UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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HOMOCISTINÚRIA CLÁSSICA NO BRASIL: UM ESTUDO CLÍNICO E GENÉTICO COM FOCO NA INVESTIGAÇÃO DA RELAÇÃO ENTRE COMPOSIÇÃO CORPORAL E METABOLISMO LIPÍDICO EM PACIENTES TRATADOS

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LISTA DE ABREVIATURAS

ACC: acetil CoA-carboxilase/ *acetyl CoA carboxylase*

AdoHcy: *S*-adenosilhomocisteína/ *S-adenosylhomocysteine*

AdoMet: *S*-adenosilmetionina/ *S-adenosylmethionine*

AG: ácidos graxos

ALA: ácido *δ*-aminolevulínico/ *δ-aminolevulinic acid*

AMPK: proteína quinase ativada por AMP/ *AMP-activated protein kinase*

ANCOVA: análise de covariância/ *analysis of covariance*

BHMT: betaína:homocisteína metiltransferase/ *betaine-homocysteine methyltransferase*

BHT: hidroxitolueno butilado/ *butylated hydroxytoluene*

BIA: bioimpedância elétrica/ *bioelectrical impedance analysis*

BMD: densidade mineral óssea/ *bone mineral density*

BMI: índice de massa corporal/ *body mass index*

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/ *National Coordination for Improvement of Higher Education Personnel*

CHDH: colina desidrogenase/ *choline dehydrogenase*

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico/ *National Counsel of Technological and Scientific Development*

CPT-1: carnitina-palmitoil transferase 1/ *carnitine palmitoyltransferase 1*

Cys: cisteína/ *cysteine*

CβS: cistationina β-sintase/ *cystathionine β-synthase*

CγL: cistationina gama-liase/ *cystathionine γ-lyase*

DAAD: Serviço Alemão de Intercâmbio Acadêmico/ *Deutscher Akademischer Austauschdienst*

DMG: dimetilglicina/ *dimethylglycine*

DNA: ácido desoxirribonucleico

DXA: absorciometria de raios-x de dupla energia/ *dual X-ray absorptiometry*

EIM: erros inatos do metabolismo

FAME: éster metílico de ácido graxo*/ fatty acid methyl ester*

FIPE: fundo de incentivo à pesquisa/ *research incentive fund*

GCLM: glutamato cisteína ligase subunidade modificadora*/ glutamate-cysteine ligase modifier subunit*

GSH: glutationa/ *glutathione*

HCPA: Hospital de Clínicas de Porto Alegre

HCU: homocistinúria clássica/ *classical homocystinuria*

HDL: lipoproteína de alta densidade/ *high density lipoprotein*

HOMA-IR: *homeostasis model assessment*

HPLC: cromatografia líquida de alta eficiência/ *high performance liquid chromatography*

IGF-1: Fator de crescimento semelhante à insulina tipo 1/ *insulin-like growth fator*

IPTG: isopropiltiogalactosídeo */ isopropyl-β-D-thiogalactoside*

LDL: lipoproteína de baixa densidade/ *low density lipoprotein*

MAT: metionina adenosiltransferase/ *methionine adenosyltransferase*

MR: dieta restrita em metionina/ *methionine-restricted diet*

MS: metionina sintase/ *methionine synthase*

MTHFR: 5,10 metilenotetrahidrofolato redutase/ *5,10-methylene-tetrahydrofolate reductase*

PEMT: fosfatidiletonolamina N-metiltransferase/ *phosphatidylethanolamine N-methyltransferase*

PLP*:* piridoxal 5'-fosfato/ *pyridoxal 5'-phosphate*

PolyPhen2: *polymorphism phenotyping*

PPARs: receptores ativados por proliferadores de peroxissoma/ *peroxisome proliferator-activated receptors*

qRT-PCR: PCR quantitativo em tempo real/ *real-time quantitative PCR*

RFLP: *restriction fragment length polymorphism*

RNA: ácido ribonucleico

SAA: aminoácidos sulfurados/ *sulfur amino acids*

SCD-1: estearoil-CoA desaturase-1/ *stearoyl-CoA desaturase-1*

SD: desvio padrão/ *standard deviation*

SHMT: serina hidroximetiltransferase/ *serine hydroxymethyltransferase*

SIFT: *sorting intolerant from tolerant*

SNC: sistema nervoso central

SPE: extração de fase sólida/ *solid phase extraction*

SREBP-1c: proteína 1c ligadora do elemento regulatório de esterol/ *sterol regulatory element binding protein*

Tau: tarina/ *taurine*

tHcy: homocisteína total/ *total homocysteine*

THF: tetrahidrofolato/ *tetrahydrofolate*

VLDL: lipoproteína de muito baixa densidade/ *very low density lipoprotein*

WT: selvagem/ *wild type*

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

SUMÁRIO

1. RESUMO

Introdução: A homocistinúria clássica (HCU) é uma doença genética causada pela atividade deficiente da enzima cistationina β-sintase (CβS). Bioquimicamente, é caracterizada por distúrbios no metabolismo dos aminoácidos sulfurados (SAA), com elevação plasmática de homocisteína e metionina e redução de cistationina e cisteína. Clinicamente afeta principalmente os sistemas ocular, vascular, nervoso central e ósseo. O tratamento inclui dieta pobre em metionina e fórmula metabólica, doses farmacológicas de piridoxina (co-fator da CβS) e betaína; e suplementação com ácido fólico e vitamina B12. Pacientes que respondem à piridoxina geralmente apresentam fenótipos mais brandos. No Brasil, o perfil clínico e genético dos pacientes com a doença é pouco conhecido. Evidências recentes sugerem que os SAA, especialmente a cisteína, têm influência também no metabolismo lipídico e na composição corporal. Estas alterações parecem estar ligadas à supressão hepática da SCD-1 (estearoil-CoA desaturase-1), enzima chave na síntese de ácidos graxos monoinsaturados. Redução de massa adiposa e da expressão da SCD-1 hepática são descritas em camundongos com deficiência de CβS não tratados, mas este fenótipo é revertido com o tratamento. Estas alterações metabólicas ainda não foram estudadas em pacientes com HCU.

Objetivos: 1) Descrever o perfil clínico e genético de pacientes brasileiros com HCU; e 2) Avaliar a relação entre SAA, composição corporal, atividade da SCD-1, resistência insulínica e metabolismo lipídico em pacientes com HCU em tratamento.

Métodos: Estudo transversal. Dados clínicos de 66 pacientes (57 famílias) provenientes das 5 regiões do país (sul n=12, sudeste n=35, nordeste n=8, norte n=1 e centro-oeste n=1) foram analisados. Amostras de sangue de 30 pacientes não relacionados foram enviadas e o DNA genômico foi isolado para sequenciamento do gene *CBS*. Amostras de RNA de 6 pacientes foram coletadas para análise de expressão gênica por qRT-PCR. Estudos de expressão em *E.coli* foram realizados para três mutações nunca descritas. Para investigação de alterações na composição corporal e metabolismo lipídico, dois estudos foram conduzidos. No primeiro, composição corporal foi avaliada por bioimpedância elétrica (BIA) em 9 pacientes e 18 controles; e níveis plasmáticos de metionina, homocisteína total, cisteína, colina, betaína, dimetilglicina e etanolamina foram determinados nos pacientes. No segundo estudo, 11 pacientes e 16 controles foram avaliados. Composição corporal foi

avaliada por *Dual X-ray Absorptiometry* (DXA). Os compostos sulfurados metionina, S-adenosilmetionina, S-adenosilhomocisteína, homocisteína total, cisteína e glutationa; além de lipoproteínas, ácidos graxos livres, acilcarnitinas, leptina, adiponectina, isoprostanos, glicose e insulina foram medidos em plasma. Resistência insulínica foi determinada por HOMA-IR. Os índices SCD-16 e SCD-18, que estimam a atividade hepática da SCD-1 em humanos, foram calculados através das razões produto/precursor dos ácidos graxos correspondentes.

Resultados: A análise de dados clínicos revelou predominância de não responsividade à piridoxina (82%). A mediana de idade atual foi de 19 anos (mín: 5; máx: 45 anos) ao diagnóstico, de 10 anos (mín: 1; máx: 39 anos); e ao primeiro sintoma, de 5 anos (mín: 0; máx: 20 anos). As manifestações oculares foram os sintomas mais precoces, o principal motivo da suspeita diagnóstica e a manifestação mais prevalente ao diagnóstico (69% dos casos). Apenas 28% dos pacientes em tratamento tinham homocisteína total <60 µmol/L. Quanto às estratégias de tratamento, 89% dos pacientes utilizavam ácido fólico e piridoxina, 76% betaína, 32% vitamina B12 e 26% e dieta pobre em metionina com fórmula metabólica. O sequenciamento do gene *CBS* revelou 21 mutações diferentes, sendo as mais prevalentes: p.Ile278Thr (18,2% dos alelos), p.Thr191Met (11,3%), r.[737_828del92, 828_931ins104] (11,3%) e p.Trp323Ter (11,3%). Oito novas mutações foram encontradas. As atividades enzimáticas expressas em *E.coli* foram de: 1,5% para a mutação c.329A>T; 17,5% para a c.284T>C e 206% para a c.2T>C. A análise de qRT-PCR revelou redução de expressão gênica (54-95%) em todos os pacientes avaliados. Não houve diferença no percentual de gordura em ambos os métodos utilizados para avaliação da composição corporal. Já a massa magra foi menor nos pacientes na avaliação por DXA (p=0,008). Leptina plasmática, colesterol LDL e índice SCD-16 foram significativamente mais baixos nos pacientes (p<0,05). Lipoproteínas apresentaram associações com colina, etanolamina e leptina nos pacientes. Cisteína foi significativamente mais baixa nos pacientes e apresentou correlação positiva com IMC (r=0,912, p=0,001), e tendência de associação com o índice SCD-16 (r=0,624, p=0,054). Não houve diferença nos demais parâmetros avaliados.

Conclusões: este estudo traz o panorama clínico e genético mais abrangente da HCU já realizado no Brasil. Observa-se que a maioria dos pacientes apresenta fenótipo grave, o que sugere subdiagnóstico das formas atenuadas/responsivas de HCU. Uma grande

variabilidade alélica foi observada, mas as quatro mutações mais prevalentes são responsáveis por mais da metade dos alelos mutados. A redução de leptina, SCD-1 e colesterol LDL, e suas associações com os SAA, corroboram estudos observacionais e experimentais prévios que apontam para um papel destes aminoácidos (em especial a cisteína), na modulação do metabolismo lipídico.

ABSTRACT

Introduction: Classical homocystinuria (HCU) is a genetic disease caused by cystathionine β-synthase (CβS) deficiency. Biochemically, it is characterized by disrupted metabolism of sulfur amino acids (SAA), with elevated homocysteine and methionine and decreased cystathionine and cysteine in plasma. Clinical signs are mainly related to ocular, vascular, central nervous and skeletal systems. Treatment includes a methionine-restricted diet + metabolic formula, pharmacologically high doses of pyridoxine (cofactor of CβS) and betaine, and supplementation with folic acid and vitamin B12. Usually, patients responsive to pyridoxine present milder phenotypes. In Brazil, little is known about the clinical and genetic profile of patients diagnosed with HCU. Interestingly, recent evidence suggests that SAA, especially cysteine, modulate lipid metabolism and body composition. These changes appear to be linked to the suppression of liver SCD-1 (stearoyl-CoA desaturase-1), a key enzyme for the synthesis of monounsaturated fatty acids. Reduced fat mass and low expression of liver SCD-1 are described in HCU mice, and treatment reverses this phenotype. This phenomenon has not been studied in patients with HCU yet.

Objectives: 1) to describe the clinical and genetic profile of Brazilian patients with HCU; and 2) to assess the relationship between SAA, body composition, SCD-1 activity, insulin resistance, and lipid metabolism in treated patients with HCU. **Methods:** A cross-sectional study was conducted. Clinical data of 66 patients (57 families) from 5 regions of Brazil (south n=12, southeast n=35, northeast n=8, north n=1, and mid-west n=1) were analyzed. Blood samples from 30 unrelated patients were collected and DNA was isolated and the CBS gene sequenced. RNA samples from 6 patients were collected and gene expression was analysed by qRT-PCR. Expression studies in *E.coli* of three novel mutations were performed. To study body composition and lipid metabolism, two studies were conducted. In the first, body composition was assessed by bioelectrical impedance analysis (BIA) in 9 patients and 18 controls; and plasma levels of methionine, total homocysteine, cysteine, choline, betaine, dimethylglycine, and ethanolamine were measured in patients. In the second study, 11 patients and 16 controls were evaluated. Body composition was assessed by Dual X-ray Absorptiometry (DXA). The sulfur compounds methionine, S-adenosylmethionine, S-adenosylhomocysteine, total homocysteine, cysteine, and glutathione; besides lipoproteins, free fatty acids, acylcarnitines, leptin, adiponectin, isoprostanes, glucose, and insulin were measured in plasma. Insulin resistance was

determined by HOMA-IR. SCD-16 and SCD-18 indices, which estimate liver SCD-1 activity in humans, were calculated by product/precursor ratios of the corresponding fatty acids.

Results: Clinical data analysis showed a high prevalence of nonresponsive patients (82%). Patients had a median age at evaluation of 19 years (range: 5-45 years); at diagnosis: 10 years (range: 1- 39 years); and first symptoms at the age of 5 years (range: 0-20 years). Eye manifestations were the earliest symptoms, the main reason for diagnostic suspicion, and the most prevalent symptom at diagnosis (69% of the cases). Only 28% of the treated patients had total homocysteine <60 µmol/L. Regarding treatment strategies, 89% of the patients used folic acid and pyridoxine, 76% betaine, 32% vitamin B12, and 26% methionine-restricted diet + formula. *CBS* sequencing revealed 21 distinct mutations, among which the most prevalent are: p.Ile278Thr (18.2%), p.Thr191Met (11.3%), r [737_828del92, 828_931ins104] (11.3%), and p.Trp323Ter (11.3%). Eight new mutations were found. Enzymatic activities expressed in *E.coli* were: 1.5% of normal for c.329A>T mutation; 17.5% for c.284T>C, and 206% for c.2T>C. qRT-PCR analysis showed reduced gene expression (54-95%) in all patients studied. Body composition analysis showed no difference in adiposity between patients and controls. However, lean mass was lower in patients according to DXA assessment ($p = 0.008$). Plasma leptin levels, LDL cholesterol, and SCD-16 index were significantly reduced in patients ($p < 0.05$). Lipoproteins showed associations with choline, ethanolamine, and leptin levels in patients. Cysteine was significantly lower in patients, and positively correlated with BMI ($r = 0.912$, $p = 0.001$), and with SCD-16 index ($r = 0.624$, $p = 0.054$). No differences were observed for the remaining variables.

Conclusions: This study provides the most comprehensive clinical and genetic profile of HCU ever held in Brazil. Most of the patients studied here presented a severe phenotype, suggesting underdiagnosis of milder/responsive types of HCU. A great number of private mutations was observed, but the four most prevalent mutations account for more than half of the mutated alleles. Low leptin, SCD-1 and cholesterol, and their association with SAA, are in agreement with previous observational and experimental studies that show that SAA (mainly cysteine) have an important role in the modulation of lipid metabolism.

2. INTRODUÇÃO

A homocistinúria clássica (HCU; OMIM +236200) é uma doença genética rara causada pela deficiência da enzima cistationina β-sintase (CβS; EC 4.2.1.22) e é herdada de maneira autossômica recessiva. A HCU pertence a um grupo de doenças genéticas denominadas erros inatos do metabolismo (EIM), que são doenças causadas por alterações na estrutura e/ou função de moléculas proteicas, em sua maioria enzimas. A deficiência da enzima CβS resulta em alterações importantes no metabolismo dos aminoácidos sulfurados (SAA), com elevação em fluidos corporais dos níveis de homocisteína, metionina, *S*-adenosil homocisteína e redução de cistationina e cisteína (Wilcken, 2006).

A primeira descrição de HCU foi em 1962, na Irlanda. Enquanto realizavam testes de urina a fim de detectar distúrbios metabólicos em indivíduos com retardo mental, os pesquisadores Nina Carson e Desmond Neill verificaram que duas irmãs excretavam grande quantidade de homocistina na urina. As irmãs apresentavam também luxação de cristalino, deformidades esqueléticas e alterações na pele e cabelos. Os pesquisadores denominaram este novo distúrbio metabólico de homocistinúria (Carson e Neill, 1962). Dois anos depois, foi descoberto o defeito enzimático resposável pela doença (Mudd *et al.*, 1964). As primeiras estratégias de tratamento - restrição dietética de metionina e suplementação de piridoxina - foram descritas poucos anos depois (Komrower, 1967; Turner, 1967; Perry *et al.*, 1968). O gene *CBS* humano foi mapeado em 1998 por Kraus e colaboradores (Kraus *et al.*, 1998). Mais de 160 variantes patogênicas diferentes já foram identificadas no gene (Kraus, 2016).

A história natural da doença foi descrita em 1985, em uma grande compilação de dados de vários centros no mundo (Mudd *et al.*, 1985). Este trabalho incluiu mais de 600 pacientes, e foi fundamental para a caracterização clínica e entendimento da progressão da HCU. Além disso, demonstrou claramente a eficácia do tratamento - especialmente no período neonatal - na prevenção e progressão das complicações (Mudd *et al.*, 1985).

Alterações das proporções corporais (p.ex. alta estatura, biótipo magro e dolicostenomelia) são frequentes em pacientes com HCU, tornando estes pacientes fenotipicamente semelhantes aos portadores da Síndrome de Marfan (Gibson *et al.*, 1964; Brenton *et al.*, 1972). Acreditava-se que as alterações ósseas eram responsáveis pelo

"fenótipo marfanóide" na HCU (Brenton, 1977). No entanto, estudos recentes em indivíduos saudáveis e em modelos animais sugerem que alterações no metabolismo da homocisteína impactam de forma significativa a composição corporal, em especial a massa adiposa (Elshorbagy *et al.*, 2008; Elshorbagy, Refsum, *et al.*, 2009; Elshorbagy *et al.*, 2010; Elshorbagy, 2014). Redução significativa de adiposidade e de ganho de peso está descrita no modelo animal de HCU (Gupta e Kruger, 2011).

Os mecanismos pelo qual alterações no metabolismo da homocisteína afetam a composição corporal não estão completamente elucidados. Evidências de estudos em modelos animais sugerem que hormônios moduladores de composição corporal (como leptina e adiponectina) e a enzima lipogênica estearoil-CoA desaturase-1 (SCD-1) medeiem estas alterações (Cohen e Friedman, 2004; Gupta e Kruger, 2011; Elshorbagy, 2014). Estas relações, no entanto, ainda não foram investigadas em pacientes com HCU.

Os objetivos do presente estudo foram: descrever o perfil clínico e genético de pacientes brasileiros com HCU; e avaliar a relação entre SAA, composição corporal, atividade da SCD-1, resistência insulínica e metabolismo lipídico em pacientes com HCU em tratamento.

3. REVISÃO DA LITERATURA

3.1 METABOLISMO DOS AMINOÁCIDOS SULFURADOS (SAA)

Os principais SAA em humanos são a metionina, cisteína, homocisteína 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 (Castro *et al.*, 2006; Blom e Smulders, 2011). O metabolismo dos SAA é resumido na Figura 1.

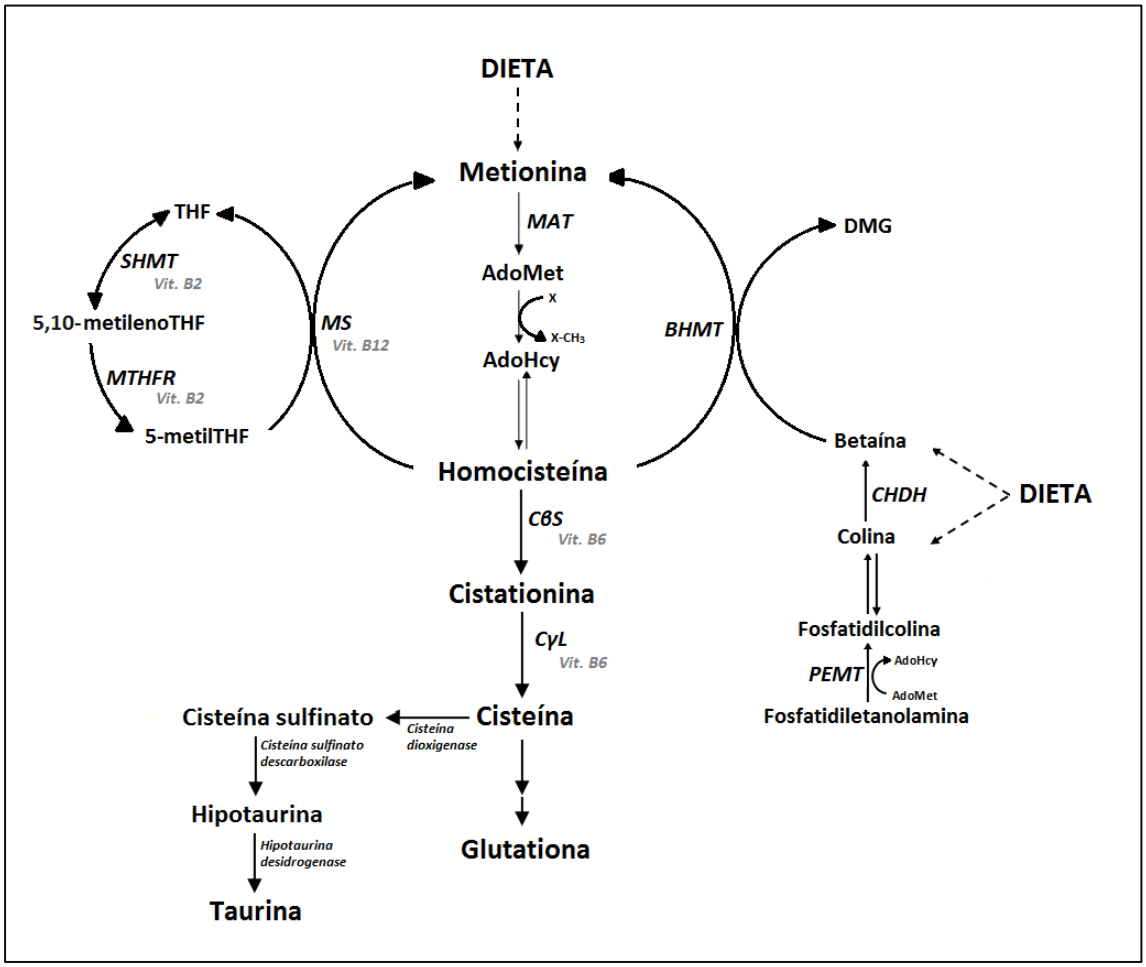

Figura 1. Metabolismo dos aminoácidos sulfurados. MAT: metionina adenosiltransferase; AdoMet: *S*-adenosilmetionina; AdoHcy: *S*-adenosilhomocisteína; THF: tetrahidrofolato; MTHFR: 5,10 metilenotetrahidrofolato redutase; SHMT: serina hidroximetiltransferase; MS: metionina sintase; BHMT: betaína:homocisteína metiltransferase; DMG: dimetilglicina; CHDH: colina desidrogenase; PEMT: fosfatidiletonolamina N-metiltransferase; CβS: cistationina β-sintase. CγL: cistationina gama-liase. Enzimas são descritas em itálico e seus cofatores em cinza (Poloni *et al.*, 2015).

A homocisteína está em um ponto de ramificação importante no metabolismo dos SAA. Estima-se que metade da homocisteína formada seja convertida em cistationina pela CβS, enquanto os outros 50% são remetilados à metionina (Kraus, 1998).

A metionina é um aminoácido essencial e é metabolizada no fígado, onde é desmetilada através de dois compostos intermediários, a *S*-adenosilmetionina (AdoMet) e a *S*-adenosilhomocisteína (AdoHcy). Após esta reação, é formada a homocisteína, um aminoácido tóxico que não é incorporado a proteínas. A remoção plasmática da homocisteína pode ocorrer de duas maneiras: através da via de transsulfuração, onde a é irreversivelmente degradada; ou da remetilação, onde é reconvertida a metionina (Finkelstein, 1998).

A remetilação da homocisteína à metionina pode ser feita por duas rotas alternativas. A primeira é catalisada pela metionina sintase, uma enzima dependente de vitamina B12 e de folato. A segunda é catalisada pela betaína: homocisteína metiltransferase (BHMT), enzima que utiliza betaína, formada pelo catabolismo da colina, como doador do grupamento metil (Blom e Smulders, 2011). Pela rota de transsulfuração a homocisteína é convertida a cistationina, e posteriormente a cisteína. Estas reações são catalisadas por duas enzimas dependentes de piridoxal 5'-fosfato (PLP), forma ativa da vitamina B_6.

A cisteína pode ser degradada por diversas reações de dessulfuração que dão origem ao sulfeto de hidrogênio (H_2S) e sulfano. A rota oxidativa leva à produção de taurina e sulfato na proporção de 2:1. Além da produção de taurina, a cisteína é utilizada na síntese proteica e de glutationa, um potente antioxidante. A taurina é o aminoácido mais abundante em tecidos animais e é utilizada na síntese de sais biliares, além de possivelmente atuar como antioxidante, estabilizador de membrana e neurotransmissor (Stipanuk e Ueki, 2011).

O controle do metabolismo dos SAA é complexo e opera em diversos níveis. As concentrações enzimáticas são influenciadas por fatores como a ingestão proteica, níveis hormonais, deficiências nutricionais, idade e mudanças em longo prazo nos níveis de substrato (Finkelstein, 1998). A AdoMet possui papel central nesta regulação. Quando os níveis de metionina aumentam, a concentração de AdoMet aumenta e favorece a reação pela rota de transsulfuração, ativando a enzima CβS e inibindo a MTHFR. Se os níveis de

metionina estão baixos, como no jejum, os níveis reduzidos de AdoMet não vão ativar a CβS e inibir a MTHFR, resultando na remetilação da homocisteína a metionina (Blom e Smulders, 2011).

3.2 DEFINIÇÃO DE HOMOCISTINÚRIA E HIPERHOMOCISTEINEMIA

O termo homocistinúria refere-se a um grupo de EIM que resultam em aumento marcante dos níveis de homocisteína no plasma ou soro, e foi assim denominada devido à eliminação excessiva de homocistina (homocisteína dissulfeto) na urina observada nos pacientes. Homocistinúria clássica (HCU) refere-se exclusivamente ao EIM causado pela atividade deficiente da enzima CβS (Mudd *et al.*, 1985; Mudd *et al.*, 2001; Wilcken, 2006).

Já o termo hiperhomocisteinemia refere-se ao aumento de homocisteína no plasma ou soro que pode ser de origem genética, ambiental, ou multifatorial; e que está presente em 5 – 10% da população (Booth e Wang, 2000; Mudd *et al.*, 2000). Em indivíduos saudáveis, os níveis plasmáticos de homocisteína mantém-se abaixo de 15 μmol/L, com pequenas 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).

3.3 HOMOCISTINÚRIA CLÁSSICA (HCU)

A HCU constitui o mais frequente distúrbio do metabolismo dos SAA (Mudd *et al.*, 2001; Wilcken, 2006). Os principais aspectos da epidemiologia, diagnóstico e manejo da doença serão discutidos nas seções a seguir.

3.3.1 Epidemiologia

A prevalência mundial estimada de HCU é de 1:100,000 a 1:344,000 indivíduos, embora acredita-se que estes números estejam amplamente subestimados (Mudd *et al.*,

2001; Skovby *et al.*, 2010; Moorthie *et al.*, 2014). Em uma meta-análise publicada recentemente, relatou-se uma prevalência no mundo de 1.09:100,000 quando diagnóstico foi realizado por MS/MS e 0.82:100,000 por critérios clínicos (Moorthie *et al.*, 2014).

Diferentes métodos de triagem neonatal, como a determinação de homocisteína ou a genotipagem de mutações comuns, têm encontrado incidências de HCU tão altas quanto 1:6,400 na Noruega, 1:20,500 na Dinamarca e 1:1,800 no Catar (Gaustadnes *et al.*, 1999; Refsum *et al.*, 2004; Gan-Schreier *et al.*, 2010). Outras populações com alta frequência da doença incluem a Irlanda (incidência de 1:65,000 recém-nascidos vivos), a Alemanha (prevalência 1:17,800) e a tribo Tao, em Taiwan, com uma prevalência de 1:240, sendo esta a mais alta no mundo (Picker e Levy, 1993-2016; Naughten *et al.*, 1998; Linnebank *et al.*, 2001; Lu *et al.*, 2012). No Brasil, não há dados sobre incidência ou prevalência de HCU.

3.3.2 Aspectos bioquímicos

Os achados bioquímicos mais marcantes na HCU são a elevação acentuada de homocisteína em fluidos corporais, especialmente plasma/soro (>100 µmol/L) e hipermetioninemia (Mudd *et al.*, 2001). O grupamento –SH da homocisteína reage rapidamente com outras moléculas, levando à formação de diversos componentes dissulfeto, como a homocistina (que posteriormente é eliminada e pode ser detectada na urina), o dissulfeto homocisteína-cisteína ou homocisteína ligada a proteínas. A homocisteína e alguns de seus metabólitos, como a homocisteína-tiolactona, possuem ação danosa em diversos tecidos, e acredita-se que estes compostos tenham papel central na fisiopatologia da doença (Kraus, 1998; Mudd *et al.*, 2001).

Redução nos níveis plasmáticos de cisteína também é observada, uma vez que a CβS está envolvida diretamente em sua síntese. Outros metabólitos envolvidos na rota dos SAA (Figura 1) também são afetados: observam-se altas concentrações circulantes de AdoMet e AdoHcy, compostos envolvidos na transferência de grupamentos metil; e baixas de cistationina, o produto primário da CβS; e ainda depleção de betaína, folato e vitamina B12, que participam das vias de remetilação (Wilcken, 2006; Imbard *et al.*, 2015).

3.3.3 Manifestações clínicas

A HCU é uma doença multissistêmica, que pode se manifestar em uma ampla gama de sintomas e em qualquer idade. Manifestações intrauterinas, no entanto, são desconhecidas (Mudd *et al.*, 1985; Mudd *et al.*, 2001). Os sinais clínicos mais frequentes envolvem principalmente quatro sistemas que serão discutidos em detalhes a seguir.

Sistema ocular: A luxação ou subluxação de cristalino (*ectopia lentis*) é o achado mais consistente da HCU, sendo altamente prevalente e surgindo precocemente na maioria dos pacientes. No levantamento realizado por Mudd et al. (1985), este sintoma contribuiu para a investigação de HCU em 85% dos casos, e ocorreu mais precocemente em pacientes não responsivos à piridoxina (mediana = 6 anos). O deslocamento do cristalino ocorre bilateralmente e geralmente na direção inferior. Outras alterações oftalmológicas comuns são: alta miopia, glaucoma, iridodonese, deslocamento e degeneração da retina, catarata e atrofia do nervo óptico (Mudd *et al.*, 1985; Burke *et al.*, 1989; Taylor *et al.*, 1998; Rajappa *et al.*, 2010).

Sistema vascular: a importância da doença vascular na HCU dá-se pela sua alta morbimortalidade. Trombose de médias e grandes artérias, em especial carótidas e artérias renais, está entre as principais causas de morte em pacientes com HCU. A cardiopatia isquêmica é rara (Wilcken e Wilcken, 1997; Yap *et al.*, 2000; Andria G, 2006; Magner *et al.*, 2011). Oclusões vasculares podem ocorrer em qualquer veia e em qualquer idade. Em pacientes não tratados, a chance de sofrer um evento tromboembólico até os 29 anos de idade é de 50% (Mudd *et al.*, 1985). Situações como gestação, puerpério ou pós-operatório; bem como a associação com outros genótipos de risco, como mutações nos genes do Fator V e MTHFR, aumentam o risco vascular (Mandel *et al.*, 1996; Kluijtmans *et al.*, 1998; Mudd *et al.*, 2001). Eventos vasculares podem ainda apresentar-se isoladamente na HCU. Homozigotos para a mutação p.Ile278Thr, por exemplo, podem ser clinicamente normais durante a infância e a adolescência, e a partir da terceira década de vida apresentar eventos tromboembólicos como única manifestação clínica de HCU (Skovby *et al.*, 2010; Magner *et al.*, 2011).

Sistema nervoso central (SNC): Déficit cognitivo é um achado frequente na HCU, porém uma grande variabilidade nos escores de QI é relatada (10 - 138 pontos) (Mudd *et al.*, 1985). No estudo de Mudd et al. (1985), em pacientes não tratados apenas 4% dos não

responsivos à piridoxina apresentavam escores de QI >90, enquanto que entre os responsivos esta parcela era de 22%. Convulsões ocorrem em aproximadamente 20% dos pacientes, e sinais extrapiramidais como distonia podem estar presentes. Além do efeito tóxico da homocisteína sobre o SNC, acidentes vasculares encefálicos recidivos também podem agravar os sintomas neurológicos (Mudd *et al.*, 2001; Andria G, 2006). Distúrbios psiquiátricos também são frequentes, sendo os principais relatados na literatura: esquizofrenia, distúrbios de personalidade, ansiedade, depressão, comportamento obsessivo-compulsivo e episódios psicóticos (Kaeser *et al.*, 1969; Bracken e Coll, 1985; De Franchis *et al.*, 1998; Colafrancesco *et al.*, 2015). Em uma pesquisa com 63 pacientes, a taxa de distúrbios psiquiátricos clinicamente relevantes foi de 51%, sendo que comportamento agressivo e outros distúrbios de conduta foram mais comuns entre pacientes com déficit cognitivo e não responsivos à piridoxina (Abbott *et al.*, 1987). É descrita também a apresentação isolada de sintomas psiquiátricos em pacientes sem outras manifestações clínicas de HCU (Li e Stewart, 1999; Ryan *et al.*, 2002).

Sistema ósseo: redução da densidade mineral óssea culminando em osteoporose é o achado ósseo mais comum: quando não tratados, metade dos pacientes desenvolve osteoporose até o fim da segunda década de vida (Mudd *et al.*, 1985). A osteoporose atinge mais frequentemente coluna vertebral e ossos longos. Também podem ocorrer escoliose, dolicostenomelia, alta estatura, aracnodactilia, *genu valgum*, *pectus excavatum* ou *carinatum*, entre outras (Mudd *et al.*, 2001; Weber *et al.*, 2016). Outro achado frequente é o alargamento das metáfises e epífises dos ossos longos, facilmente detectado nos joelhos (Brenton, 1977). Com exceção da osteoporose, estas manifestações ósseas e a luxação de cristalino também são observadas na síndrome de Marfan, doença genética do tecido conjuntivo (Brenton *et al.*, 1972).

Alterações na molécula de fibrilina e nas moléculas de colágeno parecem estar envolvidas na fisiopatologia da doença óssea na HCU (Masse *et al.*, 2003). A fibrilina, molécula deficiente na síndrome de Marfan, pertence a uma família de proteínas de matriz extracelular e é essencial para a formação das fibras elásticas no tecido conjuntivo. A homocisteína é capaz de incorporar-se na molécula de fibrilina *in vitro*, alterando sua função. Além disso, a fibrilina é rica em resíduos de cisteína (~14%), e a deficiência deste aminoácido na HCU também pode estar envolvida na patogênese das manifestações ósseas (Mudd *et al.*, 2001; Hubmacher *et al.*, 2010).

Outros sistemas: Podem ocorrer manifestações dermatológicas como a hipopigmentação de pele e cabelos, *malar flush* e *livedo reticularis*, uma vez que a homocisteína prejudica a atividade da tirosinase, principal enzima da rota de síntese da melanina (Picker e Levy, 1993-2016; Reish *et al.*, 1995). Complicações gastrointestinais como pancreatite aguda e diarreia crônica já foram descritas na HCU, mas são raras (Ilan *et al.*, 1993; Makins *et al.*, 2000).

3.3.4 Diagnóstico e triagem neonatal

No Brasil, o diagnóstico normalmente é guiado pela suspeita clínica, uma vez que a HCU não está inclusa no Programa Nacional de Triagem Neonatal. Usualmente, os achados oftalmológicos típicos, o fenótipo marfanóide e o déficit cognitivo são as características que levam à investigação de HCU (Mudd *et al.*, 1985; Mudd *et al.*, 2001).

Há alguns anos, quando as medidas de homocisteína em sangue ainda eram pouco disponíveis, testes de triagem como o do cianeto-nitroprussiato que detecta a eliminação de compostos sulfurados na urina; ou a determinação de homocistina na urina, eram bastante utilizados. Atualmente, opta-se pela determinação direta dos níveis plasmáticos de homocisteína total (tHcy), metionina e cisteína, uma vez que o padrão de alteração destes aminoácidos (↑Metionina e tHcy e ↓Cisteína) é bastante sugestivo de HCU (Picker e Levy, 1993-2016; Andria G, 2006).

Entretanto, indivíduos com formas mais leves de HCU podem apresentar uma resposta importante a baixas doses de piridoxina, como 2 mg/dia. Assim, o uso de suplementos vitamínicos pode normalizar os testes bioquímicos, levando a um diagnóstico falso-negativo (Mudd *et al.*, 2001). A mensuração da atividade enzimática da CβS é o padrão-ouro para confirmação do diagnóstico de HCU. Ela pode ser feita através de cultura de fibroblastos, biópsias hepáticas ou linfócitos estimulados por fitohemaglutinina. A atividade enzimática em pacientes com HCU varia de 0 a 1.8 U/mg proteína, enquanto em controles fica na faixa de 3.7-60 U/mg proteína (Picker e Levy, 1993-2016).

O diagnóstico por análise de DNA também pode ser realizado, especialmente em famílias/populações com mutações conhecidas. (Fowler e Jakobs, 1998; Huemer *et al.*, 2015). O diagnóstico pré-natal pode ser realizado através da extração e cultura de células do fluido amniótico e determinação da atividade da CβS (Fowler *et al.*, 1982).

Triagem neonatal para HCU normalmente é realizada pela determinação de metionina em papel filtro, já que sua dosagem é de mais fácil execução do que a tHcy. No entanto, um grande percentual falso-negativos (até 50%) pode ser obtido utilizando este método, já que formas mais leves de HCU, responsivas à piridoxina, podem não cursar com hipermetioninemia nos primeiros dias de vida (Naughten *et al.*, 1998; Sokolova *et al.*, 2001; Refsum *et al.*, 2004). Além disso, outras doenças também podem ocasionar hipermetioninemia. Para melhorar a sensibilidade do teste pode-se reduzir o ponto de corte para detecção de metionina ou utilizar a razão metionina/fenilalanina, que ajusta o resultado para o consumo proteico (Peterschmitt *et al.*, 1999; Bowron *et al.*, 2005; Mchugh *et al.*, 2011; Huemer *et al.*, 2015).

Também pode ser realizada para triagem neonatal a determinação de tHcy em papel filtro ou triagem de mutações comuns em populações de alto risco. Ambos os métodos têm custo mais elevado e exigem maior capacidade técnica, o que limita seu uso (Refsum *et al.*, 2004; Gan-Schreier *et al.*, 2010; Huemer *et al.*, 2015).

3.3.5 Estratégias de tratamento

O principal objetivo do tratamento da HCU é a redução nos níveis de homocisteína. Para isto, três estratégias principais têm sido utilizadas: 1) estimulação da atividade residual da CβS, 2) aumento da remetilação à metionina e 3) redução da sobrecarga de substrato (metionina) (Walter *et al.*, 1998; Schiff e Blom, 2012).

O nível ótimo de homocisteína a ser atingido não está plenamente estabelecido. Estudos observacionais sugerem que níveis de tHcy <20 µmol/L em pacientes responsivos à piridoxina e <60-70 µmol/L em não responsivos estão associados a melhores desfechos (Yap, Boers, *et al.*, 2001; Wilcken, 2006; Schiff e Blom, 2012). As principais estratégias de tratamento são detalhadas a seguir.

Piridoxina (vitamina B6): A CβS utiliza como cofator o piridoxal fosfato, forma ativa da piridoxina. Na HCU, a suplementação por via oral de piridoxina aumenta a atividade residual da enzima (Mudd *et al.*, 1970). A dose a ser utilizada é variável de acordo com a resposta; em geral inicia-se com 100 mg/dia podendo-se atingir até 1000 mg/dia em adultos (Picker e Levy, 1993-2016; Schiff e Blom, 2012). Embora efeitos adversos sejam

raros, doses >400 mg/dia podem ocasionar neuropatia periférica e devem ser utilizadas com cautela (Wilcken, 2006).

A resposta à piridoxina depende da localização e gravidade das mutações: sabe-se que cerca de 50% dos pacientes apresentam algum nível de responsividade à piridoxina, e em geral, pacientes não responsivos desenvolvem a forma mais grave da doença (Mudd *et al.*, 1985). Assim, após o diagnóstico de HCU, deve ser realizado o teste de responsividade à piridoxina. Existem diversos protocolos propostos, mas em geral, suplementa-se piridoxina oral em doses de 100-500 mg/dia (para bebês, no máximo 250 mg/dia), sendo considerados responsivos os pacientes que atingirem os níveis alvo de homocisteína (Picker e Levy, 1993-2016; Walter *et al.*, 1998; Mudd *et al.*, 2001; Schiff e Blom, 2012). Aos pacientes que apresentarem resultados insatisfatórios com a suplementação de piridoxina, outras estratégias de tratamento devem ser incluídas.

Folato (vitamina B9): O ciclo do folato está estreitamente ligado ao da homocisteína e esta vitamina tem papel central na rota de remetilação (Figura 1). Assim, recomenda-se suplementar ácido fólico ou folínico (este último é a forma reduzida mais estável) para prevenir sua depleção, uma vez que os requerimentos parecem estar aumentados na HCU pelo aumento do fluxo pela rota de remetilação. Além disso, a resposta à piridoxina é reduzida em situações de depleção de folato (Wilcken e Turner, 1973). Doses de 1 a 5 mg/dia são recomendadas (Wilcken, 2006; Schiff e Blom, 2012).

Cobalamina (vitamina B12): A vitamina B12 participa da rota de remetilação pela mesma via do folato (Figura 1). A deficiência de B12 não é rara na HCU e recomenda-se suplementação por via oral (1 mg por dia ou por semana) ou parenteral (1 mg/mês) (Wilcken, 2006; Schiff e Blom, 2012).

Betaína (*N,N,N*-trimetilglicina): A betaína participa da rota de remetilação por via independente do folato e B12 (Figura 1). A suplementação por via oral de betaína pode reduzir em mais de 70% os níveis de homocisteína, mas deve-se atentar para o aumento concomitante das concentrações de metionina. Doses acima de 1000 μmol/L podem ocasionar edema cerebral. A inalação acidental da vitamina em pó também pode causar sérios problemas pulmonares (Lawson-Yuen e Levy, 2006; Wilcken, 2006). A dose recomendada é de até 150 - 250 mg/kg/dia ou 6-9 g/dia em adultos, divididas em 2 – 3 tomadas diárias (Matthews *et al.*, 2002; Wilcken, 2006; Schiff e Blom, 2012).

Restrição dietética de metionina: a restrição dietética de metionina faz parte da terceira estratégia de tratamento, a redução da sobrecarga de substrato. Reduzindo a ingestão de metionina, uma importante queda nos níveis de homocisteína é observada (Komrower, 1967). A restrição de metionina é feita através da limitação do aporte de proteínas naturais na dieta. Para atingir as necessidades proteicas diárias utiliza-se um suplemento de aminoácidos (também chamado fórmula metabólica) isento de metionina e suplementado com cisteína, vitaminas, minerais e elementos-traço (Pons *et al.*, 2004; Frangipani *et al.*, 2006). A quantidade de metionina liberada na dieta varia de 15 – 60 mg/kg/dia, dependendo do sexo, faixa etária e tolerância do paciente (Frangipani *et al.*, 2006; Thomas, 2015). A má adesão à dieta é comum, principalmente em pacientes com diagnóstico tardio, uma vez que exige uma mudança drástica nos hábitos alimentares. Além disso, devido à baixa palatabilidade, a ingestão insuficiente do suplemento de aminoácidos também é frequente (Walter *et al.*, 1998; Adam *et al.*, 2013).

Outras opções terapêuticas: Para pacientes de alto risco vascular (história de eventos tromboembólicos ou portadores de mutação do Fator V de Leiden), o uso de agentes antitrombóticos como o ácido acetilsalicílico é recomendado (Wilcken, 2006).

Uma nova alternativa terapêutica em desenvolvimento é o uso de chaperonas, moléculas de baixo peso molecular que protegem proteínas de várias condições desnaturantes. Uma vez que o enovelamento anormal da CβS parece ser um importante mecanismo patogênico na HCU (Kozich *et al.*, 2010; Hnizda *et al.*, 2012), as chaperonas tornam-se uma possibilidade terapêutica promissora. Estudos *in vitro* com chaperonas químicas têm apresentado resultados positivos (Singh *et al.*, 2007; Majtan *et al.*, 2010; Kopecka *et al.*, 2011).

O transplante hepático já foi realizado com sucesso na HCU, com completo desaparecimento dos sintomas bioquímicos da doença (Lin *et al.*, 2012). Esta, porém, não é considerada uma opção terapêutica rotineira devido ao alto risco de morte envolvido. Estudos para desenvolvimento de terapia de reposição enzimática para HCU foram iniciados recentemente por Kraus e colaboradores (Kraus *et al.*, 2014).

3.3.6 Prognóstico

Os benefícios do tratamento na HCU estão bem estabelecidos. O tratamento desde o período neonatal, associado a um bom controle metabólico, é capaz de prevenir todas as manifestações clínicas (Mudd *et al.*, 1985; Yap e Naughten, 1998; Yap, Rushe, *et al.*, 2001; Lim e Lee, 2013). Em pacientes com diagnóstico tardio, o tratamento também é efetivo na redução da morbimortalidade. Importante redução de eventos vasculares é descrita em pacientes tratados, mesmo quando on níveis de tHcy permanecem elevados (Wilcken e Wilcken, 1997; Yap, 2003; Skovby *et al.*, 2010). Já baixa densidade mineral óssea é comum em pacientes tratados com controle metabólico inadequado (Weber *et al.*, 2016).

3.3.7 Estrutura e regulação da CβS

A CβS é uma hemeproteína, piridoxal fosfato dependente, sujeita a complexa regulação. O produto translacional primário da CβS humana é um polipeptídeo de peso molecular de 63 kDa, que posteriormente forma tetrâmeros. Cada subunidade de 551 aminoácidos se liga a duas moléculas: a homocisteína e a serina, para formar a L-cistationina (Oyenarte *et al.*, 2012). A regulação da atividade da enzima ocorre primariamente através da ligação de três componentes: o piridoxal fosfato, a AdoMet e o grupamento heme (Banerjee e Zou, 2005).

Em humanos, a CβS contém uma região N-terminal de ~70 aminoácidos que liga ao grupamento heme. Esta ligação é essencial para a ativação completa da CβS, uma vez que sob dissociação completa do grupamento heme, a enzima retém apenas 20% de sua atividade original (Meier *et al.*, 2001; Miles e Kraus, 2004). Acredita-se que o heme atue como sensor redox e/ou no enovelamento e estabilidade da enzima (Kery *et al.*, 1994; Janosik *et al.*, 2001; Majtan *et al.*, 2010).

O sítio ativo representa a porção conservada da proteína, e isoladamente forma dímeros de 45 kDa que são cerca de duas vezes mais ativos do que a forma tetramérica (Kraus, 2016). O cofator piridoxal fosfato atua em uma fenda entre os domínios N- e C-terminal, sendo o sítio ativo acessível apenas por um estreito canal (Meier *et al.*, 2001).

O domínio regulatório C-terminal contém aproximadamente 140 resíduos de aminoácidos e inclui o chamado “domínio CβS”. Trata-se de uma porção conservada da proteína, composta por cerca de 60 aminoácidos, que exerce um efeito autoinibitório no sítio ativo da enzima (Banerjee e Zou, 2005). Esta inibição é reduzida pela ligação da AdoMet, o ativador alostérico da CβS que aumenta em até três vezes a atividade catalítica da enzima (Miles e Kraus, 2004; Ereno-Orbea *et al.*, 2013). Dois sítios de ligação da AdoMet estão presentes no domínio C-terminal da CβS: um de alta afinidade, que liga duas moléculas de AdoMet por tetrâmero, parece ser responsável pela estabilização cinética do domínio. O segundo, de baixa afinidade, liga quatro moléculas por tetrâmero e parece ser responsável pela ativação da enzima (Pey *et al.*, 2013). Defeito na ligação da AdoMet tem se mostrado um mecanismo patogênico frequente na HCU (Ereno-Orbea *et al.*, 2013; Pey *et al.*, 2013; Mendes *et al.*, 2014; Majtan *et al.*, 2016).

3.3.8 Gene *CBS*

Em humanos, o gene *CBS* localiza-se no braço longo do cromossomo 21 (21q22.3) e sua sequência completa foi descrita em 1998 (Kraus *et al.*, 1998). Um total de 28.046 nucleotídeos compõe o gene, e um fragmento adicional de 5 kpb forma a sequência flanqueadora. O gene é composto por 23 éxons, sendo a região codificadora composta pelos éxons 1-16; região que codifica o polipeptídeo de 551 aminoácidos (Figura 2).

A região 5’-UTR do mRNA da CβS humana é formada por um de cinco éxons alternativos, denominados -1a a -1e; mais o éxon 0, invariavelmente presente. A região 3’-UTR é composta por parte do éxon 16 e pelo éxon 17. O éxon 15 codifica 14 aminoácidos e sofre *splicing* alternativo, sendo incorporado em poucas moléculas humanas maduras de mRNA, e sua funcionalidade é desconhecida. Em estudos *in vitro*, a presença ou ausência do éxon 15 não altera a atividade catalítica da CβS. Além disso, sua presença não foi detectada em nenhum tecido humano avaliado até hoje (Kraus *et al.*, 1998; Kraus, 2016).

Existem pelo menos dois promotores utilizados alternativamente no gene *CBS* humano. Eles estão localizados acima dos éxons -1a e -1b, são ricos em CG (~80%), e contém diversos sítios de ligação para a Sp1, Ap1, Ap2 e c-myb, mas ausência de TATA *box* (Kraus, 2016).

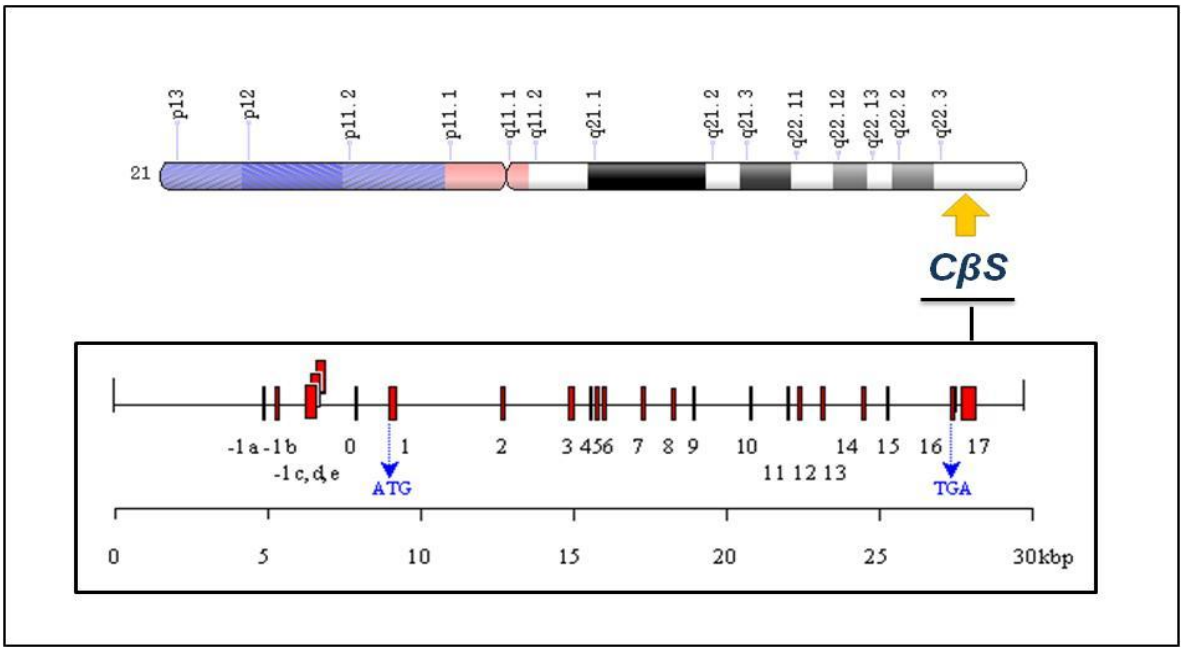

Figura 2. Localização e organização do gene *CBS* em humanos (Kraus, 2016). Os éxons são representados por caixas vermelhas sólidas. O início da sequência codificante é representado pelo códon ATG; e o fim, pelo códon TGA.

3.3.8.1 Variantes patogênicas

Mais de 160 variantes patogênicas são descritas no gene *CBS*, sendo a maioria delas do tipo *missense* (85%) (Kraus, 2016). Mutações no gene *CBS* podem alterar a estabilidade ou atividade da enzima ou do mRNA, a ligação do piridoxal fosfato ou do grupamento heme, ou prejudicar a regulação alostérica (Miles e Kraus, 2004; Chen *et al.*, 2006).

Devido à alta frequência de variantes do tipo *missense* na HCU, o enovelamento anormal da proteína (*misfolding*) é um mecanismo patogênico importante. Estas proteínas mutantes são propensas a formar agregados de alto peso molecular e corpos de inclusão. Em estudo desenvolvido por Kozich e colaboradores (2010) com as 27 variantes mais prevalentes em pacientes com HCU, observou-se que 18 delas resultavam em *misfolding* moderado a grave (formação de <25% de tetrâmeros em relação ao tipo selvagem). Além disso, verificou-se que mutações no interior da enzima parecem exercer efeitos mais deletérios no enovelamento da enzima do que aquelas acessíveis ao solvente (Kozich *et al.*, 2010).

As variantes patogênicas mais comuns no gene *CBS* são: p.Ile278Thr, p.Thr191Met, p.Gly307Ser e p.Arg336Cys. Juntas, correspondem a 51% dos alelos mutados (Kraus, 2016).

p.Ile278Thr (c.833T>C): variante pan-étnica, responsável por cerca de 16% dos alelos de HCU no mundo. Altamente prevalente em populações europeias (Sebastio *et al.*, 1995; Shih *et al.*, 1995; Sperandeo *et al.*, 1995; Kluijtmans *et al.*, 1999; Sokolova *et al.*, 2001). Também já foi descrita na Austrália, Argentina, Estados Unidos e Israel (Tsai, Bignell, *et al.*, 1996; Gat-Yablonski *et al.*, 2000; Kruger *et al.*, 2003; Cozar *et al.*, 2011). Em um estudo com pacientes brasileiros da região sudeste, foi detectada em 6/28 alelos estudados (frequência em não relacionados de 13,64%) (Porto *et al.*, 2005). A variante está associada a fenótipos atenuados e responsividade à piridoxina, tanto em homozigotos como em heterozigotos compostos (Mudd *et al.*, 2001; Kraus, 2016).

A mutação localiza-se no núcleo ativo da enzima, em região não exposta ao solvente. A CβS mutante retém aproximadamente 2% da atividade da enzima selvagem (Kruger *et al.*, 2003). A enzima mutante apresenta reduzida ativação pela AdoMet (Kozich *et al.*, 2010; Mendes *et al.*, 2014). Além disso, a variante parece afetar mais o enovelamento do que a atividade catalítica da enzima, uma vez que *in vitro* o uso de chaperonas aumenta significativamente a atividade enzimática da CβS mutante (Singh *et al.*, 2007).

p.Thr191Met (c.572C>T): responsável por cerca de 16% dos alelos mutados no mundo (Kraus, 2016). Esta variante é altamente prevalente em países da península ibérica (Portugal e Espanha) e América do Sul (Venezuela, Colômbia e Argentina). As maiores frequências foram encontradas na Colômbia (75% dos alelos mutados) e Espanha (52% dos alelos mutados) (Urreizti *et al.*, 2003; De Lucca e Casique, 2004; Bermudez *et al.*, 2006; Urreizti *et al.*, 2006; Cozar *et al.*, 2011). Em pacientes do sudeste brasileiro, foi detectada 3/28 alelos (frequência em não relacionados de 13,64%) (Porto *et al.*, 2005).

Também localizada no núcleo ativo da enzima, esta variante está em região exposta ao solvente e retém cerca de 1% da atividade normal da CβS (Urreizti *et al.*, 2003). *In vitro*, a enzima mutante apresenta resposta anormal aos reguladores AdoHcy e AdoMet (Kozich *et al.*, 2010). Em homozigotos, está associada a não responsividade à piridoxina; enquanto que em heterozigotos compostos a responsividade é variável. Uma ampla variabilidade fenotípica é observada nos portadores desta variante (Urreizti *et al.*, 2006).

p.Gly307Ser (c.919G>A): está presente em aproximadamente 9,5% dos alelos de HCU no mundo (Kraus, 2016). É altamente prevalente em grupos populacionais de origem céltica, sendo responsável por 71% dos alelos mutados na Irlanda (Gallagher *et al.*, 1995; Gallagher *et al.*, 1998). Também já foi detectada em diversas populações européias e norte-americanas (Hu *et al.*, 1993; Tsai, Bignell, *et al.*, 1996; Tsai, Hanson, *et al.*, 1996; Kim *et al.*, 1997; Kozich *et al.*, 1997; De Franchis *et al.*, 1999; Gaustadnes *et al.*, 2002; Kruger *et al.*, 2003), mas em nenhum país latino-americano, incluindo o Brasil (Porto *et al.*, 2005; Urreizti *et al.*, 2006; Cozar *et al.*, 2011).

A enzima mutante retém uma atividade residual de cerca de 1% da enzima selvagem (Kruger *et al.*, 2003). A variante localiza-se no sítio ativo da enzima, em região não exposta ao solvente, e também parece resultar em prejuízo na ativação pela AdoMet (Kozich *et al.*, 2010). O fenótipo associado é moderado a grave e a variante não confere responsividade à piridoxina (Gallagher *et al.*, 1998; Mudd *et al.*, 2001).

p.Arg336Cys (c.1006C>T): esta variante foi detectada em cerca de 10% dos alelos de HCU no mundo, sendo especialmente prevalente em países árabes. No Catar, é responsável pela grande maioria dos casos de HCU, cuja incidência da doença é de 1:1800 nv (El-Said *et al.*, 2006; Zschocke *et al.*, 2009; Gan-Schreier *et al.*, 2010). A variante já foi detectada em outras regiões como Coréia (Lee *et al.*, 2005), Austrália (Gaustadnes *et al.*, 2002), península ibérica (Urreizti *et al.*, 2003) e Inglaterra (De Franchis *et al.*, 1999). A atividade enzimática da CβS mutante é nula (Urreizti *et al.*, 2003), e fenótipos graves associados à não responsividade à piridoxina são observados (Urreizti *et al.*, 2003; El-Said *et al.*, 2006; Zaidi *et al.*, 2012).

3.4 RELAÇÃO ENTRE SAA E METABOLISMO LIPÍDICO

Associação entre os SAA e metabolismo de lipoproteínas é descrita em diversos estudos experimentais e populacionais (El-Khairy *et al.*, 2001; Hirche *et al.*, 2006; Chen *et al.*, 2012; Elshorbagy, Valdivia-Garcia, Refsum, *et al.*, 2012). Na HCU, estudos em modelos animais e pacientes apontam para redução das frações de colesterol, em particular de HDL (Moat *et al.*, 1999; Namekata *et al.*, 2004; Liao *et al.*, 2006; Nuno-Ayala *et al.*, 2010; Poloni *et al.*, 2012). O papel do aumento de lipoperoxidação na patogênese da HCU

não está estabelecido, uma vez que os achados são inconsistentes (Dudman *et al.*, 1993; Blom *et al.*, 1995; Davi *et al.*, 2001; Vanzin *et al.*, 2011; Poloni *et al.*, 2012; Vanzin *et al.*, 2015).

No entanto, a associação mais intrigante que tem sido descrita é entre os níveis plasmáticos de cisteína e o desenvolvimento de obesidade e síndrome metabólica. As principais evidências serão revisadas a seguir.

3.4.1 Evidências em estudos populacionais

Uma das primeiras evidências da associação entre cisteína e composição corporal foi o estudo de Elshorbagy et al. (2008), que analisou a associação entre os níveis plasmáticos de cisteína e o percentual de gordura corporal (avaliado por DXA) em uma população de mais de 5000 indivíduos saudáveis. Os autores encontraram uma forte associação positiva e independente entre os níveis de cisteína e a quantidade de gordura corporal (Elshorbagy *et al.*, 2008). Esta associação vem sendo corroborada por diversos outros estudos populacionais (Elshorbagy, Refsum, *et al.*, 2009; Nuno-Ayala *et al.*, 2010; Elshorbagy, Kozich, *et al.*, 2012; Elshorbagy, Valdivia-Garcia, Graham, *et al.*, 2012; Elshorbagy, Valdivia-Garcia, Refsum, *et al.*, 2012; Vinknes *et al.*, 2013). Associações de cisteína com resistência insulínica (Elshorbagy, Valdivia-Garcia, Refsum, *et al.*, 2012) e lipoproteínas (Elshorbagy, Valdivia-Garcia, Graham, *et al.*, 2012; Da Silva *et al.*, 2013) também são descritas.

3.4.2 Evidências em modelos animais

Diversos modelos animais com metabolismo alterado de SAA apresentam alterações na composição corporal e metabolismo lipídico. Em uma revisão recente modelos animais *knockouts* para enzimas envolvidas no metabolismo dos SAA, Elshorbagy descreve que todos modelos analisados apresentam um fenótipo protetor de obesidade, com baixo ganho de peso apesar de não haver redução na ingestão energética. Outras características comuns nestes *knockouts* são: massa adiposa reduzida, gasto energético basal aumentado, sensibilidade à insulina aumentada e proteção contra

obesidade induzida pela dieta. Estas características estiveram associadas a baixas concentrações plasmáticas de cisteína em 6/8 modelos estudados (Elshorbagy, 2014).

Ratos submetidos a uma dieta restrita em metionina (MR) apresentam níveis reduzidos de metionina, taurina, cisteína e cistationina; além de elevação de 2,5 vezes na concentração de tHcy (Elshorbagy *et al.*, 2010). Estes animais apresentam baixo ganho de peso apesar do maior consumo energético; além de redução de triglicerídeos, leptina, insulina, IGF-1 e expressão hepática da da enzima SCD-1; e elevação de LDL e adiponectina. Todas estas alterações foram revertidas após o tratamento com suplementação de cisteína, ainda que as concentrações de tHcy tenham permanecido moderadamente elevadas (Elshorbagy *et al.*, 2011).

No modelo animal de HCU - camundongos TgI278T CβS$^{-/-}$ - alterações metabólicas semelhantes são observadas. Este modelo apresenta fenótipo clínico e bioquímico semelhante à doença em humanos (Wang *et al.*, 2005). Camundongos TgI278T CβS$^{-/-}$ apresentam baixo ganho de peso e adiposidade (>50% em relação aos heterozigotos). Também foi observada redução >90% de mRNA e de 54% dos níveis de proteína da enzima SCD-1 hepática (Gupta e Kruger, 2011). O tratamento com dieta restrita em metionina nestes camundongos reduz a tHcy e aumenta cisteína plasmática, além de resultar em aumento significativo de peso (28%), massa adiposa (130%), expressão da SCD-1 hepática (82%) e de densidade mineral óssea (10%) (Gupta *et al.*, 2014).

Alterações no metabolismo da colina/betaína, altamente interligado com o dos SAA, também estão associadas a mudanças no metabolismo energético, lipídico e glicídico (Teng *et al.*, 2011; Teng *et al.*, 2012). A deficiência de BHMT, enzima responsável pela remetilação de homocisteína, é caracterizada por elevação de betaína e homocisteína e redução de colina e cisteína em camundongos (Zeisel, 2013). Camundongos com deficiência de BHMT apresentam menor massa adiposa, síntese reduzida de triglicerídeos, melhor sensibilidade à insulina e maior gasto energético. Estas alterações não estão associadas à mudança na ingestão alimentar, absorção de lipídeos, lipólise ou termogênese (Teng *et al.*, 2012).

3.4.3 Papel da enzima SCD-1

A enzima SCD-1 é responsável pela síntese de ácidos graxos monoinsaturados, sendo seus principais substratos os ácidos palmítico (C16:0) e esteárico (C18:0), embora outros substratos contendo de 9-20 carbonos também possam se ligar à enzima (Paton e Ntambi, 2009; Hodson e Fielding, 2013). Seus produtos – os ácidos graxo palmitoleico (C16:1) e oleico (C18:1) são os principais ácidos graxos presentes nos triglicerídeos, ésteres de colesterol e fosfolipídeos de membrana (Miyazaki *et al.*, 2001; Hodson e Fielding, 2013).

A SCD-1 é expressa principalmente no fígado e tecido adiposo, e em menor proporção no rim, baço, coração e pulmões. O gene contém diversos sítios de ligação para fatores de transcrição que regulam sua expressão; como o SREBP-1c (*sterol regulatory element binding protein*), os PPARs (*Peroxisome proliferator-activated receptors*), e o receptor de estrogênio (Hodson e Fielding, 2013). A leptina, hormônio central na regulação do apetite e gasto energético, também modula a expressão da SCD-1. Acredita-se que maior parte das ações da leptina no fígado ocorra através da supressão desta enzima (Cohen e Friedman, 2004; Biddinger *et al.*, 2006).

A tríade baixa concentração plasmática de cisteína, supressão da SCD-1 hepática e baixa adiposidade vem sendo consistentemente observada em estudos experimentais e populacionais (Ntambi *et al.*, 2002; Rizki *et al.*, 2006; Elshorbagy *et al.*, 2011; Gupta e Kruger, 2011; Vinknes *et al.*, 2013; Gupta *et al.*, 2014). Isto sugere um papel da enzima como mediador do efeito dos SAA no metabolismo lipídico e composição corporal (Poloni *et al.*, 2015). Os principais mecanismos propostos são resumidos na Figura 3 e no Capítulo 2.

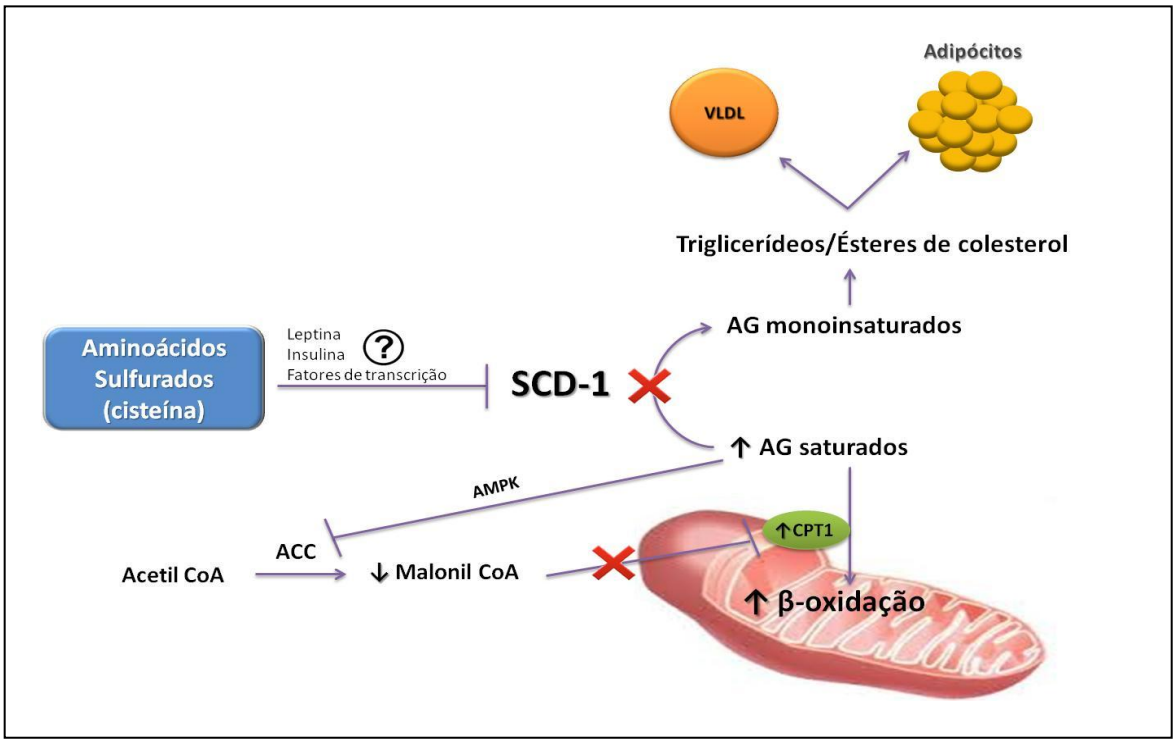

Figura 3. Efeitos da deficiência de SCD-1 no metabolismo lipídico. Estudos sugerem que os SAA, em particular a cisteína, sejam capazes de suprimir a SCD-1 hepática por mecanismos ainda não esclarecidos. Na deficiência de SCD-1, a síntese de VLDL e triglicerídeos é prejudicada pela redução da disponibilidade de ácidos graxos monoinsaturados, e isto acaba por limitar o depósito de gordura no tecido adiposo. Já o aumento de acil CoAs saturadas inibe alostericamente a enzima acetil CoA carboxilase, que faz a conversão de acetil CoA para malonil CoA. A redução nos níveis de malonil CoA suprime a enzima Carnitina-Palmitoil Transferase 1 (CPT-1), que por sua vez permitiria a entrada dos ácidos graxos na mitocôndria, onde seriam oxidados (Ntambi *et al.*, 2002; Cohen e Friedman, 2004). VLDL: lipoproteína de muito baixa densidade; SCD-1: Estearoil-CoA desaturase-1; AG: ácidos graxos; AMPK: proteína quinase ativada por AMP; ACC: acetil CoA-carboxilase.

4. JUSTIFICATIVA

A HCU representa a forma grave de uma condição frequente na população - a hiperhomocisteinemia, que está associada a diversas situações patológicas como doenças cardiovasculares, demência, Alzheimer e fraturas ósseas (Mudd *et al.*, 2001). A HCU constitui ainda a anomalia congênita mais prevalente do metabolismo dos SAA (Wilcken, 2006).

No Brasil, há apenas um estudo descrevendo o perfil genético e clínico de pacientes com HCU (Porto *et al.*, 2005), sendo a amostra proveniente da região sudeste e, portanto, pouco representativa do perfil da HCU no restante do Brasil. Em um país caracterizado por extensa dimensão territorial e ancestralidade diversificada e heterogênea entre regiões (Ruiz-Linares *et al.*, 2014; Moura *et al.*, 2015), uma ampla variabilidade genotípica pode ser esperada. Com a amostra obtida no presente estudo, auxiliaremos a traçar o perfil cínico e genético da doença no Brasil, o que irá contribuir para melhorias no diagnóstico e manejo da HCU no país. Além disso, o conhecimento do genótipo pode auxiliar na escolha do tratamento e na predição do curso da doença, uma vez que boa relação genótipo-fenótipo está descrita para algumas variantes.

A investigação da relação entre os SAA e o metabolismo lipídico trará benefícios não apenas para o conhecimento da patogênese da HCU, mas também pelo entendimento de mecanismos bioquímicos envolvidos na etiologia da obesidade, doença crônica que atinge mais de 35% da população mundial (Who, 2010). O papel da cisteína na modulação do metabolismo lipídico e energético vem sendo relatado em diversos estudos; e a enzima hepática SCD-1 parece ser o principal mediador de seus efeitos. A relação da SCD-1 com o desenvolvimento de obesidade, resistência insulínica e esteatose hepática é conhecida. A enzima já é inclusive considerada um alvo terapêutico: somente até 2009, mais de 70 patentes para desenvolvimento de inibidores da SCD-1 já haviam sido publicadas (Liu, 2009). Estudos sobre a enzima são abundantes em modelos animais, mas limitados em humanos, sendo este pioneiro em avaliar a atividade da enzima em pacientes com HCU.

5. OBJETIVOS

5.1 OBJETIVOS GERAIS

5.1.1 Descrever o perfil clínico e genético de pacientes brasileiros com HCU.

5.1.2 Avaliar a relação entre SAA, composição corporal, atividade da SCD-1, resistência insulínica e metabolismo lipídico em pacientes com HCU em tratamento.

5.2 OBJETIVOS ESPECÍFICOS

5.2.1 Realizar sequenciamento de toda a região codificante do gene *CBS* em uma amostra representativa de pacientes brasileiros com HCU.

5.2.2 Caracterizar o perfil clínico da HCU no Brasil.

5.2.3 Descrever a composição corporal de pacientes com HCU em tratamento.

5.2.4 Caracterizar e comparar marcadores de metabolismo lipídico (níveis de lipoproteínas, isoprostanos, acilcarnitinas, leptina, adiponectina) entre pacientes com HCU em tratamento e controles saudáveis.

5.2.5 Determinar resistência insulínica, através do índice HOMA-IR, em pacientes com HCU em tratamento.

5.2.6 Verificar a associação entre os SAA (metionina, homocisteína e cisteína), colina e betaína com a composição corporal e marcadores de metabolismo lipídico em pacientes e controles.

5.2.7 Estimar a atividade da SCD-1 hepática através dos índices SCD-16 e SCD-18; e analisar a associação dos índices com composição corporal, níveis de lipoproteínas, leptina e adiponectina e concentrações de SAA em pacientes e controles.

6. CAPÍTULOS

Os resultados da tese serão apresentados nos capítulos a seguir, no formato de quatro artigos científicos.

6.1 CAPÍTULO 1**:** "*Body composition in patients with classical homocystinuria: body mass relates to homocysteine and choline metabolism*"

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

Autores: Soraia Poloni, Sandra Leistner-Segal, Isabel Cristina Bandeira, Vânia D'Almeida, Carolina Fischinger Moura de Souza, Poli Mara Spritzer, Kamila Castro, Tássia Tonon, Tatiéle Nalin, Apolline Imbard, Henk J. Blom, Ida V.D. Schwartz.

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Short Communication

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

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ABSTRACT

Introduction: Classical homocystinuria is a rare genetic disease caused by cystathionine β-synthase deficiency, resulting in homocysteine accumulation. Growing evidence suggests that reduced fat mass in patients with classical homocystinuria may be associated with alterations in choline and homocysteine pathways. This study aimed to evaluate the body composition of patients with classical homocystinuria, identifying changes in body fat percentage and correlating findings with biochemical markers of homocysteine and choline pathways, lipoprotein levels and bone mineral density (BMD) T-scores.

Methods: Nine patients with classical homocystinuria were included in the study. Levels of homocysteine, methionine, cysteine, choline, betaine, dimethylglycine and ethanolamine were determined. Body composition was assessed by bioelectrical impedance analysis (BIA) in patients and in 18 controls. Data on the last BMD measurement and lipoprotein profile were obtained from medical records.

Results: Of 9 patients, 4 (44%) had a low body fat percentage, but no statistically significant differences were found between patients and controls. Homocysteine and methionine levels were negatively correlated with body mass index (BMI), while cysteine showed a positive correlation with BMI ($p < 0.05$). There was a trend between total choline levels and body fat percentage ($r = 0.439$, $p = 0.07$). HDL cholesterol correlated with choline and ethanolamine levels ($r = 0.757$, $p = 0.049$; $r = 0.847$, $p = 0.016$, respectively), and total cholesterol also correlated with choline levels ($r = 0.775$, $p = 0.041$). There was no association between BMD T-scores and body composition.

Conclusions: These results suggest that reduced fat mass is common in patients with classical homocystinuria, and that alterations in homocysteine and choline pathways affect body mass and lipid metabolism.

1. Introduction

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

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

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

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

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

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

2. Subjects and methods

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

2.1. Study sample

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

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

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

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

2.2. Assessment of body composition

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

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

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

2.4. Laboratory assessment

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

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

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

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

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

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

2.5. Statistical analysis

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

3. Results

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

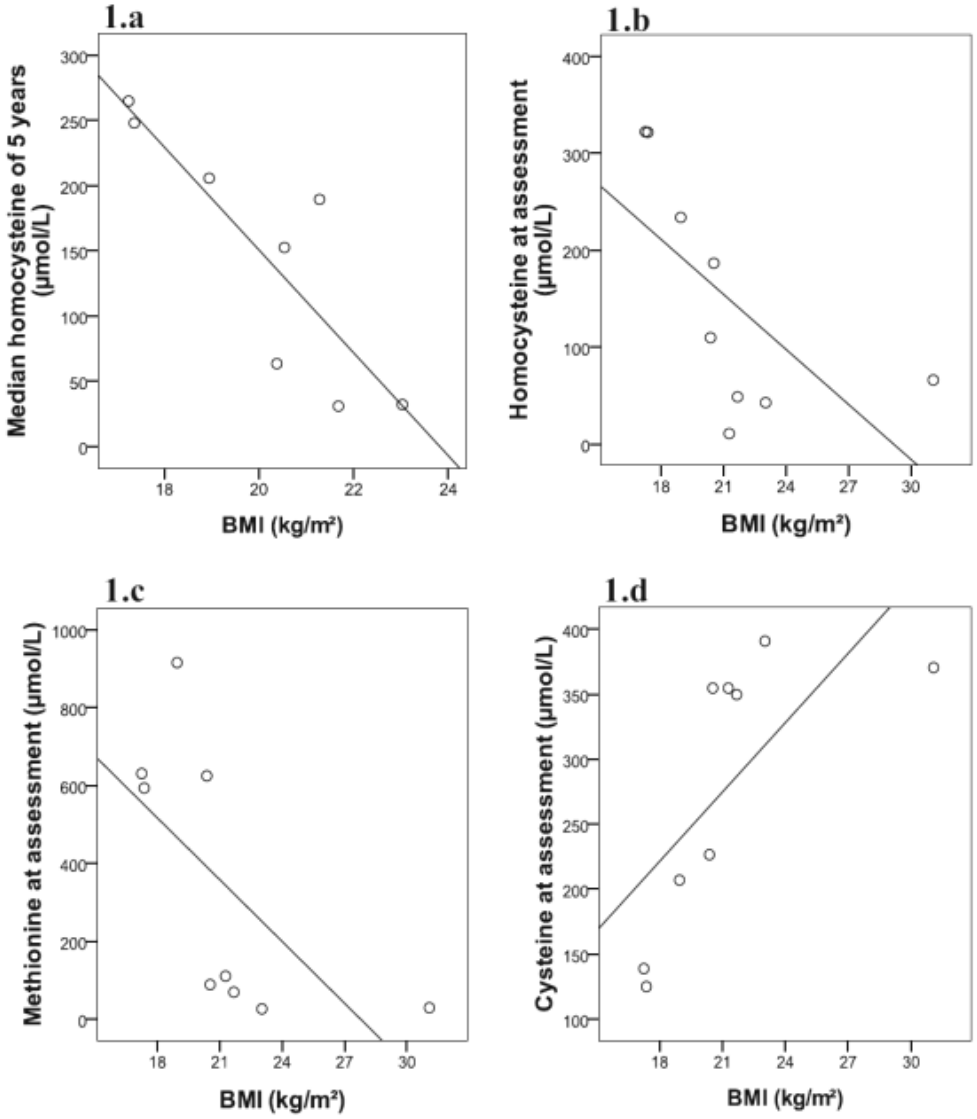

Fig. 1. Correlations between the main metabolites of homocysteine pathway and the body mass index (BMI). Solid lines represent the trend lines. 1.a) Median homocysteine values of the last 5 years correlate negatively with BMI ($r = -0.881$, $p = 0.004$). 1.b) Homocysteine levels at assessment also correlate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

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

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

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

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

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

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

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

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

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

Conflict of interest

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

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6.2 CAPÍTULO 2: "*Stearoyl-CoA Desaturase-1: Is It the Link between Sulfur Amino Acids and Lipid Metabolism*?"

Título do artigo: "Stearoyl-CoA Desaturase-1: Is It the Link between Sulfur Amino Acids and Lipid Metabolism?"

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Review

Stearoyl-CoA Desaturase-1: Is It the Link between Sulfur Amino Acids and Lipid Metabolism?

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Abstract: An association between sulfur amino acids (methionine, cysteine, homocysteine and taurine) and lipid metabolism has been described in several experimental and population-based studies. Changes in the metabolism of these amino acids influence serum lipoprotein concentrations, although the underlying mechanisms are still poorly understood. However, recent evidence has suggested that the enzyme stearoyl-CoA desaturase-1 (SCD-1) may be the link between these two metabolic pathways. SCD-1 is a key enzyme for the synthesis of monounsaturated fatty acids. Its main substrates C16:0 and C18:0 and products palmitoleic acid (C16:1) and oleic acid (C18:1) are the most abundant fatty acids in triglycerides, cholesterol esters and membrane phospholipids. A significant suppression of SCD-1 has been observed in several animal models with disrupted sulfur amino acid metabolism, and the activity of SCD-1 is also associated with the levels of these amino acids in humans. This enzyme also appears to be involved in the etiology of metabolic syndromes because its suppression results in decreased fat deposits (regardless of food intake), improved insulin sensitivity and higher basal energy expenditure. Interestingly, this anti-obesogenic phenotype has also been described in humans and animals with sulfur

amino acid disorders, which is consistent with the hypothesis that SCD-1 activity is influenced by these amino acids, in particularly cysteine, which is a strong and independent predictor of SCD-1 activity and fat storage. In this narrative review, we discuss the evidence linking sulfur amino acids, SCD-1 and lipid metabolism.

Keywords: lipoproteins; stearoyl CoA desaturase-1; SCD-1; homocysteine; cysteine; sulfur amino acids; homocystinuria

1. Introduction

Methionine, homocysteine, cysteine and taurine metabolism are highly linked. These main sulfur amino acids are involved in several metabolic pathways such as glutathione synthesis, protein synthesis and the methylation of several substances, such as DNA, RNA, proteins and lipids [1–3]. Several reports have suggested that sulfur amino acids play a role in the regulation of lipid metabolism and body composition [4–7]. In this narrative review, we describe the possible connections between sulfur amino acids, SCD-1 and lipid metabolism.

The metabolism of sulfur amino acids is depicted in Figure 1. Methionine is an essential amino acid that is demethylated via two intermediate compounds, *S*-adenosylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy). Homocysteine is a product of the transmethylation pathway, an amino acid that is not incorporated into proteins, and is considered toxic. Homocysteine may be metabolized by two different methods: through the transsulfuration pathway, where it is irreversibly degraded to cysteine; or through remethylation, where it is converted back to methionine.

Remethylation of homocysteine into methionine can occur through two alternative pathways. Homocysteine can be catalyzed by methionine synthase, which is a vitamin B_{12}- and folate-dependent enzyme, or it can be catalyzed by betaine-homocysteine methyltransferase (BHMT), an enzyme present in liver and kidney that uses as methyl group donor betaine, which on its turn is formed by choline catabolism [3,8]. The transsulfuration pathway converts homocysteine into cystathionine and subsequently into cysteine. These reactions are catalyzed by two pyridoxal 5'-phosphate (PLP)-dependent enzymes.

Cysteine can be degraded through oxidative reactions that generate taurine or sulfate in a 2:1 ratio [2]. In addition, cysteine is used in the synthesis of proteins and glutathione, a powerful antioxidant. Taurine is the most abundant amino acid in animal tissues and used in the synthesis of bile salts, and it potentially acts as an antioxidant, membrane stabilizer and neurotransmitter [2,9].

The control of sulfur amino acid metabolism is a complex process that operates on several levels. AdoMet plays a central role in this regulation. When methionine levels increase, the concentration of AdoMet increases, favoring sulfur amino acid metabolism through the transsulfuration pathway, via activating cystathionine β-synthase (CβS) and inhibiting 5,10-methylene-tetrahydrofolate reductase (MTHFR). If methionine levels are low, such as during fasting, the reduced AdoMet levels do not activate CβS or inhibit MTHFR, thus resulting in the remethylation of homocysteine into methionine [3,8]. The enzyme activity is also influenced by factors such as protein intake, hormone levels, nutrient deficiencies, age and long-term changes in substrate levels [10].

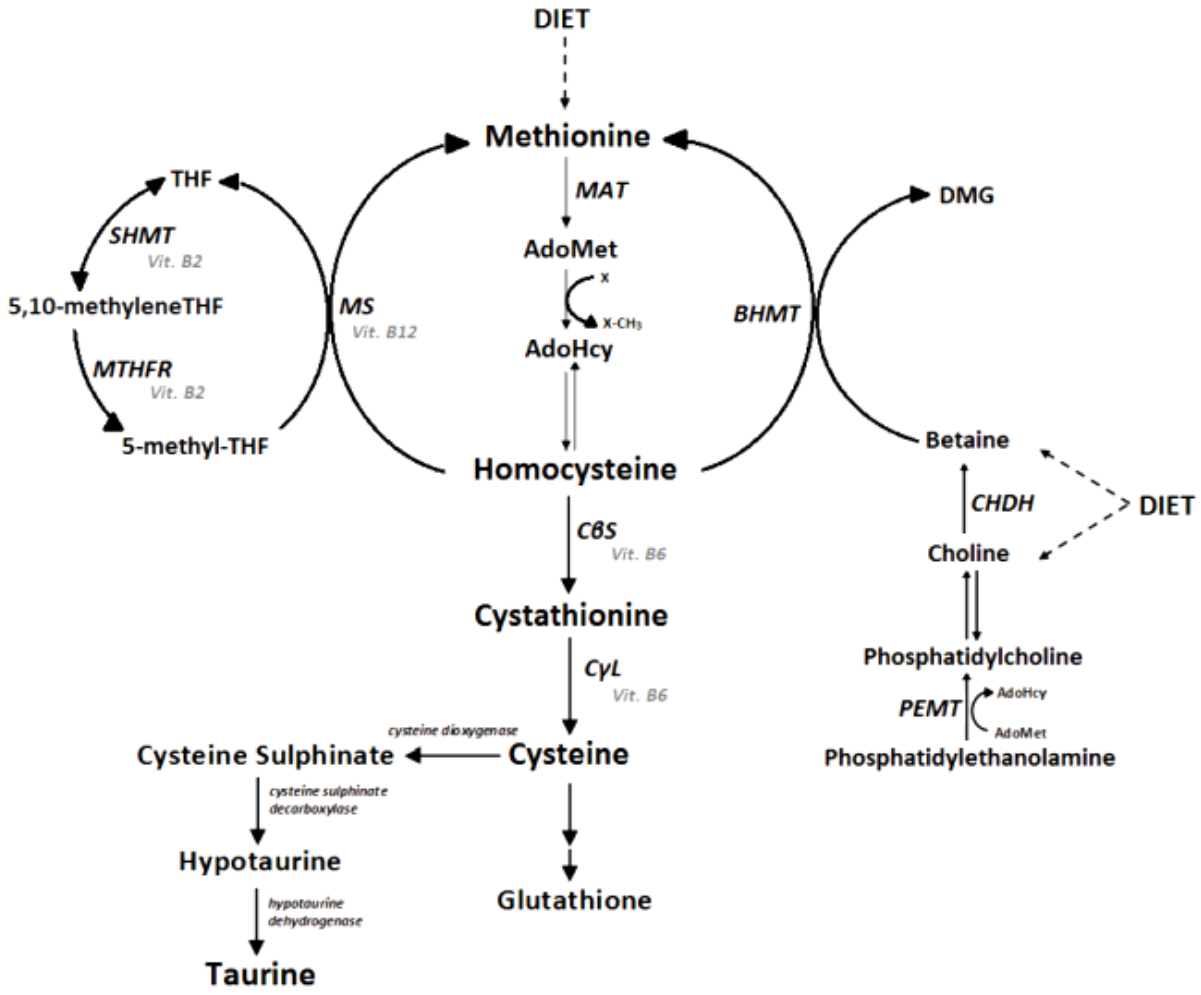

Figure 1. Metabolism of sulfur amino acids. MAT, methionine adenosyltransferase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; X, methyl acceptor; CβS, cystathionine β-synthase; CγL, cystathionine γ-lyase; MS, methionine synthase; THF, tetrahydrofolate; MTHFR, 5,10-methylene-THF reductase; SHMT, serine hydroxymethyltransferase; BHMT, betaine-homocysteine *S*-methyltransferase; DMG, dimethylglycine; CHDH, choline dehydrogenase; PEMT, phosphatidylethanolamine *N*-methyltransferase. Enzymes are shown in italics, and their cofactors are shown in gray.

Hyperhomocysteinemia is a condition characterized by elevated circulating levels of homocysteine (>15 μmol/L). Mild and moderate forms are frequent and present in 5%–10% of the population [11]. These forms are generally of multifactorial origin, and they are associated with higher mortality and incidence of several chronic diseases, such as stroke, dementia, Alzheimer's disease, bone fractures and heart failure [12]. Causes of mild/moderate hyperhomocysteinemia are described in Table 1.

The severe form of hyperhomocysteinemia (homocysteine >50 μmol/L) is rare, usually monogenic (autosomal recessive inheritance), and is caused by pathogenic variations in genes involved in homocysteine clearance, such as *CβS*, *MTHFR* and *methylmalonic aciduria and homocystinuria type C* (*MMACHC*). CβS deficiency is the most common type of severe hyperhomocysteinemia, with an estimated worldwide prevalence of 1 in 344,000 individuals [13]. In humans, this deficiency results in

classical homocystinuria, a disease characterized by increased plasma levels of homocysteine, methionine and AdoMet and decreased levels of cystathionine and cysteine. The classical clinical manifestations are lens dislocation, thromboembolism, osteoporosis, cognitive deficit and psychiatric disorders [13,14]. The so-called Marfanoid phenotype, which includes increased height, dolichostenomelia and lean biotype, is also common [13,15].

Table 1. Summary of the main causes of mild and moderate hyperhomocysteinemia.

Etiology	Characteristics
Vitamin B12 deficiency	Vitamin B12 is used in homocysteine remethylation. The groups at higher risk for this deficiency are vegetarians and the elderly, with the deficiency rates reaching 20% for the latter. Deficient absorption is likely the main etiological factor in elderly individuals because intrinsic-factor deficiency, which is fundamental for the absorption of B12, is common in this age group [62].
Folate deficiency	Folate is also involved in the remethylation of homocysteine, and its deficiency is more common in individuals with increased needs, such as pregnant women and alcoholics. In addition, a frequent polymorphism in the *MTHFR* gene (c.677C > T) makes the enzyme thermolabile and decreases enzymatic activity in homozygotes to 70%. If their folate status is low to normal, homocysteine can increase dramatically [63].
Medicines	The chronic use of certain drugs, such as methotrexate, theophylline, phenytoin, carbamazepine and valproic acid, is known to increase homocysteine levels [64,65].
Other causes	Additional causes include chronic renal disease, cancer, hypothyroidism, liver failure, smoking, alcohol and coffee intake, age and hormonal disorders [64,65].

MTHFR: 5,10 methylenetetrahydrofolate acid reductase.

Severe MTHFR deficiency is characterized by hyperhomocysteinemia, homocystinuria and low-to-normal levels of methionine. This form of homocystinuria is rare with approximately 100 cases described [16]. The main clinical manifestations include seizures, cognitive impairments, gait disturbances and isolated thromboembolic episodes. In addition to the mutations that cause severe deficiency, polymorphisms in the *MTHFR* gene are highly prevalent in the population. The most prevalent are the c.677C > T and c.1298A > C. The first is associated with mild hyperhomocysteinemia, especially when associated with low folic acid status [14].

Another cause of severe hyperhomocysteinemia (over 500 cases described) [17] is the *cblC* type of homocystinuria with methylmalonic aciduria, which is caused by pathogenic variations in the *MMACHC* gene, which codes for a protein involved in the binding and intracellular trafficking of cobalamin. The most frequent symptoms are developmental delays, psychiatric disorders, microangiopathy, and ocular and hematologic abnormalities [13,14].

2. Sulfur Amino Acids and Lipid Metabolism

Several studies have indicated the role of sulfur amino acids in the regulation of lipoprotein metabolism. In wild-type mice, methionine supplementation promotes hypercholesterolemia [18]. In turn, taurine is inversely related to very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol in humans and rodents, and its supplementation has hypocholesterolemic action in rats [4,5]. In population-based studies, plasma

cysteine levels were found to be positively associated with levels of several lipoproteins, such as total cholesterol, LDL, apolipoprotein B (ApoB) and triglycerides [6,7].

Regarding homocysteine, an inverse association between this amino acid and lipoproteins, especially HDL, has been well described in humans [19–21] and various animal models of hyperhomocysteinemia. CβS homozygous deficient mice ($C\beta S^{-/-}$) present plasma homocysteine levels that are 40-fold higher than that of the wild type, which is comparable to the levels observed in patients with classical homocystinuria. They also present low weight gain and have low survival rates [22]. In this mouse model, increased hepatic levels of triglycerides and non-esterified fatty acids, low levels of ApoB100 and ApoA-I and reduced serum concentrations of total cholesterol and HDL were observed [23,24].

Mice with MTHFR deficiency have excess homocysteine but normal levels of cysteine. They also exhibit lipid metabolism changes, including reduced levels of ApoA-I and an inverse association of homocysteine with ApoA-I and HDL [25]. The clinical features include developmental retardation with cerebellar pathology, growth failure and abnormal aortic lipid deposition [26]. Despite consistent evidence of lipid metabolism modulation by these sulfur amino acids, their mechanism of action is not well understood. Recently, several studies have suggested that sulfur amino acids modulate the expression of stearoyl-CoA desaturase-1 (SCD-1), a key enzyme in the hepatic synthesis of monounsaturated fatty acids [27,28]. The putative mechanisms by which sulfur amino acids might regulate lipid metabolism and SCD-1 expression are summarized in Table 2.

Table 2. Proposed mechanisms by which sulfur amino acids influence lipid metabolism and may potentially regulate SCD-1 expression.

Amino Acid	Effect on Lipid Metabolism	Effect on SCD-1 Regulators
Methionine	– Induction of ApoA1 synthesis in rats [66]	– Unknown
Homocysteine	– Suppression of ApoAI synthesis in mice [27] – Regulation of ApoB100 levels in mice [24] – Induction of *CYP7A1* expression in rats [66]	– Induction of leptin secretion in humans [68,69] – Activation of the transcription factors SREBP-1c and PPARα in humans and mice [70,71]
Cysteine	– Regulation of ApoE and ApoA1 levels in mice [25,67]	– Modulation of PPARγ, SREBP-1c and estrogen receptor-α expression in humans and animals [72–75]
Taurine	– Reduction in ApoB and VLDL secretion in human liver cells [4] – Modulation of gene expression (*CYP7A1*, *LXR*) in animal models [66] – Increased excretion of fecal bile acid in several animal models [4]	– Modulation of LXR-α (direct ligand) and *SREBP-1* expression in macrophages [76] – Regulation of insulin and leptin secretion in animal models [5,77]

SCD-1, stearoyl-CoA desaturase-1; ApoA1, Apolipoprotein A1; ApoB100, Apolipoprotein B100; CYP7A1, cytochrome P450, family 7, subfamily A, polypeptide 1; ApoE, apolipoprotein E; LXR, liver X receptor; SREBP-1c, sterol regulatory element binding transcription factor 1; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma.

3. SCD-1: Localization, Function and Regulation

The enzyme SCD-1 is bound to the endoplasmic reticulum and responsible for the synthesis of monounsaturated fatty acids. Together with NAPH, cytochrome b5 reductase and cytochrome b5,

SCD-1 introduces a single double bond at the Δ9,10 of long-chain acyl-CoA substrates, and its main substrates are palmitic (C16:0) and stearic (C18:0) acids, although other substrates containing 9–20 carbons can also bind to the enzyme [23,24]. The products palmitoleic acid (C16:1) and oleic acid (C18:1) are the main fatty acids present in triglycerides, cholesterol esters and membrane phospholipids [28–30].

In humans and rodents, SCD-1 is mainly expressed in liver and adipose tissue (brown and white); however, expression at lower levels is also observed in the kidneys, spleen, heart and lungs [27]. The half-life of the enzyme is only 3–5 h, and its degradation occurs in the microsomes [31]. Its gene contains several binding sites for transcription factors that regulate its expression. Among the main factors is SREBP-1c (sterol regulatory element binding protein), a transcription factor that regulates the biosynthesis of fatty acids, LXRα and LXRβ receptors (which activate genes associated with cholesterol transport), PPARs (peroxisome proliferator-activated receptors), nuclear receptors involved in adipocyte differentiation and lipid storage and estrogen receptors, which also regulate lipogenesis [28,32].

Expression of the *SCD-1* gene is regulated by several intrinsic and extrinsic factors, including leptin, a hormone involved in appetite regulation and energy expenditure. It is believed that most of leptin's actions in the liver occur through SCD-1 suppression, and that this effect is independent of insulin and SREBP-1c [32,33]. Diet is also an important modulator of SCD-1 expression, with high consumption of polyunsaturated fatty acids suppressing the enzyme and consumption of saturated fatty acids, cholesterol and carbohydrates increasing its expression. Alcohol consumption and smoking appear to upregulate SCD1 activity, whereas physical activity has the opposite effect [28,34–37].

The effects of SCD-1 deficiency have been reported in animal models. *SCD-1* global knockout mice (SCD-1 −/−) exhibit reduced levels of cholesterol esters and triglycerides, and dietary supplementation with monounsaturated fatty acids is not capable of normalizing the production of these compounds [30]. SCD-1 −/− mice also exhibit lower fat accumulation in adipose tissue regardless of higher energy consumption as well as higher basal energy expenditure and improved insulin sensitivity. In addition, these animals are resistant to weight gain and diet-induced fat accumulation [38].

SCD-1's effect on the phenotype, however, appears to be related to SCD-1 expression in specific tissues. Mice that only present SCD-1 deficiency in the liver have a similar phenotype to that of the wild type fed a chow diet. However, on a high-carbohydrate diet, SCD-1 −/− mice showed less weight gain and a reduction in plasma triglycerides compared with the controls, and they were also protected from diet-induced liver steatosis. The same changes did not occur when the mice were fed a high-fat diet, and they presented similar gain weight gain as their littermate controls and developed liver steatosis. The level of insulin sensitivity did not differ between treatments [39]. In addition, the food intake, insulin sensitivity and fat mass in mice with the adipose SCD-1 deletion were similar to that of the controls, and they were not protected from diet-induced obesity [40]. In contrast, mice with skin-specific deletions of SCD-1 showed significantly increased energy expenditures and were protected from high-fat diet-induced obesity [41].

Increased β-oxidation is the main proposed mechanism through which SCD-1 deficiency results in this anti-obesogenic phenotype. Because SCD-1 deficiency has negative effects on the synthesis of VLDL and triglycerides, an increase in the pool of saturated acyl CoAs would result and allosterically inhibit acetyl CoA carboxylase. This enzyme converts acetyl CoA into malonyl CoA. The decrease in

malonyl CoA levels counteracts the inhibition of carnitine palmitoyltransferase 1 (CPT-1), thus allowing the entry of fatty acids into the mitochondria for oxidation [32].

In humans, product/precursor ratios in either plasma or serum have been used to estimate SCD-1 activity. Population-based studies have found strong positive associations between the indices 16:1n-7/16:0 and 18:1n-9/18:0 and obesity markers such as the body mass index (BMI), waist circumference and body fat mass as evaluated by dual-energy X-ray absorptiometry [35,36,42]. Other cardiovascular risk factors are also associated with SCD-1 indices. In a study with 134 healthy men, plasma levels of palmitoleic acid, one of the main products of SCD-1, were strongly and independently associated with triglyceridemia [43]. In another study, high SCD-1 activity (estimated through the indices mentioned above) was found to be a predictor of the development of metabolic syndrome in a cohort of 1558 middle-aged men [44]. A similar association was observed for the development of hyperglycemia [45]. The development of other diseases, such as cancer, bone fractures and hepatic steatosis, has also been associated with SCD-1 expression [28].

4. Evidence that Sulfur Amino Acids Influence SCD-1 Expression

Knowledge of the effects of sulfur amino acids on the regulation of SCD-1 expression has increased in recent years, especially because of studies using animal models. A recent review [46] of animal model knockouts for several enzymes (CβS, glutamate cysteine ligase modifier subunit, cystathionase, BHMT, PEMT, gamma glutamyltransferase and cysteine dioxygenase) involved in sulfur amino acid metabolism showed that alterations in these pathways, especially when related to low cysteine and choline levels, resulted in similar anti-obesogenic phenotypes in rodents. However, because of the complexity of this regulation and its interdependence with other metabolic pathways, the individual role of each amino acid remains unclear.

Low weight gain and a significant decrease in body fat mass (46% in females and 62% in males) were observed in a study using TgI278T CβS$^{-/-}$ mice, an animal model of classical homocystinuria that has a mutant human *CβS* gene containing the common p.I278T pathogenic variation. This mutation was found in 16% of the homocystinuria alleles studied in the world, presents a pan-ethnic distribution and is associated with pyridoxine responsiveness (http://cbs.lf1.cuni.cz/index.php). A liver microarray analysis revealed SCD-1 to be the transcript with the largest magnitude change in these mice, with a seven-fold decrease in gene expression relative to the control. The hepatic levels of the enzyme were also reduced by 54% for group TgI278T CβS$^{-/-}$, but no changes were observed in visceral and subcutaneous fat [47].

The effects of sulfur amino acids on body composition and lipid metabolism have also been evaluated in mice subjected to a methionine-restricted (MR) diet. These animals exhibited reduced levels of methionine (62%), taurine (64%), cysteine (44%) and cystathionine (44%) and a 2.5-fold increase in homocysteine concentration [48]; and they displayed lower weight gains despite their higher energy consumption. The effect of treatment with cysteine (MR + Cys) was tested on this animal model. Following 12 weeks of treatment, the MR + Cys group exhibited weight gain and adiposity similar to that of the control group. Certain lipoprotein changes observed in the MR group (low levels of triglycerides and higher levels of LDL) were reverted by the cysteine treatment. In addition, the reduced levels of leptin, insulin growth factor (IGF)-1 and insulin and elevated levels

of adiponectin observed for the MR group were normalized following treatment with cysteine. The decreases in the SCD1-16 and SCD1-18 indices and low hepatic expression of SCD-1 observed in the MR group were also reverted in the MR + Cys group. Supplementation with cysteine completely normalized the plasma levels of cysteine and cystathionine but only partially corrected the taurine and homocysteine concentrations [49].

The role of taurine was also tested in this animal model. The remaining sulfur amino acid concentration, weight gain, body composition, lipid profile, hepatic activity and SCD-1 expression were all unaffected by supplementation with taurine (MR + Tau). In fact, the taurine treatment presented further decreases of body fat, suggesting that this amino acid is not a direct mediator of adipogenesis [50]. In another study, mice lacking the glutamate-cysteine ligase modifier subunit gene (Gclm(−/−)) were studied. These animals present glutathione deficiency and low cysteine plasma levels of cysteine [51]. The Gclm(−/−) mice presented lower body weight (primarily because of lower fat mass), improved glucose tolerance and higher basal metabolic rates compared with that of the wild type, and a high-fat diet did not induce significant changes in these phenotypes. The SCD-1 expression was markedly downregulated in the liver at only 17% of the wild type [52].

Changes in choline/betaine metabolism, which is highly interconnected with sulfur amino acid metabolism, have also been associated with changes in energy, lipid and glucose metabolism [53]. Deficiency of the enzyme BHMT (Figure 1), which remethylates homocysteine to methionine, results in increased levels of betaine and homocysteine and decreased levels of choline and cysteine in mice [54]. BHMT-deficient mice exhibit lower body-fat mass, reduced triglyceride synthesis, improved sensitivity to insulin and higher energy expenditure. These changes are not associated with changes in food intake, lipid absorption, lipolysis or thermogenesis [55]. Dietary deprivation of methionine and choline in mice also results in decreased body-fat mass; higher energy expenditures; lower serum triglyceride, leptin, insulin and glucose concentrations; and suppressed hepatic SCD-1 expression. In this animal model, downregulation of SCD-1 was associated with hepatic steatosis [56].

Studies of the relationship between sulfur amino acids and SCD-1 activity in humans are scarce. In the Hordaland Health Studies (HUSK) and Hoorn European cohort studies, a positive and independent association was observed between plasma cysteine levels and the SCD-16 index. The plasma levels of total cholesterol and triglycerides were also positively associated with the SCD-16 index. No consistent associations were found between the SCD-16 index and homocysteine, methionine, AdoMet, cystathionine or glutathione plasma concentrations [57]. These findings are consistent with other epidemiological studies indicating that cysteine is an independent predictor of obesity [58–60]. In addition, patients with classical homocystinuria who also have low cysteine concentrations have reduced body mass and exhibit lipid metabolism abnormalities [21,61]. A positive association between BMI and cysteine levels has also been observed in these individuals [61]. This phenotype has not been described in other forms of homocystinuria in humans who have normal cysteine levels.

5. Conclusions

The regulation of lipid metabolism by sulfur amino acids has gained more attention in recent years. Although the mechanisms are far from fully understood, animal and population-based studies have suggested that one or more sulfur amino acids affect lipoprotein production and lipid storage in

adipose tissues and indicated that this action is possibly mediated by SCD-1. Although the most consistent evidence indicates cysteine to be the main modulator, other sulfur amino acids or choline and its derivatives may produce independent or additional effects on lipid metabolism.

Because SCD-1 appears to be strongly involved in the etiology of metabolic syndrome, understanding the regulation of this enzyme may assist in the development of new therapies and prevention strategies for several chronic diseases, such as obesity, diabetes, dyslipidemia and arteriosclerosis. Changes in sulfur amino acid metabolism, especially hyperhomocysteinemia, are frequent in the human population, further reinforcing the importance of these findings.

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Author Contributions

Soraia Poloni was responsible for the conception, literature review and writing of the manuscript. Henk J. Blom and Ida V. D. Schwartz participated in the conception and review of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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6.3 CAPÍTULO 3: "*Leptin levels and SCD-1 activity in classical homocystinuria: evidence for the role of sulfur amino acids in the regulation of lipid metabolism*"

Título do artigo: "Leptin levels and SCD-1 activity in classical homocystinuria: evidence for the role of sulfur amino acids in the regulation of lipid metabolism"

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Leptin levels and SCD-1 activity in classical homocystinuria: evidence for the role of sulfur amino acids in the regulation of lipid metabolism

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ABSTRACT

Classical homocystinuria (HCU) is a rare genetic disease associated with disrupted sulfur amino acids (SAA) metabolism, increased levels of homocysteine and methionine, and low levels of cysteine. HCU mice have low adiposity and are protected from diet-induced obesity, with marked suppression of the lipogenic enzyme stearoyl-CoA desaturase-1 (SCD-1) in liver. The aim of this study was to describe alterations in body composition, lipid metabolism, and SCD-1 activity in patients with HCU. **METHODS:** Eleven HCU patients receiving homocysteine-lowering treatment and 16 healthy controls were included in the study. Body composition and bone mineral density were assessed by DXA. Fasting blood samples were obtained to measure sulfur-containing compounds (total homocysteine, cysteine, methionine, S-adenosylmethionine, S-adenosylhomocysteine, and glutathione), lipids (free fatty acids, acylcarnitines, triglycerides, high-density lipoprotein, and total cholesterol), glucose and insulin in plasma. Insulin resistance was determined by homeostasis model assessment (HOMA-IR). To estimate liver SCD-1 activity, SCD-16 [16:1 (n-7)/16:0] and SCD-18 [18:1 (n-9)/18:0] indices were determined. Plasma leptin, adiponectin, and isoprostane levels were determined by ELISA. **RESULTS:** In HCU patients, SCD-16 index was significantly reduced ($p=0.03$) and correlated with cysteine levels ($r= 0.477$; $p=0.01$). HCU patients presented lower lean mass ($p<0.05$), but fat mass percentage was not significantly different between the groups. Leptin levels were lower in HCU (median levels: 1.79 *vs* 5.59 ng/dL; $p=0.027$) and correlated with plasma methionine ($r: -0.558$) and total homocysteine ($r: -0.466$) ($p<0.05$). Low-density lipoprotein was also reduced in HCU patients ($p<0.01$). Insulin resistance was similar in both groups. No other differences were found between patients and controls. **CONCLUSIONS**: Our results provide evidence of an important role of SAA (especially cysteine) in the modulation of lipid metabolism. SAA action on lipid metabolism is probably mediated by leptin and liver SCD-1.

INTRODUCTION

In the past few years, increasing attention has been paid to the role of sulfur amino acids (SAA) methionine, homocysteine, and cysteine in regulating lipid metabolism. These amino acids are involved in several metabolic pathways, such as glutathione and protein synthesis, and methylation of several molecules (Figure 1) (Stipanuk *et al.*, 2006). They have also been linked to metabolic syndrome and obesity in epidemiologic and experimental studies (Narin *et al.*, 2005; Elshorbagy *et al.*, 2008; Vinknes *et al.*, 2013; Elshorbagy, 2014). Among SAA, cysteine has been shown to have the most consistent and independent associations with obesity (Elshorbagy *et al.*, 2011; Elshorbagy, Kozich, *et al.*, 2012), insulin resistance (Elshorbagy, Valdivia-Garcia, Refsum, *et al.*, 2012), and lipid homeostasis (Bettzieche *et al.*, 2008; Da Silva *et al.*, 2013; Tanaka *et al.*, 2014).

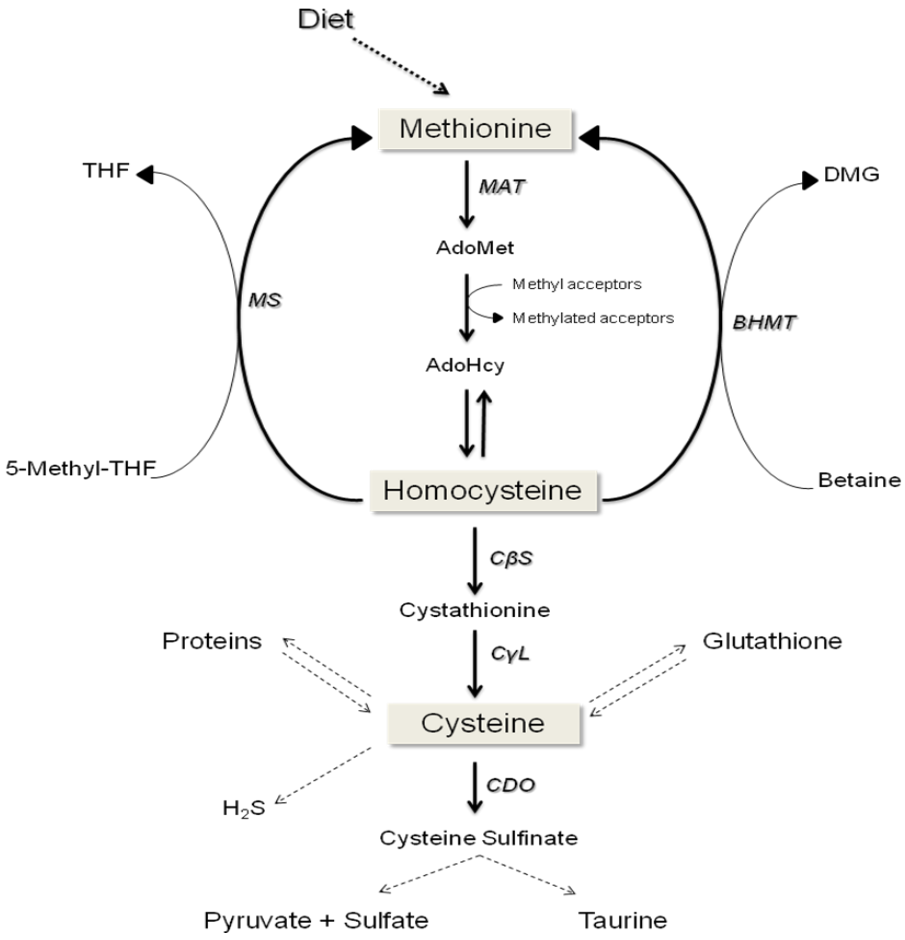

Figure 1. Sulfur amino acid pathway: cysteine metabolism. Dotted lines indicate pathways with omitted steps. MAT: methionine adenosyltransferase; AdoMet: S-adenosylmethionine; AdoHcy: S-adenosylhomocysteine; CβS: cystathionine β-synthase; CγL: cystathionine γ-lyase; CDO: *cysteine dioxygenase*; MS: methionine synthase; THF: tetrahydrofolate; BHMT: betaine-homocysteine S-methyltransferase; DMG: dimethylglycine. Adapted from Stipanuk *et al* (Stipanuk *et al.*, 2006).

A recent review (Elshorbagy, 2014) has shown reduced fat mass, increased energy expenditure, improved insulin sensitivity, and protection against dietary-induced obesity in many knockout mouse models used to study enzymes involved in SAA metabolism, especially in the presence of low cysteine and/or choline levels. Reduced stearoyl-CoA desaturase-1 (SCD-1) expression was another common feature of some of these models (Teng *et al.*, 2011; Teng *et al.*, 2012). SCD-1 is a key enzyme in lipid metabolism, because its major products, palmitoleic acid (C16:1) and oleic acid (C18:1), are the main fatty acids in triglycerides, cholesterol esters, and membrane phospholipids. When SCD-1 is deficient, monounsaturated fatty acids synthesis is reduced, which impairs very low density lipoprotein (VLDL) and triglycerides synthesis and so, fat accumulation in the adipose tissue (Kim *et al.*, 2011). SCD-1 global knockout mice (SCD-1 −/−) exhibit lower triglycerides levels, reduced fat deposition in adipose tissue, higher energy intake, higher basal energy expenditure, and improved insulin sensitivity (Miyazaki *et al.*, 2001; Ntambi *et al.*, 2002).

Classical homocystinuria (HCU; 236200) is a rare genetic disease in which cystathionine β-synthase (CβS, EC 4.2.1.22) deficiency causes marked accumulation of homocysteine and methionine and decrease in cysteine plasma levels (Mudd *et al.*, 2001; Wilcken, 2006). The biochemical and clinical phenotype of the transgenic HCU mouse model (TgI278T *CβS*-/-) is comparable to the human disease (Wang *et al.*, 2005). The clinical picture in humans includes abnormalities in ocular, vascular, central nervous, and skeletal systems. HCU phenotype also resembles that of Marfan syndrome, with affected individuals being unusually tall and lean (Brenton *et al.*, 1972; Wilcken, 2006). Transgenic HCU mice have low weight gain and a significant decrease in body fat mass (46% in females and 62% in males), while lean mass is only slightly reduced. A 34% decrease in liver glutathione (GSH) and a marked decrease in SCD-1 expression in liver has also been described in transgenic HCU mice (Gupta e Kruger, 2011). Treatment with a low-methionine diet restored weight gain, fat mass, SCD-1 activity, and bone mineral density in HCU mice. However, the nearly 10-fold increase in homocysteine plasma levels was not reversed, while cysteine concentrations were restored to normal levels. Liver SCD-1 expression increased 82% (Gupta *et al.*, 2014).

In humans, a good correlation has been reported between C18:1/C18:0 and C16:1/C16:0 fatty acid ratios in plasma and liver SCD-1 activity. Fatty acid ratios have been strongly

associated with adiposity and metabolic syndrome in epidemiologic studies (Dobrzyn e Ntambi, 2005; Paton e Ntambi, 2009; Vinknes *et al.*, 2013). However, little is known about body composition and lipid metabolism specifically in patients with HCU. We have recently studied 9 patients with poorly controlled HCU. Four of these patients presented low fat mass percentage according to bioelectrical impedance analysis (BIA). We also found significant associations between BMI and SAA plasma concentrations (Poloni *et al.*, 2014). In another study with five well-controlled HCU patients, no alterations in body composition measured by dual X-ray absorptiometry (DXA) were found (Lim e Lee, 2013). Alterations in lipoprotein levels, especially reduction in high-density lipoprotein (HDL) concentration, have been described in humans and in animal models of HCU (Moat *et al.*, 1999; Namekata *et al.*, 2004; Poloni *et al.*, 2012).

To explore the link between SAA and lipid homeostasis, we studied SCD-1 indices and body composition by DXA in treated patients with HCU. In addition, we investigated the existence of a relationship between SCD-1 and lipid metabolism markers (lipoproteins, isoprostanes, acylcarnitines), hormones (leptin and adiponectina), bone mineral density (BMD), and insulin resistance in this group of patients.

MATERIALS AND METHODS

A cross-sectional study was conducted at Hospital de Clínicas de Porto Alegre (HCPA), 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.

Subjects

Eleven patients with HCU from 10 unrelated families were included. Nine of these patients had participated in a previous study focusing on aspects of body composition (Poloni *et al.*, 2014) and had been included in a retrospective study about lipid metabolism (Poloni *et al.*, 2012).

All 11 patients were receiving homocysteine-lowering treatment, including pyridoxine (n = 9/11), betaine (n = 9/11), folic acid (n = 9/11), B12 vitamin (n = 4/11), and a combination of low-methionine diet and metabolic formula (n= 5/11). Additionally, 4/11 patients were

instructed to avoid meat and high amounts of dairy products, but were not taking metabolic formula. HCU metabolic formula is a methionine-free, amino acid-based mixture supplemented with vitamins and minerals. It also contains high amounts of L-cystine to prevent cysteine deficiency. Despite treatment, most patients were not able to maintain good metabolic control - only 4/11 presented total homocysteine (tHcy) <60 µmol/L at the time of inclusion. Regarding pyridoxine responsiveness, eight patients were considered nonresponsive, one was partially responsive, and two were fully responsive.

In addition, 16 healthy volunteers of similar sex, age, and BMI were included as controls. Exclusion criteria for the control group were 1) chronic diseases, 2) pregnancy, and 3) recent or current intake of vitamins or other medicines that can alter sulfur metabolism.

Assessment of body composition and bone mineral density

Body composition was assessed in a single appointment by DXA. Of the 11 patients included in the present study, one did not attend the DXA evaluation, and therefore only biochemical and clinical data were analyzed for this patient. Using a whole-body scanner (GE- Lunar Prodigy®), fat and lean mass were determined as previously described by Mario et al (Mario *et al.*, 2012). Since absolute lean mass is highly related to height, the Baumgartner index [lean mass (kg)/height² (m)] (Baumgartner *et al.*, 1998) was employed to provide a measure of relative lean mass. BMD was assessed in lumbar spine (L1– L4), proximal femur (total and femoral neck), and whole body. Precision error was assessed as recommended by the International Society for Clinical Densitometry taking into consideration the minimum acceptable precision for individual technologists (Baim *et al.*, 2008).

Biochemical analysis

Blood samples were taken after a 12-h overnight fast on the same day of DXA. Immediately after collection, samples were centrifuged and plasma was isolated and stored at -80 ˚C for further analysis.

Plasma total homocysteine, cysteine, and methionine were measured by LC-MS/MS following a protocol adapted from Rafii *et al* (Rafii *et al.*, 2007), Persichilli *et al* (Persichilli *et al.*, 2010), and Bártl *et al* (Bartl *et al.*, 2014). 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, the supernatant was evaporated and butylated, and was subsequently injected into the LC-MS/MS system (Waters UPLC Acquity/Waters Quattro Premier XE).

S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) were measured by a stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry method described by Gellekink *et al* (Gellekink *et al.*, 2005). Initially, sample cleanup was performed with solid-phase extraction (SPE) columns containing phenylboronic acid. The isotopically labelled samples were then loaded onto the SPE cartridge and washed with ammonium acetate. Finally, AdoMet and AdoHcy were eluted with formic acid from the SPE column, and then the samples were injected into the LC-MS/MS system. Plasma total glutathione (GSH) concentrations were analyzed by high performance liquid chromatography (HPLC) through fluorescence detection and isocratic elution, following the protocol described by da Silva *et al* (Da Silva *et al.*, 2014).

Plasma free fatty acids assessment was performed as described by Tucci *et al* (Tucci *et al.*, 2015). First, total lipids were extracted from plasma with a protocol adapted from Folch *et al* (Folch *et al.*, 1957). Initially, a solution containing 0.9% NaCl and 0.1% butylated hydroxytoluene (BHT) was added to the samples before homogenization. Lipids were extracted with a mixture of methanol/chloroform (2:1, v/v) followed by a second extraction with methanol/chloroform (3:1, v/v). Trimethylsulfonium hydroxide was added to the total lipid extract for conversion of lipids into fatty acid methyl esters (FAMEs) as reported by Müller *et al* (Muller *et al.*, 1998). FAMEs were analyzed using an Agilent gas chromatograph 7890A equipped with an MS 5975C detector and a 7963 autosampler. Detection of FAMEs was conducted with the Agilent MSD ChemStation E02.02.1431 software. The relative amount of each fatty acid (% of total fatty acids) was determined by dividing the peak area of each single fatty acid by the sum of the peak areas of all fatty acids. FAMEs standard for the identification of the products was obtained by Supleco/Sigma-Aldrich (Steinheim, Germany). To estimate liver SCD-1 activity, two product/precursor ratios were used: SCD-16 [16:1 (n-7)/16:0] and SCD-18 [18:1 (n-9)/18:0].

Acylcarnitine profile was measured with a protocol adapted from Matern (Matern, 2008). Briefly, 50 µl of a 1:10 solution of plasma in water was mixed with 500 µL of acetonitrile to precipitate proteins. Next, an isotope labeled standard was added to the mixture. After

centrifugation, the supernatant was transferred to another vial and evaporated with nitrogen. Following that step, 3 N butanolic HCl was added and incubated for 15 minutes at 65 °C to form butyl esters corresponding to acylcarnitines. After incubation, residues were evaporated with nitrogen. Finally, the sample was reconstituted in 400 µL acetonitrile/water (80:20), and then 20 µl was injected and measured via flow-injection and tandem mass spectrometry. Acylcarnitines were quantified by comparison to internal standards.

Triglycerides, HDL, and total cholesterol were measured by enzymatic colorimetric assay. The Friedewald equation was used to calculate LDL-cholesterol. To evaluate lipid peroxidation, we measured plasma isoprostanes levels using a commercially available ELISA kit (Stat-8-Isoprostane EIA Kit - Cayman Chemical, Michigan, USA). Plasma concentrations of leptin (ng/dL), and adiponectin (ng/mL) were determined using ELISA commercial kits (Millipore, St. Charles, MO, USA) with sensitivities of 0.5 ng/dL and 0.78 ng/mL, respectively.

Fasting plasma glucose was measured by an enzymatic hexokinase assay, and insulin by chemiluminescence. To evaluate insulin resistance, the homeostasis model assessment (HOMA-IR) method was chosen, with a cutoff of 2.71, which is considered a reliable threshold to detect insulin resistance in the Brazilian population (Geloneze *et al.*, 2006).

Statistical analysis

Statistical analysis was carried with SPSS version 19.0 for Windows®. 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. Analysis of covariance (ANCOVA) was employed to compare variables controlling for the effects of age, sex, BMI, and plasma cysteine levels. Variables with asymmetrical distribution were log-transformed to approach normality for ANCOVA analysis, and were presented as geometric mean ± geometric SD. The chi-square test was used for comparison of categorical data. Associations between body fat percentage, biochemical markers, and BMD Z-scores were evaluated with Spearman's correlation coefficient. All tests were two-tailed, and $p<0.05$ was considered significant.

RESULTS

Table 1 presents baseline features, BMD Z-scores, and plasma levels of sulfur-containing compounds for patients and controls. As expected, patients presented lower BMD Z-scores and altered levels of sulfur-containing compounds, with the exception of GSH, which was similar in both groups. Two patients had been previously diagnosed as having osteoporosis and were receiving sodium alendronate. Another five patients were taking calcium supplements.

Table 2 shows the results of ANCOVA analysis. No significant differences were found in fat mass between the groups either before or after adjustments for sex, age, BMI, and plasma cysteine levels. In the HCU group, fat mass percentage was negatively correlated with plasma GSH levels ($r= -0.717$; $p=0.03$) (Figure 2), while in the overall group cysteine was positively associated with fat mass ($r= 0.596$; $p=0.002$). Lean mass and lean mass index were significantly reduced in patients after adjustment for sex (Table 2). Fat mass was not associated with BMD in either group, while lean mass was positively associated with spine, femur, and whole-body BMD Z-scores only in the control group ($p<0.05$). In patients, femur BMD Z-scores were highly correlated with plasma cysteine ($r= 0.829$; $p=0.04$) and tHcy ($r= -0.829$; $p=0.04$) levels.

SCD-16 index was reduced in the HCU group in univariate analysis and after adjustments for sex, age, and BMI. In the overall group, SCD-16 index was positively associated with cysteine levels ($r=0.477$, $p=0.012$). Significance was lost when the groups were analyzed separately, but a trend towards association was still observed for HCU (patients: $r=0.624$, $p=0.054$; controls: $r=0.109$, $p=0.688$) (Figure 2). The groups had a similar acylcarnitine profile (data not shown). Relative amounts of fatty acids in plasma were similar in the two groups, with the exception of C16:1, which was lower in the HCU group (Table 3).

Leptin levels were reduced in HCU patients in univariate analysis and after adjustments for sex, age, and BMI. Significance was lost after adjustment for cysteine levels (Table 2). Leptin was inversely associated with plasma methionine and tHcy levels in the overall group ($r: -0.558$ and $r: -0.466$, respectively, $p<0.05$). The trends were similar in both groups but non-significant when analyzed independently. Leptin was strongly associated with fat mass percentage in both groups (patients: $r=0.879$, $p=0.001$; controls: $r=0.864$,

p<0.001). There was also a trend towards association between cysteine and leptin levels in the overall group (r=0.378; p=0.057).

Adiponectin concentrations tended to be lower in HCU patients, with marginally significant values in univariate and multivariate analyses (Table 2). Adiponectin was associated with neither body composition nor sulfur and/or lipid compounds.

In HCU patients, plasma LDL-cholesterol was reduced in univariate and multivariate analyses, and the difference between the groups was lost only after adjustment for cysteine (Table 2). Total cholesterol and triglycerides were inversely associated with leptin plasma levels in HCU patients (r=0.879 and r=0.806, respectively; p<0.05). Isoprostanes, glucose, insulin, and HOMA-IR were similar in the two groups (Table 2) and were not associated with body composition, SCD indices, or sulfur compounds.

Table 1. Plasma sulfur-containing compounds and BMD in patients with homocystinuria and controls

	Patients (n=11*)	***Controls (n=16)***	***P***
Male n (%)	6 (60)	7 (44)	0.74
Age (years)	24.6±7	26±4	0.58
Weight (kg)	65±15	71±16	0.15
Height (cm)	173±8	176±10	0.82
BMI (kg/m²)	21.5±4	23.1±3	0.07
Z-score proximal femur	-1.1 (-1.6;2.1)	0.1 (-0.7;1.5)	0.07
Z-score lumbar spine	-0.9 (-1.4;-0.4)	-0.1 (-0.5;1.8)	0.009
Z-score whole body	-0.6 (-1.1;-0.1)	0.5 (-0.7;1.1)	0.006
Methionine (µmol/L)	162 (31;477)	23 (17;25)	<0.001
AdoMet (nmol/L)	756 (208;1028)	83 (73;104)	<0.001
AdoHcy (nmol/L)	135 (58;253)	18 (16;22)	<0.001
tHcy (µmol/L)	130 (36;191)	7 (6;8)	<0.001
Cysteine (µmol/L)	158 (90;224)	222 (210;233)	0.007
GSH (µmol/L)	12±4.5	12±4	0.77

*BMD Z-scores available for 10 patients. Variables are presented as means±SD or medians (interquartile range: p25-p75), except for sex. BMD: bone mineral density; BMI: body mass index; tHcy: total homocysteine; GSH: glutathione. AdoMet: S-adenosylmethionine; AdoHcy: S-adenosylhomocysteine.

Table 2. Body composition, SCD-1 activity, and lipid and glucose markers in patients with homocystinuria and controls

			Univariate analysis	Multivariate analysis			
	Patients n=11‡	**Controls** n=16	***t test*** ***p***	***Adj sex*** ***p***	***Adj age*** ***p***	***Adj BMI*** ***p***	***Adj cys*** ***P***
Fat mass (%)	24.6 ± 11.0	27.7 ± 8.9	0.442	0.546	0.592	0.751	0.858
Lean mass (kg)	43.6 ± 8.5	48.3 ± 14	0.275	0.008	0.299	0.932	0.893
Lean mass index (kg/m²)	14.4 ± 1.9	15.9 ± 3.2	0.024	0.001	0.105	0.886	0.704
SCD-16 *	55.0 ± 1.3	39 ± 1.6	0.011	0.004	0.011	0.023	0.214
SCD-18	1.7 ± 0.5	1.7 ± 0.3	0.824	0.470	0.827	0.953	0.843
Leptin (ng/dL)*	2.0 ± 2.1	4.6 ± 2.4	0.016	0.008	0.022	0.016	0.062
Adiponectin (ng/mL)*	18.2 ± 2.6	34.4 ± 1.9	0.057	0.057	0.049	0.056	0.440
LDL cholesterol (mg/dL)	81.0 ± 22.0	103.0 ± 25.0	0.017	0.022	0.028	0.044	0.222
Total cholesterol (mg/dL)	148.0 ± 41.0	169.0 ± 37.0	0.171	0.200	0.253	0.303	0.713
HDL cholesterol (mg/dL)	49.0 ± 16.0	47.0 ± 11.0	0.629	0.083	0.610	0.873	0.700
Triglycerides (mg/dL)*	67.0 ± 2.0	92.0 ± 1.5	0.175	0.190	0.324	0.753	0.611
Isoprostanes (pg/mL)	49.7 ± 2.0	50.2 ± 2.0	0.439	0.415	0.440	0.442	0.151
Glucose (mg/dL)	87.2 ± 9.0	85.1 ± 6.0	0.516	0.852	0.509	0.199	0.386
Insulin (µmol/L)*	8.7 ± 2.0	9.5 ± 1.6	0.718	0.531	0.661	0.767	0.835
HOMA-IR*	1.9 ± 2.0	2.0 ± 1.7	0.787	0.559	0.736	0.673	0.884

‡Body composition measurements were available for 10 patients. Variables are presented as mean±SD or as (*) geometric mean±geometric SD (variables having asymmetrical distribution which were log-transformed for ANCOVA analysis).

BMI: body mass index; Cys: cysteine; Adj: Adjusted for; SCD-1: stearoyl-CoA desaturase-1; SCD-16: 16:1 (n-7)/16:0 product/precursor ratio; SCD-18: 18:1 (n-9)/18:0 product/precursor ratio; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HOMA-IR: homeostasis model assessment for insulin resistance.

Table 3. Relative amounts of plasma fatty acids in homocystinuria patients and controls

	Patients (n=11)	***Controls (n=16)***	***P***
C14 (%)	0.84 (0.7;1.1)	1.14 (0.9;1.3)	0.07
C16 (%)	20.7 (18.8;22.8)	22.1 (20.7;23.2)	0.84
C16:1 (%)	0.39 (0.32;0.43)	0.53 (0.4;0.73)	0.02
C17 (%)	0.30 (0.27;0.33)	0.34 (0.07;0.39)	0.72
C18 (%)	10.1 (8.8;11.5)	9.3 (8.5;9.9)	0.17
C18:1n9 (%)	16.0 (14.7:18.6)	15.1 (14.2;17.2)	0.09
C18:2n6 (%)	21.0 (19.1;23.8)	21.1 (20.1;24.1)	0.61
C18:3n3 (%)	0.72 (0.46;0.79)	0.59 (0.45;0.65)	0.14
C20:3n6 (%)	1.6 (1.33;1.84)	1.3 (0.86;1.58)	0.96
C20:4 (%)	6.3 (4.0;7.4)	5.2 (4.4;6.3)	0.31
Free cholesterol (%)	19.3 (15.9;24.2)	20.6 (19.5;22.1)	0.20

Variables are presented as medians (interquartile range: p25-p75). Fatty acids are presented as % of total fatty acids.

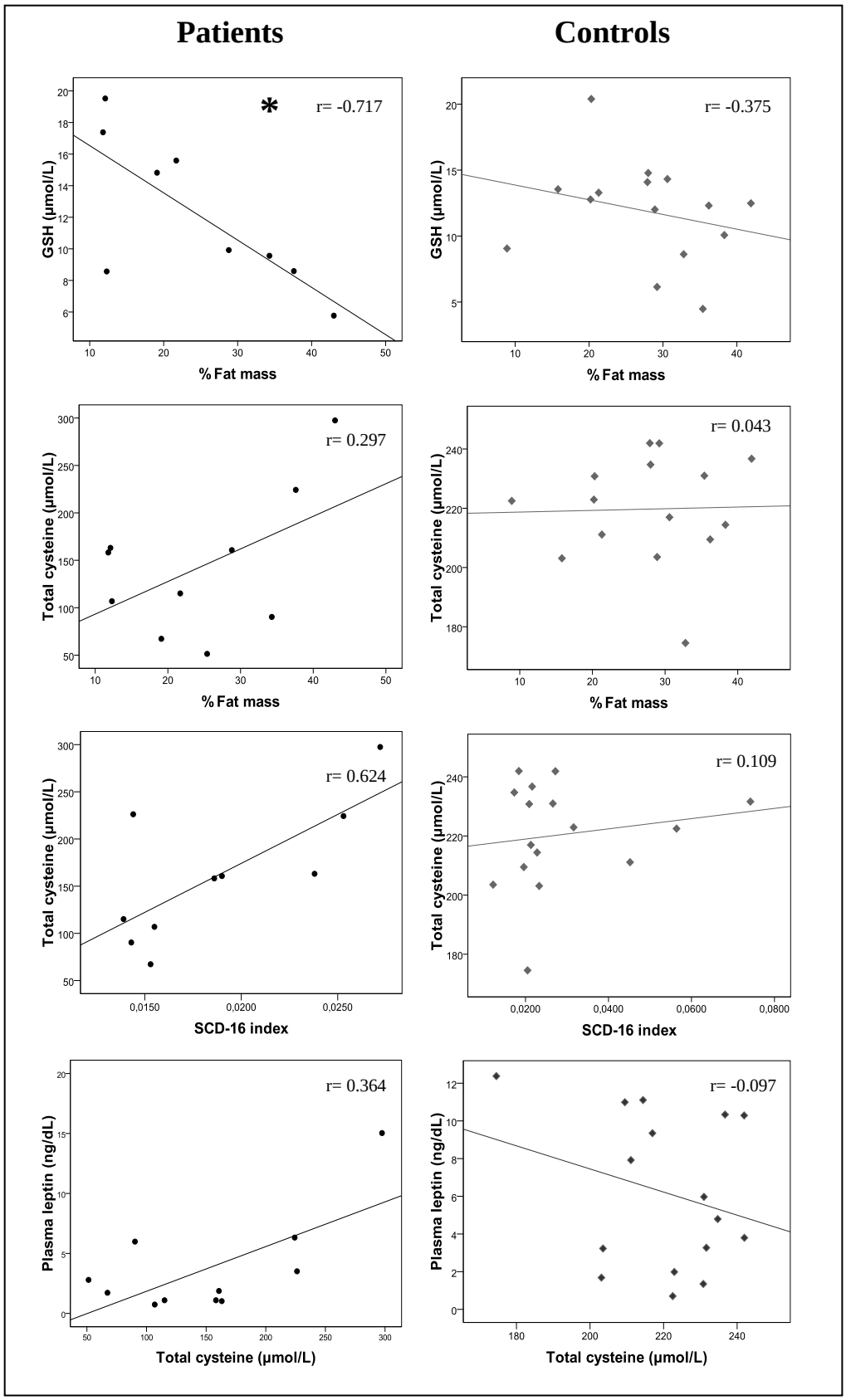

Figure 2. Correlations between GSH, cysteine, fat mass, SCD-16 index, and leptin in homocystinuria patients and controls. Significant correlations are flagged with an asterisk (*). Patients = black dot; Controls = black diamond.

DISCUSSION

In the present study, we observed alterations in lean mass, SCD-1, LDL, and leptin concentrations in treated HCU patients. Our results suggest that liver SCD-1 activity is reduced in HCU, which might impair fatty acid and lipoprotein synthesis in these patients. We found significant associations between plasma SAA levels and SCD-16 index, leptin, and body fat mass; this supports the hypothesis that SAA play an important role in the control of lipid and energy metabolism (Narin *et al.*, 2005; Bettzieche *et al.*, 2008; Elshorbagy *et al.*, 2008; Elshorbagy *et al.*, 2011; Elshorbagy, Kozich, *et al.*, 2012; Elshorbagy, Valdivia-Garcia, Refsum, *et al.*, 2012; Da Silva *et al.*, 2013; Vinknes *et al.*, 2013; Elshorbagy, 2014; Tanaka *et al.*, 2014).

Adiposity was similar in our groups of HCU patients and controls, perhaps as a result of homocysteine-lowering treatment. In HCU mice, treatment has been shown to restore the low fat mass phenotype (Gupta *et al.*, 2014). Selection bias might also explain this finding, since the BMI of HCU patients was similar to that of controls. Overweight subjects were under-represented in our sample (one patient and two controls). A previous study by our group has also failed to detect significant differences in body fat percentage by BIA, although 4/11 patients presented low body fat (Poloni *et al.*, 2014). BIA, however, is a less reliable method than DXA for measuring body composition, and has not been validated for HCU.

Interestingly, we found reduced lean mass in HCU patients. In transgenic HCU mice, a slight decrease in lean mass (~10%) has been reported in non-treated animals, which was not recovered after treatment (Gupta e Kruger, 2011; Gupta *et al.*, 2014). The decrease observed in our patients might have resulted from limited protein intake, since nine of our 11 patients were following a low or low to normal natural protein diet. Those with more protein-restricted diets (n= 5/11) received metabolic formula to meet amino acid requirements. Yet, compliance with the use of metabolic formula was not optimal, and long-term amino acid deficiencies cannot be ruled out. In treated patients with Phenylketonuria (OMIM 261600), natural protein intake and compliance with diet have been found to be directly correlated with muscle mass (Huemer *et al.*, 2007; Adamczyk *et al.*, 2011).

Several experimental models with disrupted SAA metabolism, including transgenic HCU mice, display abnormalities in lipid metabolism. Fatty acid synthesis seems to be markedly

impaired in these rodents; not only are they slimmer than their littermates on a regular diet, they are also protected from diet-induced obesity (Paton e Ntambi, 2009; Elshorbagy *et al.*, 2011; Teng *et al.*, 2012). Liver SCD-1 seems to play a central role in this phenotypic feature: SCD-1 expression is highly reduced in liver of untreated HCU. In treated animals, with restored cysteine concentrations, SCD-1 expression is rescued and the anti-obesogenic phenotype is completely prevented (Paton e Ntambi, 2009; Gupta *et al.*, 2014).

SCD-1 plays a central role in the control of lipid metabolism, since its products — palmitoleic acid (C16:1) and oleic acid (C18:1) — are the main fatty acids in triglycerides, cholesterol esters, and membrane phospholipids (Dobrzyn e Ntambi, 2005; Paton e Ntambi, 2009). In line with this, we observed low LDL-cholesterol in HCU patients. Reduced lipoprotein concentration is a common feature of HCU in humans and animal models (Moat *et al.*, 1999; Namekata *et al.*, 2004; Poloni *et al.*, 2012), and a strong inverse relationship between plasma homocysteine and lipoproteins has been described in HCU patients (Moat *et al.*, 1999). In HCU mice, a positive association between APOA1 and cysteine levels has been reported (Nuno-Ayala *et al.*, 2010).

SCD-1 deficiency also results in an increased pool of saturated acyl CoAs, which are believed to be targeted to mitochondria for oxidation (Dobrzyn e Ntambi, 2005; Paton e Ntambi, 2009; Poloni *et al.*, 2015). This mechanism explains the reduced adipogenesis and increased energy expenditure seen in experimental models of SCD-1 deficiency (Ntambi *et al.*, 2002; Kim *et al.*, 2011). In our study, we observed that treated patients with HCU had lower SCD-16 index, indicating reduced SCD-1 activity. SCD-18 index was not different between the groups. SCD-16 index is considered a better predictor of liver SCD-1 activity in humans than SCD-18, since the latter is more prone to be affected by dietary intake (Vessby *et al.*, 2013). SCD-16 was positively associated with plasma cysteine levels in our sample, while the remaining SAA were not. Even with treatment, most of our HCU patients had not achieved adequate metabolic control, and cysteine levels remained significantly lower when compared to controls (Table 1). This might explain the reduced SCD-1 activity in our HCU group.

There is evidence of a major role played by cysteine in the regulation of lipid metabolism and fat deposition (Elshorbagy *et al.*, 2011; Elshorbagy, Kozich, *et al.*, 2012; Poloni *et al.*, 2015). In different animal models with disrupted SAA metabolism, restoring cysteine supply reverses the anti-obesogenic phenotype, including SCD-1 expression (Elshorbagy *et*

al., 2011; Gupta *et al.*, 2014), leptin and insulin levels (Elshorbagy *et al.*, 2011), and body weight and fat mass (Lieberman *et al.*, 1996; Mani *et al.*, 2011; Gupta *et al.*, 2014). In an epidemiologic study with more than 2500 subjects, cysteine was the only SAA independently associated with SCD-16 index (Vinknes *et al.*, 2013). In Down syndrome, CβS is overexpressed (since the gene is located at chromosome 21), leading to increased plasma cysteine levels. Individuals with Down syndrome are also more prone to obesity (Pogribna *et al.*, 2001; Elshorbagy, Kozich, *et al.*, 2012). Finally, a study with obese patients undergoing gastric bypass surgery showed that, at baseline, patients had modestly higher mean plasma total cysteine concentrations. Cysteine remained unchanged in serial measurements after surgery, while other SAA decreased, accompanying a 30% weight loss (Aasheim *et al.*, 2011). Taken together, these data support a causative effect of cysteine on obesity. In our study, all altered lipid metabolism measurements in HCU patients (SCD-16 index, leptin, and LDL-cholesterol) became non-significant after adjustment for cysteine, reinforcing the role of this SAA in these metabolic abnormalities.

Cysteine plasma levels were also associated with body fat mass in our sample, with a stronger trend observed in HCU patients. Also, we found an inverse association between GSH and fat mass among patients, although plasma GSH levels were not significantly different in the two groups. GSH is an important antioxidant formed from cysteine, and its relationship with obesity is controversial. While *in vitro* and epidemiologic studies have found and inverse association between GSH and both obesity and energy expenditure (Di Renzo *et al.*, 2010; Lettieri Barbato *et al.*, 2015), genetically GSH-deficient mice (*Gclm(−/−)*) are protected from diet-induced obesity and have higher basal metabolic rate. However, these animals also display decreased cysteine levels (Kendig *et al.*, 2011; Elshorbagy, 2014), which may account for this phenotype.

Leptin, an important regulator of SCD-1 expression, was reduced in HCU patients, and showed inverse associations with SAA levels and fat mass in our study. Leptin is a hormone secreted mainly by adipocytes; it regulates several aspects of energy balance, such as appetite, energy expenditure, insulin action, and lipoprotein synthesis. Several effects of leptin on metabolism are believed to be mediated by SCD-1 (Cohen e Friedman, 2004). Although genetic leptin deficiency increases fat mass in humans and rodents, our findings agree with the experimental model described by Elshorbagy *et al* (Elshorbagy *et al.*, 2011). In rats, a methionine-restricted induces cysteine deficiency and

hyperhomocysteinemia. Low SCD-1 activity was associated with reduced leptin levels and low adiposity in these rats. This phenotype was reversed with a cysteine-supplemented diet (Elshorbagy *et al.*, 2011). It should be noted that in humans, leptin concentration fluctuates with fat mass (Cohen e Friedman, 2004; Ferrannini *et al.*, 2014).

Adiponectin concentrations tended to be lower in our HCU patients, with marginal significance between the groups after adjustment for sex. In the diet-induced model described by Elshorbagy *et al* (Elshorbagy *et al.*, 2011), non-treated rats presented increased adiponectin levels, while cysteine-treated animals presented the lowest levels, even when compared with control-fed rats. In humans, low adiponectin levels have been associated with obesity, type 2 diabetes, cardiovascular disease and metabolic syndrome, although a causal link has not been established (Weyer *et al.*, 2001; Belalcazar *et al.*, 2015; Bluher e Mantzoros, 2015).

No abnormalities in insulin action were found in our study. To the best of our knowledge, only one study so far has evaluated glucose homeostasis in HCU patients. In that study (Holmgren *et al.*, 1973), 2/5 patients presented reduced glucose tolerance. The authors do not describe whether these patients were on treatment or not. Improved insulin action has been described in non-treated animals with disrupted SAA metabolism, but this has not studied in the HCU mouse model (Elshorbagy *et al.*, 2011; Kendig *et al.*, 2011; Teng *et al.*, 2012)

We also evaluated other aspects of lipid metabolism, such as acylcarnitines profile and plasma lipid peroxidation. Acylcarnitines are required for the transport of long-chain fatty acids into mitochondria, where the enzymes of β-oxidation degrade them to acetyl-CoA for energy production. When oxidation of one or more fatty acids is defective, specific acylcarnitines accumulate in body fluids and tissues (Foster, 2004). In the present study, the plasma acylcarnitines profile was similar in the two groups, suggesting that fatty acid β-oxidation was not impaired in our sample. Plasma isoprostanes were also not affected in our patients. Isoprostanes are considered the most specific markers of lipid peroxidation *in vivo*, and their association with several pathological conditions has been established (Janicka *et al.*, 2010). We were able to identify only one study in the literature evaluating isoprostanes in treated HCU patients. In that study, urinary 8-iso-PGF2α excretion was observed to be significantly higher in HCU patients than in age-matched healthy subjects (Davi *et al.*, 2001). Regarding lipid peroxidation in HCU patients, contradictory findings

have been reported, with some authors describing increased lipid peroxidation (Blom *et al.*, 1995; Davi *et al.*, 2001; Vanzin *et al.*, 2011; Vanzin *et al.*, 2015), while others describe no alterations (Dudman *et al.*, 1993; Poloni *et al.*, 2012).

We also investigated whether alterations in lipid metabolism and body composition could be associated with BMD. In transgenic HCU mice, cysteine-restoring treatment prevents osteoporosis. An association between cysteine plasma levels and BMD has been shown in previous studies with healthy individuals (Baines *et al.*, 2007; Elshorbagy, Gjesdal, *et al.*, 2009). Premature osteoporosis does not occur in other types of homocystinuria that are not accompanied by cysteine deficiency (Wilcken, 2006), suggesting a central role of this SAA in the development of osteoporosis. In our study, we found an association of femur BMD Z-scores with cysteine and tHcy levels. However, it should be noted that two patients were receiving treatment for osteoporosis, and five were taking calcium supplements. This might have been a confounding factor in our analysis. In controls, BMD was associated with lean mass, which is a well-known predictor of BMD in humans (Ho-Pham *et al.*, 2014).

Our study has some limitations. Firstly, the small sample size limited the statistical power of our analysis. Secondly, our patients presented a heterogeneous profile in terms of age, disease severity, treatment strategies and compliance, and SAA concentrations. Thirdly, the fact that SAA levels fluctuate widely in HCU patients in a short period of time must be considered; thus, the SAA values obtained here might not reflect long-term mean concentrations. Finally, many of our patients were on a low-methionine diet combined with an amino acid formula enriched with micronutrients and cystine. These distinct diets and nutrients intake on our sample might have also contributed to the differences found.

In conclusion, our results support previous findings from epidemiologic and animal studies showing that SAA play an important role in the regulation of lipid metabolism. Among SAA, cysteine has been shown to have the strongest, most consistent, and independent associations with lipid metabolic alterations. Our findings also support the role of leptin and SCD-1 as mediators of SAA effects on lipid homeostasis.

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6.4 CAPÍTULO 4: *"Classical Homocystinuria in Brazil: clinical and genetic findings"*

Título do artigo: Classical Homocystinuria in Brazil: clinical and genetic findings

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CLASSICAL HOMOCYSTINURIA IN BRAZIL: clinical and genetic findings

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ABSTRACT

INTRODUCTION: Classical homocystinuria (HCU) is a rare genetic disease caused by deficient activity of cystathionine β-synthase (CβS). Treatment aims at reducing homocysteine levels, which delays or prevents clinical manifestations, especially when started early in life. In Brazil, HCU is not included in the National Neonatal Screening Program, and no specific treatment is provided through the national Unified Health System. The objective of the present study was to establish a broad clinical and genetic characterization of HCU in Brazil.

METHODS: This concerns a cross-sectional, observational study with a convenience sampling strategy. Clinical data from 66 patients (57 families) from all 5 regions of Brazil (South, n=12; Southeast, n=35; Northeast, n=8; North, n=1; Midwest, n=1) were analyzed. *CBS* gene sequencing was performed on DNA from blood samples of 35 of 66 patients. RNA was collected from 6 patients for gene expression analysis by qRT-PCR, and three novel mutations were expressed in *E. coli* by site-directed mutagenesis.

RESULTS: Parental consanguinity was reported in 27 of 57 families. Complete pyridoxine responsiveness was observed in 17.5% of patients. The median age was 19 (5-45) years at the time of the study, 10 (1-39) years at diagnosis, and 5 (0-20) years at onset of first symptoms. Ocular manifestations were the most common earliest-detected symptoms (in 58% of cases), the main reason for diagnostic suspicion (58% of cases), and the most prevalent manifestation at diagnosis (69% of cases). Only 28% of treated patients had total homocysteine levels <60 µmol/L. Twenty-one different mutations were detected; those most prevalent were p.Ile278Thr (18.2%), p.Thr191Met (11.3%), r.[737_828del92, 828_931ins104] (11.3%), and p.Trp323Ter (11.3%). Eight novel mutations were found (c.329A>T, c.284T>C, c.2T>C, c.444delG, c.209+1delG, c.989_991delAGG, c.864_868delGAG and c.1223+5G>T). Enzyme activity in the *E. coli*-expressed mutations was 1.5% for c.329A>T, 17.5% for c.284T>C, and 206% for c.2T>C, and qRT-PCR analysis revealed reduced gene expression in all evaluated genotypes.

CONCLUSIONS: Most patients diagnosed with HCU in Brazil express a severe phenotype. However, we believe that milder forms of the disease are underdiagnosed. Many private mutations were observed, but the four most prevalent mutations together accounted for over 50% of mutated alleles. These findings should contribute to the development of protocols for diagnosis and management of HCU in Brazilian patients.

INTRODUCTION

Classical homocystinuria (HCU; OMIM 236200) is an inborn error of metabolism caused by deficient activity of the enzyme 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 severely 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 at 1:100,000 to 1:344,000 (1-3).

The natural history of this disease was first described in 1985 by Mudd et al., in an international multicenter study of more than 600 patients (4). Clinical signs are mainly related to ocular, vascular, central nervous and skeletal systems. Ocular anomalies are among the earliest and most prevalent manifestations, with ectopia lentis (luxation or subluxation of the lens) being one of the classical symptoms. Cognitive deficits, seizures, and psychiatric disorders are common neurological complications. Frequent skeletal manifestations include reduced bone mineral density and various Marfanoid features. Vascular disease, particularly thromboembolism, is also prevalent and associated with high morbidity and mortality (3-5).

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 (6, 7). Over 160 different mutations in the *CBS* gene have been reported, most of private origin (8). 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 (8). While the first of these mutations is panethnic, the other three follow rather well-demarcated geographic and ethnic distributions (9-13). The molecular bases of HCU in Brazil are poorly characterized; only one publication has addressed this topic. In a series of 14 patients (11 unrelated) described by Porto et al., the common mutations p.Ile278Thr and p.Thr191Met were detected at a frequency of 13.6% by screening techniques (RFLP and SSCP) (14).

Brazil is a rather large country with over 200 million inhabitants, and characterized by intense admixture. The country also has one of the world's largest publicly funded health

care systems, the Unified Health System (*Sistema Único de Saúde*, SUS), which was established to provide equitable, comprehensive care to all users. However, no specific treatment for HCU is available through the SUS, and only few centers across the country offer biochemical testing for diagnosis and management of this condition. Furthermore, the prevalence of HCU in Brazil is unknown, and the disease is not included in the National Neonatal Screening Program. These various factors make diagnosis and management of HCU within the Brazilian health system a major challenge, and many patients with the condition are believed to remain undiagnosed and untreated.

Within this context, the present study sought to establish a broad clinical and genetic characterization of HCU in Brazil, using a combination of *CBS* gene analysis and a survey on diagnosis and management of a representative patient population that is being followed at several centers nationwide.

METHODS

The present study was approved by was approved by the local research ethics committee (Hospital de Clínicas de Porto Alegre). Collection procedures for the study were conducted only after participants had agreed to take part in the investigation and provided written informed consent.

Patients

The study sample comprised 66 Brazilian patients with an established diagnosis of HCU, from 57 different families. Families from all five regions of Brazil were represented: South (n=12), Southeast (n=35), 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. Overall, 15 medical centers participated in the study.

Of the 66 patients included, 35 underwent all study procedures (clinical data collection + blood sampling for DNA analysis). Complete clinical data, but no DNA samples, were available for 6 patients. In 26 patients, only clinical data regarding diagnosis were

available. RNA samples were obtained from 6 patients. In 10 families, the genetic variant(s) found in the probands were also confirmed in at least one parent.

Except for one patient diagnosed through neonatal screening, all had received delayed diagnoses. Fifty-five 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 responsive if they achieved target homocysteine levels (<60 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. Treatment adherence was determined by the subjective impressions of the care team at each medical center.

CBS gene 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* gene were amplified by conventional PCR, using primers and reaction conditions previously described elsewhere (15). The following primers were designed to amplify exon 15: forward, CCACAGGAAGAGTTGGGAGG; reverse, TGAGAGCCATTCTGAGGGGT. 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) softwares. In addition, a group of 100 controls was 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 (íntron 7). Testing for c.2T>C was performed by the restriction fragment length polymorphism (RFLP) method with the *NIaIII* restriction

enzyme, whereas the other mutations were analyzed by sequencing of the mutation-containing exon.

qRT-PCR

For qRT-PCR analysis of gene expression, blood samples from 6 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*); (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 (16).

Expression of mutations in *E. coli*

The novel mutations c.284T>C, c.2T>C, and c.329A>T were expressed in *E. coli* using a protocol adapted after Mendes et al. (17), 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 16h 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. (18). All experiments were performed in triplicate, with the arithmetic mean of the resulting measurements considered for analysis.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows, Version 18.0 (Chicago: SPSS Inc). Continuous variables were expressed as median (range). The Mann–Whitney *U* test (continuous variables) or chi-square test (categorical variables) were used to assess between-group differences. P-values < 0.05 were deemed significant.

RESULTS

Of the 66 patients included, 59% were male. Two patients (1c and 8) were already deceased at the time of inclusion in the study. The patients belonged to 57 families; and parental consanguinity was reported in 27 families (57%). Median age at assessment was 19 years. The youngest patient was aged 5, and the oldest was 45. Regarding pyridoxine responsiveness, 52 patients (82.5%) were classified as nonresponsive and 11 (17.5%) as responsive. In three patients, pyridoxine responsiveness was not reported/determined (Table 1).

Diagnosis

Table 1 reports diagnostic information for the sample as a whole and stratified by pyridoxine responsiveness. The main clinical findings leading to investigation of HCU are represented in Figure 1. Eye problems accounted for 58% of referrals for HCU investigation, and were the most prevalent manifestations at diagnosis (present in 69% of cases). The most commonly reported first symptom was ectopia lentis (35% of cases), followed by unspecified visual abnormalities (23% of cases), developmental delay (20% of cases), and seizures (12% of cases). The median time elapsed between symptom onset and diagnosis was 5 years (maximum, 34 years). Overall, 40% of patients already had three or more organ systems affected at diagnosis.

Management

Current clinical and biochemical data were available for 39 patients, of whom 6 were responsive and 30 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 (66 vs. 97%, p=0.01). Ectopia lentis was the most common complication, affecting 89% of patients at the time of study inclusion.

Responsive patients had significantly lower total homocysteine and methionine levels at study inclusion ($p<0.05$). Only 28% of patients had total homocysteine levels <60 µmol/L. Treatment adherence was reported in 44% of patients (71% of those responsive and 41% of those nonresponsive).

Regarding treatment strategies, 89% of patients were on folic acid + pyridoxine supplementation, 76% on betaine, 32% on vitamin B12, and 26% on a low-methionine diet + metabolic formula. Considering the nonresponsive group alone, 87% were on betaine and 33% on low-methionine diet + metabolic formula.

Analysis of mutations

The genotypes of the 35 patients in whom *CBS* gene analysis was performed are shown in Table 3. In only one allele no mutation could be identified (patient 17; Table 3). The most prevalent mutations were: p.Ile278Thr (allele frequency 18.2%), p.Thr191Met (allele frequency 11.3%), r.[737_828del92, 828_931ins104] (allele frequency 11.3%), and p.Trp323Ter (allele frequency 11.3%). Eight novel mutations were detected: c.2T>C (exon

1), c.209+1delG (intron 1), c.284T>C (exon 2), c.329A>T (exon 3), c.444delG (exon 3), c.864_868delGAG (exon 8), c.989_991delAGG (exon 9), and c.1223+5G>T (intron 11). In addition, 100 control samples were tested for the c.284T>C, c.2T>C, c.329A>T, and c.828+1G>A mutations. No mutant alleles were detected, which provides further evidence of the pathogenic potential of the mutations tested.

The p.Ile95Thr and p.Glu110Val mutations were analyzed in the PolyPhen-2 and SIFT softwares 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.

Enzyme activity in the *E. coli*-expressed mutant proteins in relation to the WT control was 1.5% for c.329A>T, 17.5% for c.284T>C, and 206% for c.2T>C. Relative *CBS* mRNA levels measured by qRT-PCR in 6 patients are described in Figure 2. Reduced expression was observed in all, with the highest expression level detected in the sole pyridoxine-responsive patient (P4).

Table 1. Classical homocystinuria: a summary of clinical findings (n=66)

		Total (n=66)	***Pyridoxine-responsive***		
			Yes (n=11)	***No (n=52)***	***P***
Current age (years)		19 (5-45)	24 (15-35)	18 (5-45)	0.052
Age at first symptom onset (years)		5 (0-20)	3 (0.2-15)	5 (0.7-20)	0.359
Age at diagnosis (years)		10 (1-39)	11 (4-34)	10 (1-39)	0.566
Manifestations at diagnosis	Ocular (%)	69	50	71	0.190
	CNS (%)	61	70	62	0.641
	Skeletal (%)	52	40	56	0.360
	Vascular (%)	12	20	11	0.465

Data expressed as median (range) or percentage. In three patients, pyridoxine responsiveness could not be determined; data from these patients were used in whole-group analysis alone.

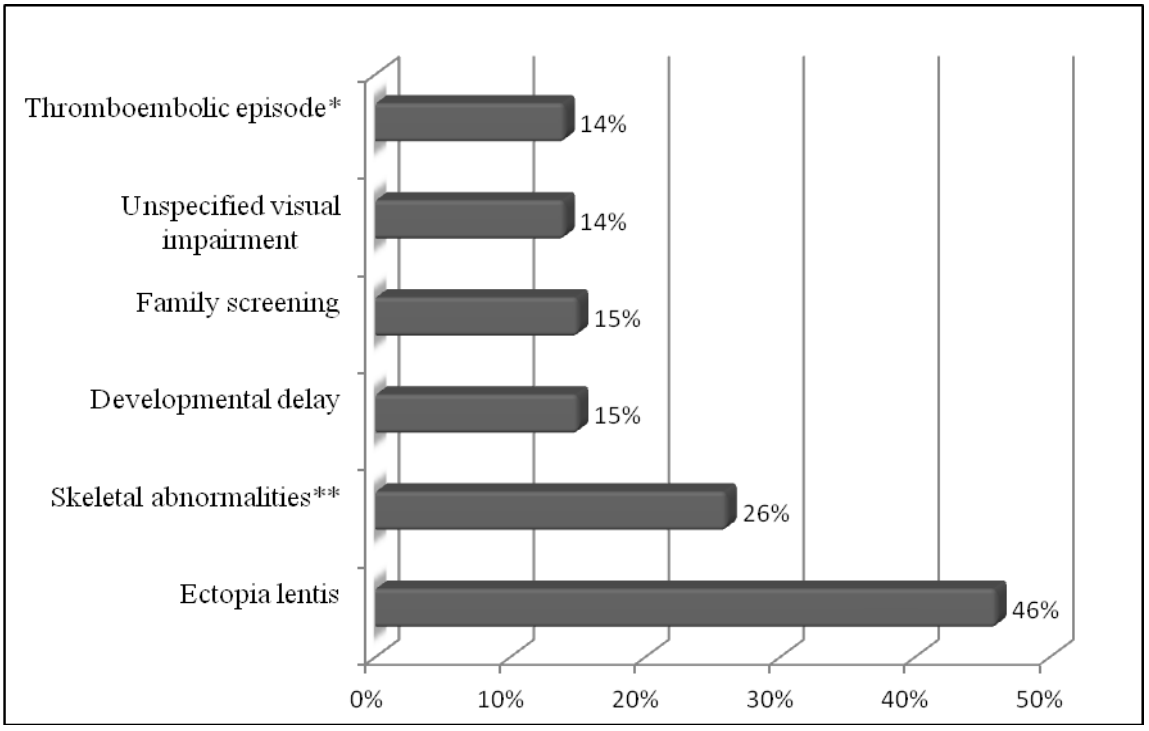

Figure 1. Reasons for clinical suspicion of Classical Homocystinuria in this study (n=66). *Including stroke. **Including marfanoid habitus.

Table 2. Clinical and biochemical profile of patients on treatment at the time of study inclusion.

		Total (n=39)	***Pyridoxine-responsive***		
			Yes (n=6)	***No (n=30)***	***p***
Current age (years)		17	23 (15-35)	18 (5-45)	0.083
Homocysteine (µmol/L)		139	25 (14-141)	168 (9-454)	0.005
Methionine (µmol/L)		117	29 (19-69)	144 (6-881)	0.025
Clinical manifestations	Ocular, n (%)	92	66	97	0.015
	CNS, n (%)	74	83	76	0.720
	Skeletal, n (%)	69	50	10	0.343
	Vascular, n (%)	24	17	27	0.606

Data expressed as median (range) or percentage. In three patients, pyridoxine responsiveness could not be determined; data from these patients were used in whole-group analysis alone. Reference ranges: homocysteine, 5-15 µmol/L; methionine, 5-30 µmol/L (5).

Table 3. Pathogenic mutations in patients who underwent *CBS* gene analysis (n=35).

	Allele 1		Allele 2			Responsiveness to	Symptom onset
Patient	**cDNA**	**Protein**	**cDNA**	**Protein**	**Consang.**	**B6**	**(years)**
1a	c.253G>A*	p.Gly85Arg	c.253G>A	p.Gly85Arg	Yes	No	6
1b	c.253G>A*	p.Gly85Arg	c.253G>A	p.Gly85Arg	Yes	No	NA
1c	c.253G>A*	p.Gly85Arg	c.253G>A	p.Gly85Arg	Yes	No	7
2	c.828+1G>A*	p.?	c.1126G>A	p.Asp376Asn	No	No	5
3	**c.284T>C***	**p.Ile95Thr**	**c.284T>C**	**p.Ile95Thr**	Yes	No	1
4	c.1058C>T*	p.Thr353Met	c.146C>T	p.Pro49Leu	No	Yes	3
5	c.833T>C	p.Ile278Thr	**c.989_991delAGG**	**p.(Glu330del)**	No	No	20
6	c.691G>C	p.Ala231Leu	c.828+1G>A	p.?	No	No	1
7	**c.209+1delG***	**p.?**	c.572C>T	p.Thr191Met	No	No	8
8	c.1126G>A*	p.Asp376Asn	c.1126G>A†	p.Asp376Asn	Yes	No	1.5
9	c.833T>C	p.Ile278Thr	c.833T>C	p.Ile278Thr	Yes	Yes	0.2
10	c.833T>C	p.Ile278Thr	c.833T>C	p.Ile278Thr	No	Yes	7
11	**c.444delG***	**p.(Asn149*fs*)**	**c.444delG**	**p.(Asn149*fs*)**	Yes	No	1.5
12	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Yes	No	5
13a	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Yes	No	7.5
13b	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Yes	No	NA
14	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Yes	No	19
15	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Yes	No	5
16	c.828+1G>A†	p.?	**c.2T>C***	**p.?**	No	No	1
17	c.209+1G>A	p.?	NI	NI	No	No	6
18	c.833T>C	p.Ile278Thr	c.28delG	p.Val10*fs*	No	Yes	1
19	c.451G>A	p.Gly151Arg	c.833T>C	p.Ile278Thr	No	Yes	4
20	c.833T>C*	p.Ile278Thr	c.833T>C†	p.Ile278Thr	Yes	Yes	15
21a	c.969G>A*	p.Trp323Ter	c.969G>A†	p.Trp323Ter	Yes	No	6
21b	c.969G>A*	p.Trp323Ter	c.969G>A†	p.Trp323Ter	Yes	No	1
22	c.572C>T	p.Thr191Met	c.572C>T	p.Thr191Met	Yes	No	4
23	c.451G>A	p.Gly151Arg	c.451G>A	p.Gly151Arg	Yes	No	3
24	c.969G>A	p.Trp323Ter	c.969G>A	p.Trp323Ter	No	No	1
25	**c.329A>T**	**p.Glu110Val**	c.770C>T	p.Thr257Met	No	No	3
26	c.828+1G>A	p.?	c.833T>C	p.Ile278Thr	No	No	4
27	c.828+1G>A	p.?	c.828+1G>A	p.?	Yes	No	1
28a	**c.1223+5G>T**	**p.?**	**c.1223+5G>T**	**p.?**	Yes	NA	3
28b	**c.1223+5G>T**	**p.?**	**c.1223+5G>T**	**p.?**	Yes	NA	3
29	c.969G>A	p.Trp323Ter	c.969G>A	p.Trp323Ter	No	No	7
30	**c.864_868delGAG**	**p.(Glu289del)**	**c.864_868delGAG**	**p.(Glu289del)**	Yes	No	6

Novel mutations are set in bold. Patients represented by the same number belong to the same family. Consang: consanguinity; B6: pyridoxine. NA: not available; NI: not identified. (*) mother heterozygous for mutation; (†): father heterozygous for mutation.

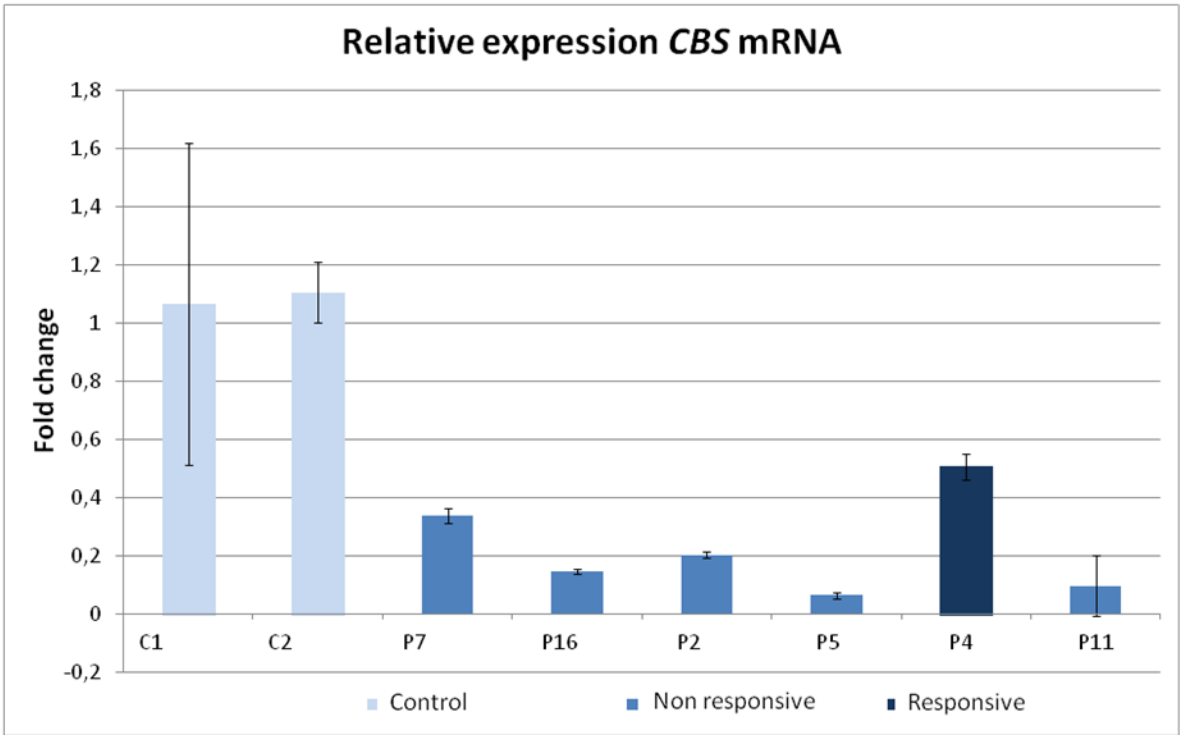

Figure 2. *CBS* mRNA expression as determined by qRT-PCR. Genotypes: P7 [c.209+1delG; c.572C>T]; P16 [c.2T>C; c.828+1G>A]; P2 [c.828+1G>A; c.1126G>A]; P5 [c.833T>C; c.989_991delAGG]; P4 [c.1058C>T; c.146C>T]; P11 [c.444delG; c.444delG]

DISCUSSION

The present report provides a genetic and clinical analysis of the largest series of HCU cases ever studied in Brazil to date. Clinical data from 66 patients (57 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 have higher rates of access to health care services and procedures as compared with the other regions of Brazil (19). The fact that patients from other regions, particularly the North (n=1) and Midwest (n=1), were underrepresented suggests that high numbers of HCU patients remain underdiagnosed likely due to limited access to health care in these regions.

A high proportion of pyridoxine-nonresponsive HCU was observed (82.5%) in the study population. Nonresponsiveness to pyridoxine is associated with a more severe phenotype and challenging management (1, 3, 4). In our study, this proportion exceeded rates described worldwide (approximately 50%) (3). 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 (12, 13). No such association was observed in our study. This suggests underdiagnosis of pyridoxine-responsive patients, who express attenuated phenotypes with fewer symptoms and later onset of clinical presentation (4). In our sample, there was no difference in age at symptom onset or age at diagnosis between responsive and nonresponsive patients. However, we believe our analysis was underpowered due to the small number of responsive patients (n=10).

The high prevalence of ocular manifestations at diagnosis and their predominant contribution to diagnostic suspicion reinforce the importance of these findings 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 (4). Although the difference was not significant, the prevalence of ocular manifestations at diagnosis was higher in pyridoxine-nonresponsive patients (71% vs. 50%). This is consistent with previous reports (4).

Skeletal and neurologic manifestations were also highly prevalent at diagnosis, affecting more than half of patients' population. The lower prevalence of vascular events at

diagnosis is consistent with the natural history of HCU, in which such manifestations usually occur late in the disease course (4). However, vascular disease may have been underrepresented due to the high lethality of thromboembolic events. The long time from symptom onset to diagnosis (median, 5 years) and the presence of multiple clinical manifestations attest to the difficulty of establishing a definitive diagnosis in these patients.

The main strategies for HCU management include supplementation with pyridoxine (the cofactor of CβS), folic or folinic acid, and betaine (trimethylglycine), as well as a low-methionine diet supplemented with a specific metabolic formula (20). Early treatment is associated with good metabolic control can prevent all clinical manifestations of HCU (4, 21-25). Patients who respond to pyridoxine supplementation usually express milder phenotypes and present a better prognosis.

In the present study, current clinical and biochemical data were obtained from 39 treated patients. Again, a high prevalence of ocular manifestations was observed, affecting nearly all of the pyridoxine-nonresponsive group (66% of responsive vs. 97% of nonresponsive patients, p=0.03). Skeletal and neurologic manifestations were also highly prevalent. There was also a clear difference in metabolic control between the groups: responsive patients had near-normal homocysteine and methionine levels, whereas nonresponsive patients had persistently high homocysteine levels (>100 μmol/L) despite multiple treatment strategies. This difficulty achieving metabolic control in pyridoxine-nonresponsive patients has been reported in other studies (21, 25, 26).

The high rate of betaine supplementation and comparatively low use of methionine-restricted diet in these nonresponsive patients may be attributed to several factors, including: 1) difficulty obtaining the metabolic formula, which is expensive and not provided by the Unified Health System; 2) low adherence to dietary treatment, 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—i.e., twice as many as in the present study (27). Whereas dietary therapy can effectively reduce methionine and homocysteine, betaine supplementation reduces homocysteine but increases methionine levels (28, 29). In animal models, betaine is less effective at preventing clinical manifestations (30), and its efficacy declines over time (31). In humans, there are no studies of the long-term efficacy and safety of betaine supplementation in HCU.

Genetic analysis

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 (32). European ancestry has the higher contribution to the genetic background of Brazilians (0.62), followed by African (0.21) and Amerindian (0.17) (33). 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 (33, 34).

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 (8). It is particularly frequent in Central and Northern Europe (9, 35-38). In the Brazilian study conducted by Porto et al., this mutation was detected in 6 of 28 alleles (frequency in unrelated alleles, 13.6%) (14). The p.Ile278Thr mutation is associated with mild phenotypes and pyridoxine responsiveness, in both homozygous and compound heterozygous (3, 8). In the present study, the majority of patients harboring this mutation were pyridoxine-responsive, and all were from the South or Southeast regions of Brazil.

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%) (14), but lower than those reported in other Latin American countries (75% in Colombia, 25% in Venezuela, 20% in Argentina) and in the Iberian Peninsula (52% in Spain and 33% in Portugal) (10, 39, 40). 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 (32, 41-43). Wide phenotypic variability has been observed for this mutation, with mild to severe phenotypes and pyridoxine responsiveness ranging from partial to absent (8, 11).

The r.[737_828del92, 828_931ins104] (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 (44). This 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 (44). 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. 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 four geographically distant states in three different 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 (26). 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 in three different software programmes estimated a damaging functional effect for the mutations c.329A>T and c.284T>C. Furthermore, no allele containing these mutations was detected in 100 controls. *E. coli* expression assays demonstrated reduced enzyme activities consistent with HCU for both mutations (<20% activity relative to controls) (8, 45). The residual enzyme activity also correlated with the clinical phenotype.

The c.2T>C mutation was not detected in the 100 tested controls, but expression assays revealed even a two-fold activity when compared with the WT control. This mutation alters the start codon, being the first of its kind ever described in a patient with HCU (8). 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 (46). One hypotheses for the result obtained in the expression assay is that the vector machinery may have been used to initiate translation, as the His-tag portion of the vector harbors an ATG codon. Furthermore, high or normal enzyme activity has also been described in other pathogenic mutations in the *CBS* gene (8, 47).

Finally, qRT-PCR analysis of samples from 4 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 or RNA instability 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 observed clinical phenotype.

In conclusion, this study provides the most wide-ranging clinical and genetic characterization of HCU patients in Brazil to date. Most patients described in this study express a severe phenotype, associated with nonresponsiveness to pyridoxine, early and multisystem manifestations, and poor metabolic control. However, we believe responsive patients were underrepresented in this sample as a result of underdiagnosis of the milder forms of HCU. Many private mutations were observed, but the four most prevalent mutations together accounted for over 50% of mutated alleles. These findings will contribute to the development of protocols and guidelines for better diagnosis and management of HCU in Brazil.

Acknowledgments

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7. DISCUSSÃO

Apesar das graves manifestações clínicas da HCU, a doença dispõe de tratamento efetivo, especialmente quando iniciado precocemente (Mudd *et al.*, 1985; Mudd *et al.*, 2001; Yap, Boers, *et al.*, 2001; Yap, Rushe, *et al.*, 2001; Yap, 2003). No Brasil, não há dados epidemiológicos sobre a HCU. Utilizando a prevalência mundial estimada de 1:344,000 indivíduos, esperaria-se mais de 500 pacientes portadores da doença no país. Este número está muito aquém da amostra obtida no estudo (n=66) 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 entre o início dos sintomas e o diagnóstico 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 o baixo uso de dieta pobre em metionina/fórmula metabólica revelaram deficiências também 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 a baixa compreensão 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; Mudd *et al.*, 2001). Dificuldade de adquirir os medicamentos, problemas relacionados à manipulação dos mesmos, e recusa por parte do paciente a seguir ao tratamento foram situações relatadas por diversos centros.

O perfil genético do grupo de pacientes estudado revelou grande heterogeneidade alélica, com grande número de mutações privadas, o que está de acordo com o descrito na literatura (Kraus, 2016). No entanto, as 4 mutações mais prevalentes correspondem a mais da metade dos alelos não relacionados analisados. Este dado contribuirá para a

investigação molecular de novos pacientes brasileiros diagnosticados com HCU, uma vez que protocolos de triagem por mutações comuns poderão ser criados, poupando tempo e recursos. Sugere-se que os éxons do gene *CBS* sejam analisados na seguinte ordem: primeiramente éxons 8, 5, 7 e 9 (portadores das mutações frequentes em pacientes brasileiros); e após, éxons: 3, 12, 10, 11, 4, 6, 1, 2, 16, 13, 14 e 15 (em ordem decrescente: éxons com maior número de mutações patogênicas em nível mundial) (Kraus, 2016).

Para a maioria das 8 novas mutações detectadas a patogenicidade pode ser inferida com razoável grau de confiança. Resultados conflitantes foram obtidos para a mutação c.2T>C. Única mutação em códon de iniciação de translação descrita na HCU até o momento, os achados obtidos no estudo de expressão não revelaram efeito negativo da atividade enzimática. O paciente portador desta mutação é heterozigoto [c.828+1G>A; c.2T>C], e possui fenótipo clínico e bioquímico compatível com a forma grave de HCU. A análise de DNA dos pais confirmou a herança parental das mutações.

O presente estudo é inédito na avaliação da associação entre composição corporal, atividade da SCD-1, leptina e lipoproteínas em pacientes com HCU. Os resultados obtidos corroboram aqueles descritos no modelo animal de HCU (Gupta e Kruger, 2011). Os achados relativos à composição corporal e metabolismo lipídico são de interesse não apenas para o entendimento da fisiopatologia da HCU, mas também para o estudo da obesidade e síndrome metabólica, uma vez que associações semelhantes têm sido observadas em indivíduos saudáveis (Elshorbagy *et al.*, 2008; Elshorbagy, Kozich, *et al.*, 2012; Vinknes *et al.*, 2013).

Os estudos descritos no capítulo 1 e 3 foram realizados em dois momentos diferentes e com técnicas distintas para aferição da composição corporal (BIA e DXA). A amostra de de pacientes foi semelhante (8/9 participantes do estudo de BIA participaram também do segundo estudo). Já o grupo controle foi composto por indivíduos diferentes entre os trabalhos. A técnica de BIA é menos precisa na aferição de composição corporal (método duplamente indireto), e acredita-se se que os resultados obtidos na análise por DXA sejam mais acurados. Além disso, o grupo controle do primeiro estudo realizou apenas avaliação por BIA, não sendo possível a comparação dos dados laboratoriais entre pacientes e controles. Em ambas as análises não foram detectadas diferenças na adiposidade entre pacientes e controles. Redução de massa adiposa é um dos achados mais marcantes nos modelos animais não tratados (Gupta e Kruger, 2011; Teng *et al.*, 2012;

Elshorbagy, 2014). Além da possível interferência do tratamento nos pacientes avaliados em nosso estudo, deve-se considerar que a composição corporal é influenciada por diversos fatores ambientais e genéticos. Diferentemente dos estudos experimentais, a maioria destes fatores não poderá ser controlada em estudos com pacientes. Estudos longitudinais, avaliando níveis plasmáticos de SAA ao longo do tempo e sua relação com mudanças na composição corporal poderão aprimorar o entendimento desta associação em humanos.

Em ambos os estudos realizados verificou-se que poucos pacientes apresentavam bom controle metabólico. Os níveis de cisteína foram significativamente mais baixos nos pacientes. No modelo animal, o tratamento recuperou os níveis de cisteína e revertou todas as alterações metabólicas, incluindo a supressão da SCD-1 hepática (Gupta *et al.*, 2014). Para melhor compreensão do(s) efeito(s) dos SAA no metabolismo lipídico na HCU, a análise comparativa de pacientes com bom e mau controle metabólico poderia ser realizada. Devido ao pequeno tamanho amostral, esta análise não foi possível em nossos estudos.

Entre os SAA, a cisteína possui as associações mais consistentes e independentes com as alterações no metabolismo lipídico relatadas. No entanto, ainda não são conhecidos os mecanismos bioquímicos pelo qual a cisteína influencia a atividade/expressão da SCD-1 e/ou seus reguladores. Estudos *in vitro* e em modelos animais são necessários para esta investigação. O efeito adjuvante ou independente da colina e/ou betaína, situadas acima da rota da homocisteína, também deve ser esclarecido. Há evidência de desequilíbrio destes metabólitos na HCU (Imbard *et al.*, 2015). Distúrbios no metabolismo da colina em modelos animais também resultam em fenótipo anti-obesogênico semelhante ao modelo animal de HCU (Teng *et al.*, 2012; Zeisel, 2013; Elshorbagy, 2014).

A relação entre deficiência de cisteína, composição corporal e densidade mineral óssea também merece maior investigação. A cisteína parece ter papel central na patogênese da osteoporose na HCU e associação positiva entre cisteína e massa óssea é relatada em estudos epidemiológicos (Baines *et al.*, 2007; Elshorbagy, Gjesdal, *et al.*, 2009). Não é claro se a cisteína possui ação direta no desenvolvimento de osteoporose ou se é mediada pela redução de adiposidade, como sugerem alguns trabalhos (Elshorbagy, Gjesdal, *et al.*, 2009; Gupta *et al.*, 2014). Neste caso, a baixa adiposidade adquire maior importância na HCU, como mecanismo fisiopatológico na gênese da osteoporose.

8. CONCLUSÕES

As conclusões serão apresentadas de acordo com cada objetivo específico do trabalho.

Objetivo específico 1. Realizar sequenciamento de toda a região codificante do gene *CBS* em uma amostra representativa de pacientes brasileiros com HCU.

O gene *CBS* foi sequenciado para 35 pacientes com HCU (30 não relacionados) provenientes de 9 estados diferentes do Brasil (RS, SP, MG, BA, SC, RJ, DF, MA, ES). Um grande número de mutações privadas foi encontrada; sendo 8 nunca descritas na literatura. Juntas, as quatro mais frequentes correspodem a mais da metade dos alelos estudados. Protocolos de triagem com estas mutações poderão ser elaborados para investigação molecular de novos pacientes brasileiros diagnosticos com HCU. Análises *in silico*, expressão de mRNA por qRT-PCR e mutagênese sítio-dirigida estimaram com grau satisfatório de confiança a patogenicidade maioria das mutações novas encontradas. O efeito da mutação c.2T>C ainda não pode ser determinado. O perfil genético da amostra parece refletir a variada ancestralidade e miscigenação dos brasileiros, diferindo do encontrado em outros países sul-americanos.

Objetivo específico 2. Caracterizar o perfil clínico da HCU no Brasil.

Dados clínicos de 66 pacientes (57 não relacionados) em acompanhamento em 15 diferentes centros de genética do Brasil foram analisados. Observou-se maior prevalência de pacientes não responsivos à piridoxina e apresentação clínica multissistêmica ao diagnóstico, o que sugere subdiagnóstico das formas atenuadas da doença. Observou-se também atraso importante entre o início dos sintomas e diagnóstico definitivo. Assim como descrito na literatura, as manifestações oculares foram as mais precoces e prevalentes na amostra, afetando a grande maioria dos pacientes. Em relação ao manejo, observou-se baixo uso de dieta restrita em metionina e elevado de betaína entre os pacientes não responsivos, indicando dificuldades no acesso/prescrição/adesão à dieta. O controle metabólico foi deficiente no grupo de pacientes não responsivo à piridoxina.

Objetivo específico 3. Descrever a composição corporal de pacientes com HCU em tratamento.

A composição corporal foi estimada por dois métodos diferentes: BIA e DXA. A análise por BIA revelou que 4/9 pacientes estudados apresentou baixo percentual de gordura, porém não houve diferença quando comparado ao grupo controle. A avaliação por DXA também não demonstrou diferença no percentual de gordura entre os grupos; mas a massa magra foi significativamente menor nos pacientes. Isto pode ter ocorrido por diferenças no consumo de proteína natural entre os grupos, uma vez que a maioria dos pacientes avaliados era orientado a controlar a ingestão proteica como parte do tratamento da HCU. A falta de diferença na adiposidade entre pacientes e controles pode ser explicada por diversos fatores, entre eles: não haver alteração na composição corporal em pacientes com HCU (diferentemente do observado no modelo animal); efeito do consumo alimentar distinto entre pacientes e controles; efeito do tratamento nos pacientes; viés de seleção dos controles que possuíam IMC semelhante aos pacientes; entre outros.

Objetivo específico 4. Caracterizar e comparar marcadores de metabolismo lipídico (níveis de lipoproteínas, isoprostanos, acilcarnitinas, leptina, adiponectina) entre pacientes com HCU em tratamento e controles saudáveis.

Níveis reduzidos de leptina e colesterol LDL foram observados em pacientes na análise univariada e multivariada, exceto quando ajustados para as concentrações plasmáticas de cisteína, o que sugere um papel deste aminoácido nas diferenças observadas. Os demais marcadores não apresentaram diferença significativa entre os grupos. Os achados estão de acordo com o descrito em modelo animal que mostra que a deficiência de cisteína leva à redução de leptina, que por sua vez, é um dos principais reguladores da SCD-1 hepática. A redução das concentrações de lipoproteínas é bem descrita em modelos animais e pacientes com HCU, e pode estar relacionada ao prejuízo em sua síntese quando a SCD-1 hepática está suprimida.

Objetivo específico 5. Determinar resistência insulínica, através do índice HOMA-IR, em pacientes com HCU em tratamento.

Não houve diferença no índice HOMA-IR entre pacientes e controles; bem como associação com composição corporal e outros marcadores bioquímicos avaliados. Embora não estudada no modelo animal de HCU, outros modelos com metabolismo alterado de SAA mostraram melhor sensibilidade à insulina nos animais não tratados. Em nosso estudo, não pode ser excluído que tratamento tenha anulado possíveis diferenças entre os grupos. Novos estudos são necessários para avaliar esta hipótese.

Objetivo específico 6. Verificar a associação entre os SAA (metionina, homocisteína e cisteína), colina e betaína com a composição corporal e marcadores de metabolismo lipídico em pacientes e controles.

Verificou-se associação de lipoproteínas com colina e leptina; cisteína com IMC, percentual de gordura corporal e SCD-16; metionina e cisteína com leptina; e glutationa com percentual de gordura corporal. Os achados reforçam a hipótese de que o metabolismo alterado dos SAA influencia a composição corporal, níveis de lipoproteínas e de leptina na HCU. No entanto, por se tratar de um estudo transversal, relações de causalidade não podem ser determinadas.

Objetivo específico 7. Estimar a atividade da SCD-1 hepática através dos índices SCD-16 e SCD-18; e analisar a associação dos índices com composição corporal, níveis de lipoproteínas, leptina e adiponectina e concentrações de SAA em pacientes e controles.

Observou-se índice SCD-16 significativamente mais baixo nos pacientes, e associação positiva com os níveis plasmáticos de cisteína. Não houve associação com outras variáveis. Estes achados indicam atividade enzimática reduzida da SCD-1 hepática nos pacientes, apesar do tratamento. A associação positiva com a cisteína plasmática corrobora estudos experimentais e populacionais que sugerem que a cisteína regule, por mecanismos ainda não elucidados, a expressão da SCD-1 hepática. O índice SCD-18 sofre maior influência da ingestão dietética de ácidos graxos, o que pode explicar a ausência de diferença entre os grupos.

9. PERSPECTIVAS

A partir dos resultados obtidos no estudo, novos trabalhos deverão ser conduzidos para aprofundar e/ou elucidar os achados. Para entender a alta frequência da mutação c.828+1G>A em nossa amostra, pretende-se conduzir a investigação genética dos pais e/ou outros familiares dos portadores da mutação a fim de determinar se a mutação foi herdada se trata-se de mutação "de novo". Novos estudos *in vitro* serão realizados para elucidar o efeito da mutação c.2T>C sobre a síntese/função da CβS. No momento, um construto mutante para a c.2T>C e também com o códon ATG do vetor mutado está sendo testado na Universidade de Freiburg. Além disso, fibroblastos dos pais e do paciente foram coletados para mensuração da atividade da CβS.

Pretende-se também ampliar a análise clínica e genética para outros tipos de homocistinúria. Um dos projetos já em fase de aprovação é da caracterização clínica e molecular de pacientes brasileiros com homocistinúria por deficiência de MTHFR. O Serviço de Genética Médica do HCPA já integra também o E-HOD (Registro Europeu de Homocistinúrias e Defeitos de Metilação); sendo o primeiro centro latino-americano a participar do registro e o centro coordenador no Brasil. O E-HOD foi criado em 2013, sob coordenação do Prof. Henk Blom, sendo um registro multicêntrico que busca construir uma ampla base de dados epidemiológicos sobre homocistinúrias e defeitos de metilação no mundo, que permita ampliar o conhecimento sobre a história natural destas doenças. Um dos objetivos do registro é o desenvolvimento de consensos e diretrizes baseados nos dados clínicos coletados e nas evidências disponíveis para melhoria do diagnóstico e manejo deste grupo de doenças.

Outro trabalho em desenvolvimento pelo grupo é o estudo *in vitro* do efeito da creatina em fibroblastos de pacientes com diferentes tipo de homocistinúria. Uma vez que a síntese endógena de creatina é responsável pela formação de uma parcela considerável de homocisteína, a suplementação de creatina poderia poupar a síntese endógena, resultando na redução da síntese de homocisteína. Se comprovada sua eficácia, a creatina poderia ser um adjuvante no tratamento de pacientes com homocistinúria.

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APÊNDICE

Cartas de aprovação dos projetos no Comitê de Ética em Pesquisa do 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: 130063
Data da Versão do Projeto:

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
POLI MARA SPRITZER
SANDRA LEISTNER SEGAL
SORAIA POLONI

Título: INVESTIGAÇÃO DE ALTERAÇÕES NA COMPOSIÇÃO CORPORAL E NO METABOLISMO LIPÍDICO EM PACIENTES 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, 12 de julho de 2013.

Prof. Jeso Roberto Goldim
Coordenação 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: 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