon 14	1	c.1477dupA	?	Li, 2018 (0/16)	China
140	Exon 14	1	c.1498_1499delT	?	Voskoboeva, 2018 (3/22)	Russia
141	Intron 14	1	c.IVS14-1G>C	?	Li, 2018 (0/16)	China
142	Exon 16	1	c.1560-1569del CACCGGGAAG	?	Voskoboeva, 2018 (3/22)	Russia
143	Exon 16	10	c.1566delG	fs, stop at aa 540	Cozar 2011 (1/46); Urreizti 2003 (0/32); Castro (0/6)	Spain; Portugal
144	Exon 16	1	c.1591delTTCG	?	Katsushima, 2006 (0/24)	Japan
145	Exon 16	1	c.1622insTGGA	?	Gaustadnes, 2002 (1/56)	Australia
146	Exon 16	3	c.1627_1645del19	fs, stop at aa 568	Yablonski, 2000 (0/12)	Israel

6.4 CAPÍTULO 4: "*Creatine as a treatment for homocystinuria"*

O capítulo 4 consiste em estudo *in vitro* com células controle e com defeitos de remetilação tratadas com creatina monohidatrada, avaliando aspectos de inflamação e stress oxidativo. Este capítulo será apresentado na forma de artigo, o qual será submetido para a revista Physiological Research, cujo fator de impacto é 1,697 e o Qualis CAPES da área de genética (Ciências biológicas I) é B3. O formato obedece às normas de submissão da revista.

Creatine as a treatment for homocystinuria: an in vitro study

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Abstract

Several studies have indicated an association between high levels of homocysteine (Hcy) and the formation of reactive oxygen species, which may have an impact on the clinic and treatment of diseases such as homocystinuria. Endogenous hepatic synthesis of creatine (Cre) is responsible for the formation of a substantial portion of Hcy, and Cre is pointed as an antioxidant compound. Therefore, we hypothesized that Cre supplementation would decrease the endogenous Cre synthesis and thus, consequently, Hcy production, and act as an antioxidant, reducing the harmful effects of Hcy, which would in theory be an alternative treatment strategy in patients with homocystinuria. In this sense, we evaluated the oxidative markers: DCFH, SOD, CAT and SH, as well as inflammatory interleukins IL-1β, TNF-α and IL-6 in fibroblasts from patients with remethylation defects and healthy control cells, submitted or not to supplementation of the culture medium with Cre. Our results showed that the intracellular content of Cre increased in the treated groups. And we observed in the healthy cell group after Cre treatment there was a decrease in CAT, SOD, SH and DCFH, but there was no statistically significant difference. In conclusion, the present study showed that Cre has the potential to reduce oxidative stress markers, even if only in some cells, which may suggest an action individual dependent. In patients with homocystinuria, Cre supplementation may be an adjunctive treatment to decrease Hcy levels and to restore the redox homeostasis probably induced by hiperhomocysteinemia

Key-words: Homocystinuria; creatine; oxidative stress; homocysteine.

Introduction

Classical homocystinuria (OMIM +236200) is caused by the deficient activity of the enzyme cystathionine-β-synthase (CβS; EC 4.2.1.23), which results in the severe elevation of homocysteine (Hcy) in body fluids, as well as elevation of methionine (Met) (Figure 1), a characteristic that differentiates it from other Hcy remethylation disorders such as methylenetetrahydrofolate reductase (MTHFR) deficiency and cobalamin defects, where Met levels are normal or decreased (Picker & Levy, 2014; WILCKEN, 2006). Neurological complications, thromboembolic events, bone abnormalities, and eye lenses dislocation (ectopia lentis) are the most common symptoms (Mudd et al., 1985; Mudd, Levy, & Kraus, 2001). Treatment is based on pyridoxine plus folate supplementation, but most patients have to be treated with a diet restricted in met, which is very difficult to maintain, particularly after childhood (Yap & Naughten, 1998).

MTHFR deficiency (OMIM # 236250; EC 1.5.1.20) results in the abnormal intracellular metabolism of folic acid and prevents the reduction of 5-10 methylenetetrahydrofolate to methyl 5-methylenetetrahydrofolate, which donates the methyl group for the re-methylation of Hcy in Met (Figure 1). As a result, the disease leads to methyletrahydrofolate deficiency and, consequently, homocystinuria with hypomethioninemia. This disorder is associated with slow brain growth, severe neurological disability, and untimely death (Rosenblatt & Erbe, 2001).Treatment of severe deficiency involves the administration of high doses of betaine, in combination with Met, pyridoxine, vitamin B12 and folic acid supplementation (Huemer et al., 2015).

Methylmalonic acidemia with homocystinuria are a hereditary vitamin B12 (cobalamin) metabolism defect characterized by megaloblastic anemia, lethargy, growth retardation, developmental delay, intellectual deficit and seizures. There are three groups

of complementation of cobalamin defects (cblC, cblD and cblF) that are responsible for methylmalonic acidemia - homocystinuria. The onset of the disease may be between infancy and adulthood. Patients who become symptomatic after childhood may have ataxia, dementia or psychosis (Carrillo-Carrasco, Chandler, & Venditti, 2012).

Methylmalonic acidemia - homocystinuria is caused by abnormalities in the synthesis of adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) resulting from genetic defects in the complement classes Cbl, D and F. CblC (OMIM # 277400) is caused by mutations in the *MMACHC* gene (GenBank ID: 25974). Patients are treated with intramuscular injections of hydroxocobalamin, oral betaine, and folic acid. Good metabolic control and correction of hematological problems can sometimes be achieved with this treatment, but most patients continue to have signs of motor retardation and language, intellectual deficit and abnormal ophthalmic findings. Early diagnosis and treatment are important. The prognosis is better in patients with later onset disease (Carrillo-Carrasco et al., 2012; Carrillo-Carrasco & Venditti, 2012).

The synthesis of Cre occurs especially in the liver, but also in the kidneys and pancreas, and requires the action of two enzymes. The first enzyme, L-arginine: glycine amidinotransferase (AGAT, EC 2.1.4.1) catalyzes the formation of guanidinoacetate and ornithine from arginine and glycine. The second enzyme, guanidinoacetate N-methyltransferase (GAMT, EC 2.1.1.2) uses S-adenosylmethionine (AdoMet) for guanidinoacetate methylation, producing Cre and S-adenosylhomocysteine (AdoHcy) (Figure 1) (Brosnan et al., 2007). The synthesis of Cre and phosphatidylcholine consume many methyl groups, approximately 40% of the methyl groups provided by AdoMet are consumed in the Cre synthesis, therefore it accounts for 40% of the body's production of hcy (Stead et al., 2006). Theoretically, Met can be spared and the accumulation of Hcy

avoided if fewer methyl groups are donated by reactions using AdoMet as substrate for methyltransferase (Finkelstein, 2006; Schiff & Blom, 2012).

Several studies have indicated an association between elevated Hcy levels and the formation of reactive oxygen species, especially the superoxide anion (O_2-) and H_2O_2 (Huang et al., 2001; Weiss, 2005; Weiss et al., 2002). Intracellular reactive oxygen species production may cause cellular damage (Streck et al., 2003) and disorders of the antioxidant defense system, demonstrating a mechanistic connection between hyperhomocysteinaemia, oxidative stress and disease (Faverzani et al., 2017). Cre is able to act as a direct antioxidant against radical and reactive species (Kolling et al., 2014; Lawler et al., 2002; Sestili et al., 2006).

Alternative treatment strategies are urgently needed. Endogenous hepatic synthesis of Cre is responsible for the formation of a substantial portion of Hcy and homocystinuric patients present alterations of oxidative stress markers. So we hypothesized that Cre supplementation will decrease the endogenous Cre synthesis and thus, consequently, Hcy production, acting as an antioxidant, reducing the harmful effects of Hcy, which would in theory be an alternative treatment strategy in patients with homocystinuria.

Methodology

Cell Culture:

Culture of immortalized commercial cell lines, three healthy control fibroblasts, five cblC deficiency and one MTHFR deficiency cell lines. Culture medium DMEM (Dulbecco's modified Eagle's medium), low glucose, GlutaMAX ™, pyruvate was used. For experiments the DMEM medium, high glucose, no glutamine, no methionine and no cysteine, was manipulated by supplementation with 153uM of cysteine, 1% of GlutaMAX

(Invitrogen, CA, USA) and 10uM of Met, plus 10% FBS (Invitrogen, CA, USA) and 1% of Penicillin-Streptomycin (10,000 U/mL) (Invitrogen, CA, USA). The cell lines were cultivated T-25 and T-75 flasks at 37°C in atmosphere of 5% CO_2. When cells had 90-100% confluence, the regular DMEM medium was replaced to the culture medium manipulated with 10uM of Met and cultivated for 48 hours, after this time the culture medium was replaced by medium with 10uM of Met plus or not 10mM Cre, and the cells were cultured for more 72 hours.

We tested doses of 2.5 mM, 5 mM and 10 mM of Cre, in the times of 1h, 3h, 24h and 72h. After the cultivation under these conditions, the cell viability assay (MTT) was perfomed (quadruplicate) to evaluate the cytotoxicity of Cre. Plate of 96 wells was used at a concentration of 5000 cells/well and incubated with or without Cre in the culture medium. The color intensity was determined in a spectrophotometer at wave length 570 nm. A dose of 10mM of Cre per 72h was determined to the further analysis.

Cell culture supernatant was collected at 72h for the measurement of interleukins 1-β, 6 and 10. Cell pellet was collected for the analysis of oxidative stress. Immediately after collection, samples were stored at −80°C for further analysis.

Oxidative stress analysis

2′7′Dichlorofluorescein oxidation assay (DCFH): Reactive species production was measured following LeBel et al., (1992) method based on 2′7′-dichlorofluorescein (H2DCF) oxidation. The reaction produces the fluorescent compound DCF which was measured at 488nm excitation and 525nm emission and the results were represented by nmol DCF/mg protein. A calibration curve was performed with purified DCF as standard.

Superoxide dismutase assay (SOD): The activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA) (Marklund, 1985). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD and the specific activity is represented as units/mg protein.

Catalase assay (CAT): activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The method used is based on the disappearance of H2O2 at 240 nm in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/ml. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein (Aebi, 1984).

Total sulfhydryl content (SH): This assay was performed as described by Aksenov & Markesbery (2001), which is based on the reduction of 5,5′-dithio-bis (2- nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Results were reported as nmol TNB/mg protein.

Cytokines assay

The culture medium was collected at 72h and the production of cytokines IL-6, IL-10 and TNF-α in were measured using ELISA kits (R&D) according to the manufacturer's instructions.

Statistics:

Statistical analysis was carried with SPSS 23. Continuous variables with a normal distribution were expressed as means ± standard deviation (SD). Differences between groups were evaluated by Student's t-test and the mixed-model ANOVA. Associations were evaluated with Spearman's correlation coefficient. All tests were 2-tailed, and $p < 0.05$ was considered significant.

Results

Treatment with Cre was not cytotoxic, with no differences in the cell viability of treated cells with 2.5mM, 5mM or 10mM of Cre when compared with control culture without Cre in times of 1, 3, 24, 48 and 72h. The cells lines were 90-100% confluent, when treated or not with 10mM of Cre. As expected Cre levels were increase in the pellet of cells in the control and patients cell lines when compared the treated and non-treated with Cre (Figure 2a).

On the oxidative stress markers in the healthy cell group after Cre treatment there was a 32% decrease in CAT levels, 27% decrease in SOD levels, 31% decrease in SH levels and 37% decrease in DCFH, but there was no statistically significant difference (Figure 2 b,c). In the patient group of cells the results were very similar with or without Cre treatment (Table 1). SH presented positive correlations with DCFH (rs: 0.796; p=

0.001), SOD (rs: 0.808; p<0.001) and CAT (rs: 0.832; p<0.001). SOD also correlated positively with DCFH (rs: 0.549; p = 0.042) and CAT (rs: 0.840; p<0.001). Regarding the cytokines, IL-1β and TNF-α were not detected in the supernatant of any cell group, and the levels of IL-6 were not statistical difference among groups.

Discussion

Several studies have indicated an association between hyperhomocysteinemia and the formation of reactive oxygen species, which may have an impact on the clinic and treatment of diseases such as homocystinuria. Endogenous hepatic synthesis of Cre is responsible for the formation of a substantial portion of Hcy, and Cre is pointed as an antioxidant compound. In this sense, we evaluated the oxidative markers: DCFH, SOD, CAT and total SH content, as well as IL-1β, TNF-α and IL-6 inflammatory interleukins in fibroblasts from patients with remethylation defects and control cells, submitted or not to supplementation of the culture medium with Cre.

After the Cre treatment the intracellular content of Cre increased in the treated groups. And in the healthy cell group there was a decrease in CAT, SOD, total SH levels and DCFH, but there was no statistically significant difference.

In homocystinuric patients Vanzin et al., (2015) found that SH content and the total antioxidant status (TAS) were significantly lower at diagnosis and in patients under treatment, suggesting that the treatment was not able to prevent the decrease in the antioxidant defenses found at diagnosis. They demonstrated that IL-6 was significantly higher in nontreated patients and a moderate reduction of these levels occurs in patients under treatment. A significant positive correlation between IL-6 levels and carbonyl group

content was observed, suggesting an association between inflammation and oxidative protein damage (Vanzin et al., 2015).

Kolling et al., (2014) showed that chronic hyperhomocysteinemia increased the DCF oxidation, an index of production of reactive species, and the TBARS levels, an index of lipid peroxidation in skeletal muscle of rats. Antioxidant enzyme, SOD, and CAT activities were also increased, but GPx activity was not altered. The contents of glutathione (GSH), SH, and carbonyl were decreased, as well as nitrite levels. Cre concurrent administration prevented the increase in SOD and CAT activities caused by Hcy. However, Machado et al., (2011) revealed that chronic administration of Hcy significantly reduced the antioxidant CAT and SOD enzyme activities in the hippocampus of rats. The same treatment increased DCFH oxidation.

Deminice et al., (2009) showed a reduction of plasma Hcy levels by Cre supplementation associated with a significant reduction of plasma lipid peroxidation biomarkers. They found significant negative correlations between plasma Cre and the plasma lipid peroxidation biomarkers (TBARS and total peroxide) which indicates the potential of Cre to remove reactive oxygen species and act as an antioxidant in a dose dependent response.

As was analyzed fibroblasts in the present study, it was not possible to assess the change in Hcy levels after Cre supplementation, but this is an important aspect of Cre as a treatment for homocystinuria. Petr et al., (2013) showed a 50% decrease in serum Hcy levels, almost reaching normal levels, in a mutant homozygous patient for a c.677C>T in the *MTHFR* gene (GenBank ID:4524) after 30 days with a daily supplement of 5g Cre monohydrate. Korzun (2004) found a 10% decrease in healthy subjects who, each day, ingested an amount of Cre equal to twice their daily creatinine excretion for 28 weeks. Van

Bavel et al., (2018) analyzed strict vegan subjects with three weeks of placebo or oral Cre supplementation, and found that Cre reduces Hcy levels among the hyperhomocysteinemic individuals.

Stead et al., (2001) supplemented healthy rats with 0.4% Cre monohydrate for two weeks and observed a 25% decrease in plasma levels of Hcy, with no difference in Met levels. In the cell culture of cardiomyocytes, Santacruz et al., (2015) used the 5mM dose of Cre supplementation, and observed a decrease in cytotoxicity, apoptosis and production of reactive oxygen species by the cells when exposed to chemotherapeutic treatment.

Other studies did not found a decreased in Hcy levels after Cre supplementation. Moraes et al., (2014) studied 40 healthy young men underwent Cre monohydrate supplementation at a dose of 20 g/day for 7 days, with no alteration in Hcy levels. Steenge et al., (2001) found no decrease in plasma Hcy in subjects who ingested 3 g Cre monohydrate/day for 61 days. Deminice et al., (2014) observed that Cre supplementation (22g/day for 7 days) in teenage athletes significantly lowered plasma guanidinoacetate compared with placebo. However, there was no effect on plasma Hcy or AdoMet and AdoHcy.

In conclusion, the present study showed that Cre seems to have the potential to reduce oxidative stress markers, even if only in some cells, which may suggest an action individual dependent, however a larger number of patients must be studied. In patients with homocystinuria, Cre supplementation may be an adjunctive treatment for decrease Hcy levels and to restore the redox homeostasis problaby induced by hiperhomocysteinemia. However, the limitations of this *in vitro* study should be considered, and the need to evaluate Cre supplementation in homocystinuric patients, since Cre is a widely used supplement with few side effects described.

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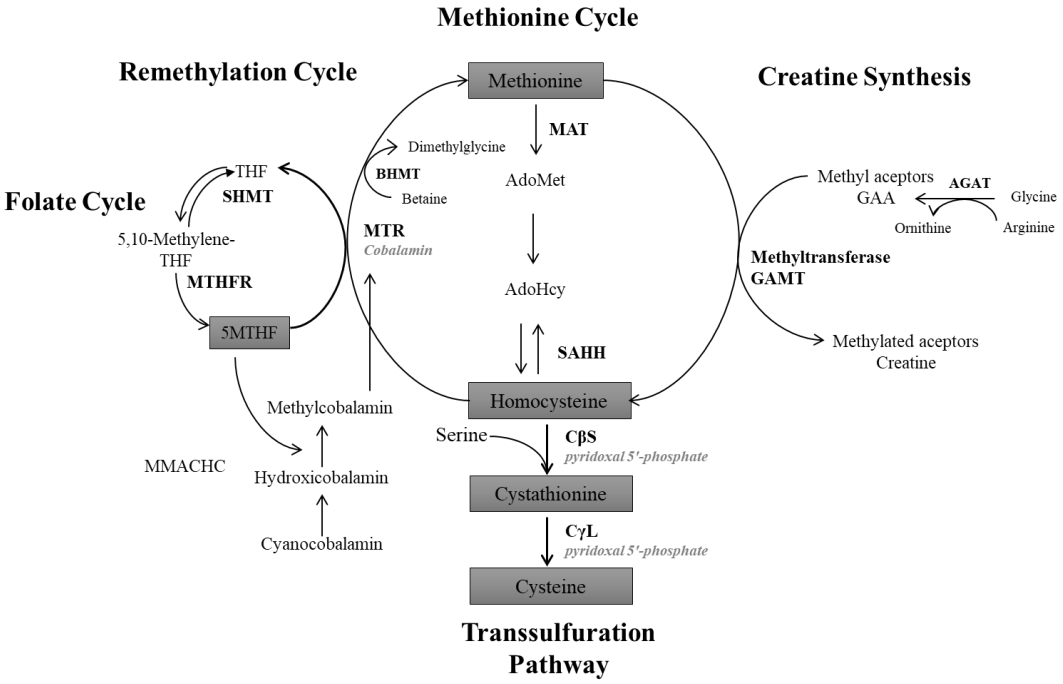

Figure 1. Homocysteine metabolism and creatine synthesis pathway. MAT: Methionine adenosyltransferase; THF: tetrahydrofolate; MTHFR: 5,10 Methylenetetrahydrofolate reductase; MTR: methionine synthase; SAHH - S-adenosyl homocysteine hydrolase; AdoHcy: S-adenosyl-homocysteine; AdoMet: S-adenosyl-l-methionine; CBS: Cystathionine-β-synthase; BHMT: Betaine-homocysteine S-methyltransferase; AGAT: L-Arginine:glycine amidinotransferase; GAA: Guanidinoacetic acid; GAMT: Guanidinoacetate methyltransferase.

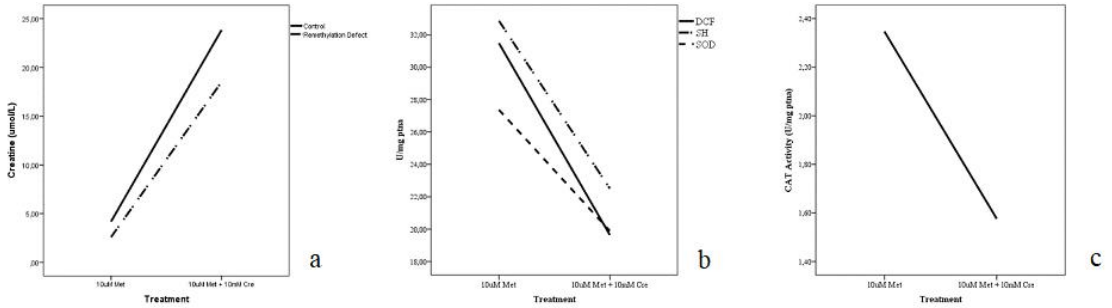

Figure 2. Creatine treatment in cell culture, a) creatine levels before and after treatment; b) DCFH, SH and SOD levels before and after treatment in control group; c) CAT levels before and after treatment in control group.

Table 1. Creatine, guanidinoacetic acid, oxidative markers and IL-6 in cell culture of remethylation defects and control groups, before and after creatine treatment.

Variable	*Control (n=3)*			*Remethylation Defect (n=6)*		
	10mM Creatine	**No Creatine**	**P**	**10mM Creatine**	**No Creatine**	**P**
Creatine	23.82 (4.50)	4.18 (1.25)	0.052	18.49 (5.77)	2.57 (0.34)	0.001
GAA	0.076 (0.035)	0.096 (0.024)	0.353	0.098 (0.028)	0.107 (0.037)	0.340
CAT	1.57 (0.34)	2.35 (0.46)	0.483	1.84 (0.75)	1.84 (0.68)	0.457
SOD	19.89 (3.38)	27.37 (2.29)	0.523	27.26 (9.86)	25.60 (7.37)	0.649
SOD/CAT	12.72 (1.02)	11.89 (1.82)	0.274	15.19 (4.01)	14.37 (1.95)	0.257
DCFH	19.65 (11.68)	31.48 (5.51)	0.149	24.60 (9.22)	27.29 (2.31)	0.108
SH	22.51 (3.37)	32.86 (6.76)	0.419	27.17 (10.06)	28.46 (4.22)	0.218
IL-6	249.0 (123.5)	283.9 (134.5)	0.906	216.4 (102.6)	246.6 (127.5)	0.482

6.5 CAPÍTULO 5: *"Homocysteine and vitamin B12 in PKU patients"*

O capítulo 5 consiste em estudo bioquímico de pacientes com PKU em diferentes momentos, avaliando os níveis de Hcy, MMA, vitamina B12 e outros, comparados a apresentação clínica. Este capítulo será apresentado na forma de artigo (*short report*), o qual será submetido para a revista Clinica Acta, cujo fator de impacto é 2,926 e o Qualis CAPES da área de genética (Ciências biológicas I) é B1. O formato obedece às normas de submissão da revista.

Homocysteine and vitamin B12 in PKU patients

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Abstract

Hyperhomocysteinemia and vitamin B12 deficiency have been reported in patients with phenylketonuria (PKU). In our study total homocysteine (tHcy) and methylmalonic acid (MMA) were measured in PKU patients in pre treatment and post treatment; patients at moments adhering to the hypoproteic diet, compared to non-adherent periods; and PKU women in the pre, post and during pregnancy stages. THcy concentrations were reduced after treatment for PKU with metabolic formula ($p=0.014$). None of the patients, except for pregnant women, had tHcy values above the normal range. In contrast, 34% of the samples had tHcy values lower than 5 umol/L. We observed a decrease in Phe, tHcy, and tyrosine levels during pregnancy time. MMA levels were not statistically different, with values within the normal range. These data indicates no B12 deficiency in patients compliant to the diet. In conclusion, in PKU patients treated with metabolic formula, tHcy is not elevated, in some patients is even in the lower normal levels, so clinical follow-up and adherence to dietary treatment are very important.

Key-words

Phenylketonuria; Homocysteine; Metilmalonic Acid; Vitamin B12.

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive metabolic disorder characterized by phenylalanine hydroxylase (PAH) deficiency, in which the essential amino acid phenylalanine (Phe) cannot be converted to tyrosine. Mutations in the gene *PAH* lead to accumulation of toxic metabolites [1-3].

The treatment for PKU is mainly dietary and consists of: restriction of dietary Phe by restricting natural proteins, typically less than 10g / day in combination with a Phe-free amino acids mixture, supplemented with vitamins and trace minerals (metabolic formula) [4]. The metabolic formula supplies 50-85% of total daily protein requirements [5]. The early initiation, shortly after birth, of a Phe restricted diet in PKU avoids neuropsychological complications [2], but nutritional deficiencies, as impaired growth, reduced bone mineral density and micronutrient deficiencies have been described [1, 2].

Vitamin B12 is mainly present in animal food and so patients treated for a protein restricted diet like PKU patients are at risk developing B12 deficiency [6, 7]. Although the metabolic formula is supplemented with B12, many patients do not take the formula as prescribed due to its low palatability. Studies on PKU patients on dietary treatment show conflicting results: some report even higher levels of B12, when compared to healthy controls [8-10]. In line Kose et al. (2018) observed higher levels of B12 in PKU patients, and that B12 deficiency was present in 15% of PKU patients versus 30% in healthy controls [11]. On the other hand, Schulpis et al. (2012) described higher levels of Hcy and lower levels of vitamin B12, B6 and folate in PKU patients adhering to the diet compared to non-adherent patients [12].

Vitamin B12 is essential for hematologic and neurologic processes, and serves as a cofactor of two enzymatic reactions: 1) conversion of methylmalonyl-CoA to succinyl-

CoA; 2) homocysteine in methionine. In vitamin B12 deficiency, methylmalonyl-coA is converted to methylmalonic acid (MMA) [14, 15]. As a consequence in B12 deficiency total homocysteine (tHcy) and methylmalonic acid (MMA) increase in plasma and are so important functional biomarkers of the vitamin B12 status [6].

PKU women with poor Phe control during pregnancy are at high risk to have a child, with mental retardation, congenital heart defects, intrauterine growth retardation, and other defects because Phe is teratogenic. Therefore, Phe levels lower than 360 umol/L prior to conception and throughout pregnancy are indicated [13, 14]. In general pregnant women with low levels of vitamin B12 and folate and/or high levels of homocysteine are at higher risk for pregnancy complications, like neural tube defects, recurrent pregnancy loss, preeclampsia, prematurity and poor birth outcomes [15, 16]. Plasma tHcy is usually lower during the first two trimesters of pregnancy, and returns to preconception concentrations during late pregnancy [17]. Murphy et al. (2002) concluded that this variation is not explained by pregnancy factors such as haemodilution, and folic acid supplementation, but may be related to hormone levels [18].

Because of the conflicting results of the B12 status in PKU patients we studied the levels of vitamin B12, MMA, Hcy, Met and other nutrients in PKU patients 1) pre and post dietary treatment 2) adhering to the low protein diet, compared to non-adherent patients; 3) in PKU women pre, during and post pregnancy stages.

2. Materials and Methods

A cross-sectional study was conducted at Hospital de Clínicas de Porto Alegre, Brazil. The study was approved by the local research ethics committee. All participants or their caretakers gave written informed consent prior to inclusion in the study.

2.1 Patients

Twenty three patients with PKU were included. Three patients had samples analyzed before and after treatment. And three pregnant patients had samples collected before, during and after pregnancy. All patients had more than one sample analyzed, with at least one moment of good metabolic control and thus possibly good adherence to the diet, and one with inadequate metabolic control not related to intercurrent illness. Patients were diagnosed by neonatal screening, except two patients who were diagnosed after a mental retardation investigation, and are under clinical follow-up at HCPA. Table 1 presents main clinical features of the PKU patients studied.

A structured form containing queries regarding diagnosis, treatment strategies, adherence to treatment, metabolic control and current health condition of PKU patients was used in the study to access information in the medical record. We correlate the values of amino acids and analytes related to Hcy with Phe levels and adherence to dietary treatment. Target Phe levels on treatment were set to < 360 μmol/L for good control and >360 μmol/L for poor metabolic control. Treatment adherence was determined by the subjective impressions of the care team at each medical center.

2.2 Biochemical analysis

Blood samples were taken after a 12-h overnight fast. Immediately after collection, samples were centrifuged for 20 min at 3000×g and plasma was isolated and stored at −80 °C for further analysis. Plasma tHcy, cysteine, and Met were measured by LC-MS/MS following a protocol adapted from Rafii et al.[19], Persichilli et al. [20], and Bártl et al. [21]. For quantification, stable isotope-labelled standards were added to the samples. Dithiothreitol (DDT) was used to reduce disulfide bonds. Next, methanol was added to the

mixture to precipitate proteins. After centrifugation (10,000 ×g), the supernatant was evaporated and butylated, and was subsequently injected into the LC-MS/MS system (Waters Quattro Premier XE, Waters Corp.).

Plasma MMA was determined by LC/MS-MS method described by Blom; van Rooij and Hogeveen [22]. After deproteinization by ultra filtration an acidified aliquot of the eluate was injected into the HPLC system for separation of MMA and succinic acid and subsequently MMA was analyzed by ESI-MS/MS. Vitamin B12 levels were measured in plasma by electrochemiluminescence using the analyser Elecsys 2010 (Roche Diagnostics GmbH, Mannheim, Germany).

Phe, tyrosine, leucine, isoleucine and valine levels were determined by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS), using the multiple reaction monitoring (MRM) mode [23]. Met, leucine, isoleucine and valine are essential amino acids acquired only by diet. Therefore, these amino acids were used to evaluate the protein intake of the patients.

2.3 Statistical analysis

Statistical analysis was carried with SPSS 23. Continuous variables with a normal distribution were expressed as means ± standard deviation (SD), whereas variables with asymmetric distribution were expressed as median (interquartile range: P25-P75). Differences between groups were evaluated by Student's t-test. Variables with asymmetrical distribution were evaluated by Mann-Whitney U-test. Associations were evaluated with Spearman's correlation coefficient. The analyses in the pregnant women between before, pregnant and after were compared by using mixed-model ANOVA. All tests were 2-tailed, and $p < 0.05$ was considered significant.

3. Results

Comparing the groups before and after treatment there is a significant difference at the age on the moment of samples collection (before: 2 [0-2] and after: 27.5 [20.7 – 41.2] months; p = 0.024). A significant difference was demonstrated with higher tHcy plasma levels before treatment (7.32 ± 3.31) when compared to samples after treatment (2.98 ± 0.81) for PKU with metabolic formula (p = 0.014). MMA levels also decreased after treatment, but with no statistical difference (Figure 1). Met, tyrosine, leucine, isoleucine and valine did not present statistical difference.

Table 1 summarizes the results about sex, Phe, Met, B12, tHcy, MMA levels and the age of patients at the sample collection in moments of good and poor metabolic control, of all samples analyzed in the study. Two out of twenty patients had levels slightly below or in the lower normal range of vitamin B12, and another patient received multivitamin supplements during the study period. Phe was significantly lower in the patients at a good moment of metabolic control compared to poor metabolic control (p<0.001). None of the patients had tHcy values above the normal range. Fifteen of 43 samples had tHcy values lower than the reference range (5 umol/L), but there was no statistical difference between moments with good or poor control. No other significant differences were evident in the evaluated parameters between the groups good and poor metabolic control.

In our study there was a positive correlation between tHcy and MMA rs= 0.486 (p=0.001), between tHcy and Cys rs= 0.528 (p<0.001) and between tHcy and age rs= 0.435 (p=0.004). Met correlated positively with tyrosine rs=0.535 (p<0.001), leucine rs=0.707 (p<0.001), isoleucine rs=0.730 (p<0.001) and valine rs=0.542 (p<0.001).

The analyses in pregnant woman showed tHcy significantly different (p<0.001) between before (22.72 ± 11.36) and pregnancy (7.66 ± 3.96) and before (22.72 ± 11.36)

and after (6.84 ± 1.73). And in tyrosine levels (p=0.001), with higher levels at the samples collected before pregnancy (79.3 ± 17.4), pregnancy (43.5 ± 18.8) and after (31.3 ± 18.7). Phe levels were significantly lower (p<0.001) during pregnancy (342.6 ± 293.1), with no statistical difference between before (1241.2 ± 409.8) and after pregnancy (879.4 ± 343.2). MMA, cysteine, methionine, leucine, isoleucine and valine showed no differences. MMA levels were not statistically different among the analyzed periods. A few samples, about 10%, presented slightly increased MMA levels, the great majority were within normal values. There were no statistical differences between the gestational trimesters.

4. Discussion

In our study a reduction in tHcy levels after treatment for PKU with metabolic formula was found. None of the PKU patients had tHcy values above the normal range, indicating no overt deficiencies of folate or B12 among PKU patients on treatment, likely due to the enrichment of the PKU formulas with B12 and folate as showed by Kose et al. (2018) [11]. The enrichment may also explain that even 34% of PKU patients had tHcy values lower than 5 umol/L.

Vugteveen, et al (2011) analyzed 75 patients with PKU on treatment, and 12 patients showed increased MMA and /or Hcy indicative of functional deficiency of vitamin B12, . There was no consistent relationship between MMA and Hcy levels and metabolic control, but they showed significant correlations between serum vitamin B12 and 1) levels of Hcy and 2) MMA, besides correlation between serum vitamin B12 and metabolic control [6].

Similar to our study, Stolen et al. (2014) showed that 68% of 34 PKU children on dietary treatment had plasma Hcy concentrations below the reference range. In this study, 91% and 53% of the studied children had plasma levels of folate and vitamin B12 above

the upper reference level, respectively [10]. At the same line, Huemer et al. (2012) reported lower levels of Hcy in plasma of 16 children and adolescents with PKU treated compared to age-matched controls, and no difference was found for folate levels [24]. However, in another study of this group, no difference was observed in Hcy levels in subjects with PKU on treatment (age: 4-20 years) and controls, although folate and vitamin B12 levels were higher in PKU patients [8].

In addition, Karam et al. (2015) showed no difference in Hcy levels among nine patients with PKU (eight children and one adult) and control group (30 healthy subjects, mean age: 12.1 years) [25]. While, Schulpis, Karikas and Papakonstantinou (2002) compared patients with non-adherent PKUs and healthy individuals, and concluded that PKU patients non-adherent to diet had low levels of vitamin B6, vitamin B12 and folate resulting in moderate hyperhomocysteinemia [12].

By analyzing pregnant women before, during, and after gestation, we observed a decrease in Phe, tHcy, and tyrosine levels during pregnancy time. THcy levels are especially elevated in the PKU women included in this study before pregnancy, mean of 22 umol/L. During pregnancy tHcy levels were within normal range, and were similar than those described in healthy pregnant women [15, 17, 18]. MMA levels were not statistically different, with values within the normal range.

Chowdhury et al. (2011) reported that pregnant women whose child had congenital heart defects, had higher levels of Hcy and s-adenosylhomocysteine and lower levels of methionine and s-adenosylmethionine. These differences were accompanied by maternal DNA hypomethylation, compared with controls [26].

5. Conclusions

In conclusion, in PKU patients treated with metabolic formula, tHcy is not elevated, in some patients is even below reference range, so clinical follow-up and adherence to dietary treatment are very important. Managing PKU patients should address adequacy of protein intake, and in addition to monitoring blood Phe, plasma amino acids, Hcy, and MMA should be evaluated to detect B12 deficiency, especially before conception to minimize risks to the fetus.

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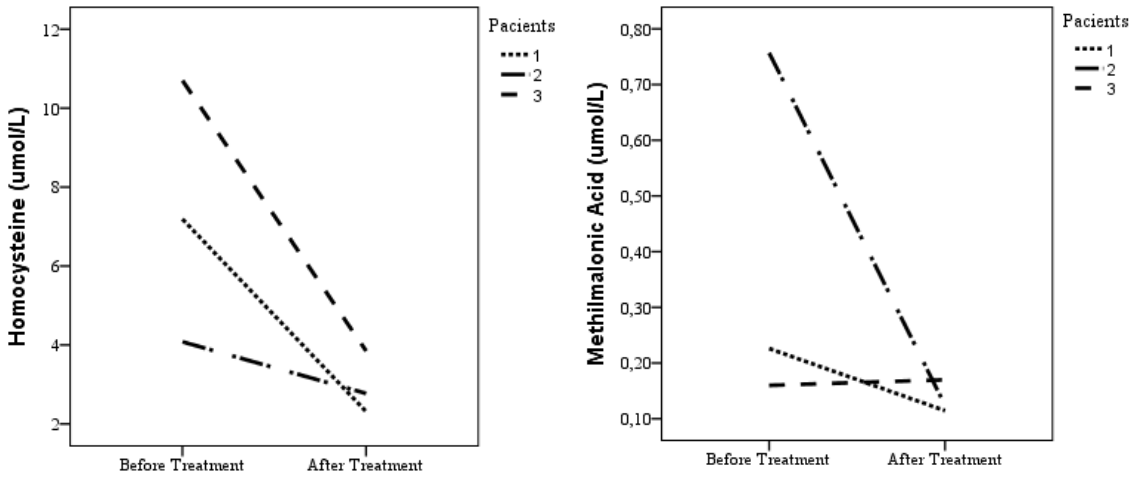
Table 1. Individual phenylalanine, methionine, B12, homocysteine and methylmalonic acid levels in patients at a moment of good and poor metabolic control.

Patient (Sex)	Good and Poor Control	Age (Years)	Phe (Umol/L)	Met (Umol/L)	B12 (Pg/Ml)	Thcy (Umol/L)	MMA
1 (F)	GC	4.5	24.2	63.9	-	10.5	0.21
	PC	3.5	556	41.3	1480	7.28	0.18
2 (M)	GC	1.7	26.8	18.2	649	2.86	0.12
	PC	2.6	371	22.4	1199	1.77	0.11
3 (M)	GC	3.5	43.8	30.1	1431	7.42	0.17
	PC	5.0	671	36.4	-	3.89	0.14
4 (F)	GC	8.0	81.6	76.2	-	10.0	0.30
	PC	7.8	413	38.6	1595	12.5	0.32
5 (F)	GC	0.1	84.4	32.9	-	3.92	0.42
	PC	0.25	1180	29.5	-	5.05	0.18
6 (F)	GC	4.3	98.6	42.9	1233	3.12	0.13
	PC	3.15	396	31.5	-	2.43	0.12
7* (M)	GC	35.5	105	23.3	760	6.80	0.11
	PC	35.8	447	16.5	-	6.05	0.10
8 (F)	GC	5.25	113	27.1	524**	6.34	0.13
	PC	5.15	512	24.1	-	4.45	0.10
9 (M)	GC	0.3	167	51.1	-	4.75	0.44
	PC	1.75	483	28.8	933	5.36	0.79
10 (M)	GC	1.0	224	61.1	965	6.24	0.15
	PC	1.1	537	18.7	-	4.92	0.14
11 (F)	GC	20	280	21.4	292	10.2	0.27
	PC	18	521	23.6	326	10.9	0.32
12* (F)	GC	60.7	289	122	1084	5.28	0.22
	PC	59.6	597	24.5	-	4.88	0.20
13 (F)	GC	6.25	317	20.8	720	7.26	0.16
	PC	6.1	565	25.0	-	9.37	0.21
14 (M)	GC	4.25	327	26.4	836	4.71	0.15
	PC	4.6	492	46.5	-	5.00	0.20
15 (F)	GC	3.8	329	31.1	846	5.91	0.28
	PC	2.3	789	41.4	780	6.76	0.21
16 (M)	GC	15.1	333	29.2	-	10.0	0.18
	PC	16.1	637	42.7	457	8.37	0.16
17 (M)	GC	4.4	333	53.8	-	8.23	0.22
	PC	2.8	544	17.1	1060	4.89	0.20
18 (M)	GC	15.3	343	24.5	306	7.52	0.34
	PC	14.5	464	24.2	-	11.1	0.31
19 (M)	GC	2.0	358	18.9	-	3.94	0.16
	PC	1.75	456	18.5	660	3.76	0.18
20 (M)	GC	8.0	359	39.2	1036	6.40	0.25
	PC	9.5	723	73.6	-	5.93	0.22
MEAN (SD)	GC	10.2 (2.4-13.3)	284 (99.6-333)	30.59 (23.6-53.1)	836 (586-1060)	6.57 (2.35)	0.18 (0.15-0.28)
	PC	10.0 (2.4-13.2)	502 (449 – 585)	26.8 (22.7 – 40.6)	933 (558-1339)	6.23 (2.89)	0.20 (0.14-0.24)
P		**NS**	**<0.001**	**NS**	**NS**	**NS**	**NS**

* Patients diagnosed after clinical presentation of mental retardation; ** Patient supplemented with polyvitamin; - Data not available.

Figure 1. Homocysteine and methylmalonic acid in PKU patients before and after treatment



7. DISCUSSÃO

Homocistinúria é um grupo de EIM dos aminoácidos sulfurados. Ao comparar as principais causas de homocistinúria (deficiência de CBS, cblC e MTHFR) observa-se que a manifestação bioquímica em comum é o acúmulo de Hcy, enquanto os outros aminoácidos sulfurados apresentam perfis diversos e até inversos. Pacientes com HCU apresentam altos níveis de Met com baixa cistationina e Cys, enquanto pacientes com defeitos de remetilação apresentam Met normal ou diminuída e a cistationina aumentada. Eventos tromboembólicos, atraso no desenvolvimento e convulsões são apresentações clínicas encontradas em todas as homocistinurias. No entanto, as complicações do SNC diferem em uma ampla variedade de apresentações e severidades e são aparentemente menos pronunciadas na HCU. Além disso, pacientes com defeitos de remetilação tipicamente não apresentam *ectopia lentis* e distúrbios ósseos como alta estatura e osteoporose.

O principal objetivo do tratamento em todas as formas de homocistinúria é reduzir a Hcy plasmática. Na HCU isso resulta em uma melhora clínica marcante (Morris *et al.*, 2017), incluindo a redução de eventos vasculares (Yap *et al.*, 2000; Yap *et al.*, 2001a) e a prevenção do retardo mental (Yap *et al.*, 2001b) e anomalias ósseas (Lim & Lee, 2013). No entanto, mesmo em tratamento, os níveis de Hcy plasmática permaneceram claramente elevados, em particular nos pacientes não-responsivos à piridoxina (Wilcken & Wilcken, 1997; Yap *et al.*, 2000). Os pacientes tratados com cblC têm, em geral, uma redução acentuada de tHcy, abaixo dos níveis encontrados em pacientes com HCU que são não-responsivos. No entanto, problemas neurológicos e oftalmológicos geralmente permanecem em cblC (Fischer *et al.*, 2014).

O significado de níveis elevados de Hcy plasmática também é comprometido por numerosos estudos sobre a mortalidade e o risco de doenças cardiovasculares. Embora o nível elevado de Hcy esteja relacionado a um risco aumentado de aterosclerose e trombose venosa, em grandes estudos controlados randomizados, a redução de Hcy por tratamento com folato e vitamina B12 não reduziu o risco de eventos tromboembólicos (Martí-Carvajal *et al.*, 2017). As observações em homocistinúrias e a elevação da Hcy como fator de risco para doença vascular causam fortes dúvidas se os níveis elevados de Hcy estão direta e causalmente relacionados às apresentações clínicas.

A prevalência de HCU é estimada em ao menos 0.29 a 1 a cada 100,000 indivíduos, mas varia dramaticamente entre regiões de 416:100.000 uma ilha habitada por uma tribo Tao em Taiwan e 55:100.000 no Catar para menos de um em um milhão na população Han de Taiwan (El-Said *et al.*, 2006; Gan-Schreier *et al.*, 2010; Lu *et al.*, 2012). Através da frequência de heterozigotos para o gene *CBS*, reportados no banco dados genéticos, gnomAD, estimamos a incidência mínima mundial de HCU em cerca de 0,38 em 100.000 indivíduos. Estratificando populações por ancestralidade, a maior incidência foi encontrada em europeus e latino americanos. Uma incidência muito menor foi encontrada em asiáticos. As incidências nestes grupos etnicos se aproximam das encontradas pelo *screening* bioquímico neonatal. Por exemplo, em Europeus estimamos 0,72:100.000 indivíduos, calculados pelo número de heterozigotos no gnomAD, versus 0,77:100.000 de acordo com a triagem neonatal e em Asiáticos 0,02:100.000 indivíduos versus 0,07:100.000, respectivamente.

No Brasil, não há dados epidemiológicos sobre a HCU. Utilizando a prevalência mundial mínima estimada em nosso estudo de 0,38:100.000 indivíduos, se esperaria quase 800 pacientes portadores da doença no país. Este número está muito aquém da amostra obtida em nosso estudo clínico (n=72) e do número de pacientes em acompanhamento estimado pela Associação Brasileira de Homocistinúria (80-100 pacientes, relato verbal). Isto sugere uma elevada taxa de subdiagnóstico no país.

Os dados clínicos obtidos no estudo reforçam esta hipótese. Observamos maior proporção de pacientes com fenótipo grave (não responsivos à piridoxina e múltiplas manifestações clínicas). Além disso, o atraso do diagnóstico após o início dos sintomas sugere baixo acesso aos serviços de saúde capacitados para o diagnóstico e/ou baixo conhecimento dos profissionais de saúde sobre a doença. Também deve-se considerar que os testes bioquímicos e moleculares para diagnóstico de HCU estão disponíveis em poucos centros do país, o que dificulta e atrasa a confirmação diagnóstica.

A dificuldade no controle metabólico nos pacientes não responsivos à piridoxina, bem como a baixa adesão à dieta pobre em Met/fórmula metabólica revelaram problemas no manejo deste grupo de pacientes. Além das dificuldades na obtenção da fórmula, podem ter contribuído para este resultado o preparo insuficiente dos profissionais de saúde para prescrição deste tipo de dieta e dificuldade de compreensão da importância da adesão ao tratamento por parte dos pacientes e familiares. Estes achados também reforçam a

necessidade de protocolos clínicos e diretrizes para o diagnóstico de manejo da HCU no país. A alta prevalência de déficit cognitivo e distúrbios psiquiátricos na HCU também pode prejudicar a adesão ao tratamento (Abbott *et al.*, 1987; S.H. *et al.*, 2001).

Diversos pacientes com HCU, incluídos em nosso artigo sobre descrição clínica, foram diagnosticados através de pesquisa molecular realizada durante o doutorado. Além disso, publicamos um estudo com descrição molecular de 35 pacientes, e no momento temos dados de mais 18 pacientes, para publicação de um *update*. Nos últimos quatro anos identificamos cinco novas variantes ou ainda não descritas em pacientes com HCU. A variante p.Trp323Ter é descrita apenas na Arábia Saudita, sendo muito frequente nesse país e foi identificada em homozigose em sete pacientes brasileiros, todos da região Nordeste; iremos investigar e descrever com detalhes esses achados. Além disso, iniciamos a pesquisa molecular para identificação de pacientes com deficiência de MTHFR, e o sequenciamento de cDNA do gene *CBS* para caracterização de alterações de splicing e também identificação de variantes possivelmente não detectadas pelo sequenciamento de DNA.

Pacientes com homocistinúria, mesmo em tratamento, mantêm altos níveis de Hcy, e diversos estudos indicam uma associação entre a hiper-homocisteinemia e a formação de espécies reativas de oxigênio, que podem ter impacto na clínica e no tratamento desses pacientes. Estudamos o efeito da suplementação de Cre em cultivo celular de pacientes com homocistinúria, uma vez que a síntese hepática endógena de Cre é responsável pela formação de uma porção substancial de Hcy, e a Cre é apontada como um composto antioxidante. Em nosso estudo *in vitro* observamos aumento do conteúdo intracelular de Cre nos grupos tratados. No grupo de células saudáveis após o tratamento com Cre houve uma diminuição em CAT, SOD, SH e DCFH, mas sem diferença estatística, possivelmente devido ao pequeno tamanho amostral.

Em pacientes com homocistinúria, Vanzin *et al.* (2015) relataram que o teor de SH e o estado antioxidante total são baixos no diagnóstico e em pacientes em tratamento, sugerindo que o tratamento não é capaz de prevenir a diminuição das defesas antioxidantes encontradas no diagnóstico. Em ratos submetidos a hiper-homocisteinemia crônica, a oxidação de DCFH, um índice de produção de espécies reativas, e os níveis de TBARS, um índice de peroxidação lipídica no músculo esquelético, aumentaram. A atividade das enzimas antioxidantes, SOD e CAT também aumentaram, mas a atividade da GPx não foi

alterada. Os teores de glutationa, SH e carbonila foram diminuídos, assim como os níveis de nitrito. A administração concomitante de Cre impediu o aumento das atividades de SOD e CAT causadas pela hiper-homocisteinemia (Kolling *et al.*, 2014). No entanto, Machado *et al.*, (2011) relataram que a administração crônica de Hcy reduziu significativamente as atividades antioxidantes de CAT e SOD no hipocampo de ratos.

Hiper-homocisteinemia e deficiência de vitamina B12 também foram relatadas em pacientes com PKU. Em nosso estudo, foi encontrada uma redução nos níveis de tHcy após o tratamento de pacientes com PKU com dieta e fórmula metabólica. Nenhum dos pacientes com PKU apresentava valores de tHcy acima do intervalo normal, indicando não haver deficiências evidentes de folato ou vitamina B12 durante o tratamento, provavelmente devido ao enriquecimento das fórmulas com esses compostos, como previamente demonstrado (Kose & Arslan, 2018). O enriquecimento também pode explicar que até 34% dos pacientes com PKU tinham valores de tHcy menores que 5 umol/L.

Semelhante ao nosso estudo, Stølen *et al.* (2014) mostraram que 68% de 34 crianças com PKU em tratamento dietético tinham concentrações plasmáticas de Hcy abaixo do intervalo de referência. Neste estudo, 91% e 53% das crianças estudadas apresentaram níveis plasmáticos de folato e vitamina B12 acima do nível de referência, respectivamente. No entanto, em outro estudo nenhuma diferença foi observada nos níveis de Hcy em indivíduos com PKU em tratamento (idade: 4-20 anos) e controles, embora os níveis de folato e vitamina B12 fossem maiores em pacientes com PKU (Huemer *et al.*, 2008).

Ao analisar as gestantes antes, durante e após a gestação, observamos uma diminuição nos níveis de Phe, tHcy e tirosina durante o período gestacional. Os níveis de tHcy foram especialmente elevados nas mulheres com PKU incluídas neste estudo antes da gravidez, com média de 22 umol/L. Durante a gravidez, os níveis de tHcy estavam dentro da faixa normal e eram similares aos descritos em gestantes saudáveis (Murphy *et al.*, 2002; Murphy *et al.*, 2004; Gaiday *et al.*, 2018). Os níveis de MMA não foram estatisticamente diferentes dentre os períodos comparados, com valores dentro da faixa normal. Chowdhury *et al.* (2011) relataram que gestantes de crianças com cardiopatias congênitas apresentavam níveis mais elevados de Hcy e AdoHcy e menores níveis de Met e AdoMet. Essas diferenças foram acompanhadas por hipometilação do DNA materno, em comparação com os controles.

8. CONCLUSÕES

As conclusões do trabalho serão apresentadas por objetivo.

I. Caracterizar e comparar as principais causas de homocistinúria: homocistinúria por deficiência de CBS, cblC e MTHFR

A manifestação bioquímica comum às deficiência de CBS, cblC e MTHFR é o acúmulo de tHcy e o tratamento da homocistinúria tem se concentrado principalmente na redução dos níveis de Hcy. Os outros aminoácidos sulfurados apresentam perfis diversos e até inversos. A deficiência de CBS mostra metionina alta com baixa cistationina e Cys, enquanto nos defeitos de remetilação, a metionina é normal ou diminuída e a cistationina aumentada. No momento do diagnóstico, o folato é tipicamente baixo ou (funcionalmente) deficiente em todas as homocistinúrias. Alguns achados clínicos comuns dentre as homocistinúrias, são atraso no desenvolvimento e convulsões, bem como eventos tromboembólicos. No entanto, as complicações do SNC diferem em uma ampla variedade de apresentações e severidades entre as diferentes formas de homocistinúria e são aparentemente menos pronunciadas na deficiência de CBS. Além disso, pacientes com defeitos de remetilação tipicamente não apresentam *ectopia lentis* e distúrbios ósseos, estatura alta e osteoporose, sintomas comuns na HCU. Alterações hematológicas, como anemia megaloblástica, neutropenia e trombocitopenia, são achados específicos de deficiência de cblC.

II. Discutir o papel da homocisteína na patogenicidade das homocistinúrias.

Há pouca sobreposição nas apresentações bioquímicas e clínicas das três formas mais comuns de homocistinúria. A clara diferença nas apresentações clínicas nos faz questionar se a própria Hcy é o principal metabólito patológico. Na deficiência de CBS, a redução dos níveis de Hcy, após tratamento, resulta em um desfecho clínico marcante, incluindo a redução de eventos vasculares, prevenção do retardo mental e anomalias ósseas. Apesar disso, os níveis de Hcy no plasma permaneceram claramente elevados, em particular nos pacientes não responsivos à piridoxina. Já os pacientes tratados com cblC têm, em geral, uma redução acentuada de tHcy abaixo dos níveis encontrados em pacientes com

deficiência de CBS que não são responsivos. No entanto, problemas neurológicos e oftalmológicos geralmente permanecem em pacientes com deficência de cblC. O significado de níveis elevados de Hcy plasmática também é comprometido por numerosos estudos sobre a mortalidade e risco de doenças cardiovasculares. Embora o nível elevado de tHcy esteja relacionado a um risco aumentado de aterosclerose e trombose venosa, a redução de tHcy por folato e vitamina B12 não reduziu o risco de desfechos vasculares em grandes estudos controlados randomizados. As observações de homocistinúria, e elevação da homocisteína como fator de risco para doença vascular geram fortes dúvidas se os níveis elevados de homocisteína estão direta e causalmente relacionados às apresentações clínicas.

III. Caracterizar o perfil clínico da homocistinúria clássica no Brasil.

Dados clínicos de 72 pacientes (60 famílias) em acompanhamento em serviços de genética de todas as regiões do Brasil foram analisados. As manifestações oculares foram as mais precoces e prevalentes na amostra, bem como a principal suspeita diagnóstica, corroborando com dados da literatura. Observou-se maior prevalência de pacientes não responsivos à piridoxina, e uma pequena porção dos pacientes não responsivos manteve os níveis de homocísteina indicados (<100umol/L). Os tratamentos mais comuns foram respectivamente: piridoxina, ácido fólico, betaína, vitamina B12 e dieta restrita em metionina associada ao uso de fórmula metabólica. A maioria dos pacientes teve diagnóstico tardio, mesmo após apresentação de sintomas.

IV. Estimar a incidência de homocistinúria clássica com base no número de heterozigotos em bancos de dados genômicos.

Dentre as mutações no gene *CBS*, p.Ile278Thr é a variante geograficamente mais difundida. Conforme a frequência de heterozigotos para *CBS* no banco de dados *GnomAD*, a incidência mundial de HCU foi calculada em aproximadamente 0,38:100.000 indivíduos. Estratificando populações por ancestralidade, as maiores incidências foram encontradas em Europeus e Latino Americanos, e a menor incidência foi encontrada em Asiáticos. As incidências nestas populações corroboram com as encontradas por *screening* bioquímico neonatal. O grande número de indivíduos analisados e a cobertura populacional do banco de dados utilizado permitem grande precisão na estimativa de incidência. A incidência

estimada de HCU, que é um distúrbio tratável, apóia a implementação da triagem neonatal na Europa (~ 0,72:100.000) e na América Latina (~ 0,45:100.000).

V. Avaliar a ação antioxidante da creatina em cultura celular de fibroblastos com defeito de remetilação da homocisteína e controles.

Observamos que o conteúdo intracelular de Cre aumentou nos grupos tratados, e no grupo de células saudáveis após o tratamento com Cre houve uma redução na CAT, SOD, SH e DCFH, mas não houve diferença estatisticamente significativa, provavelmente devido ao pequeno número amostral. Portanto, Cre tem o potencial de reduzir marcadores de estresse oxidativo, mesmo que apenas em algumas células, o que pode sugerir uma ação individuo-dependente. Em pacientes com homocistinúria, a suplementação de Cre pode ser um tratamento adjuvante para restaurar a homeostase redox causada por hiperhomocisteinemia.

VI. Avaliar os níveis de homocisteína, ácido metilmalônico e vitamina B12 em pacientes com fenilcetonúria.

Nossos resultados indicam que o tratamento de pacientes PKU com fórmula metabólica reduz nos níveis de tHcy. Nenhum dos pacientes com PKU apresentou valores de tHcy acima da faixa normal, indicando que não há deficiências evidentes de folato ou vitamina B12 entre os pacientes em tratamento, provavelmente devido ao enriquecimento das fórmulas metabólicas com vitamina B12 e folato. Nas pacientes grávidas, os níveis de fenilalanina foram menores durante a gestação, bem como os níveis de Hcy, indicando melhor aderência ao tratamento. Concluímos que o acompanhamento clínico e adesão ao tratamento dietético são muito importantes. O manejo de pacientes com PKU deve abordar a adequação da ingestão de proteínas, além de monitorar os níveis de fenilalanina, os aminoácidos plasmáticos, Hcy e MMA, que devem ser avaliados para detectar a deficiência de vitamina B12, especialmente antes da concepção, para minimizar os riscos para o feto.

9. PERSPECTIVAS

Como continuidade dessa pesquisa, os seguintes estudos serão ou estão sendo desenvolvidos pelo grupo de pesquisa:

- Ensaio clínico com suplementação de creatina em pacientes com homocistinúria;
- Sequenciamento de cDNA de pacientes com homocistinúria clássica onde foi encontrado apenas uma mutação, ou ainda que apresentem variantes próximas à sítios de splicing;
- Caracterização clínica e molecular de pacientes brasileiros com suspeita de homocistinúria por deficiência de MTHFR. Até o momento realizamos a análise molecular de 16 pacientes, foram identificados polimorfismos em todos os indivíduos, além de uma mutação ainda não descrita e uma variante com possível alteração de splicing. Assim, nenhum dos pacientes com suspeita de deficiência de MTHFR teve seu diagnóstico confirmado.

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11. APÊNDICES

Apêndice 1 - Artigo publicado no Molecular Genetics & Genomic Medicine, 2018: "*CBS mutations are good predictors for B6-responsiveness: A study based on the analysis of 35 Brazilian Classical Homocystinuria patients*"

Received: 20 May 2017 | Revised: 13 September 2017 | Accepted: 22 September 2017

DOI: 10.1002/mgg3.342

ORIGINAL ARTICLE

WILEY Molecular Genetics & Genomic Medicine Open Access

CBS mutations are good predictors for B6-responsiveness: A study based on the analysis of 35 Brazilian Classical Homocystinuria patients

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Abstract

Background: Classical homocystinuria (HCU) is a monogenic disease caused by the deficient activity of cystathionine β-synthase (CβS). The objective of this study was to identify the *CBS* mutations in Brazilian patients with HCU.

Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Deutscher Akademischer Austauschdienst

Methods: gDNA samples were obtained for 35 patients (30 families) with biochemically confirmed diagnosis of HCU. All exons and exon-intron boundaries of *CBS* gene were sequenced. Gene expression analysis by qRT-PCR was performed in six patients. Novel missense point mutations were expressed in *E. coli* by site-directed mutagenesis.

Results: Parental consanguinity was reported in 16 families, and pyridoxine responsiveness in five (15%) patients. Among individuals from the same family, all presented the same phenotype. Both pathogenic mutations were identified in 29/30 patients. Twenty-one different mutations were detected in nine exons and three introns; being six common mutations. Most prevalent were p.Ile278Thr (18.2%), p.Trp323Ter (11.3%), p.Thr191Met (11.3%), and c.828+1G>A (11.3%). Eight novel mutations were found [c.2T>C, c.209+1delG, c.284T>C, c.329A>T, c.444delG, c.864_868delGAG c.989_991delAGG, and c.1223+5G>T]. Enzyme activity in *E. coli*-expressed mutations was 1.5% for c.329A>T and 17.5% for c.284T>C. qRT-PCR analysis revealed reduced gene expression in all evaluated genotypes: [c.209+1delG; c.572C>T]; [c.2T>C; c.828+1G>A]; [c.828+1G>A; c.1126G>A]; [c.833T>C; c.989_991delAGG]; [c.1058C>T; c.146C>T]; and [c.444delG; c.444delG]. The expected phenotype according to the genotype (pyridoxine responsiveness) matched in all cases.

Conclusions: Most patients studied were pyridoxine nonresponsive and presented early manifestations, suggesting severe phenotypes. Many private mutations were observed, but the four most prevalent mutations together accounted for over 50% of mutated alleles. A good genotype–phenotype relationship was observed within families and for the four most common mutations.

KEYWORDS
classical homocystinuria, CβS deficiency, CβS expression, *CBS* mutations, homocysteine

1 | INTRODUCTION

Classical homocystinuria (HCU; OMIM 236200) is an inborn error of metabolism caused by deficient activity of cystathionine β-synthase (CβS; EC 4.2.1.22). This enzyme catalyzes the first step of the transsulfuration pathway, whereby homocysteine is condensed with serine to form cystathionine. CβS deficiency leads to significantly elevated plasma levels of homocysteine and methionine and low levels of cysteine. HCU is inherited in an autosomal recessive pattern, and its worldwide prevalence is estimated between 1:100,000 and 1:344,000 (Moorthie, Cameron, Sagoo, Bonham, & Burton, 2014; Mudd, Levy, & Skovby, 2001; Skovby, Gaustadnes, & Mudd, 2010).

From a clinical standpoint, the classical signs of the disease are lens dislocation, thromboembolism, intellectual disability, psychiatric disorders, osteoporosis, and marfanoid features (Mudd et al., 1985, 2001). The treatment strategies include supplementation of pyridoxine (CβS cofactor), folic/folinic acid, betaine, and a methionine-restricted diet (Morris et al., 2016; Schiff & Blom, 2012). Usually, patients who respond to pyridoxine supplementation exhibit a milder phenotype and have a better prognosis (Mudd et al., 1985; Skovby et al., 2010).

The *CBS* gene is located on chromosome 21q22.3. It spans 23 exons, with exons 1–16 comprising the coding region, which encodes a 551-amino acid polypeptide. The 5′-UTR region of the gene is formed by one of five alternative exons (−1a to −1e), in addition to exon 0. The 3′-UTR region is encoded by exons 16 and 17 (Bao, Vlcek, Paces, & Kraus, 1998; Kraus et al., 1998). Over 160 different mutations in *CBS* have been reported, most of them being private. However, taken together, the four most prevalent mutations (p.Ile278Thr, p.Thr191Met, p.Gly307Ser, and p.Arg336Cys) account for more than half of all HCU alleles worldwide (Kraus, 2017). While the first of these mutations is panethnic, the other three follow rather well-demarcated geographic and ethnic distributions (Cozar et al., 2011; El-Said et al., 2006; Gallagher et al., 1995; Porto et al., 2005; Shih et al., 1995;

Urreizti et al., 2006). The molecular bases of HCU in Brazil are poorly characterized. In the only study published (Porto et al., 2005), *CBS* analysis performed by RFLP and SSCP is reported for 14 patients (11 unrelated) followed in a single medical center located in southeast Brazil. The common mutations p.Ile278Thr and p.Thr191Met were detected at a frequency of 13.6% each.

A consistent genotype–phenotype correlation is described for some frequent mutations. For instance, p.Ile278Thr mutation is usually associated with milder phenotypes and pyridoxine responsiveness (Kraus, 2017; Shih et al., 1995). It is also reported that homozygotes for this mutation may have a higher risk of developing thromboembolism instead of other HCU symptoms (Magner et al., 2011; Skovby et al., 2010). Patients carrying the Latin/Iberian p.Thr191Met mutation are usually nonresponsive to pyridoxine, but a great variability of severity and clinical symptoms can be observed (Cozar et al., 2011; Urreizti et al., 2006).

The present study sought to establish a broad genetic characterization of HCU in Brazil, performing *CBS* analysis in HCU patients that are being followed at several centers nationwide.

2 | METHODS

The present study was approved by the local research ethics committee (Hospital de Clínicas de Porto Alegre, Brazil). Collection procedures were conducted only after participants/legal guardians had agreed to take part in the investigation and provided written informed consent. To be included in the study, the patient should have been previously diagnosed as having HCU according to the following criteria: (1) presence of high levels of homocysteine in plasma; (2) presence of normal or high levels of methionine in plasma; AND (3) presence of clinical picture compatible with HCU. As public neonatal screening for HCU is not available in Brazil, all patients were late diagnosed (e.g., diagnosed after the starting of clinical manifestations).

2.1 | Patients

The study sample comprised of 35 Brazilian HCU patients, from 30 different families. Families from 4/5 regions of Brazil were represented: south (n = 12), southeast (n = 11), northeast (n = 6), and midwest (n = 1) (Table 1). In addition, RNA samples were obtained from six patients. In 10 families, the genetic variant(s) found in the probands were also confirmed in at least one parent.

Patients were recruited through contact with physicians involved in care and/or research activities at medical genetics centers across the country. Overall, 13 medical centers participated in the study. Some patients have been followed in different medical centers in Brazil and even abroad throughout their lives. Thus, it may be possible that they might have been or will be studied and described elsewhere. To the best of our knowledge, however, only one patient in our sample (patient #30) might have been already described in the *CBS* mutation database (Kraus, 2017), although no clinical data is available there.

Pyridoxine responsiveness was the clinical parameter used to evaluate genotype–phenotype relationship. For the purposes of this study, patients were classified as responsive if they achieved target homocysteine levels (<100 μmol/L) on pyridoxine alone or pyridoxine + folic acid (regardless of the number of weeks since testing) (Morris et al., 2016). All other patients were classified as nonresponsive to pyridoxine. The genotypes found were compared with other family members and with previously reported cases.

2.2 | *CBS* sequencing

Genomic DNA was extracted from whole blood using the commercially available Easy-DNA™ gDNA Purification Kit (Invitrogen), following manufacturer instructions. Exons 1–14 and 16 and the exon/intron junctions of the *CBS* were amplified by conventional PCR, using primers and reaction conditions previously described elsewhere (Kruger, Wang, Jhee, Singh, & Elsas, 2003). The following primers were designed to amplify exon 15: forward, CCA CAGGAAGAGTTGGGAGG; reverse, TGAGAGCCATTC TGAGGGGT. After amplification, fragments were purified and sequenced by the Sanger method. The sequence found was compared to the GenBank reference sequence (NG_008938.1). Any mutations identified were confirmed by repetition of amplification and sequencing reactions. Furthermore, parental DNA was used whenever available to confirm that mutations were in *trans* position.

Missense mutations not previously described in the literature were analyzed *in silico* in the PolyPhen2 (Polymorphism Phenotyping), MutPred, and SIFT (Sorting Intolerant From Tolerant) software. In addition, a group of 100 controls were tested for the novel c.2T>C (exon 1), c.284T>C (exon 2), and c.329A>T (exon 3) mutations and for the previously described c.828+1G>A mutation (intron 7). Testing for c.2T>C was performed by the restriction fragment length polymorphism (RFLP) method with the *NlaIII* restriction enzyme, whereas the other mutations were analyzed by sequencing of the mutation-containing exon.

TABLE 1 *CBS* analysis—pathogenic mutations found in patients with classical homocystinuria (n = 35)

Patient	Sex	Origin (Brazilian region)	Allele 1		Allele 2		Cons.	Age at inclusion (years)	Age of onset (years)	B6 response found	B6 response expected[a]
			cDNA	Protein	cDNA	Protein					
1a	M	S	c.253G>A[c]	p.Gly85Arg	c.253G>A	p.Gly85Arg	Y	36	6	N	N
1b	F	S	c.253G>A[c]	p.Gly85Arg	c.253G>A	p.Gly85Arg	Y	27	NA	N	N
1c	F	S	c.253G>A[c]	p.Gly85Arg	c.253G>A	p.Gly85Arg	Y	31	7	N	N
2	M	S	c.833T>C	p.Ile278Thr	c.833T>C	p.Ile278Thr	Y	35	0.2	Y	Y
3	M	S	c.833T>C	p.Ile278Thr	c.833T>C	p.Ile278Thr	N	35	7	Y	Y
4	F	SE	c.833T>C[c]	p.Ile278Thr	c.833T>C[d]	p.Ile278Thr	Y	18	15	Y	Y
5	M	SE	c.833T>C	p.Ile278Thr	c.28delG	p.Val10 *fs*	N	26	1	Y	Y/N[b]
6	M	SE	c.833T>C	p.Ile278Thr	c.451G>A	p.Gly151Arg	N	23	4	Y	Y/N[b]
7	F	S	c.833T>C	p.Ile278Thr	**c.989_991delAGG**	**p.(Glu330del)**	N	28	20	N	Y/N[b]
8	M	NE	c.833T>C	p.Ile278Thr	c.828+1G>A	p.?	N	16	4	N	N[b]
9	F	S	c.828+1G>A[c]	p.?	c.1126G>A	p.Asp376Asn	N	23	5	N	N[b]
10	M	SE	c.828+1G>A[d]	p.?	**c.2T>C**[c]	**p.?**	N	13	1	N	N[b]
11	F	S	c.828+1G>A	p.?	c.691G>C	p.Ala231Leu	N	13	1	N	N[b]
12	M	NE	c.828+1G>A	p.?	c.828+1G>A	p.?	Y	8	1	N	N[b]
13	M	SE	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Y	26	19	N	N
14	M	SE	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Y	10	5	N	N
15	F	S	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Y	18	5	N	N
16	M	SE	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Y	19	4	N	N
17	M	S	c.572C>T	p.Thr191Met	**c.209+1delG**[c]	**p.?**	N	14	8	N	N[b]
18a	M	SE	c.969G>A[c]	p.Trp323Ter	c.969G>A[d]	p.Trp323Ter	Y	17	6	N	NA
18b	M	SE	c.969G>A[c]	p.Trp323Ter	c.969G>A[d]	p.Trp323Ter	Y	6	1	N	NA
19	M	NE	c.969G>A	p.Trp323Ter	c.969G>A	p.Trp323Ter	N	15	1	N	NA
20	M	NE	c.969G>A	p.Trp323Ter	c.969G>A	p.Trp323Ter	N	10	7	N	NA
21a	M	SE	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Y	15	7.5	N	N
21b	F	SE	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Y	14	NA	N	N
22	F	SE	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Y	17	3	N	N
23	M	S	**c.284T>C**[c]	**p.Ile95Thr**	**c.284T>C**	**p.Ile95Thr**	Y	18	1	N	NA
24	M	S	c.1058C>T[c]	p.Thr353Met	c.146C>T	p.Pro49Leu	N	19	3	Y	Y[b]

(Continues)

TABLE 1 (Continued)

Patient	Sex	Origin (Brazilian region)	Allele 1		Allele 2		Cons.	Age at inclusion (years)	Age of onset (years)	B6 response found	B6 response expected[a]
			cDNA	Protein	cDNA	Protein					
25	F	S	c.1126G>A[c]	p.Asp376Asn	c.1126G>A[d]	p.Asp376Asn	Y	14	1.5	N	N
26	M	S	**c.444delG[c]**	**p.(Asn149*fs*)**	**c.444delG**	**p.(Asn149*fs*)**	Y	22	1.5	N	NA
27	M	NE	**c.329A>T**	**p.Glu110Val**	c.770C>T	p.Thr257Met	N	16	3	N	N[b]
28a	F	NE	**c.1223+5G>T**	**p.?**	**c.1223+5G>T**	**p.?**	Y	5	3	NA	NA
28b	F	NE	**c.1223+5G>T**	**p.?**	**c.1223+5G>T**	**p.?**	Y	7	3	NA	NA
29	F	CW	**c.864_868delGAG**	**p.(Glu289del)**	**c.864_868delGAG**	**p.(Glu289del)**	Y	17	6	N	NA
30	M	SE	c.209+1G>A	p.?	NI	NI	N	37	6	N	N[b]

Novel mutations are set in bold. Patients represented by the same number belong to the same family. M, male; F, female; S, south; SE, southeast; NE, northeast; CW, central-west; Cons, consanguinity; Y, yes; N, no; B6, pyridoxine; NA, not available; NI, not identified.

[a]According to previously described cases in the literature (Kraus, 2017), all partially responsive patients were considered as nonresponsive.

[b]Estimated; no identical genotype reported previously.

[c]Mother heterozygous for mutation.

[d]Father heterozygous for mutation.

2.3 | Homology modeling

Structural analyses were performed to investigate the structural and stability alteration of the novel coding *CBS* missense variants from the native CbS protein structure. The selected mutant models (c.284T>C; p.Ile95Thr and c.329 A>T; p.Glu110Val) were generated using SWISS-MODEL and its automated server based on the target-template alignment using ProMod3. The crystal structure of the CbS protein was retrieved from the Protein Data Bank (PDB ID 4COO, resolution at 2 Å) (McCorvie et al., 2014). Coordinates which are conserved between the target and the template are copied from the template to the model. Finally, the geometry of the resulting model is regularized by using a force field. In case loop modeling with ProMod3 fails, an alternative model is built with PROMOD-II (Guex and Peitsch, 1997). The Swiss-PDB viewer (version 4.1.0) was utilized for energy minimization of the modeled 3D structure.

2.4 | qRT-PCR

For qRT-PCR analysis of gene expression, blood samples from six patients were collected into PAXgene tubes (Qiagen). RNA isolation was performed with the PAXgene Blood RNA kit (Qiagen) in accordance with manufacturer instructions. cDNA was then synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). *CBS* mRNA levels were determined by qRT-PCR using the commercially available *TaqMan Expression Assay* (Hs00163925_m1 (*CBS*); Hs02758991_g1 (*GAPDH*); and (Applied Biosystems) in a StepOne system (Applied Biosystems). *GAPDH* was used as the housekeeping gene. All reactions were performed under the conditions specified in the corresponding manufacturer instructions. Relative quantification of *CBS* RNA was normalized to the *GAPDH* gene using the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008).

2.5 | Expression of mutations in *E. coli*

The novel mutations c.284T>C and c.329A>T were expressed in *E. coli* using a protocol adapted after Mendes et al., (2014), as described below.

For expression of wild-type (WT) and mutant CβS, WT cDNA was first cloned in pOTB7 vector (Thermo Scientific, Lafayette, CO, USA), between restriction sites EcoRI and XhoI. The insert was then cleaved with NdeI and XhoI, purified (QIAquick gel extraction kit, Qiagen), and ligated into pET28b (Clontech Laboratories), at the same sites, with T4 DNA ligase (New England Biolabs). The pET28b carries an N-terminal *6xHis-tag*, followed by a thrombin cleavage site which enables later removal of this tag. The pET28b-6xHis-pepT-hCBSWT expression construct was thus created

and used as a template for site-directed mutagenesis with the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies), per manufacturer instructions.

WT and mutant CβS proteins were expressed in *E. coli* (BL21 DE3). Cells without the expression vector and cells harboring an empty expression vector were used as negative controls. Cells were cultured at 37°C in LB medium and selected with kanamycin. Protein expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) and δ-aminolevulinic acid (ALA) to the medium. After 16 hr at 22°C, bacteria were resuspended in lysis buffer and sonicated. The insoluble (pellet) and soluble (supernatant) fractions were separated by centrifugation.

The proteins thus generated were analyzed by SDS-PAGE and Western blotting. Briefly, the protein content of the pellet fraction was quantitated by the Bradford method. Then, a 60-μg aliquot of protein was analyzed by SDS-PAGE. The same amount was used for Western blot analysis. This was performed using PVDF membranes, primary mouse anti-CbS polyclonal antibody (Abnova, A75-A01), and secondary polyclonal rabbit anti-His tag antibody (PAB0862, Abnova). The enzyme activity of CβS was determined by LC-MS/MS in the soluble fraction of the lysate, using the protocol described by Smith et al., (2012). All experiments were performed in triplicate, with the arithmetic mean of the resulting measurements considered for analysis.

2.6 | Statistical analysis

Statistical analysis was performed using SPSS for Windows, Version 18.0 (Chicago: SPSS Inc). Asymmetrically distributed variables were expressed as median (range), and normally distributed variables as mean and standard deviation. The Mann–Whitney *U* test (continuous variables) or chi-square test (categorical variables) was used to assess between-group differences. *p*-values <.05 were considered significant. To calculate allele frequencies, only unrelated patients were considered ($n = 30$), and when consanguinity was reported, only one allele per patient was taken into account.

3 | RESULTS

The summary of the clinical and genetic data of the sample is shown in Table 1.

Of the 35 patients included, 22 (63%) were male. Parental consanguinity was reported in 16 families (53%). Median age at inclusion was 19 years (range 5–37 years). Regarding pyridoxine responsiveness, 28 patients (85%) were classified as nonresponsive and five (15%) as responsive, with three of them homozygous for the p.Ile278Thr mutation (patients 4, 5, and 6). In two patients, pyridoxine responsiveness was not reported/determined. The median age of symptom onset was 4 years (Table 1).

3.1 | Genotype

Twenty-one different mutations were detected, with six recurrent (Figure 1). Regarding the type, most mutations were missense ($n = 11$, nine transitions and two transversions), followed by splicing site ($n = 4$), small deletions ($n = 2$), frameshift ($n = 2$) and nonsense ($n = 1$).

Exons 8, 9, 7, 5, and 3 had the higher number of nonrelated alleles mutated ($n = 9$, 7, 6, 5, and 5, respectively). Altogether, the most prevalent mutations were: p.Ile278Thr (allele frequency 18.2%; found in south, southeast, and northeast regions), p.Trp323Ter (allele frequency 11.3%; found in southeast and northeast), p.Thr191Met (allele frequency 11.3%; found in south and southeast), and c.828+1G>A (allele frequency 11.3%; found in south, southeast, and northeast). In only one allele no mutation could be identified (patient 30; Table 1). Eight novel mutations were detected: c.2T>C (exon 1), c.209+1delG (intron 1), c.284T>C (p.Ile95Thr, exon 2), c.329A>T (p.Glu110Val, exon 3), c.444delG (exon 3), c.864_868delGAG (exon 8), c.989_991delAGG (exon 9), and c.1223+5G>T (intron 11). No mutant alleles were detected in the 100 controls tested.

3.2 | Genotype versus phenotype relationship

When available in the literature, the expected phenotype according to the genotype (responsive or nonresponsive to

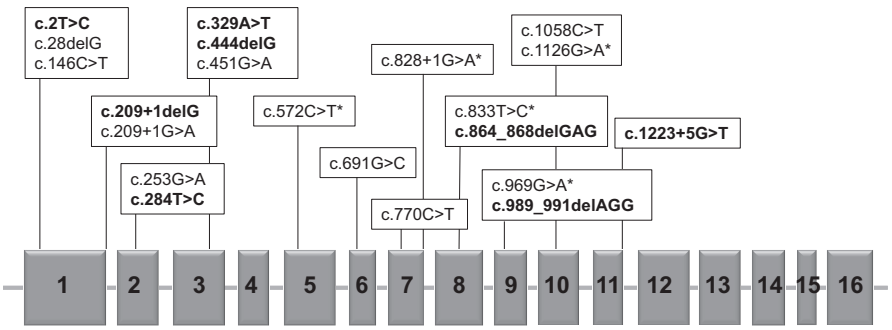

FIGURE 1 *CBS* map showing the location of the mutations found. Exons are represented by solid gray boxes. Novel mutations are shown in bold. An asterisk (*) indicates mutations found in more than one unrelated patient. Adapted from Kraus, 2017 (Kraus, 2017)

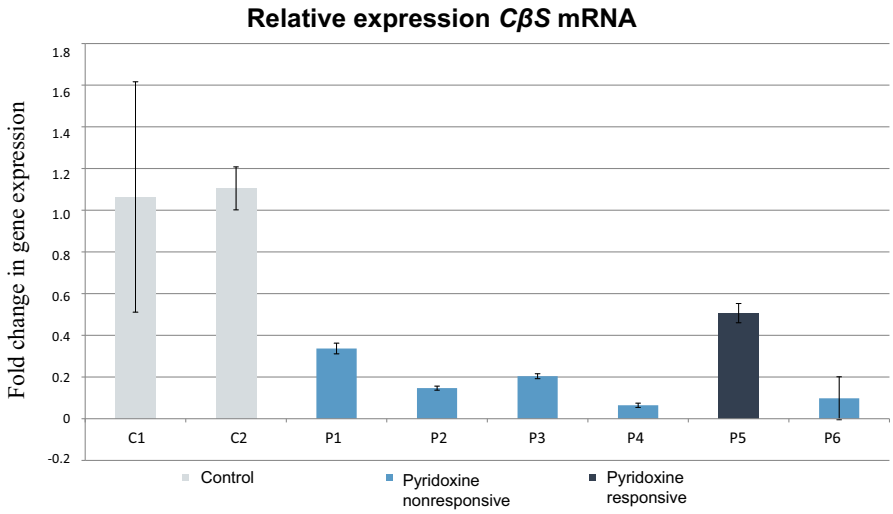

FIGURE 2 *CBS* mRNA expression as determined by qRT-PCR. Genotypes: P1 (c.209+1delG; c.572C>T); P2 (c.2T>C; c.828+1G>A); P3 (c.828+1G>A; c.1126G>A); P4 (c.833T>C; c.989_991delAGG); P5 (c.1058C>T; c.146C>T); and P6 (c.444delG; c.444delG). Fold change in gene expression is calculated through the $2^{-\Delta\Delta C}{}_{T}$ method, where $\Delta C_T = (C_{T,\text{Time x}} - C_{T,\text{Time 0}})$ (Schmittgen & Livak, 2008)

pyridoxine) matched in all cases the observed phenotypes (Table 1).

All patients from the same family (1, 18, 21, and 28) presented the same phenotype regarding pyridoxine responsiveness. All homozygous for the p.Ile278Thr mutation (n = 3) were pyridoxine responsive. In the compound heterozygous (n = 3), association with another missense mutation (p.Gly151Arg) resulted in pyridoxine responsiveness in patient #6, while patients #7 and #8, carrying the mutations c.989_991delAGG and c.828+1G>A, were nonresponsive.

Most patients (4/5) carrying the p.Thr191Met mutation were homozygous, and all five were nonresponsive to pyridoxine. Patient #17 is heterozygous for the p.Thr191Met and the novel c.209+1delG mutation.

Patients carrying the c.828+1G>A were also nonresponsive to pyridoxine. Most of them (4/5) were compound heterozygous, and 3/4 carried a missense mutation in the second allele. Patients carrying the p.Trp323Ter mutation (n = 4) were all homozygous and nonresponsive to pyridoxine.

3.3 | In silico analysis

The p.Ile95Thr and p.Glu110Val mutations were analyzed in the PolyPhen-2 and SIFT software for prediction of functional effects, and both were predicted to be pathogenic (PolyPhen-2: scores of 0.999 and 1.000, respectively; SIFT: score of 0 for both mutations). The MutPred software suite, which estimates potential changes in mutant proteins, interpreted both mutations as probably damaging, with scores of 0.827 for p.Ile95Thr and 0.892 for p.Glu110Val.

In the crystal structure of the human CbS enzyme, Glu110 and Ile95 residues are located in the catalytic domain and have a predicted severe effect. Ile95 is a conserved residue located in the H3 of the catalytic domain near the dimers interface (Figs S1 and S2); its main interactions are with Phe99 and Lys98 residues located in the same helix and Glu342 located in the H14. The alignment of the structure of the mutant model I95T with the CbS protein structure (PDB:4COO) showed no alteration of the original interactions of Ile95 in the monomers (Fig. S1). Glu110 is located in the H4 near to a highly conserved position surrounding the cofactor pyridoxal 5-phosphate (PLP). Glu110 form hydrogen bonds with the following residues: Thre87 located next to the strand A, Asn113 located in a beta turn, and Arg121 located in the H5. Salt bridge is observed with Lys108 and Arg121 residues. The central part of the dimer interface is formed by the residues Phe111 and Phe112 close to the twofold dimer axis; thus, Phe112 of monomer A interacts with Phe112 of monomer B and vice versa (Meier et al., 2001). The side chain of the Thr87 is part of the dimer interface. Alignment of the mutant model Glu110V with 4COO showed a loss of interaction with the Thr87 (Figs S1 and S2).

3.4 | Expression studies

Enzyme activity in the *E. coli*-expressed mutant proteins in relation to the WT control was 1.5% (24.7 nmol/h/mg protein) for c.329A>T (p.Glu110Val) and 17.5% (432.6 nmol/h/mg protein) for c.284T>C (p.Ile95Thr). Relative *CBS* mRNA levels measured by qRT-PCR in six patients are described in Figure 2. Reduced expression was observed in all, with the highest expression level detected in the sole pyridoxine-responsive patient (P24).

4 | DISCUSSION

The present report provides the largest genetic analysis of Brazilian HCU patients reported so far. The *CBS* gene was sequenced for 35 patients (30 unrelated) followed in 13 medical genetics centers across Brazil. Age of symptom onset varied considerably within the sample. However, more than half of the patients presented symptoms at an

early age (<5 years old), suggesting more severe forms of HCU in these patients. All patients included in the study have had delayed diagnosis, based mainly on clinical suspicion. This is partially explained by the fact that, in Brazil, HCU is not included in the National Neonatal Screening Program.

A high proportion of pyridoxine-nonresponsive HCU was observed in the study population. Nonresponsiveness to pyridoxine is associated with a more severe phenotype and challenging management (Mudd et al., 1985, 2001; Wilcken, 2006). In our study, this proportion exceeded rates described worldwide (approximately 50%) (Mudd et al., 2001). Although the proportion of nonresponsive patients is also high in some countries, such as Qatar and Ireland, these cases are associated with specific genotypes that are highly prevalent in the corresponding countries (El-Said et al., 2006; Gallagher et al., 1995). No such association was observed in our study. In fact, the most prevalent mutation in our sample (p.Ile278Thr) is a pyridoxine-responsive mutation. These findings may suggest that milder, pyridoxine-responsive forms of HCU remain largely underdiagnosed in Brasil.

Great variability in genotypes was observed in the present study. The Brazilian population is characterized by extraordinary genetic diversity as a result of centuries of admixture among Amerindians, European colonizers, and African slaves (IBGE, 2007). European ancestry has the higher contribution to the genetic background of Brazilians (0.62), followed by African (0.21) and Amerindian (0.17) (Moura, Coelho, Balbino Vde, Crovella, & Brandao, 2015). However, major regional differences exist: European contributions are even more dominant in the south of the country, whereas the northeast and north regions have the highest proportions of African and indigenous ancestry, respectively (Moura et al., 2015; Ruiz-Linares et al., 2014). This might contribute to the wide range of genotypes found. In our study, all frequent mutations found were detected in at least two different regions of Brazil, which does not support the hypothesis that they could have arisen through a founder effect.

The most prevalent mutation in our study was the p.Ile278Thr (c.833T>C). This is also the most prevalent mutation worldwide, accounting for 16% of all HCU alleles described (Kraus, 2017). It is particularly frequent in central and northern Europe (Kluijtmans et al., 1999; Sebastio et al., 1995; Shih et al., 1995; Sokolova et al., 2001; Sperandeo et al., 1995). In the Brazilian study conducted by Porto et al., this mutation was detected in 6 of 28 alleles (frequency in unrelated alleles, 13.6%) (Porto et al., 2005). Since patients carrying this mutation can exhibit very mild or isolated symptoms later in life, it is possible that the prevalence of this mutation in Brazil is even higher, and that many HCU patients may remain undiagnosed. In the present study, most patients harboring this mutation were pyridoxine responsive (4/6), with the two patients classified as nonresponsive being compound heterozygous carrying more severe mutations in the second allele. Although less frequent, several other cases of pyridoxine-nonresponsive patients carrying the p.Ile278Thr mutation are described (Kluijtmans et al., 1999; Kraus, 2017; Porto et al., 2005).

The Iberian mutation p.Thr191Met (c.572C>T) was found in 11.3% of unrelated alleles, again all in patients from the south and southeast regions. This allele frequency was similar to that reported by Porto et al. (13.64%) (Porto et al., 2005), but lower than those reported in other Latin American countries (75% in Colombia, 25% in Venezuela, and 20% in Argentina) and in the Iberian Peninsula (52% in Spain and 33% in Portugal) (Bermudez et al., 2006; Cozar et al., 2011; Urreizti et al., 2003). These findings appear to reflect the greater genetic heterogeneity of Brazil as compared with other Latin American countries and, possibly, the more limited contribution of Spanish immigration to Brazil (IBGE, 2007; Kehdy et al., 2015; Pena et al., 2011; Resque et al., 2016). Wide phenotypic variability has been observed for this mutation, with mild to severe phenotypes and pyridoxine responsiveness ranging from partial to absent (Kraus, 2017; Urreizti et al., 2006).

The c.828+1G>A mutation, detected in 11.3% of the alleles in our studies, had previously been described in only one individual, a heterozygous Czech patient [c.1146-2A>C; c.828+1G>A] (Janosik et al., 2001). Their patient was described as pyridoxine nonresponsive and had null CβS activity in fibroblasts. Furthermore, there was no mRNA expression of the allele containing the mutation. According to the authors, this suggests a nonsense-mediated mRNA decay mechanism, as a premature termination codon at exon 8 has been predicted for this mutation (Janosik et al., 2001). In our study, all patients with this mutation were classified as nonresponsive. One patient heterozygous for (c.828+1G>A; c.1126G>A) underwent qRT-PCR analysis, which showed a ~80% reduction in mRNA expression. Our clinical data support the severity of this mutation. In our sample, all patients carrying the c.828+1G>A presented early symptom onset (≤5 years) and were all nonresponsive to pyridoxine, even the compound heterozygous. The high prevalence of this mutation in Brazilian patients does not appear to be related to genetic drift effects, as the affected patients came from three geographically distant regions of the country. Furthermore, this mutation was not detected in any of the 100 healthy controls, and thus appears to be rare in the overall population.

The p.Trp323Ter (c.969G>A) mutation was also detected with an allele frequency of 11.3%. This mutation is highly prevalent in Saudi Arabia (10 of 13 families assessed) and is associated with severe phenotypes (Zaidi

et al., 2012). In our study, all patients with this mutation were homozygous and pyridoxine nonresponsive. These families were from the northeast and southeast regions of Brazil. As there was no determination of ancestry in our study, we could not infer whether the presence of this mutation in Brazilian patients might be associated with migratory events.

Eight novel mutations were detected in this study. Several tests corroborated the pathogenicity of the analyzed missense mutations. In silico analyses using three different software programmes predicted a damaging functional effect for the mutations p.Glu110Val and p.Ile95Thr. Furthermore, no allele containing these mutations was detected in 100 controls. *E. coli* expression assays demonstrated reduced enzyme activity consistent with pathogenicity for HCU of both mutations (<20% activity relative to controls) (Arruda et al., 1998; Picker & Levy, 2014). Enzyme activity in *E. coli* also correlated well with clinical phenotype (pyridoxine responsiveness and disease severity).

Homology modeling of the p.Ile95Thr mutation showed no alteration of the original interactions of Ile95 in the monomers. However, the loss of hydrophobicity due to the substitution of Ile to Thr may affect the hydrophobic character of the dimer interface. This could explain the lack of residual activity of the mutant in the protein extract. Analysis of protein oligomerization and CbS enzyme activity of the purified mutant protein could provide more information to confirm the structural predictions.

For the p.Glu110Val mutation, structural analyses showed that the substitution of Glu to Val may disturb the ionic interactions due to the charge difference of the Glu (negative) and Val (neutral). Furthermore, the size and the hydrophobicity difference may affect the hydrogen bond formation with the surrounded residues remaining just the interaction with Asn113 and Lys1083.

The c.2T>C mutation alters the translation start site codon, being the first of its kind ever described in *CBS* (Kraus, 2017). This mutation was not detected in the 100 controls we tested and not found in the 1000 genomes database (http://www.1000genomes.org/). Start-site missense mutations are relatively common in hereditary diseases; in fact, point mutations at this position are more likely to be damaging than other missense mutations (Wolf et al., 2011). In *CBS*, the next ATG codon is located in exon 2, far from the first methionine codon. However, exon 1 carries a leucine at position 28 with a flanking sequence that approximates the "Kozak consensus sequence," a specific flanking sequence that maximizes translational efficiency (Kozak, 2002; Wolf et al., 2011). This suggests that this CTG codon could be used as an alternative initiation codon in *CBS*.

Finally, qRT-PCR analysis of samples from four patients heterozygous for the novel c.2T>C, c.209+1delG, c.989_991delAGG, and c.444delG mutations revealed major reductions in mRNA levels relative to controls, which suggests reduced gene expression in the presence of these variants. This reduction is greater in patients with splice-site mutations, which disrupt reading, as premature termination codons are encoded in the mutant sequence; therefore, mRNA decay pathways eventually degrade the product of expression. Our findings were also consistent with the most severe phenotypes being found in patients with greater reductions in mRNA levels.

In general, the genotypes were consistently associated with pyridoxine responsiveness (presence or absence) within families and also agreed with previous findings worldwide (Janosik et al., 2001; Kraus, 2017; Zaidi et al., 2012). However, it is important to highlight that no clear genotype–phenotype correlation is established for most *CBS* mutations. The lack of clinical data in many studies, varied pyridoxine responsiveness protocols, and the high number of private/rare mutations in *CBS* are some of the factors that limit this analysis.

In our study, 72% of the mutated alleles were condensed in just five exons. Considering this finding, we propose a protocol for *CBS* sequencing that would be cost- and time-saving for the molecular investigation of new Brazilian HCU patients. We suggest that exons 8, 9, 7, 5, and 3 are sequenced first, since they carried the great majority of mutated alleles found in our sample. Second, exons 1, 2, 4, 10, 11, 12, and 16 should be tested. In our study, 10 different mutations were found there, and a great number of mutations reported worldwide are also located in these exons. We suggest that exons 6, 13, 14, and 16 are sequenced last, since mutations in these regions are rare. No disease-causing mutation has been reported in exon 15 (Kraus, 2017).

In conclusion, this study provides the most wide-ranging genetic characterization of HCU in Brazil to date. Most patients studied here were nonresponsive to pyridoxine and presented clinically early in life, suggesting more severe forms of HCU in our sample. A great variability in genotypes was observed; this might reflect the intense admixture and the diverse genetic background of the Brazilian population. The four most prevalent mutations together accounted for over 50% of mutated alleles. A consistent genotype–phenotype association was observed within families and for common mutation. However, for many rare and novel mutations described here, additional studies should be carried to evaluate the effect of these variants on human CβS deficiency. These findings should contribute to the development of protocols for diagnosis and molecular screening of HCU in Brazil.

ACKNOWLEDGMENTS

The authors thank Prof. Roberto Giugliani and Rede EIM Brasil for their assistance with patient diagnosis and

follow-up. Financial support for this study was provided by CNPq, CAPES, DAAD, and FIPE-HCPA. I. Schwartz is a National Council of Scientific and Technological Development (CNPq) research productivity fellow.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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How to cite this article: Poloni S, Sperb-Ludwig F, Borsatto T, et al. *CBS* mutations are good predictors for B6-responsiveness: A study based on the analysis of 35 Brazilian Classical Homocystinuria patients. *Mol Genet Genomic Med*. 2017;00:1–11. https://doi.org/10.1002/mgg3.342

Apêndice 2 - Artigo publicado no Molecular Genetics and Metabolism Reports, 2018: "*Cytokines levels in late-diagnosed Classical Homocystinuria patients*"

Molecular Genetics and Metabolism Reports 17 (2018) 43–44

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

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

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Cytokines levels in late-diagnosed Classical Homocystinuria patients

ARTICLE INFO

Keywords:
Classical homocystinuria
CBS deficiency
Inflammation
Cytokines

Classical homocystinuria (HCU; CβS deficiency) is characterized by a blockage in homocysteine (Hcy) degradation, resulting in Hcy and methionine accumulation and cysteine deficiency. Studies in healthy and chronically ill individuals have found positive associations between proinflammatory cytokines and plasma total homocysteine (tHcy) [1–3], suggesting a role for immunomodulation in HCU pathogenesis. Therefore, we aimed to investigate 20 inflammatory cytokines in plasma of poorly controlled HCU patients and healthy controls.

The study sample comprised 9 late-diagnosed HCU patients and 10 age and gender-matched healthy controls from South Brazil. tHcy, cysteine, methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were measured in plasma by LC-MS/MS. The cytokines quantification assay was performed through EMD Millipore's MILLIPEX® MAP Human Cytokine kit, accordingly manufacturer's instruction. All samples were measured in duplicates for 20 cytokines (Table 1). Measurements with divergence ≥30% between duplicates

Table 1
Homocysteine-related metabolites and cytokine levels in HCU patients and controls.

		Patients (n = 9)	Controls (n = 10)	p
		Median (range)	Median (range)	
Homocysteine-related metabolites (μmol/L)	Met	165 (22–777)	24 (14–30)	0.007
	Hcy	130 (17–300)	6.7 (5.7–12)	< 0.001
	Cys	158 (67–297)	223 (174–241)	0.014
	SAM	756 (99–3264)	82 (69–107)	< 0.001
	SAH	135 (18–591)	19 (10 − 23)	0.002
Pro-inflammatory cytokines (pg/mL)	IL-1α	0.05 (0.01–1.25)	0.24 (0.01–30.77)	0.252
	IL-1β	0.79 (0.39–1.83)	0.57 (0.37–1.05)	0.743
	IL-6	1.03 (0.45–2.05)	1.12 (0.47–11.56)	0.653
	IL-8	2.07 (0.58–15.60)	1.58 (1.12–18.14)	0.624
	IL-17	1.96 (1.04–5.48)	4.09 (1.23–12.94)	0.102
	TNF-α	7.92 (3.62–14.81)	9.17 (3.92–13.67)	0.287
	TNF-β	0.01 (0.00–1.94)	0.02 (0.00–119)	0.617
	MCP-1	254 (185–1014)	267 (216–357)	0.935
	IP-10	277 (170–1855)	489 (264–1764)	0.153
	GRO	829 (197–2473)	704 (303–1014)	0.595
	MDC	554 (299–1288)	527 (295–759)	0.744
	MIP-1α	1.00 (0.54–3.62)	2.24 (0.66–34.89)	0.077
	MIP-Iβ	18.61 (1.01–37.62)	19.57 (1.84–68.80)	0.327
	VEGF	156.71 (0.01–376)	255.33 (1.32–704)	0.102
	GM-CSF	3.62 (1.06–15.20)	3.01 (1.77–13.67)	0.744
	IFN-γ	3.62 (1.67–9.72)	10.92 (4.94–82.04)	0.007
Anti-inflammatory cytokines (pg/mL)	IL-4	0.48 (0.04–2.83)	0.68 (0.16–26.46)	0.327
	IL-10	0.75 (0.27–1.39)	0.79 (0.41–3.29)	0.935
	IL-13	0.11 (0.02–9.17)	0.15 (0.02–78)	0.368
	G-CSF	13.67 (7.69–46.01)	16.83 (7.69–135.66)	0.653

Met: methionine; Hcy: homocysteine; Cys: cysteine; SAM: S-Adenosylmethionine; SAH: S-Adenosylhomocysteine.

https://doi.org/10.1016/j.ymgmr.2018.09.003
Received 6 September 2018; Accepted 7 September 2018

were excluded from data analysis (n = 1).

Hcy-lowering treatment (pyridoxine 7/9, folic acid 8/9, betaine 7/9, methionine-restricted diet 3/9) was prescribed; but only 3/9 patients had tHcy < 100 μmol/L (target level). Most patients (7/9) were pyridoxine nonresponsive. Because of the high concentrations of tHcy with a wide range we consider this an ideal group to explore the potential relation between cytokines and tHcy. Cytokines plasma levels were similar in patients and controls, with the exception of IFN-γ, which was three-fold reduced ($p = .007$) in patients (Table 1). In line an inverse association of Hcy and SAM with IFN-γ was found (r − 0.487 and r − 0.537; $p < .05$).

To our knowledge, only one study had previously evaluated cytokines in HCU patients. Keating et al. measured 16 cytokines in plasma of HCU patients, and found that patients with tHcy > 150 μM, ($n = 5$) had increased levels of several pro-inflammatory cytokines (IL-1a, IL-6, TNF-α, IL-17 and IL-12), while well controlled patients (Hcy < 86.1 μM, n = 5) had not [4]. The authors provided no information about which patients received treatment. IFN-γ was not evaluated in this study. In previous studies, reduced IFN-γ levels have shown anti-inflammatory properties [5,6].

In summary, our study provides no evidence of increased inflammatory cytokines in HCU patients on treatment, despite poor metabolic control. Hcy may even show anti-inflammatory properties like glutathione [7], what could explain the finding of lowered IFN-γ. The potential impact of Hcy-lowering treatment on cytokines requires further study.

Acknowledgments

Financial support for this study was provided by CNPq, FAPERGS, CAPES and DAAD.

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ANEXOS – Cartas de Aprovação de Projetos pelo CEP

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: 130525
Data da Versão do Projeto:

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
SANDRA LEISTNER SEGAL

Título: DETECÇÃO DE MUTAÇÕES NOS GENES C¿S, BHMT E CHDH EM PACIENTES BRASILEIROS COM HOMOCISTINÚRIA CLÁSSICA

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, 26 de dezembro de 2013.

Prof. Eduardo Pandolfi Passos
Coordenador GPPG/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: 150636
Data da Versão do Projeto: 17/12/2015

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
NATANIEL FLORIANO LUDWIG
GIOVANA REGINA WEBER
FERNANDA SPERB LUDWIG
SORAIA POLONI

Título: Creatina em Homocistinúria Clássica: Suplementação em Cultivo de Fibroblastos

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, 15 de abril de 2016.

Prof. José Roberto Goldim
Coordenador CEP/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: 160140
Data da Versão do Projeto: 22/03/2016

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
TACIANE BORSATTO
GIOVANA REGINA WEBER
SORAIA POLONI

Título: INVESTIGAÇÃO GENÉTICA DE PACIENTES BRASILEIROS COM SUSPEITA DE HOMOCISTINÚRIA POR DEFICIÊNCIA DE MTHFR

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, 26 de abril de 2016.

Prof. José Roberto Goldim
Coordenador CEP/HCPA