Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós-Graduação em Ciências Biológicas:Bioquímica

Perfil bioquímico e inflamatório em pacientes com homocistinúria e genotoxicidade *in vitro* da homocisteína: Uma possível relação com o estresse oxidativo

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À Deus, por nunca me deixar desistir dos meus sonhos, e nunca me desamparar.

“Menor que meu sonho não posso ser”

Lindolf Bell

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RESUMO

A homocistinúria é um erro inato do metabolismo dos aminoácidos causado principalmente pela deficiência na atividade da enzima cistationina-β-sintase (CBS), resultando em acúmulo de homocisteína (Hcy) e metionina nos fluidos biológicos. As manifestações clínicas incluem retardo mental, *ectopia lentis*, episódios tromboembólicos e osteoporose. Estudos realizados em modelos animais demonstram um possível papel do estresse oxidativo na fisiopatologia da homocistinúria. Mais recentemente, foram demonstradas alterações em alguns parâmetros de estresse oxidativo em pacientes homocistinúricos. Nesse trabalho, essas investigações foram ampliadas, no intuito de melhor entender os mecanismos fisiopatológicos e a terapêutica da homocistinúria por deficiência de CBS. Dessa forma, o objetivo principal desse trabalho foi avaliar os perfis lipídico, inflamatório e oxidativo, bem como a atividade das enzimas paraoxonase (PON1) e butirilcolinesterase (BuChE) em pacientes homocistinúricos tratados (dieta hipoproteica suplementada com vitamina B_6, ácido fólico, betaína e vitamina B_{12}) e não tratados, em comparação com indivíduos saudáveis, e adicionalmente avaliar a indução *in vitro* de mutagenicidade causada pela Hcy, através do teste de micronúcleos em leucócitos, e ainda avaliar o efeito *in vitro* do antioxidante *N*-acetil-L-cisteína na redução do dano ao DNA causado pelas altas concentrações de Hcy. No que se refere ao perfil sérico, foi verificada uma redução nos níveis de colesterol HDL, apolipoproteína A, bem como na atividade da enzima (PON1) em pacientes homocistinúricos tratados e não tratados. A atividade da enzima BuChE e os níveis de interleucina 6 (IL-6) estavam aumentados apenas nos pacientes não tratados. Correlações significativas positivas foram encontradas nos dois grupos de pacientes estudados entre a atividade da PON1 e o conteúdo de sulfidrilas; entre a atividade da PON1 e os níveis de vitamina B_{12}; entre os níveis de HDL e apolipoproteína A1; entre os níveis de apolipoproteína A1 e vitamina B_{12}; e entre os níveis de IL-6 e os níveis de carbonilas. Correlação significativa negativa foi encontrada entre os níveis de ácido fólico e de homocisteína total (tHcy). Em um segundo momento foram avaliados parâmetros de estresse oxidativo e níveis de nitritos na urina dos pacientes. Foram demonstrados níveis aumentados de 15-F2t-isoprostanos, di-tirosina e nitritos na urina de pacientes homocistinúricos tratados. Os níveis de 15-F2t-isoprostanos apresentaram uma correlação significativa positiva com os níveis de tHcy e os níveis de di-tirosina apresentaram correlação significativa negativa com os níveis de sulfidrilas. A atividade da enzima catalase encontrou-se aumentada no sangue de pacientes homocistinúricos tratados. Finalmente, demonstrou-se em estudo *in vitro* que a Hcy causa um aumento no dano cromossômico, avaliado pelo método de micronúcleos. Além disso, foi observado um efeito *in vitro* do antioxidante *N*-acetil-L-cisteína na redução do dano ao DNA causado pelas altas concentrações de Hcy. Avaliados em conjunto, nossos resultados indicam que o estresse oxidativo, acompanhado por alterações nos níveis urinários de nitritos, e por alterações nos perfis inflamatório e lipídico são fatores que possivelmente contribuam para o dano vascular encontrado na homocistinúria. Esses eventos parecem estar interconectados, e parecem ser decorrentes dos altos níveis de tHcy encontrados no sangue dos pacientes. Novas abordagens terapêuticas, além das atualmente utilizadas que incluem ácido fólico e vitamina B_{12}, como o uso de antioxidantes, poderiam ser alternativas para atingir melhores resultados no tratamento de pacientes homocistinúricos.

ABSTRACT

Homocystinuria is an inborn error of metabolism of amino acids primarily caused by deficiency in the activity of cystathionine-β-synthase (CBS), resulting in accumulation of homocysteine (Hcy) and methionine in biological fluids. Clinical manifestations include mental retardation, *ectopia lentis*, thromboembolic events and osteoporosis. Studies in animal models show a possible role of oxidative stress in the pathophysiology of homocystinuria. More recently, changes in some parameters of oxidative stress have been demonstrated in homocistinuric patients. In this work, we expanded these investigations, in order to understand the mechanisms that lead to the development of clinical manifestations of disease and the therapeutic for homocystinuria due CBS deficiency. Thus, the main objective of this study was to evaluate the lipid, inflammatory and oxidative profiles as well as the activity of paraoxonase (PON1) and butyrylcholinesterase (BuChE) in treated homocistinuric patients (hypoproteic diet supplemented with vitamin B_6, folic acid, betaine and vitamin B_{12}) and untreated patients compared with healthy individuals, and to evaluate the *in vitro* induction of mutagenicity caused by Hcy, through the micronucleus test in leukocytes, and further, to evaluate the *in vitro* effect of N-acetyl-L-cysteine in reducing DNA damage caused by high concentrations of Hcy. With regard to the serum profile, was observed a reduction in HDL cholesterol, apolipoprotein A, and in enzyme activity (PON1) in both group of CBS-deficient patients. The activity of butyrylcholinesterase and levels of interleukin 6 (IL-6) were increased only in untreated patients. Positive significant correlations were found in both patients groups between PON1 activity and the content of sulfhydryl; between PON1 activity and vitamin B_{12} levels; between the levels of HDL and apolipoprotein A1; between the levels of apolipoprotein A1 and B_{12}; and between IL-6 levels and levels of carbonyls. Negative significant correlation was found between folic acid levels and total homocysteine (tHcy). In a second moment were evaluated parameters of oxidative stress and the nitrite levels in the urine of patients. Increased levels of 15-F2t-isoprostanes, di-tyrosine and nitrite were demonstrated in the urine of treated homocistinuric patients. The 15-F2t-isoprostanes levels showed a significant positive correlation with tHcy levels; and di-tyrosine levels showed a significant negative correlation with the sulfhydryl levels. The catalase activity was found increased in the blood of treated homocistinuric patients. Finally, it was shown the *in vitro* effect of Hcy on increase of chromosomal damage assessed by micronuclei method. Furthermore, it was observed the *in vitro* effect of N-acetyl-L-cysteine antioxidant in reducing DNA damage caused by high concentrations of Hcy. Evaluated together, our results indicate that oxidative stress accompanied by changes in urinary nitrite levels and changes in inflammatory and lipid profiles are factors possibly contributing to vascular damage found in homocystinuria. These events appear to be interconnected, and appear to be due to the high tHcy levels found in the blood of patients. New therapeutic approaches in addition to the currently used which include folic acid and vitamin B_{12}, as the use of antioxidants, could be alternatives to achieve best results in the treatment of homocistinuric patients. Furthermore, an early diagnosis and, consequently, an early treatment, could avoid that the patients remain for a long time subjected to exposure of high concentrations of tHcy.

LISTA DE ABREVIATURAS

AdoMet - S-adenosilmetionina

BH4 - tetrahidrobiopterina

BuChE - butirilcolinesterase

CBS - cistationina-β-sintase

DNA – ácido desoxirribonucleico

EIM - erros inatos do metabolismo

eNOS - óxido nítrico sintase endotelial

ERO – espécies reativas de oxigênio

Hcy - homocisteína

HDL – lipoproteína de alta densidade

IL - interleucina

LDL – lipoproteína de baixa densidade

MAT - metionina-adenosiltransferase

MDA - malondialdeído

Met - metionina

MTHFR - 5,10-metilenotetra-hidrofolato redutase

NAC - *N*-acetil-L-cisteína

NO - óxido nítrico

PON1 - paraoxonase-1

RNA - ácido ribonucléico

SNC – sistema nervoso central

TAS – status antioxidante total

tHcy - Hcy total

X-ALD – Adrenoleucodistrofia ligada ao X

1. INTRODUÇÃO

1.1 Erros Inatos do Metabolismo

Archibald Garrod fez a primeira menção ao termo erros inatos do metabolismo (EIM) em 1908, ao descrever um grupo de doenças – alcaptonúria, pentosúria benigna e albinismo – aparentemente causadas por defeitos pontuais no metabolismo de aminoácidos e monossacarídeos. Garrod observou que essas condições duravam a vida toda, não eram significativamente alteradas por nenhum tratamento, e eram transmitidas através de um padrão de herança recessivo. Hoje, o número de doenças que são atribuídas a defeitos pontuais no metabolismo excede 500 (Scriver et al., 2001). Apesar de individualmente raros, coletivamente os EIM atingem proporções altas, especialmente em crianças. Os EIM apresentam uma variedade de sinais e sintomas, envolvendo vários órgãos e tecidos, sendo o diagnóstico precoce muito importante para o tratamento, bem como para a prevenção da doença em outros membros da família (Clarke, 2004). A recente aplicação da espectrometria de massas em *tandem* para triagem neonatal, tem permitido o diagnóstico pré-sintomático para alguns EIM (Saudubray et al., 2006).

A partir de uma perspectiva fisiopatológica, os EIM podem ser divididos em três grupos:

Grupo 1: Inclui os erros inatos do metabolismo intermediário que levam à intoxicação aguda ou progressiva pelo acúmulo de compostos tóxicos próximos ao bloqueio metabólico. Fazem parte desse grupo os erros inatos do metabolismo de aminoácidos (homocistinúria, fenilcetonúria, doença da urina do xarope do bordo, tirosinemia, etc), a maioria das acidúrias orgânicas (metilmalônica, propiônica, isovalérica, etc), os defeitos do ciclo da uréia, as intolerâncias aos açúcares (galactosemia, intolerância hereditária a frutose, etc), as intoxicações por metais

(Wilson, hemocromatose, etc) e as porfirias. Todas estas condições apresentam alguma semelhança em relação às manifestações clínicas: não prejudicam o desenvolvimento embriofetal, os pacientes apresentam um intervalo variável livre de sintomas desde o nascimento até que os sinais e sintomas de "intoxicação" se manifestem, quer de forma aguda (vômitos, coma, falência hepática, complicações tromboembólicas) ou crônica (déficit de crescimento e desenvolvimento, alterações visuais, cardiomiopatia, sintomas psiquiátricos). Os sintomas podem ser desencadeados por alterações do estado catabólico (febre, infecções virais ou ingestão alimentar reduzida). O diagnóstico na maioria das vezes é feito em plasma ou urina através da cromatografia de aminoácidos, ácidos orgânicos ou acilcarnitinas (Saudubray et al., 2006). É de fundamental importância o reconhecimento precoce deste grupo de patologias, pois, para a maioria delas, há uma possibilidade de tratamento, seja pela suplementação de determinadas vitaminas ou por alterações da dieta alimentar. Na maioria desses casos, o tratamento, se instituído precocemente, pode reverter o quadro clínico e promover um desenvolvimento adequado com sequelas mínimas ou mesmo ausentes (Leonard e Morris, 2000).

Grupo 2: Consiste em doenças com sintomas decorrentes à deficiência na produção de energia ou na sua utilização pelo fígado, coração, músculo, cérebro ou outros tecidos. Esse grupo pode ser dividido em defeitos energéticos citoplasmáticos e mitocondriais. Os defeitos mitocondriais são mais graves e compreendem as acidemias láticas congênitas, as doenças da cadeia respiratória mitocondrial e os defeitos da oxidação de ácidos graxos e corpos cetônicos. Os defeitos citoplasmáticos são geralmente menos graves e incluem as doenças da glicólise, do metabolismo do glicogênio, e da gliconeogênese, o hiperinsulinismo, as doenças do metabolismo da

creatina e os defeitos da via das pentoses. O diagnóstico é baseado em análises enzimáticas realizadas em biópsia ou cultura de células, e em análises moleculares.

Grupo 3: Envolve organelas celulares e inclui doenças que alteram a síntese ou o catabolismo de moléculas complexas. Todas as doenças lisossomais, peroxissomais, de glicosilação e os erros inatos da síntese do colesterol pertencem a esse grupo. Os sintomas são permanentes, progressivos e não relacionados com a ingestão de alimentos (Saudubray et al., 2006).

Essa tese de doutorado abordará a homocistinúria, um erro inato do metabolismo de aminoácidos.

1.2 Homocistinúria Clássica

A homocistinúria foi primeiramente descrita em 1962 através de uma pesquisa entre indivíduos com retardo mental (Carson e Neill, 1962). A descoberta da causa da doença como sendo uma deficiência enzimática se deu logo em seguida (Mudd et al., 1964). A homocistinúria clássica ocorre devido a uma deficiência na enzima cistationina-β-sintase (CBS), o que leva ao acúmulo no organismo dos aminoácidos homocisteína (Hcy) e metionina (Met), bem como de outros metabólitos da Hcy (Mudd et al., 2001). A figura 1 mostra, esquematicamente, a via metabólica normal da Hcy.

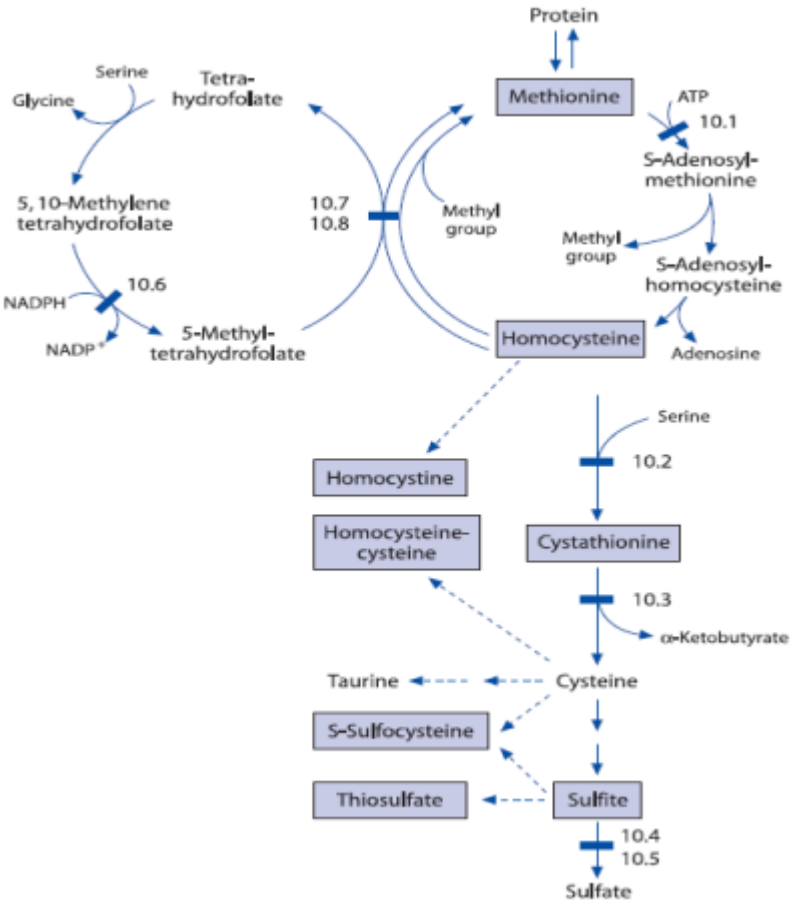

Figura 1. Enzimas das vias de transmetilação (metionina → homocisteína), remetilação (homocisteína → metionina) e transulfuração (homocisteína → metionina → sulfato) envolvidas no metabolismo da Hcy: 10.1 metionina adenosiltransferase; 10.2 cistationina-β-sintase; 10.3 ɣ-cistationase; 10.4 sulfito oxidase; 10.5 co-fator molibdênio; 10.6 metilenotetrahidrofolato redutase; 10.7 e 10.8 metionina sintase. Adaptado de Skovby (2003).

1.2.1 Homocisteína: aspectos bioquímicos

A Hcy é um pequeno aminoácido sulfidrílico com um peso molecular de 135,1g derivada da metionina proveniente da dieta e é um intermediário na via biossintética que converte metionina em cisteína. A metionina dietética é convertida a S-adenosilmetionina (AdoMet) pela enzima metionina-adenosiltransferase (MAT). Uma pequena quantidade da AdoMet é utilizada para a síntese de poliaminas, e a maior parte doa seu grupo metil a outros compostos, incluindo creatina, DNA e RNA; deste modo, AdoMet é metabolizada em S-adenosil-homocisteína que, por sua vez, é hidrolisada à

Hcy e adenosina. A Hcy sintetizada, a partir da metionina, tem dois destinos possíveis: metabolização pela via da transulfuração a cistationina, ou pela via de remetilação, a metionina. Aproximadamente 50% da Hcy é convertida, irreversivelmente, à cistationina, reação catalizada pela enzima CBS, dependente do fosfato de piridoxal (vitamina B_6), como co-fator, pela chamada via de transulfuração. A enzima seguinte na reação, ɤ-cistationase, hidrolisa a cistationina para gerar cisteína e α-cetobutirato. Esta enzima, como a CBS, é dependente da vitamina B_6. A Hcy remanescente, formada da metionina, é remetilada para regenerar metionina. Duas diferentes enzimas catalizam esta reação, mas a mais importante é a metionina sintase. Esta enzima é encontrada em todas as células e requer, como co-fator, a vitamina B_{12}. A reação é acoplada à conversão do 5-metiltetra-hidrofolato (a forma circulante do folato reduzido) a tetrahidrofolato, que então, entra nas células. A geração do 5-metiltetrahidrofolato por esta reação requer a redução do 5,10-metilenotetrahidrofolato a 5-metiltetrahidrofolato, que é catalisada pela 5,10-metilenotetrahidrofolato redutase (MTHFR). Em seres humanos, a remetilação pode ser efetivada por vias alternativas. A betaína, que é derivada da colina da dieta, também pode atuar como um doador de grupamento metil na conversão da Hcy em metionina. Esta reação é catalizada pela betaína-homocisteína metiltransferase, que é uma outra enzima dependente da vitamina B_{12}, encontrada em quantidades significantes somente no fígado (Mudd et al., 2001; Selhub, 1999).

Na deficiência de CBS, a rota metabólica não segue seu fluxo normal, devido a um bloqueio na via de transulfuração. Sob essas condições, a taxa da síntese de metionina é aumentada, levando a um temporário aumento na concentração de AdoMet intracelular. Esse aumento continuará até que o nível desse metabólito seja suficiente para inibir por feedback negativo a enzima MTHFR, tornando assim, a via de remetilação inibida. Consequentemente, ambas as vias do metabolismo da Hcy são

prejudicadas. A primeira consequência é a tendência para acumular a Hcy intracelularmente; sob essas condições, as células exportam Hcy e isso se reflete no plasma pelas concentrações anormais de uma variedade de derivados da Hcy. Pacientes CBS deficientes não tratados apresentam níveis plasmáticos de Hcy livre acima de 200 μM. Dissulfetos mistos como Hcy-cisteína e Hcy-cisteinilglicina, também são encontrados (Mudd et al., 2001; Selhub, 1999).

No plasma de indivíduos saudáveis, a Hcy existe em várias formas: a forma reduzida (aproximadamente 1%), ligada a resíduos de cisteína em proteínas (aproximadamente 70%) e ligada à cisteína livre, formando o dissulfeto misto Hcy-cisteína (aproximadamente 30%). Quando os níveis de Hcy estão elevados, o dissulfeto homocistina (Hcy-Hcy) é formado. Todas essas formas podem ser convertidas em Hcy pela redução química e, então, medidas como Hcy total (tHcy) (Fowler, 2008). A faixa normal para tHcy em adultos é de 5-15 μM, crianças tem valores ligeiramente menores que adultos (Mudd et al., 2001).

1.2.2 Homocistinúria: manifestações clínicas

A homocistinúria devido a deficiência de CBS apresenta um padrão de herança autossômica recessiva. A doença manifesta-se através de uma variedade de anormalidades clínicas e patológicas, principalmente no que envolve os olhos e os sistemas esquelético, vascular e nervoso central. Outros órgãos, incluindo o fígado e a pele, podem também estar envolvidos. Dados indicam que o risco de desenvolvimento das manifestações aumenta com a idade, sendo que os pacientes responsivos a terapia (B_6 responsivos) apresentam-se mais levemente afetados que os pacientes não responsivos a terapia (B_6 não responsivos) (Mudd et al., 2001). Seguem descritas

abaixo as manifestações clínicas que podem ser apresentadas por pacientes portadores de homocistinúria por deficiência de CBS.

- *Olhos: Ectopia lentis* – Provavelmente o achado mais consistente na homocistinúria seja a *ectopia lentis* (deslocamento do cristalino). Esgaçamento e ruptura das fibras zonulares levam ao afrouxamento do cristalino e, eventualmente, ao deslocamento (Mudd et al., 2001). *Ectopia lentis* pode ser notada precocemente em pacientes com 4 semanas (Cruysberg et al., 1996), no entanto, na maioria das vezes em pacientes não tratados há uma demora de aproximadamente 2 anos desde o nascimento até o deslocamento ser notado (Mudd et al., 2001).

- *Ossos: Osteoporose* – Osteoporose é mais comum na coluna, seguido pelos ossos longos. Escoliose também é frequente, possivelmente como consequência da osteoporose na coluna. Os pacientes homocistinúricos apresentam um grande número de outras anormalidades esqueléticas, notavelmente a dolicostenomelia (alongamento dos ossos longos), que resulta em indivíduos altos e magros, frequentemente associados a aparência "marfanóide".

- *Sistema Vascular: Tromboembolismo* – A maior causa de morbidade, e a mais frequente causa de morte em pacientes homocistinúricos é o tromboembolismo. A oclusão vascular pode ocorrer em qualquer vaso, incluindo a veia porta (Hong et al., 1997), e em qualquer idade, incluindo a infância (Kerrin et al., 1996). Gravidez e estado pós-parto parecem aumentar o risco de tromboembolismo (Mudd et al., 2001).

- *Sistema Nervoso Central:*

Retardo mental – A mais frequente anormalidade do SNC é o retardo mental, o qual é frequentemente o primeiro sinal reconhecido da homocistinúria, apresentando-se como atraso no desenvolvimento durante o primeiro ou o segundo ano de vida (Mudd et al., 2001).

Anormalidades neurológicas – Cerca de 21% dos pacientes com homocistinúria não tratados apresentam convulsões (Mudd et al., 1985). Outros distúrbios neurológicos incluem eletroencefalogramas anormais e distúrbios extrapiramidais. Sinais neurológicos focais sugerem a presença de oclusão cerebrovascular (Mudd et al, 2001).

Anormalidades psiquiátricas – Doenças mentais tem sido descritas frequentemente em indivíduos com deficiência de CBS. Entre as mais recorrentes estão: distúrbios de personalidade, distúrbios de comportamento, episódios de depressão e transtorno obsessivo compulsivo (Mudd et al.,2001).

1.2.3 Homocistinúria: diagnóstico

A presença de um ou mais dos sinais clínicos típicos podem levar a suspeita de homocistinúria. Estudos enfatizam a frequência do diagnóstico tardio na homocistinúria por deficiência de CBS, podendo chegar a 11 anos de atraso entre o primeiro sinal significativo da doença e o diagnóstico propriamente dito (Cruysberg et al., 1996). Esses autores sugerem que em adição a *ectopia lentis*, miopia de 1 dióptero ou mais associada com sinais de doença sistêmica tais como eventos vasculares, anormalidades esqueléticas, ou envolvimento do SNC, devem alertar para a possibilidade de homocistinúria por deficiência de CBS. No entanto, o diagnóstico definitivo é baseado na presença de certas anormalidades bioquímicas. Um achado bioquímico importante é a presença de homocistina na urina, o que é mais facilmente suspeitada quando a reação urinária do cianeto-nitroprussiato é positiva. Como esse teste detecta a maioria dos dissulfetos, o mesmo pode positivar com outras doenças, como cistinúria e/ou β-mercaptolactato-cisteinúria. Consequentemente, a presença de homocistina na urina não é suficiente para estabelecer o diagnóstico da homocistinúria por deficiência de CBS. Sendo assim, aminoácidos devem ser medidos no plasma ou soro de todos os indivíduos

suspeitos. Na deficiência de CBS, essa medida deve revelar níveis plasmáticos elevados de tHcy, usualmente acompanhados por uma concentração marcadamente reduzida de cisteína. Além disso, uma concentração aumentada de metionina é encontrada na maioria dos pacientes. A hipermetioninemia é um achado importante já que, nos defeitos metabólicos da metilação da Hcy (que são causas alternativas de homocistinúria) a concentração sanguínea de metionina é baixa ou normal (Mudd et al., 2001). A medida direta da atividade da enzima CBS confirma o diagnóstico de homocistinúria. A mesma pode ser realizada em biópsia de fígado (Finkelstein et al., 1964) e em cultura de fibroblastos (Uhlendorf e Mudd, 1968).

Usando o critério da elevação dos níveis da metionina sanguínea, a homocistinúria tem sido detectada em programas de triagem neonatal em diferentes países a uma taxa de 1:58.000 – 1:1.000.000 nascidos vivos, com um frequência média de 1:344.000 nascidos vivos (Mudd et al., 2001). Países como Noruega e Irlanda apresentem frequências mais elevadas, como 1:6.400 e 1:65.000 nascidos vivos, respectivamente (Yap e Naughten, 1998; Refsum et al., 2004).

Mais de 60 mutações foram detectadas no gene da enzima CBS, no entanto algumas parecem ser relativamente mais comuns entre os pacientes com homocistinúria (Kluijtmas et al., 1999). O diagnóstico molecular da homocistinúria é importante no sentido de direcionar o tratamento dos pacientes. Sabe-se que a responsividade ao tratamento com vitamina B_6 pode variar de acordo com o genótipo do paciente. Assim, a mutação I278T usualmente confere responsividade à vitamina B_6, se em homozigotos ou heterozigotos compostos. O fenótipo clínico na maioria desses pacientes parece ser leve. Em adição à mutação I278T, as mutações A114V, R266K, R336H, K384E e L539S, também parecem correlacionar-se com a responsividade à vitamina B_6 *in vivo*. Por outro lado, as mutações T191M, R121L, R125Q, C165Y, E176K, T257M e T262M,

parecem ser incompatíveis com a responsividade à vitamina B_6. Além disso, pacientes portadores da mutação G307S parecem ter fenótipos moderados a graves, exceto aqueles pacientes que são tratados desde o nascimento (Mudd et al., 2001).

1.2.4 Homocistinúria: tratamento

Uma vez estabelecido bioquimicamente o diagnóstico de homocistinúria, é importante determinar se o paciente apresenta ou não responsividade à vitamina B_6. Aqueles que são responsivos, podem ser metabolicamente controlados com vitamina B_6 isolada ou combinada com uma dieta moderadamente restrita em metionina; o que se torna uma grande vantagem, considerando a dificuldade da imposição de uma dieta severamente restrita em metionina, a qual é prescrita para aqueles pacientes que não são responsivos à vitamina B_6 (Mudd et al., 2001). Alguns autores consideram a forma de como essa responsividade pode ser avaliada. Kluijtmans et al. (1999) deram vitamina B_6 por 6 semanas em doses de 750 mg/dia para adultos e 200 a 500 mg/dia para crianças. Aqueles cuja Hcy livre diminuiu para menos de 20 μM, ou cuja tHcy diminuiu para menos de 50 μM, foram classificados como "B_6-responsivos". Wilcken e Wilcken (1997) também determinaram a responsividade à vitamina B_6 com base nos níveis de Hcy livre abaixo de 20 μM, mas os pacientes, nesse caso, receberam doses de vitamina B_6 de somente 100 a 200 mg/dia. Yap e Naughten (1998) determinaram a responsividade à vitamina B_6 em crianças com base em uma rápida queda nos níveis de Met e de homocistina livre no plasma, enquanto os pacientes recebiam 150 mg de B_6/dia por 3 dias.

O tratamento na homocistinúria clássica tem dois objetivos principais: (1) controlar ou eliminar as anormalidades bioquímicas com o objetivo de prevenir as manifestações clínicas, parar a progressão das manifestações existentes ou melhorar

aquelas manifestações reversíveis; e (2) tratar as complicações. Sempre que possível, a terapia para alcançar o controle bioquímico deve começar antes que as manifestações clínicas ocorram, já que muitas dessas complicações são irreversíveis. Mesmo antes de os efeitos clínicos tornarem-se reconhecíveis, é possível que o dano tecidual já tenha ocorrido. Assim, o benefício máximo da terapia pode ser possível quando essa doença é diagnosticada no período neonatal, como resultado do conhecimento da doença na família, ou através da triagem neonatal (Mudd et al., 2001).

Estratégias para o tratamento na homocistinúria incluem: (1) aumento da atividade residual da enzima CBS através da administração de vitamina B_6 nos pacientes responsivos à essa vitamina; (2) reduzir a carga da via metabólica prejudicada através de uma dieta restrita em Met suplementada com cisteína; (3) administração de betaína no intuito de utilizar uma via alternativa para a remoção do excesso de Hcy (Walter et al., 1998).

A enzima CBS requer vitamina B_6 (piridoxina) como um grupo prostético para sua atividade catalítica. Com a administração de vitamina B_6, cerca de 50% dos pacientes deficientes em CBS apresentam uma diminuição significativa nos níveis de Met e Hcy no plasma, bem como uma diminuição nas complicações tromboembólicas. As dosagens variam de 250-500 mg por dia (Clarke, 2004). Alguns médicos rotineiramente optam por administrar a piridoxina mesmo para aqueles pacientes aparentemente não responsivos, neste caso é usada tipicamente nas doses de 100-200mg/dia (Mudd et al., 2001; Rao et al., 2008).

Para os pacientes B_6 não responsivos, o acúmulo de Hcy pode ser tratado pela administração de betaína (N,N,N-trimetilglicina). A betaína promove a metilação da metionina, com a produção de N,N-dimetilglicina, numa reação catalisada pela enzima betaína-homocisteína metiltransferase. Dessa forma, a administração de betaína causa

aumento nos níveis de metionina e diminuição nos níveis de Hcy, permitindo um controle nos níveis de Hcy para aqueles pacientes não responsivos à terapia com vitamina B_6 (Clarke, 2004). A betaína é administrada via oral na dose máxima de 6 – 9g/dia dividida em 3 doses. (Schwahn et al., 2003; Andria et al., 2006). A figura 2 mostra esquematicamente o ciclo da remetilação.

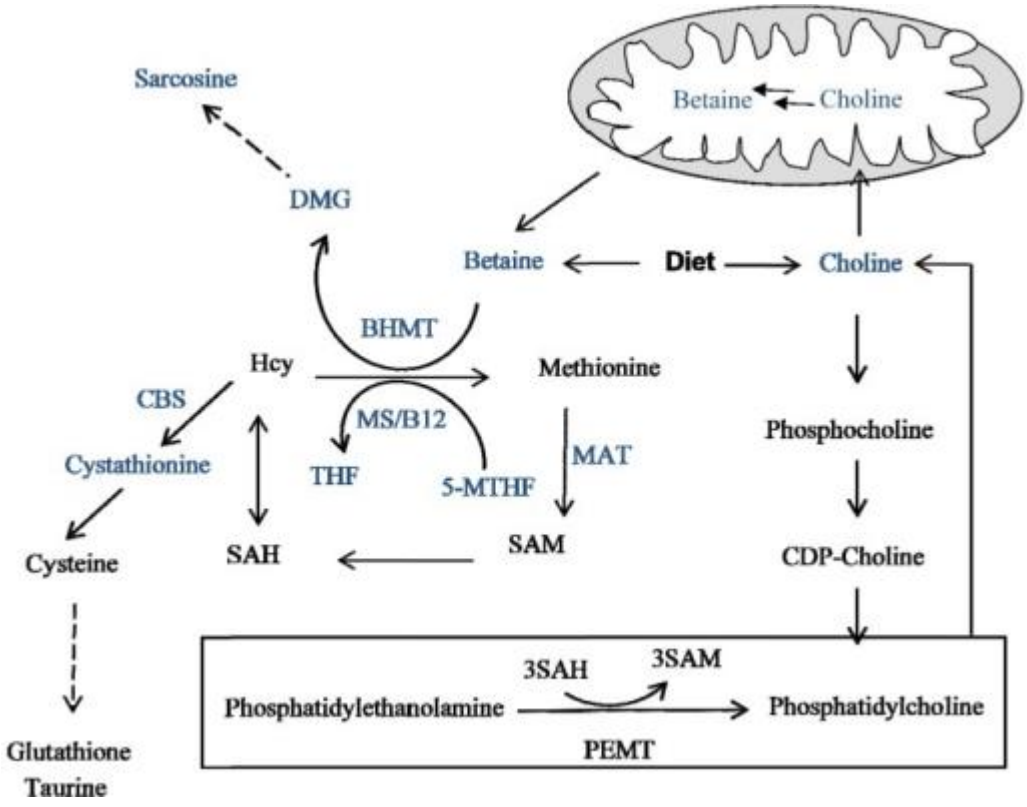

Figura 2. Ciclo da remetilação. BHMT: betaína homocisteína metiltransferase; CBS: cistationina-β-sintase; DMG: dimetilglicina; Hcy: homocisteína; MAT: metionina S-adenosiltransferase; MS: metionina sintase; PEMT: fosfatidiletanolamina metiltransferase; 5-MTHF: 5-metiltetrahidrofolato; SAH: S-adenosilhomocisteína; SAM: S-adenosilmetionina; THF: tetrahidrofolato. Adaptado de Obeid (2013).

O folato e a vitamina B_{12} otimizam a conversão da Hcy em Met pela metionina sintase, ajudando assim, a diminuir a concentração plasmática de Hcy. A dose usual de

folato a ser administrada é de 5mg/dia. A vitamina B_{12} é administrada como hidroxicobalamina na dose de 1mg intramuscular 1 vez por mês (Rao et al., 2008).

Dieta severamente restrita em Met é indicada para os pacientes B_6 não responsivos, e uma dieta menos restrita em Met pode ser necessária para os pacientes B_6 responsivos. Misturas sintéticas de aminoácidos "metionina free" estão comercialmente disponíveis e são especialmente úteis para crianças. Após a infância, alimentos contendo proteína "pobre" em Met podem ser introduzidos, incluindo gelatina, lentilha e soja. Em adição à vitamina B_6, folato e, possivelmente à vitamina B_{12}, a suplementação de vitaminas e minerais é recomendada (Andria et al., 2006)

Qualquer que seja a combinação terapêutica empregada, alcançar níveis normais de tHcy é muito difícil na maioria dos pacientes. A prevenção das anormalidades clínicas graves causadas por essa desordem requer o tratamento por toda a vida; quando a administração de uma dieta pobre em Met é iniciada no período neonatal, o retardo mental é prevenido, o início e a progressão do deslocamento do cristalino é atrasado, e a incidência de convulsões é diminuída (Andria et al., 2006). De fato, um QI normal foi reportado em pacientes homocistinúricos adolescentes B_6 não responsivos, que tiveram boa aderência ao tratamento desde o nascimento (Yap et al., 2001).

Outra abordagem terapêutica explorada em diferentes EIM é baseada no "resgate" de enzimas mutantes pelas chaperonas; exemplos incluem a administração de sapropterina na fenilcetonúria ou outras pequenas moléculas testadas em doenças lisossomais (Leandro e Gomes 2008; Muntau et al., 2014). Essas moléculas possuem diversas estruturas químicas e atuam como ligantes proteicos, interagindo diretamente com a proteína alvo e facilitando deu dobramento na forma correta. Melenovská et al. (2015) demonstraram em fibroblastos humanos que o heme arginato aumenta a atividade residual e proporciona o dobramento adequado de CBS mutantes

selecionadas. Esses autores propõem que a bolsa de ligação na CBS em enzimas mutantes pode ser um promissor alvo para o desenvolvimento de novos agentes terapêuticos para algumas formas de deficiência de CBS.

Embora para muitas condições as novas terapias ainda não representam a cura, certamente significam um passo importante para melhorar a qualidade de vida dos afetados e dar a muitos deles uma chance de sobrevida enquanto novos tratamentos, mais eficazes e definitivos, estão sendo desenvolvidos (Schwartz et al., 2008). Assim, novas abordagens terapêuticas incluem estratégias que apesar de não afetarem especificamente e diretamente o mecanismo principal da doença, podem melhorar a qualidade de vida dos pacientes portadores de EIM, tais como o uso de antioxidantes.

Finalmente, o sucesso do tratamento na deficiência de CBS claramente depende de um diagnóstico e tratamento precoce, reforçando a importância da triagem neonatal em massa.

1.3 Outras Formas de Hiper-Homocisteinemia

Além da deficiência de CBS, outros defeitos enzimáticos podem levar à homocistinúria. Entre esses estão incluídos defeitos de remetilação devido às deficiências das enzimas MTHFR e metionina sintase, bem como defeitos no metabolismo intracelular da cobalamina (Fowler, 2008). Os principais achados bioquímicos dos defeitos de remetilação são a alta excreção de homocistina na urina e a hiper-homocisteinemia com níveis baixos ou relativamente normais de metionina plasmática, o que diferencia essas doenças da homocistinúria clássica devido à deficiência de CBS, na qual os níveis de metionina plasmática estão elevados (Rosenblatt e Fenton, 2001).

Elevados níveis de Hcy também podem ocorrer em deficiências nutricionais de vitamina B_{12} e folato. Vários medicamentos, tais como o composto antifolato metotrexato e o anestésico óxido nitroso podem interferir com o metabolismo da metionina e levar a leves aumentos de Hcy. Além disso, a função renal anormal também pode levar a níveis plasmáticos aumentados de Hcy (Fowler, 2008).

1.4 Homocistinúria: fisiopatologia e possíveis mecanismos de lesão

1.4.1 Estresse oxidativo

Estresse oxidativo pode ser definido como um desequilíbrio pró-oxidante/antioxidante, causado pelo aumento da produção de espécies reativas e/ou pela depleção das defesas antioxidantes, o que leva a um dano tecidual. Tal dano é frequentemente chamado de dano oxidativo e pode ser definido como o dano biomolecular causado pelo ataque de espécies reativas aos constituintes de organismos vivos. O estresse oxidativo pode produzir uma série de desequilíbrios no metabolismo celular, incluindo quebras de fita no DNA, aumento do Ca^{2+} intracelular, dano a proteínas de membrana e peroxidação de lipídeos (Halliwell e Chirico, 1993; Halliwell e Gutteridge, 2007).

- *Radicais livres / Espécies reativas:*

Radical livre é definido como uma espécie capaz de existência independente, que possui um ou mais elétrons desemparelhados ocupando um orbital atômico ou molecular sozinho. Exemplos de radicais livres incluem os radicais superóxido ($O_2^{\cdot -}$) e hidroxil ($OH^{\cdot}$), os quais são altamente reativos e instáveis, existindo por somente micro ou nanosegundos antes de desencadear reações em cadeia que acabam por danificar componentes biológicos. O termo espécies reativas de oxigênio (ERO) inclui não somente os radicais livres, mas também alguns derivados não radicalares do oxigênio,

como o peróxido de oxigênio (H_2O_2) (Halliwell e Gutteridge, 2007). Além das ERO, existem ainda as espécies reativas de cloro, as espécies reativas de bromo e as espécies reativas de nitrogênio, como o óxido nítrico ($NO^{\cdot}$), uma importante molécula sinalizadora em animais e plantas (Halliwell, 2006).

As ERO estão constantemente sendo formadas na célula; aproximadamente 3 a 5% do oxigênio consumido por um indivíduo são convertidos em radicais livres de oxigênio. Alguns são produzidos como produtos acidentais de reações enzimáticas normais, que escapam do sítio ativo de enzimas que contêm metais durante reações de oxidação. Outros, como o H_2O_2, são produtos fisiológicos de oxidases nos peroxissomos. A produção deliberada de radicais livres tóxicos ocorre na resposta inflamatória. Medicamentos, radiação, poluentes do ar e outros agentes químicos, também podem aumentar a produção de radicais livres nas células (Smith et al., 2005).

- *Sistema antioxidante:*

Para a proteção contra o dano causado pelas espécies reativas, o organismo desenvolveu sistemas de defesa antioxidante, bem como sistemas de reparo, para prevenir o acúmulo de moléculas oxidativamente modificadas (Halliwell e Gutteridge, 2007).

O sistema antioxidante inclui vários tipos de agentes. Por exemplo, agentes que removem cataliticamente as espécies reativas, como fazem as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). A SOD remove o $O_2^{\cdot -}$ por catalisar sua dismutação, uma vez que um $O_2^{\cdot -}$ é reduzido a H_2O_2 enquanto o outro é oxidado a O_2 (Halliwell e Gutteridge, 2007). A CAT, a qual é encontrada principalmente nos peroxissomos e em menor extensão no citosol e na fração microssomal da célula, reduz o H_2O_2 formado à H_2O. As glutationas-peroxidases existem como uma família de enzimas contendo selênio e são a principal maneira de

remover do H_2O_2 produzido fora dos peroxissomos (Smith et al., 2005). A GPx elimina o H_2O_2 pelo acoplamento da sua redução à H_2O com a oxidação da glutationa reduzida (GSH). O produto dessa reação, glutationa oxidada (GSSG), consiste de duas moléculas de GSH ligadas por uma ponte dissulfeto, e pode ser convertido novamente a GSH pela enzima glutationa redutase (GR) (Halliwell, 2006).

Outra linha de agentes antioxidantes atua diminuindo a formação de espécies reativas. Nessa categoria podem-se incluir proteínas que minimizam a disponibilidade dos pró-oxidantes, tais como íons ferro, íons cobre ou heme. Como exemplos estão transferrina, albumina, haptoglobina, hemopexina e ceruloplasmina. Proteínas que protegem biomoléculas contra o dano oxidativo por outros mecanismos, como fazem as chaperonas, também são consideradas antioxidantes (Halliwell e Gutteridge, 2007).

Compostos como os carotenóides, podem exercer efeitos antioxidantes, bem como de *quench* de oxigênio *singlet*. Carotenóides é um termo aplicado ao β-caroteno (o precursor da vitamina A) e aos compostos similares como zeaxantina e luteína (Smith et al, 2005).

Por fim, também fazem parte do sistema de defesa antioxidante aqueles agentes que são preferencialmente oxidados pelas espécies reativas a fim de preservar biomoléculas mais importantes. São exemplos desse grupo GSH, bilirrubina, urato, albumina, plasmalogênios, α-tocoferol (vitamina E) e ascorbato (vitamina C).

As defesas antioxidantes não são 100% efetivas, já que o dano oxidativo ao DNA, proteínas, lipídios e outras moléculas pode ser demonstrado nos sistemas vivos em ambiente aeróbio. Sendo assim, alguns autores classificam como defesa antioxidante os sistemas de reparo necessários para lidar com moléculas danificadas (reparo ao DNA) ou para degradar lipídios e proteínas danificados (Halliwell e Gutteridge, 2007).

- *Estresse oxidativo em EIM:*

Evidências de danos por radicais livres foram descritas em mais de 100 estados patológicos. Em alguns desses, o dano por radicais livres é a causa primária da doença; em outros, ele aumenta as complicações da doença (Halliwell e Gutteridge, 2007). Nos EIM, o estresse oxidativo pode ser causado pelo acúmulo de metabólitos tóxicos, que levam à produção excessiva de radicais livres ou à depleção da capacidade antioxidante. Trabalhos recentes demonstram que o estresse oxidativo pode estar envolvido na fisiopatologia de vários EIM, como nas acidemias propriônica e metilmalônica (Fontella et al., 2000; Ribas et al., 2010a, 2010b); em aminoacidopatias, como na doença da urina do xarope do bordo (Bridi et al., 2005; Barschak et al., 2006, 2008; Mescka et al., 2011) e na fenilcetonúria (Kienzle Hagen et al., 2002; Sirtori et al. 2005; Sitta et al., 2006, 2009, 2011; Preissler et al., 2015); em doenças peroxissomais, como na adrenoleucodistrofia ligada ao X (Vargas et al., 2004; Powers et al., 2005; Deon et al., 2006, 2007) ou na mucopolissacaridose tipo II (Pereira et al., 2008; Filippon et al., 2011a, 2011b).

Com relação à homocistinúria, o estresse oxidativo vem sendo considerado um possível mecanismo através do qual a Hcy exerce seus efeitos tromboembólicos (Mudd et al., 2001). Estudos em modelos animais reforçam essa hipótese e demonstram uma possível associação entre as altas concentrações de Hcy e a produção de espécies reativas (Streck et al., 2003; Robert et al., 2005; Matté et al., 2009; da Cunha et al., 2011). Adicionalmente, foi demonstrado recentemente a ocorrência de estresse oxidativo no sangue de pacientes homocistinúricos, evidenciado através do aumento do dano oxidativo a proteínas, lipídeos e ao DNA, bem como através da diminuição das defesas antioxidantes (Vanzin et al., 2011; Vanzin et al., 2014). Sabe-se que o DNA é um importante alvo para oxidação e seu dano gera uma gama de produtos que podem ser medidos, tais como produtos da oxidação de bases e produtos de fragmentação

(quebras de fita), ligações cruzadas inter e intra-fitas, ligações cruzadas DNA-proteína e produtos da fragmentação de açúcares (Cooke et al., 2006). Além disso, o estudo dos danos no DNA ao nível de cromossomo é uma parte essencial da toxicologia genética porque mutação cromossômica é um evento importante na carcinogênese. O ensaio de micronúcleos tem emergido como um dos métodos preferidos para avaliar o dano cromossômico, porque avalia tanto a perda cromossômica quanto quebras cromossômicas, de maneira confiável (Fenech, 2000).

- *N-acetilcisteína (NAC):*

Levando em consideração a possível relação do estresse oxidativo na fisiopatologia da doença, cabe salientar que a suplementação com antioxidantes pode ser benéfica e deve ser estudada em pacientes homocistinúricos. Há um aumento no interesse com relação à N-acetilcisteína (NAC), uma vez que essa molécula vem demonstrando efeitos antioxidantes importantes em algumas doenças, como na adrenoleucodistrofia ligada ao X (Marchetti et al., 2015) e na β-talassemia (Ozdemir et al., 2014). A NAC vem sendo utilizada na prática terapêutica por várias décadas como agente mucolítico e para tratamento de numerosas condições incluindo intoxicação com paracetamol. Sua ação antioxidante direta se dá pela sua função como "scavenger" de radicais livres, e indiretamente a NAC atua como antioxidante aumentando a concentração intracelular de cisteína e glutationa. Além disso, a NAC parece exercer um efeito anti-inflamatório, limitando a liberação de citocinas, tais como TNFα, IL-6 e IL-1β durante a fase inicial da resposta imune (Dhouib et al., 2016).

1.4.2 Alterações no metabolismo de lipídeos

O tromboembolismo é uma das principais características clínicas da homocistinúria por deficiência de CBS e muitas investigações tem sido conduzidas

nesse sentido com objetivo de definir as causas da coagulação anormal e das tendências ateroscleróticas que são observadas em pacientes homocistinúricos. Entre os possíveis mecanismos, o dano a lipoproteína(a) ou a outras lipoproteínas plasmáticas tem sido descrito como contribuinte (Mudd et al., 2001). O termo lipoproteín(a) refere-se a uma família de lipoproteínas plasmáticas, que em concentrações elevadas possuem correlação com risco aumentado de doenças cardiovasculares (Scanu, 1992). Harpel e colaboradores (1992) demonstraram uma afinidade aumentada da lipoproteína(a) pelo fibrinogênio e pela fibrina na presença de Hcy, o que, segundo os autores, poderia potencializar a incorporação da lipoproteína(a) nos vasos.

De acordo com estudos, a Hcy causa dano às células e aos tecidos das artérias através da liberação de citocinas, ciclinas e outros mediadores inflamatórios e de divisão celular (McCully, 1983). Por afetar células musculares lisas, a Hcy produz mudanças no tecido conectivo das placas ateroscleróticas, causando fibrose, calcificação e deposição de proteinoglicanos (McCully, 2007).

A Hcy-tiolactona é um anidrido reativo da Hcy que interage com LDL, causando agregação, aumento da densidade, e aumento da captação desses componentes por macrófagos vasculares, formando células espumosas (Naruszewicz et al., 1994). A formação desses agregados leva a deposição do colesterol e de outras gorduras, permitindo o desenvolvimento das placas. Em adição, a reação da Hcy-tiolactona com proteínas séricas leva a produção de novas proteínas antigênicas e anticorpos autoimunes, facilitando a resposta inflamatória (Undas et al., 2004).

Paraoxonase-1 (PON1) é uma enzima esterase e lactonase sintetizada pelo fígado e encontrada na circulação associada com lipoproteínas de alta densidade (HDL). A função fisiológica da PON1 parece ser a de degradar ésteres de colesterol e fosfolípideos oxidados em lipoproteínas e membranas celulares (Camps et al., 2009).

Alterações nos níveis circulantes da PON1 têm sido relatadas em uma variedade de doenças envolvendo o estresse oxidativo, tais como doenças cardiovasculares, doença de Alzheimer, insuficiência renal crônica, infecção por HIV, síndrome metabólica, e insuficiência hepática crônica (Marsillach et al., 2008). Além das funções já descritas, a PON1 exibe atividades fisiológicas relacionadas com o metabolismo de drogas, incluindo a hidrólise de metabólitos de inseticidas organofosforados e a detoxificação da Hcy-tiolactona (Draganov et al. 2005). A Hcy-tiolactona modifica resíduos de lisina em proteínas através de um processo chamado *N*-homocisteinilação, o qual afeta a estrutura e a função proteica (Jakubowski 1997, 1999). A descoberta da atividade lactonase da PON1 levou a hipótese de que a PON1 poderia ser ateroprotetora também devido a sua habilidade de detoxificar a Hcy-tiolactona e minimizar a *N*-homocisteinilação de proteínas (Jakubowski, 2000).

Adicionalmente, sugere-se que a Hcy-tiolactona também possa aumentar a atividade da enzima butirilcolinesterase (BuChE) (Darvesh et al., 2007). A BuChE catalisa a hidrólise de acetilcolina e outros ésteres de colina, e mais recentemente vem sendo relacionada com hiperlipidemia, e com doenças tais como acidente vascular cerebral e doença de Alzheimer (Vaisi-Raygani et al., 2009).

1.4.3 Inflamação

Estudos atuais evidenciam que a inflamação atua como uma importante força motriz nas diferentes fases do desenvolvimento da placa aterosclerótica: formação, progressão e eventual ruptura. Biomarcadores são geralmente proteínas ou enzimas – medidos em soro, plasma ou sangue – que fornecem um diagnóstico independente e/ou um valor prognóstico, por refletir o estágio da doença. No caso da doença arterial coronariana, uma gama de biomarcadores existem, e especificamente os biomarcadores

inflamatórios têm sido extensivamente investigados. Entre esses destacam-se a proteína C reativa, mieloperoxidase, moléculas de adesão celular, bem como as citocinas (interleucinas) IL-1, IL-6, IL-8 (Zakynthinos e Pappa, 2009).

Citocinas e quimiocinas são proteínas solúveis ou glicoproteínas produzidas por vários tipos de células, que atuam como moléculas de comunicação celular, exercendo efeitos regulatórios. A maioria é secretada, mas algumas podem ser expressas na membrana celular, enquanto outras podem ser sequestradas na matriz extracelular e armazenadas, ou liberadas de acordo com a demanda biológica (Hanada e Yoshimura, 2002). Acredita-se que o balanço entre citocinas pró e anti-inflamatórias, bem como a expressão de quimiocinas desempenhe um significativo papel na etiologia da trombose e da aterogênese (Ross, 1999; Zakynthinos e Pappa, 2009).

Nesse sentido, alguns autores têm investigado o papel da inflamação na fisiopatologia da homocistinúria. Da Cunha et al. (2010) demonstraram que a hiper-homocisteinemia crônica aumenta significativamente os níveis de algumas citocinas pró-inflamatórias (TNF-α, IL-1β, IL-6), proteína quimiotática de monócitos-1 (MCP-1) e prostaglandina E2 em hipocampo e soro de ratos 1h e 12h após a injeção de Hcy. Keating et al. (2011) evidenciaram anormalidades na indução da expressão de citocinas em modelo animal de homocistinúria, bem como em pacientes homocistinúricos na presença e na ausência de terapia para redução dos níveis de Hcy. Foi evidenciado em modelo animal o aumento dos níveis das citocinas IL-1α, IL-1β, TNF-α; similarmente em pacientes não tratados ou com baixa aderência ao tratamento foi demonstrado indução de múltiplas citocinas pró-inflamatórias (IL-1α, IL-6, TNF-α, IL-17, IL-12). Esses autores concluem que a homocistinúria é uma doença que apresenta inflamação crônica, e que a expressão de citocinas pode interferir em muitos aspectos da patogênese da doença.

Apesar de os possíveis mecanismos de dano observados na homocistinúria não estarem completamente esclarecidos, existem evidências do envolvimento de alterações no metabolismo lipídico, bem como alterações no perfil inflamatório e no equilíbrio pró-oxidante/antioxidante. É de grande interesse o detalhamento dessas alterações metabólicas, no intuito de melhor entender a fisiopatologia da trombose e aterosclerose observadas em pacientes homocistinúricos, bem como possibilitar novas abordagens terapêuticas, como por exemplo, o uso de antioxidantes.

2. OBJETIVOS

2.1 Objetivo geral

Levando em consideração que dados na literatura mostram um possível papel do estresse oxidativo no dano vascular observado na homocistinúria, e considerando que as causas das alterações aterotrombóticas observadas na homocistinúria permanecem ainda hoje não completamente conhecidas, o objetivo geral desse trabalho é avaliar os perfis oxidativo, lipídico e inflamatório, bem como a atividade das enzimas paraoxonase, butirilcolinesterase e catalase em pacientes homocistinúricos no momento do diagnóstico e durante o tratamento clássico, e, ainda, avaliar o efeito *in vitro* do antioxidante N-acetil-L-cisteína sobre o dano ao DNA induzido pelas altas concentrações de Hcy e o efeito *in vitro* da Hcy sobre o dano cromossômico.

2.2 Objetivos específicos

- Avaliar o perfil lipídico, a saber: colesterol total, colesterol HDL, colesterol LDL (calculado pela fórmula de Friedewald), colesterol LDL oxidado, bem como avaliar os níveis de apolipoproteína A1 no plasma de pacientes com homocistinúria no momento do diagnóstico (não tratados) e durante o tratamento preconizado (dieta hipoproteica suplementada com vitamina B_6, ácido fólico, betaína e vitamina B_{12});

- Avaliar o perfil inflamatório através da medida dos níveis das citocinas IL-1β, IL-6, INF-ɤ no plasma de pacientes com homocistinúria no momento do diagnóstico (não tratados) e durante o tratamento preconizado;

- Avaliar o dano oxidativo às proteínas, através da medida do conteúdo de sulfidrilas e carbonilas no plasma de pacientes com homocistinúria no momento do diagnóstico (não tratados) e durante o tratamento preconizado;

- Avaliar a atividade das enzimas paraoxonase-1 e butirilcolinesterase no plasma de pacientes com homocistinúria no momento do diagnóstico (não tratados) e durante o tratamento preconizado.

- Correlacionar todos os parâmetros acima citados com os níveis de tHcy, ácido fólico e vitamina B_{12} nos dois grupos de pacientes, tratados e não tratados.

- Avaliar parâmetros de estresse oxidativo, tais como níveis de 15-F2t-isoprostanos e níveis de di-tirosina, bem como níveis de nitritos na urina de pacientes homocistinúricos durante o tratamento preconizado, e correlacionar com os níveis de tHcy e de grupamentos sulfidrilas;

- Avaliar a atividade da enzima catalase no sangue de pacientes homocistinúricos durante o tratamento preconizado;

- Avaliar o efeito *in vitro* do antioxidante N-acetil-L-cisteína (NAC) sobre o dano ao DNA causado pelas altas concentrações de Hcy.

- Avaliar o efeito *in vitro* da Hcy sobre o dano cromossômico, através do teste de micronúcleos.

3. RESULTADOS

Os resultados serão apresentados na forma de artigos científicos.

3.1 Capítulo I – Artigo 01

Lipid, oxidative and inflammatory profile and alterations in the enzymes paraoxonase and butyrylcholinesterase in plasma of patients with homocystinuria due CBS deficiency: the vitamin B_{12} and folic acid importance.

Camila S. Vanzin, Caroline P. Mescka, Bruna Donida, Tatiane G. Hammerschimidt, Graziela S. Ribas, Janaína Kolling, Emilene B. Scherer, Laura Vilarinho, Célia Nogueira, Adriana S. Coitinho, Moacir Wajner, Angela T. S. Wyse, Carmen R. Vargas

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ORIGINAL RESEARCH

Lipid, Oxidative and Inflammatory Profile and Alterations in the Enzymes Paraoxonase and Butyrylcholinesterase in Plasma of Patients with Homocystinuria Due CBS Deficiency: The Vitamin B_{12} and Folic Acid Importance

Camila Simioni Vanzin[1,2] · Caroline Paula Mescka[1,2] · Bruna Donida[2,5] · Tatiane Grazieli Hammerschimidt[2] · Graziela S. Ribas[2] · Janaína Kolling[1] · Emilene B. Scherer[1] · Laura Vilarinho[3] · Célia Nogueira[3] · Adriana Simon Coitinho[4] · Moacir Wajner[1,2] · Angela T. S. Wyse[1] · Carmen Regla Vargas[1,2,5]

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Abstract Cystathionine-β-synthase (CBS) deficiency is the main cause of homocystinuria. Homocysteine (Hcy), methionine, and other metabolites of Hcy accumulate in the body of affected patients. Despite the fact that thromboembolism represents the major cause of morbidity in CBS-deficient patients, the mechanisms of cardiovascular alterations found in homocystinuria remain unclear. In this work, we evaluated the lipid and inflammatory profile, oxidative protein damage, and the activities of the enzymes paraoxonase (PON1) and butyrylcholinesterase (BuChE) in plasma of CBS-deficient patients at diagnosis and during the treatment (protein-restricted diet supplemented with pyridoxine, folic acid, betaine, and vitamin B_{12}). We also investigated the effect of folic acid and vitamin B_{12} on these parameters. We found a significant decrease in HDL cholesterol and apolipoprotein A1 (ApoA-1) levels, as well as in PON1 activity in both untreated and treated CBS-deficient patients when compared to controls. BuChE activity and IL-6 levels were significantly increased in not treated patients. Furthermore, significant positive correlations between PON1 activity and sulphydryl groups and between IL-6 levels and carbonyl content were verified. Moreover, vitamin B_{12} was positively correlated with PON1 and ApoA-1 levels, while folic acid was inversely correlated with total Hcy concentration, demonstrating the importance of this treatment. Our results also demonstrated that CBS-deficient patients presented important alterations in biochemical parameters, possibly caused by the metabolites of Hcy, as well as by oxidative stress, and that the adequate adherence to the treatment is essential to revert or prevent these alterations.

Keywords Homocysteine · Lipid profile · Inflammation · Paraoxonase · Butyrylcholinesterase · Vitamin B_{12} · Folic acid

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Introduction

Homocystinuria is a metabolic disorder characterized by the accumulation of the amino acid homocysteine (Hcy) in biological fluids of affected patients. Cystathionine β-synthase (CBS) deficiency is the most frequently encountered cause of homocystinuria. In addition to Hcy, methionine

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(Met) and a variety of other metabolites of Hcy accumulate in the body or are excreted in the urine of such patients. Dislocation of the optic lens, osteoporosis, thinning and lengthening of the long bones, mental retardation, and thromboembolism affecting large and small arteries and veins are the most common clinical features. However, thromboembolism represents the major cause of morbidity and death in CBS-deficient patients. It is unclear the cause of the cardiovascular changes found in patients with homocystinuria due to CBS deficiency, but evidence suggests that blood coagulation disorders, damage to lipoproteins, or effects on the platelets, on the endothelium and the non-vascular endothelial cells may be involved (Mudd et al. 2001).

Management of CBS-deficient patients leads to amelioration of the characteristic biochemical abnormalities (Mudd et al. 2001). Recognized modalities of treatment include pyridoxine (vitamin B_6) in combination with folic acid and vitamin B_{12}, methionine-restricted diet, cystine-supplemented diet, and betaine (Yap 2003). Treatment of patients with vitamin B_6 in combination with folate or betaine lowers plasma total homocysteine (tHcy) and improves vascular outcomes, suggesting that Hcy or its metabolites play a causal role in aterothrombosis (Yap et al. 2001). Hcy itself, *S*-adenosyl-Hcy, Hcy-thiolactone, and Hcy-thiolactone-modified protein (*N*-Hcy-protein) are potential candidates, and possible mechanisms have been assigned to each (Yap et al. 2001; Jakubowski 2001, 2007; Perla-Kaján et al. 2007). Hcy-thiolactone is a reactive metabolite that causes protein *N*-homocysteinylation what impairs or alters the protein's function (Jakubowski 2008). It was observed that Hcy-thiolactone and *N*-Hcy-protein are elevated in patients with CBS deficiency (Jakubowski et al. 2008). Paraoxonase (PON1), a calcium-dependent enzyme carried on HDL in the blood, hydrolyzes Hcy-thiolactone and protects against the accumulation of *N*-Hcy-protein in vivo and in vitro (Jakubowski et al. 2000; 2001; Perla-Kaján and Jakubowski 2010).

Butyrylcholinesterase (BuChE) is an α-glycoprotein synthesized in the liver. Its enzymatic activity is positively associated with cardiovascular risk factors. Several investigators have found significant relationships between cholinesterase activity and triacylglycerols, HDL cholesterol, and LDL cholesterol (Santarpia et al. 2013). Additionally, the knowledge that chronic inflammation is implicated in the pathogenesis of atherosclerosis established the use of inflammatory markers for assessing coronary risk. Among these biomarkers are pro-inflammatory cytokines such as IL-1, IL-6, and INF-γ (Pearson et al. 2003).

We recently demonstrated that lipid and protein oxidative damage is increased and that antioxidant defenses are reduced in plasma of CBS-deficient patients, probably due to the increase in reactive species generation induced by Hcy (Vanzin et al. 2011). Furthermore, it is known that the enzymes PON1 and BuChE contain SH-groups which are important for their activities (Nishio and Watanabe 1997; Aviram et al. 1998) and that Hcy is able to induce alterations in BuChE activity in vivo and in vitro, as demonstrated by Stefanello et al. (2003, 2005). In this context, the aim of this study was to evaluate lipid (total cholesterol, HDL cholesterol, LDL cholesterol, oxidized LDL cholesterol, apolipoprotein A-1) and inflammatory profile (IL-1β, IL-6, and INF-γ), as well as protein oxidative damage and the activities of the enzymes PON1 and BuChE in plasma of patients with homocystinuria due to CBS deficiency, analyzing the effect of the treatment, especially of folic acid and vitamin B_{12}, on these parameters. Additionally, we correlated all parameters with the total Hcy, folic acid, and vitamin B_{12} levels.

Materials and Methods

Patients and Controls

The present study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil. Informed consent was obtained according to the guidelines of the committee, number 100290.

Patients

Subjects with homocystinuria due to CBS deficiency were recruited from the Medical Genetic Service of Hospital de Clínicas de Porto Alegre, Brazil. Sex, age, and metabolic features of patients are described in Table 1. Plasma samples were obtained from 10 patients at the moment of diagnosis (median age 10 years; range 4–27 years) (Group A) and 10 patients under treatment (median age 19 years; range 12–32 years) (Group B). All patients were diagnosed after the neonatal period by identification of abnormal elevated concentrations of tHcy and Met in plasma. The major clinical manifestations were ectopia lentis, seizures, developmental delay, thinning and lengthening of the long bones (*marfanoid* appearance). The prescribed treatment consisted of a protein-restricted diet supplemented by pyridoxine (median dose: 500 mg/day; range: 100–750 mg/day), folic acid (median dose: 5 mg/day; range 2–5 mg/day), betaine (median dose: 6 g/day; range 2–6 g/day), and vitamin B_{12} (median dose: 1 mg IM/month). The average duration of treatment was 10 years (range 5–20 years).

Controls

Healthy individuals with comparable age and sex of the patients were recruited from the Laboratório de Análises Clínicas da Universidade Federal do Rio Grande do Sul.

Table 1 Clinical and metabolic features of CBS-deficient patients

Patient	Sex	Age at diagnosis (years)	tHcy (μmol/L)	Met (μmol/L)
1*	M	10	238.6	108.3
2*	M	10	189.08	551.1
3*	M	4	195.38	650.4
4*	F	9	354.5	277.0
5*	F	10	318.42	581.3
6*	F	10	341.81	377.5
7*	M	5	316.72	929.5
8*	F	27	245.7	230.5
9*	F	8	197.9	95.3
10*	M	14	393.7	599.3
11	M	12	135.82	60.4
12	M	14	179.6	735.7
13	M	15	29.3	42.0
14	M	19	164.4	822.6
15	F	23	264.8	75.8
16	F	19	48.4	539.1
17	F	28	240.61	66.0
18	M	32	280.96	141.0
19	M	20	165.25	64.2
20	M	18	24.01	21.2

* Patients with homocystinuria at diagnosis (untreated)

Plasma samples were obtained from 13 individuals (median age: 25 years; range 4–34 years).

Plasma Preparation

Plasma was separated from whole blood samples obtained from controls and CBS-deficient patients by venous puncture with heparinized vials. Whole blood was centrifuged at 3000×*g* for 10 min at 4 °C; plasma was removed by aspiration and frozen at −80 °C until analysis.

Lipid Profile

Total Cholesterol Levels

Total cholesterol was measured by a commercial kit (Labtest Diagnóstica, Minas Gerais, Brazil). The results were expressed as mg/dL.

HDL Cholesterol Levels

HDL cholesterol levels were measured by a commercial kit (Labtest Diagnóstica, Minas Gerais, Brazil). The system uses two reagents which enable selective dosage of cholesterol bound to HDL. The results were expressed as mg/dL.

Triglycerides Levels

Triglycerides levels were evaluated by a commercial kit (Labtest Diagnóstica, Minas Gerais, Brazil). The results were expressed as mg/dL.

LDL Cholesterol Levels

The LDL levels were calculated through the Friedewald formula:

$$\text{LDL cholesterol} = \text{Total cholesterol} - \text{HDL cholesterol} - \frac{\text{Triglycerides}}{5}.$$

The results were expressed as mg/dL.

Oxidized LDL Cholesterol Levels

Oxidized LDL cholesterol levels were measured by a commercial kit (Mercodia, Sweden). Sandwich ELISA based on the mouse monoclonal antibody 4E6, which is specific for a conformational epitope in oxidized ApoB-100, was utilized. The results were expressed as U/L.

Apolipoprotein A1 (ApoA-1)

ApoA-1 levels were evaluated by a commercial kit (BioTécnica, Brazil). The determination is based on an

immunoturbidimetric method in which antibodies specific to apolipoprotein A1 form an insoluble complex, and the turbidity produced is proportional to the amount of ApoA1 in sample. The results were expressed as mg/dL.

Inflammatory Profile

Plasma IL-1β, IL-6, and IFN-γ were measured by enzyme-linked immunosorbent assay (ELISA) kits (Mabtech AB, Sweden). The assay utilizes ELISA strip plates pre-coated with a capture monoclonal antibody (mAb), to which samples are added. Captured cytokine is detected by adding a biotinylated mAb followed by streptavidin-horseradish peroxidase. Addition of the enzyme substrate TMB results in a colored product. Intensity of the color is directly proportional to the concentration of cytokine in the sample, which is determined by comparison with a serial dilution of recombinant cytokine standard analyzed in parallel. The results were expressed as pg/mL.

Paraoxonase Activity (PON1)

PON1 enzyme activity was assessed according to Eckerson et al. (1983). Initially, it was prepared a solution containing 4.8 mL buffer glycine/NaOH pH 10.6, 0.9 mM $CaCl_2$, and 1.06 mL of 1.0 mM paraoxon. For each sample, it was utilized 780 µL of this solution and 20 µL of plasma. The absorbance was measured in a spectrophotometer at 412 nm in three times with intervals of 1 min, thus obtaining the release of para-nitrophenol per minute. The blank was assayed the same way, but without the sample, and was subtracted from the absorbances obtained. The results were expressed as U/mL (1 U of enzyme hydrolyzes 1 µmol of paraoxon per minute).

Butyrylcholinesterase Activity (BuChE)

BuChE activity was determined by the method of Ellman et al. (1961) with some modifications. Hydrolysis rate was measured at acetylthiocholine concentration of 0.8 mM in 1 mL assay solutions with 100 mM potassium phosphate buffer pH 7.5 and 1.0 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Fifty microliters of diluted plasma was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25 °C. All samples were run in duplicate. Specific enzyme activity was expressed as mmol acetylthiocholine hydrolyzed per hour per milligram of protein.

Total Homocysteine (tHcy) Measurement

The total homocysteine levels in plasma were measured by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS), as described by Magera et al. (1999). This method is based on the analysis of 100 µL of plasma with 20 µL of homocysteine-d_8 (2 nmol) added as internal standard. After the step of reduction with 20 µL of 500 mM dithiothreitol followed by deproteinization, the analysis was performed in the multiple reaction monitoring mode in which total Hcy and Hcy-d_4 were detected through the transition from the precursor ion (*m/z* 136–*m/z* 90 and *m/z* 140–*m/z* 94, respectively). The retention times of total Hcy and Hcy-d_4 were 1.5 and 2.5 min, respectively. The calibration was performed by a curve with 5 concentrations of Hcy. The results were expressed as µmol/L. In plasma, tHcy is the sum of free and protein-bound homocysteine, homocystine, and several other mixed disulfides.

Folic Acid Measurement

Folic acid levels were measured in plasma by electrochemiluminescence using the analyser Elecsys 2010 (Roche Diagnostics GmbH, Mannheim, Germany). The results were expressed as ng/mL.

Vitamin B_{12} Measurement

Vitamin B_{12} levels were measured in plasma by electrochemiluminescence using the analyser Elecsys 2010 (Roche Diagnostics GmbH, Mannheim, Germany). The results were expressed as pg/mL.

Sulphydryl Groups Content

This assay is based on the reduction of 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Results were reported as nmol TNB.

Carbonyl Content

Carbonyl content was measured according to the method described by Levine et al. (1990). Briefly, duplicate aliquots of plasma (100 µL) were treated with 100µL of trichloroacetic acid 28 %. The tubes were centrifuged at 8000× *g* for 10 min to obtain the protein pellet. One milliliter of 2.4-dinitrophenylhydrazine (DNPH) 10 mM prepared in 2 M HCl or 1.0 mL of 2 M HCl (blank) were added to the precipitates and incubated at 37 °C for 90 min. After, the samples were centrifuged and the DNPH excess was removed with ethanol–ethyl acetate 1:1 (v/v). The final protein pellet was dissolved in 200µL of 6 M guanidine hydrochloride. Quantification was performed using a spectrophotometer at 370 nm. The carbonyl content was calculated using a millimolar absorption coefficient of

the hydrazone (21.000 M^{-1} cm^{-1}). Values of carbonyl content were expressed in nmol carbonyl/mg protein.

Protein Determination

Protein was measured by the method of Bradford (1976), using serum bovine albumin as standard.

Statistical Analysis

Data were expressed as mean ± standard deviation. Comparisons between means were analyzed by one-way ANOVA followed by the Duncan multiple range test when the F value was significant. Correlations between variables were calculated using the Pearson correlation coefficient. A p value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

Results

In this study, we evaluated the lipid and inflammatory profiles, protein oxidation, and the activities of enzymes PON1 and BuChE in plasma of CBS-deficient patients at diagnosis (group A) and under treatment (group B). These parameters were compared to those of controls with similar ages. Furthermore, we correlated all parameters measured with the tHcy, folic acid, and vitamin B_{12} concentrations.

With regard to lipid profile (Table 2), we found a significant decrease in HDL [$F(2.26) = 9.60$, $p < 0.001$] and apolipoprotein A1 [$F(2.29) = 13.00$, $p < 0.001$] levels in both groups of CBS-deficient patients (at diagnosis and under treatment) when compared to controls. LDL and oxidized LDL levels were statistically similar in all groups. Total cholesterol levels were significantly reduced in both groups of CBS-deficient patients [$F(2.25) = 6.88$, $p < 0.01$], probably due to the decrease in HDL levels. Total Hcy levels were significantly increased in patients at diagnosis and during treatment, showing that the therapy approach was not able to adequately control the tHcy levels. Sulphydryl groups content was significantly decreased in both treated or not treated patients [$F(2.23) = 4.94$, $p < 0.05$].

Additionally, the activities of enzymes PON1 and BuChE were evaluated. PON1 activity was decreased in both groups A and B of CBS-deficient patients when compared to controls [$F(2.27) = 11.73$, $p < 0.001$] (Fig. 1). Otherwise, BuChE activity was increased only in the group of untreated patients when compared to controls and to the treated CBS-deficient patients [$F(2.25) = 7.72$, $p < 0.01$] (Fig. 2). We verified significant positive correlations between PON1 activity and sulphydryl groups content ($r = 0.596$, $p < 0.05$) (Fig. 3) and, as expected, between HDL and apolipoprotein A-1 levels ($r = 0.589$, $p < 0.05$) (Fig. 4).

Next, we evaluated the inflammatory profile in both groups of CBS-deficient patients (Table 3). We demonstrated that IL-6 was significantly higher in group A when compared to controls [$F(2.26) = 3.897$, $p < 0.05$]. It was verified a tendency to reduction (27.68 %) in the IL-6 levels in group B when compared to group A. Similar results were observed in carbonyl groups formation, which were significantly higher in group A when compared to controls [$F(2.21) = 3.565$, $p < 0.05$], presenting a tendency to reduction (15,2 %) in group B when compared to group A. We found a significant positive correlation between IL-6 levels and carbonyl groups content ($r = 0.551$, $p < 0.05$) (Fig. 5), indicating a possible association between inflammation and oxidative protein damage in plasma of CBS-deficient patients. Additionally, we also evaluated the IL-1β and INF-γ

Table 2 Lipid profile, total Hcy levels, and sulphydryl content in CBS-deficient patients at diagnosis (Group A), CBS-deficient patients under treatment (Group B) and controls

	Controls	Group A	Group B
Total cholesterol (mg/dl)	185.62 ± 56.53	118.75 ± 38.48*	125.78 ± 28.36*
HDL cholesterol (mg/dl)	59.35 ± 19.43	38.98 ± 9.62**	33.27 ± 9.99**
LDL cholesterol (mg/dl)	106.65 ± 53.95	71.14 ± 30.45	78.76 ± 29.21
Oxidized LDL cholesterol (U/L)	19.15 ± 7.44	14.77 ± 4.76	13.12 ± 5.75
Apolipoprotein A (mg/dL)	200.98 ± 29.57	156.32 ± 33.81**	141.34 ± 20.79**
tHcy (μmol/L)	5.84 ± 2.52	266.5 ± 66.71***	137.8 ± 104.3*
Sulphydryl content (nmol TNB)	384.91 ± 82.78	247.99 ± 92.52#	282.66 ± 112.11#

Data represent mean ± standard deviation

# $p < 0.05$ Statistically different from controls (ANOVA, followed by the Duncan multiple range test)

* $p < 0.01$ Statistically different from controls (ANOVA, followed by the Duncan multiple range test)

** $p < 0.001$ Statistically different from controls (ANOVA, followed by the Duncan multiple range test)

*** $p < 0.0001$ Statistically different from controls (ANOVA, followed by the Duncan multiple range test)

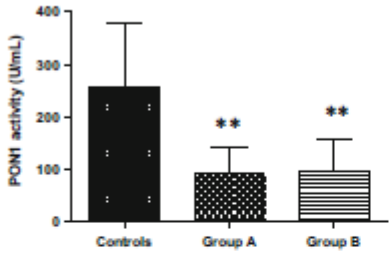

Fig. 1 PON1 activity in CBS-deficient patients at diagnosis (*Group A*, $n = 9$), CBS-deficient patients under treatment (*Group B*, $n = 9$) and controls ($n = 12$). Data represent mean ± standard deviation, ** $p < 0.001$ statistically different from controls (ANOVA, followed by the Duncan multiple range test)

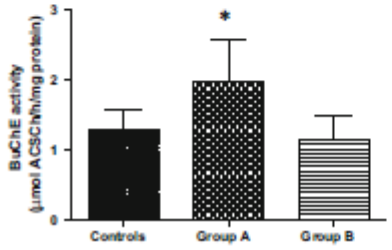

Fig. 2 BuChE activity in CBS-deficient patients at diagnosis (*Group A*, $n = 8$), CBS-deficient patients under treatment (*Group B*, $n = 8$) and controls ($n = 11$). Data represent mean ± standard deviation, *$p < 0.01$ statistically different from controls and from *group B* (ANOVA, followed by the Duncan multiple range test)

levels which were statistically similar in all groups (patients and controls).

Finally, we correlated all parameters investigated with the folic acid and vitamin B_{12} concentrations. We found a significant positive correlation between vitamin B_{12} and apolipoprotein A-1 levels ($r = 0.535$, $p < 0.05$) (Fig. 6), as well as a significant positive correlation between vitamin B_{12} levels and PON1 ($r = 0.631$, $p < 0.05$) (Fig. 7). Additionally, we demonstrated a significant negative correlation between folic acid and total Hcy concentrations ($r = -0.633$, $p < 0.05$) (Fig. 8). Folic acid was increased in treated patients (16.59 ± 5.03 ng/mL; mean ± standard deviation) when compared with not treated patients (7.85 ± 5.11 ng/mL; mean ± standard deviation) [$t(18) = 3.541$, $p < 0.01$], as expected. On the other hand, vitamin B_{12} levels were similar in both groups of patients (299,25 ± 150,88 pg/mL before treatment versus 330,32 ± 185,68 pg/mL after treatment; mean ± standard deviation) [$t(21) = 0.964$, $p > 0.05$], indicating that these patients presented a poor adherence to the treatment.

Discussion

A link between Hcy and atherothrombotic vascular disease was first suggested by McCully (1969) more than 40 years ago. Since that time, many investigations have been conducted to define the cause(s) of atherosclerosis in CBS-deficient patients. Findings suggest that Hcy or its derivatives other than Met (for example homocysteine-thiolactone) may be the major contributor(s) to the vascular damage of CBS deficiency (Mudd et al. 2001). In this sense, we evaluated in this work the lipid (total cholesterol, HDL cholesterol, LDL cholesterol, oxidized LDL cholesterol, apolipoprotein A1) and the inflammatory (IL-6, IL-1β, IFN-γ) profiles, protein oxidative damage (sulphydryl and carbonyl groups content), and the activities of enzymes PON1 and BuChE in plasma of CBS-deficient patients, treated and not treated (at diagnosis). We also correlated these measurements with tHcy, folic acid, and vitamin B_{12} concentrations.

The atherogenicity of Hcy may involve several mechanisms including LDL cholesterol oxidative modification and HDL cholesterol decrease (Xiao et al. 2011). Low HDL cholesterol is a strong independent predictor of coronary artery disease (CAD) when its level are <40 mg/dL (JAMA 2001). Studies reported that Hcy is able to inhibit ApoA-1 expression and decrease HDL cholesterol levels in vitro and in vivo (animal model) (Liao et al. 2006; Mikael et al. 2006). ApoA-1 is the major protein component of HDL cholesterol and it is known that the protective effect of ApoA-1 may be related to its association with increased HDL production. Case–control studies have provided supporting data showing that plasma total Hcy was negatively correlated with HDL cholesterol levels in patients with myocardial infarction (Qujeq et al. 2001). An effect of Hcy on HDL metabolism could be clinically important, because HDL protects against vascular disease not only by facilitating reverse cholesterol transport but also through its direct anti-inflammatory properties (Devlin and Lentz 2006). In our work, we found a reduction of HDL and ApoA-1 plasma levels in CBS-deficient patients when compared to controls. An interesting fact is the failure of the treatment to prevent or reverse these reductions, since both groups of patients, at diagnosis and during treatment, presented a decrease of HDL and ApoA-1 plasma levels. It was observed in this study a significant correlation between vitamin B_{12} and Apo A-1 levels, as well as between vitamin B_{12} and PON1. These findings suggest that vitamin B_{12} could be essential to increase the Apo A-1 levels and PON1 activity in CBS-deficient patients, which is interesting since these components demonstrate important atheroprotective effects (Liao et al. 2006; Mikael et al. 2006; Devlin and Lentz 2006) that could, at least in part,

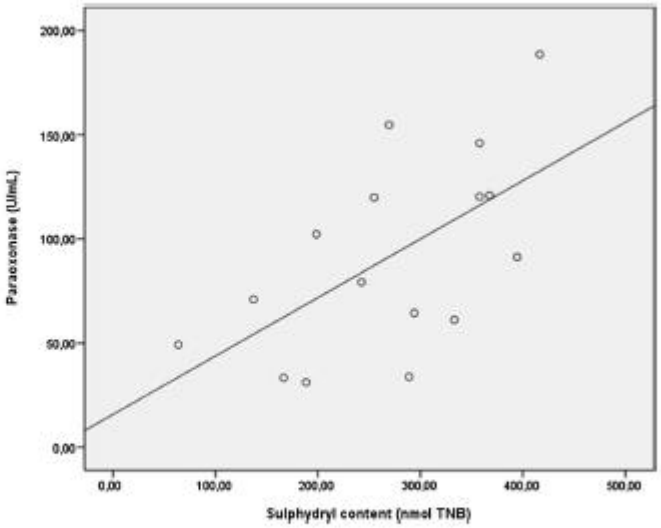

Fig. 3 Correlation between PON1 activity and sulphydryl groups content ($r = 0.596$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

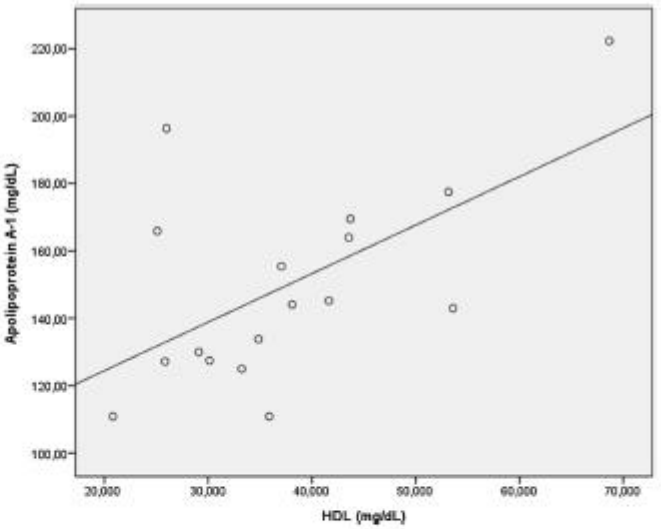

Fig. 4 Correlation between HDL levels and apolipoprotein A-1 levels ($r = 0.589$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

decrease or revert the vascular alterations found in not treated CBS-deficient patients. It is important to emphasize that probably the treated patients involved in this study have a poor adherence to the treatment with vitamin B_{12}, which was prescribed to patients in a dose of 1 mg/month intramuscularly. More studies evaluating the effect of vitamin B_{12} in patients with good adherence to therapy are necessary.

Our results are different from those found by Jiang et al. (2012) who demonstrated that ApoA-1 was decreased only in not treated CBS-deficient patients. The treatment, in the patients studied by Jiang et al. was based in protein

Table 3 Inflammatory profile and carbonyl content in CBS-deficient patients at diagnosis (Group A), CBS-deficient patients under treatment (Group B) and controls

	Controls	Group A	Group B
Interleukin-6 (pg/mL)	11.74 ± 3.54	22.87 ± 9.81*	16.54 ± 9.16
Interleukin-1β (pg/mL)	19.47 ± 12.49	17.23 ± 12.41	15.99 ± 11.63
Interferon-γ (pg/mL)	50.22 ± 12.91	51.40 ± 16.15	59.46 ± 25.16
Carbonyl content (nmol/mg protein)	0.278 ± 0.038	0.355 ± 0.091*	0.303 ± 0.366

Data represent mean ± standard deviation

* $p < 0.05$ statistically different from controls (ANOVA, followed by the Duncan multiple range test)

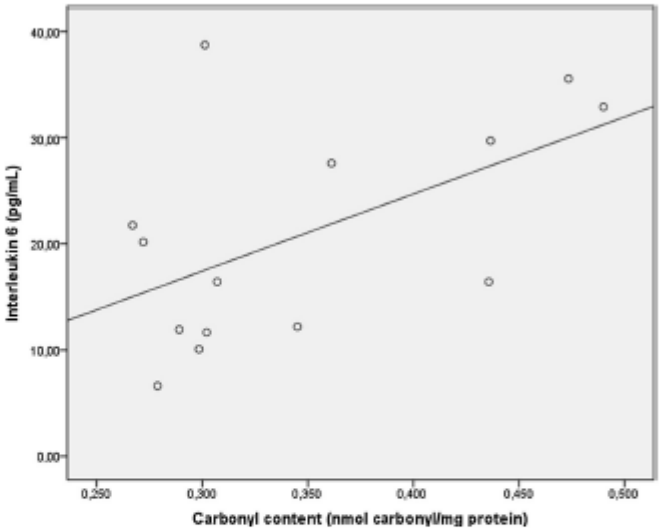

Fig. 5 Correlation between IL-6 levels and carbonyl groups content ($r = 0.551$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

restriction in addiction to cysteine and betaine supplementation. In the present work, the patients were treated by a protein-restricted diet supplemented by pyridoxine, folic acid, betaine, and vitamin B_{12}, without cysteine supplementation. This can partially explain the differences found in the two studies, since a previous study demonstrated that plasma levels of ApoA-1 were positively associated with plasma cysteine levels (Nuño-Ayala et al. 2010). Besides, in the work of Jiang et al. (2012), treated patients presented total Hcy concentrations between 24.5 and 86.1 μM, while the patients of our study had much higher levels of this metabolite.

It is possible that the effects observed on ApoA-1 and HDL cholesterol could be attributed to any metabolite of Hcy, such as Hcy-thiolactone. Hcy-thiolactone is a product of an error-editing reaction in protein biosynthesis which is formed when Hcy is mistakenly selected by methionyl-tRNA synthetase. Pathophysiological consequences of protein *N*-homocysteinylation include, among other things, protein and cell damage (Jakubowski 2008). A recent study demonstrated that *N*-Hcy-ApoA1 is present in humans (Ishimine et al. 2010). In this sense, it is important to emphasize that the adherence to the treatment is essential to reduce the tHcy levels and, consequently, to reduce the Hcy-thiolactone production, as well as their harmful effects. It is important to emphasize that folic acid supplementation in the patients of our study induced a decrease in tHcy levels, since a negative correlation was observed between this compound and Hcy, although this amino acid concentration remained higher than those expected to an adequate treatment.

In a recent work, we demonstrated that oxidative protein damage occurs in plasma of CBS-deficient patients at diagnosis and during the treatment probably due to the high tHcy levels (Vanzin et al. 2011). In this work, we found a significant positive correlation between sulphydryl groups content, a biomarker inversely proportional to protein oxidative damage, and PON1 activity. Moreover, we verified

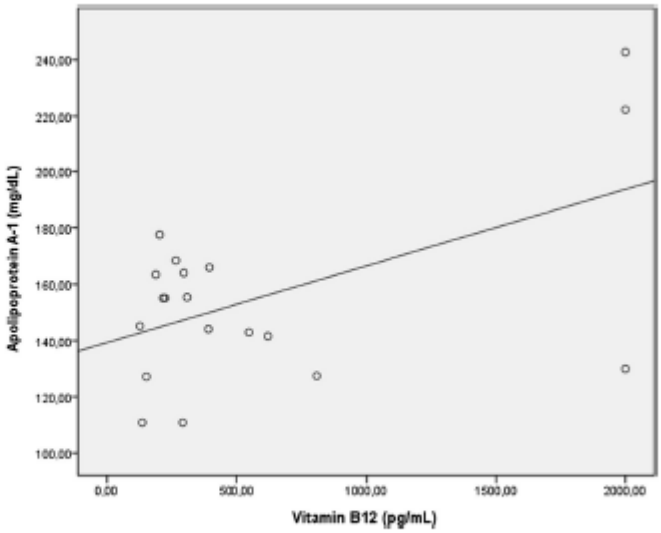

Fig. 6 Correlation between vitamin B_{12} levels and apolipoprotein A-1 levels ($r = 0.535$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

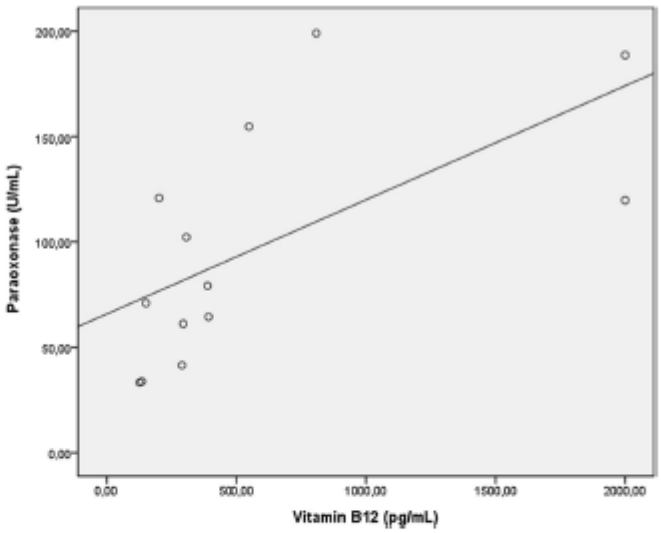

Fig. 7 Correlation between vitamin B_{12} levels and PON1 ($r = 0.631$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

a reduction in PON1 activity in the both groups of CBS-deficient patients (at diagnosis and under treatment) when compared to controls. PON1, a component of HDL cholesterol, is a calcium-dependent multifunctional enzyme that connects the metabolism of lipoproteins and Hcy. Due to its ability to reduce oxidative stress, PON1 contributes to atheroprotective functions of HDL in mice and humans. Furthermore, PON1 has the ability to hydrolyze a variety of substrates, including Hcy-thiolactone (Perla-Kaján and Jakubowski 2012). In humans, Hcy-

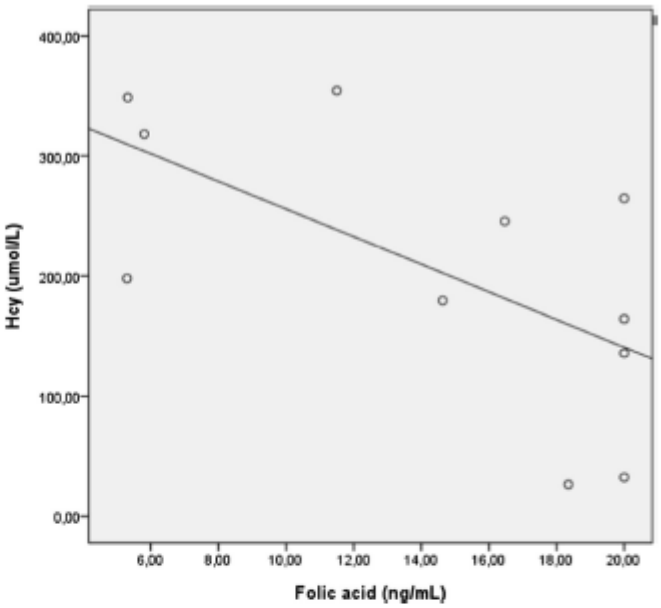

Fig. 8 Correlation between folic acid and total Hcy levels ($r = -0.633$, $p < 0.05$) in plasma from CBS-deficient patients at diagnosis and under treatment

thiolactonase activity of PON1 protects against *N*-homocysteinylation in vivo and in vitro (Jakubowski et al. 2000; Perla-Kaján and Jakubowski 2010). In this context, we can hypothesize that the decrease in PON1 activity found in the CBS-deficient patients included in our study could be caused by Hcy-dependent oxidation of any sulfhydryl group important to the PON1 catalytic activity (Aviram et al. 1998). PON's free sulfhydryl at cysteine-283 is required for its antioxidant activity. It is plausible, therefore, to hypothesize that the proatherogenic effects of Hcy may involve diminished serum PON1 activity, leading to impaired antioxidant function and decreased capacity to degrade Hcy-thiolactone. Furthermore, it was demonstrated that plasma tHcy remains high in patients during the treatment (137.8 ± 104.3 μmol/L; mean ± standard deviation). Additionally, as discussed before, we found a positive significant correlation between vitamin B_{12} levels and PON1 activity, suggesting that the fail of treatment in reversing the PON1 activity may be, at least in part, due to the insufficient doses of vitamin B_{12} used in the therapy, caused probably by a low adherence to the treatment. Recent studies demonstrating the effects of vitamin B_{12} supplementation on paraoxonase activity reinforce our hypothesis (Gu et al. 2007; Weijun et al. 2008; Koc et al. 2012). Weijun et al. (2008) demonstrated an increase of 17.59 % in HTase/PON activity in patients with type 2 diabetes after supplementation with folic acid (5 mg/day) plus vitamin B_{12} (500 μg/day, intramuscularly). These authors also found a significant inverse correlation between the changes in HTase/PON activity and Hcy levels. Additionally, Koc et al. (2012) found a decrease in PON activity in patients with vitamin B_{12} deficiency anemia, whereas PON activity was significantly increased after treatment with vitamin B_{12}.

There is increasing evidence that acute coronary syndromes are related to activation of the immune-mediated inflammatory process associated with atherosclerotic plaques. Oxidized low-density lipoprotein (ox-LDL) is thought to play a key role in the genesis of the inflammatory process in atherosclerotic lesions (Ehara et al. 2001). In this sense, we evaluated the LDL and ox-LDL levels in plasma of both groups of CBS-deficient patients. We did not find significant differences in LDL and ox-LDL levels between patients and controls. The influence of Hcy on LDL cholesterol levels is unclear; it is known that Hcy-thiolactone can cause *N*-homocysteinylation in LDL, forming *N*-Hcy-LDL, which tends to form aggregates in vitro and induces cell death in human endothelial cells (Jakubowski 2008). Thereby, it is possible to suggest that Hcy can be acting through the formation of *N*-Hcy-LDL instead of ox-LDL in the CBS-deficient patients studied.

Considering that a pro-inflammatory state associated with hyperhomocysteinemia has been demonstrated by several authors (Gori et al. 2005; da Cunha et al. 2010; Keating et al. 2011), we evaluated in this study the IL-6, IL-1β, and INF-γ levels in both groups of CBS-deficient patients and controls. We found a significant increase in IL-6 levels in group A when compared to controls and a tendency to reduction (27.68 %) in the IL-6 levels in group B when compared to group A. Furthermore, we demonstrated a significant positive correlation between IL-6 levels and carbonyl groups content, indicating a possible association between inflammation and protein oxidative damage in plasma of CBS-deficient patients. Carbonyls groups are relatively difficult to induce compared with methionine sulphoxide and cysteinyl derivatives and might, therefore, indicate a more severe oxidative stress. Indeed, elevated levels of protein carbonyl are generally a sign not only of oxidative stress but also of disease-derived protein dysfunction (Dalle-Donne et al. 2003). High carbonyl content has also been observed in diabetes and arteriosclerosis and has been implicated in the accelerated vascular damage observed in these conditions (Singh et al. 2001). Additionally to IL-6, we also evaluated the IL-1β and INF-γ levels which were statistically similar in all groups (patients and controls). Our results are close to those found by Keating et al. (2011) who demonstrated an increase in IL-6 levels in homocystinuric patients before the treatment compared to treated homocystinuric patients, as well as no changes in IL-1β levels in both groups of patients.

Finally, we evaluated in this work the BuChE activity in CBS-deficient patients. It was verified that plasma BuChE activity was increased in the group of CBS-deficient patients at diagnosis when compared to controls and patients under treatment. BuChE is present in all tissues, including serum, vascular endothelia, and nervous system (Prody et al. 1987; Mack and Robitzki 2000). Preclinical studies showed that neonates rats subjected to Hcy (400–500 μmol/L) administration presented a decrease in serum BuChe activity in vitro and in vivo (Stefanello et al. 2003, 2005). Nevertheless, the effect of Hcy and its metabolites on human cholinesterases is not well understood. BuChE seems to be involved in the pathophysiology of the metabolic syndrome. Its enzymatic activity is positively associated with cardiovascular risk factors and several investigators have found significant relationships between cholinesterase activity and triacylglycerols, HDL cholesterol and LDL cholesterol (Santarpia et al. 2013). Interestingly, Darvesh et al. (2007) demonstrated that the incubation of Hcy-thiolactone with BuChE produced an immediate stimulation of the activity of this enzyme. The authors suggested that a constant stimulation of BuChE by Hcy-thiolactone could be expected to decrease acetylcholine levels. Low acetylcholine levels are known to be responsible for symptoms in Alzheimer and vascular diseases. In this work, only CBS-deficient patients at diagnosis presented increased BuChE activity, while treated CBS-deficient patients presented BuChE activity similar to controls. It is important to emphasize that the treatment based on protein-restricted diet supplemented by pyridoxine, folic acid, betaine, and vitamin B_{12} was able to decrease the BuChE activity, demonstrating positive results. The data of this work showing alterations in BuChE activity in plasma of CBS-deficient patients are pioneers in the literature.

In conclusion, analyzing all the aspects above discussed, we can suggest that Hcy and/or its metabolites can cause important alterations in the metabolism of CBS-deficient patients that include a decrease in PON1 activity, in HDL, and in ApoA-1 levels, as well as alterations in BuChE activity, in IL-6 levels, and induction of protein oxidative damage. These findings appear to be interconnected and correlated with protein oxidative damage. Furthermore, vitamin B_{12} supplementation seems to be important to improve PON1 activity and ApoA-1 levels, probably contributing to the atheroprotective effects of these components, as well as folic acid demonstrated to be essential to decrease the total Hcy levels. This work contributes to the understanding of the responsible mechanisms of vascular lesions in CBS-deficient patients and creates perspectives to future works.

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Conflict of interest The authors declare that there is no conflict of interest disclosure associated with this manuscript.

References

Aksenov MY, Markesbery WR (2001) Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302:141–145

Aviram M, Billecke S, Sorenson R, Bisgaier CL, Newton RS, Rosenblat M et al (1998) Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different than that required for its arylesterase/paraoxonase activities: selective action of human paraoxonase allozymes Q and R. Arterioscler Thromb Vasc Biol 18:1617–1624

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-die-binding. Anal Biochem 72:248–254

da Cunha AA, Ferreira AG, Wyse AT (2010) Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration. Metab Brain Dis 25:199–206

Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A (2003) Protein carbonylation in human diseases. Trends Mol Med 9:169–176

Darvesh S, Walsh R, Martin E (2007) Homocysteine thiolactone and human cholinesterases. Cell Mol Neurobiol 27:33–48

Devlin AM, Lentz SR (2006) ApoA-I : a missing link between homocysteine and lipid metabolism? Circ Res 98:431–433

Eckerson HW, Wyte CM, La Du BN (1983) The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. Am J Hum Genet 35:214–227

Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M (2001) Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 103:1955–1960

Ellman GL, Courtney KD, Andres V, Feather-Stone RM (1961) A new and rapid determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) (2001) Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). JAMA 285:n19

Gori AM, Corsi AM, Fedi S, Gazzini A, Sofi F, Bartali B et al (2005) A proinflammatory state is associated with hyperhomocysteinemia in the elderly. Am J Clin Nutr 82:335–341

Gu WJ, Lu JM, Yang GQ, Guo QH, Dou JT, Mu YM et al (2007) Effects of intervention therapy of methylcobalamin and folic acid on plasma homocysteine concentration and homocysteine thiolactonase/paraoxonase activity in patients with type 2 diabetes mellitus. Zhonghua Yi Xue Za Zhi 87:256–258

Ishimine N, Usami Y, Nogi S, Sumida T, Kurihara Y, Matsuda K et al (2010) Identification of N-homocysteinylated apolipoprotein AI in normal human serum. Ann Clin Biochem 47:453–459

Jakubowski H (2001) Protein N-homocysteinilation: implications for atherosclerosis. Biomed Pharmacother 55:443–447

Jakubowski H (2007) The molecular basis of homocysteine thiolactone-mediated vascular disease. Clin Chem Lab Med 45:1704–1716

Jakubowski H (2008) The pathophysiological hypothesis of homocysteine thiolactone-mediated vascular disease. J Physiol Pharmacol 59:155–167

Jakubowski H, Zhang L, Bardeguez A, Aviv A (2000) Homocysteine thiolactone and protein homocysteinylation in human endothelial cells: implications for atherosclerosis. Circ Res 87:45–51

Jakubowski H, Ambrosius WT, Pratt JH (2001) Genetic determinants of homocysteine thiolactonase activity in humans: implications for atherosclerosis. FEBS Lett 491:35–39

Jakubowski H, Boers GH, Strauss KA (2008) Mutations in cystathionine β-synthase or methylenetetrahydrofolate reductase gene increase N-homocysteinylated protein levels in humans. FASEB J 22:4071–4076

Jiang H, Stabler SP, Allen RH, Maclean KN (2012) Altered expression of apoA-I, apoA-IV and PON-1 activity in CBS deficient homocystinuria in the presence and absence of treatment: possible implications for cardiovascular outcomes. Mol Genet Metab 107:55–65

Keating AK, Freehauf C, Jiang H, Brodsky GL, Stabler SP, Allen RH et al (2011) Constitutive induction of pro-inflammatory and chemotactic cytokines in cystathionine beta-synthase deficient homocystinuria. Mol Genet Metab 103:330–337

Koc A, Cengiz M, Ozdemir ZC, Celik H (2012) Paraoxonase and arylesterase activities in children with iron deficiency anemia and vitamin B_{12} deficiency anemia. Pediatr Hematol Oncol 29:345–353

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG et al (1990) Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186:464–478

Liao D, Tan H, Hui R, Li Z, Jiang X, Gaubatz J et al (2006) Hyperhomocysteinemia decreases circulating high-density lipoprotein by inhibiting apolipoprotein A-I protein synthesis and enhancing HDL cholesterol clearance. Circ Res 99:598–606

Mack A, Robitzki A (2000) The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5′-butyrylcholinesterase-DNA study. Prog Neurobiol 60:607–628

Magera MJ, Lacey JM, Casetta B, Rinaldo P (1999) Method for the determination of total homocysteine in plasma and urine by stable isotope dilution and electrospray tandem mass spectrometry. Clin Chem 45:1517–1522

McCully KS (1969) Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 56:111–128

Mikael LG, Genest J Jr, Rozen R (2006) Elevated homocysteine reduces apolipoprotein A-I expression in hyperhomocysteinemic mice and in males with coronary artery disease. Circ Res 98:564–571

Mudd SH, Levy HL, Kraus JP (2001) Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited disease. McGraw-Hill, New York, pp 2007–2056

Nishio E, Watanabe Y (1997) Cigarette smoke extract inhibits plasma paraoxonase activity by modification of the enzyme's free thiols. Biochem Biophys Res Commun 236:289–293

Nuño-Ayala M, Guillén N, Navarro MA, Lou-Bonafonte JM, Arnal C, Gascón S et al (2010) Cysteinemia, rather than homocysteinemia, is associated with plasma apolipoprotein A-I levels in hyperhomocysteinemia: lipid metabolism in cystathionine beta-synthase deficiency. Atherosclerosis 212:268–273

Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO 3rd, Criqui M et al (2003) Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals From the Centers for Disease Control and Prevention and the American Heart Association. Circulation 107:499–511

Perla-Kaján J, Jakubowski H (2010) Paraoxonase 1 protects against protein *N*-homocysteinylation in humans. FASEB J 24:931–936

Perla-Kaján J, Jakubowski H (2012) Paraoxonase 1 and homocysteine metabolism. Amino Acids 43:1405–1417

Perla-Kaján J, Twardowski T, Jakubowski H (2007) Mechanisms of homocysteine toxicity in humans. Amino Acids 32:561–572

Prody CA, Zevin-Sonkin D, Gnatt A, Goldberg O, Soreq H (1987) Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues. Proc Natl Acad Sci USA 84:3555–3559

Qujeq D, Omran TS, Hosini L (2001) Correlation between total homocysteine, low density lipoprotein cholesterol and high-density lipoprotein cholesterol in the serum of patients with myocardial infarction. Clin Biochem 34:97–101

Santarpia L, Grandone I, Contaldo F, Pasanisi F (2013) Butyrylcholinesterase as a prognostic marker: a review of the literature. J Cachexia Sarcopenia Muscle 4:31–39

Singh R, Barden A, Mori T, Beilin L (2001) Advanced glycation end-products: a review. Diabetologia 44:129–146

Stefanello FM, Zugno AI, Wannmacher CMD, Wajner M, Wyse ATS (2003) Homocysteine inhibits butyrylcholinesterase activity in rat serum. Metab Brain Dis 18:187–194

Stefanello FM, Franzon R, Tagliari B, Wannmacher CMD, Wajner M, Wyse ATS (2005) Reduction of butyrylcholinesterase activity in rat serum subjected to hyperhomocysteinemia. Metab Brain Dis 20:97–103

Vanzin CS, Biancini GB, Sitta A, Wayhs CAY, Pereira IN, Rockenbach F et al (2011) Experimental evidence of oxidative stress in plasma of homocystinuric patients: a possible role for homocysteine. Mol Genet Metab 104:112–117

Weijun G, Juming L, Guoqing Y, Jingtao D, Qinghua G, Yiming M et al (2008) Effects of plasma homocysteine levels on serum

HTASE/PON activity in patients with type 2 diabetes. Adv Ther 25:884–893

Xiao Y, Zhang Y, Lv X, Su D, Li D, Xia M et al (2011) Relationship between lipid profiles and plasma total homocysteine, cysteine and the risk of coronary artery disease in coronary angiographic subjects. Lipids Health Dis 10:137

Yap S (2003) Classical homocystinuria: vascular risk and its prevention. J Inherit Metab Dis 26:259–265

Yap S, Boers GHJ, Wilcken B, Wilcken DEL, Brenton DP, Lee PJ et al (2001) Vascular outcome in patients with homocystinuria due to cystathionine ß-synthase deficiency treated chronically: a multicenter observational study. Arterioscler Thromb Vasc Biol 21:2080–2085

3.2 Capítulo II – Artigo 02

Increase of oxidative stress parameters and nitrite levels in treated CBS-deficient patients and the *in vitro* effect of *N*-acetyl-L-cysteine upon DNA damage caused by high levels of homocysteine.

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Periódico: Molecular and Cellular Biochemistry

Status: Submetido

Increase of oxidative stress parameters and nitrite levels in treated CBS-deficient patients and the *in vitro* effect of N-acetyl-L-cysteine upon DNA damage caused by high levels of homocysteine.

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Abstract:

Tissue accumulation of homocysteine (Hcy) occurs in a metabolic disease characterized biochemically by cystathionine β-synthase (CBS) deficiency and clinically by mental retardation, vascular problems and skeletal abnormalities. Previous studies have been shown a relationship between Hcy and oxidative stress in brain and other tissues of animal models, as well as in plasma of CBS-deficient patients. In this work we evaluated oxidative stress biomarkers and nitrite levels in urine, sulphydryl groups content in plasma and activity of catalase in blood of CBS-deficient patients, as well as the *in vitro* effect of *N*-acetyl-L-cysteine (NAC) on DNA damage caused by high levels of Hcy. It was found an increase in parameters of lipid (15-F2t-isoprostanes levels) and protein (di-tyrosine levels) oxidative damage, as well as an increase in nitrite levels in urine from CBS-deficient patients when compared to controls. Moreover, it was demonstrated a significant positive correlation between 15-F2t-isoprostanes levels and total Hcy levels and a significant negative correlation between di-tyrosine and sulphydryl groups content. Additionally, it was observed an increase in catalase activity in blood of CBS-deficient patients when compared to controls and a protector *in vitro* effect of NAC at concentrations 1 and 5 mM on DNA damage caused by Hcy 50μM and 200μM. These results, when evaluated together, suggest a possible involvement of oxidative stress-mediated by Hcy in pathogenesis of CBS deficiency, highlighting the beneficial action of NAC therapy for these patients.

1. Introduction:

Cystathionine beta synthase (CBS) deficiency, classic homocystinuria, results in elevated levels of circulating Hcy and methionine, S-adenosylmethionine and S-adenosyl homocysteine, and reduced circulating cystathionine and cysteine [1]. The reported incidence of CBS deficiency varies from 1 in 344.000 worldwide to 1 in 65.000 in Ireland [2].

Hcy is a sulphydryl amino acid that oxides readily to its disulphide form homocystine. In normal plasma, Hcy exists in various forms: the sulphydryl form (approximately 1%), bound to the cysteine residues of proteins (approximately 70%), and bound to free cysteine as cysteine-Hcy mixed disulphide (approximately 30%). When the levels are elevated, the disulphide form homocystine is formed [3]. Renal tubular reabsorption of homocystine is very inefficient in patients with severe untreated CBS deficiency, more than 1 mmol of this disulfide may be excreted each day [1]. Normal plasma total Hcy (tHcy) values are less than 15 µmol/L, whereas most untreated CBS-deficient patients exhibit levels above 200 µmol/L. In plasma, tHcy is the sum of free and protein-bound homocysteine, homocystine, and several others mixed disulfides [4].

CBS deficiency is inherited as an autosomal recessive trait. Some patients have small residual activities of CBS, whereas others have no such activities detected by even the most sensitive methods [1]. The eye, skeleton, central nervous system and vascular system are all involved in the typical presentation. The patient is normal at birth and, if not treated, progressively develops the full clinical picture [4]. Recognized modalities of treatment include pyridoxine, in combination with folic acid and/or vitamin B_{12} and/or betaine; and methionine-restricted diet supplemented with cysteine [2]. Probably, both

early diagnosis and strict compliance to treatment will change the natural history of cardiovascular and mental symptoms even in pyridoxine-nonresponsive individuals. Pathophysiology of CBS deficiency has not been completely elucidated, but accumulation of tHcy probably plays a major role in determining some of the most relevant clinical manifestations [4]. We demonstrated in a recent study the importance of vitamin B_{12} upon some biochemical parameters, as well as of folic acid in reducing tHcy levels in plasma of CBS-deficient patients [5].

Free radicals and other reactive species are widely believed to contribute to the development of several diseases, causing 'nitrative and oxidative stress' and 'oxidative damage' [6]. For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients [7-9]. Other diseases in which oxidative damage has been implicated include cancer, atherosclerosis, other neurodegenerative diseases and diabetes [10-15]. Oxidative damage has been proposed as an important pathogenic feature of various inborn errors of metabolism, including organic acidurias, phenylketonuria, maple syrup urine disease and X-linked adrenoleukodystrophy [16-23]. With regard to homocystinuria, several studies realized in animal models show the involvement of oxidative stress [24-26]. Moreover, we recently demonstrated that lipid and protein oxidative damage is increased and the antioxidant defenses diminished in plasma of CBS-deficient patients, probably due to increase of reactive species generation induced by tHcy [27].

Besides, it was demonstrated that occurs DNA damage in leukocytes from CBS-deficient patients; and it was evidenced a concentration-dependent effect of Hcy on DNA damage (*in vitro* study), reinforcing the hypothesis that the Hcy could be, at least in part, responsible for the elevated DNA damage found in the CBS-deficient patients [28]. Studies have shown that Hcy can induce DNA damage [29;30], suggesting a

further mechanism by which increased levels of Hcy may contribute to the pathogenesis of the atherosclerosis and neurodegenerative diseases. In addition, recent observations indicate that plasma Hcy levels are positively correlated with baseline levels of genetic damage, as measured by the cytokinesis-block micronucleus (MN) assay [31;32].

In this sense, antioxidants can be an important alternative to prevent or revert the harmful effects of Hcy and can be tested as an adjuvant therapy for homocystinuria due to CBS deficiency. The thiol *N*-acetyl-L-cysteine (NAC) could be one of these antioxidants, since is readily deacetylated in cells to yield L-cysteine thereby promoting intracellular reduced glutathione (GSH) synthesis. Reduced glutathione plays a central physiological role in maintaining the body homeostasis and in protecting cells against oxidants, toxicants, DNA-damaging agents and carcinogens of either exogenous or endogenous source. Besides acting as a GSH precursor, NAC is, *per se*, responsible for protective effects in the extracellular environment, mainly due to its nucleophilic and antioxidant properties, which influence the toxicokinetics of xenobiotics [33].

Considering the points raised above, in this work we extended our previous investigations, analyzing oxidative stress parameters and nitrite levels in urine from CBS-deficient patients. Moreover, we evaluated sulphydryl groups content and catalase activity in blood of CBS-deficient patients, as well as the *in vitro* DNA damage and the effect of NAC on this damage caused by high Hcy levels. Finally, we correlate the biochemical parameters in urine with the tHcy levels.

2. Materials and Methods

2.1 *In vivo* study

2.1.1 Patients and controls:

Subjects with homocystinuria due CBS deficiency were recruited from the Medical Genetic Service of Hospital de Clínicas de Porto Alegre, Brazil. Plasma and urine samples were obtained from 9 CBS-deficient patients (median age: 17 years; range: 11 – 33 years) and 9 healthy individuals with comparable age and sex (median age: 22 years; range: 15 – 30 years). All patients were using the treatment, which consisted of a protein-restricted diet supplemented by pyridoxine (median dose: 500mg/day; range: 100 – 750mg/day), folic acid (median dose: 5mg/day; range: 2 – 5mg/day), betaine (median dose: 6g/day; range: 2 – 6g/day), vitamin B_{12} (median dose: 1mg IM /month). The average duration of treatment was 13 years (range: 1 – 22 years). The tHcy average levels presented by treated CBS-deficient patients was 191.3 ± 105.3 μmol/L (mean ± standard deviation).

All the patients were diagnosed by identification of abnormal elevated concentrations of tHcy and Met in plasma. Clinical presentation was predominantly characterized by *ectopia lentis*, seizures, developmental delay, thinning and lengthening of the long bones (*marfanoid* appearance).

Healthy individuals with comparable age and sex were recruited from the Laboratório de Análises Clínicas da Universidade Federal do Rio Grande do Sul. Plasma samples were obtained from 9 individuals (median age: 22 years; range: 15 – 30 years).

The present study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil, number 100290. Informed consent was obtained according to the guidelines of this committee.

2.1.2 Urine preparation

Urine was obtained from individuals (controls and CBS-deficiency patients) and frozen at -80ºC until analysis.

2.1.3 Blood preparation

Whole blood samples were obtained from individuals (controls and CBS-deficient patients) by venous puncture with heparinized vials. Whole blood was centrifuged at 3,000 xg for 10 min at 4ºC, plasma was removed by aspiration and frozen at -80ºC until analysis. Erythrocytes were washed three times with cold saline solution (sodium chloride 0.153 M). Lysates were prepared by addition of 1 mL of distilled water to 100 μL of erythrocytes, and were frozen at −80°C until determinations. For antioxidant enzyme activity determination, erythrocytes were frozen and thawn three times, centrifuged at 13,500×g for 10 min. The supernatant was diluted to approximately 0.5 mg/mL of protein.

2.1.4 15-F2t-isoprostane determination

Isoprostanes are prostaglandin-like compounds, which are produced by free radical-mediated peroxidation of lipoproteins. These by-products have a short half-life and are eliminated primarily in the urine. Urine from subjects was thawed and isoprostane levels were determined using a competitive enzyme-linked immunoassay (ELISA) (Oxford Biomed, EA85). In brief, urine samples were mixed with an enhanced dilution buffer that essentially eliminates interference because of non-specific binding. In this assay, the 15-F2t-isoprostane in the samples competes with 15-F2t-isoprostane conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-F2t-isoprostane coated on the microplate. The HRP activity results in

color development when substrate is added, the intensity of which is inversely proportional to the amount of unconjugated 15-F2t-isoprostane in the samples and may be measured at 630 nm. Results were expressed as nanograms of isoprostanes per mg urinary creatinine.

2.1.5 Di-tyrosine autofluorescence determination

Di-tyrosine (di-tyr) content, a measure of protein oxidation, was determined by autofluorescence. For di-tyr fluorescence determination, 50 µl of thawed urine was added to 950 µl, 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 min, di-tyr concentration was measured using a fluorometer (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg urinary creatinine [34].

2.1.6 Nitrite levels

Nitrite levels were determined using a nitrite colorimetric assay kit (LDH method) (Cayman Chemical Company). In brief, NADPH is an essential cofactor for the function of the NOS enzyme. In addition, nitrate reductase utilizes NADPH in the enzymatic reduction of nitrate to nitrite. Results were expressed as µmol per mg urinary creatinine.

2.1.7 Urine creatinine determination

Urine creatinine determination was performed by a picric acid method (kit Labtest Diagnóstica, Minas Gerais, Brazil). Creatinine and other serum components react with the solution picrate in alkaline conditions to form a red colored complex, which is measured photometrically. The addition of an acid promotes the decomposition

of creatinine picrate, which is also measured photometrically. The difference between the two readings gives the value of creatinine. Results were expressed as mg/dL.

2.1.8 Catalase assay (CAT)

CAT activity was assayed in lysate of erythrocytes measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 mM potassium phosphate buffer, pH 7.0 and 0.1–0.3 mg protein/mL [35]. One unit of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is reported as units per mg protein.

2.1.9 Homocysteine (tHcy) measurement

The total homocysteine levels in plasma were measured by liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS), as described by Magera et al. [36]. This method is based on the analysis of 100μL of plasma with 20 μL of homocysteine-d_8 (2nmol) added as internal standard. After the step of reduction with 20μL of dithiothreitol 500mM followed by deproteinization, the analysis was performed in the multiple reaction monitoring mode in which tHcy and Hcy-d_4 were detected through the transition from the precursor to the product ion (*m/z* 136 to *m/z* 90 and *m/z* 140 to *m/z* 94, respectively). The retention time of tHcy and Hcy-d_4 was 1.5 minutes in a 2.5-minutes analysis. The calibration was performed by a curve with 5 concentrations of Hcy. The results were expressed as μmol/L. In plasma, total homocysteine (tHcy) is the sum of free and protein-bound homocysteine, homocystine, and several others mixed disulfides.

2.1.10 Sulphydryl groups content

This assay is based on the reduction of 5.5'-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative whose absorption is measured in plasma spectrophotometrically at 412 nm [37]. Results were reported as nmol TNB.

2.2 *In vitro* study

Venous blood was collected under sterile conditions in heparinized vials from five healthy volunteers individuals. Whole blood cells from each subject were incubated without Hcy and NAC (control group), in the presence of Hcy at two concentrations (50 and 200 µM), and in a co-treatment with Hcy (50 and 200 µM) and NAC (1 and 5 mM). The Hcy concentrations were based on levels found in blood from treated CBS-deficient patients. Patients that have good adherence to treatment present tHcy levels around 50 µM, and patients that do not have good adherence to treatment present tHcy levels around 200 µM. Whole blood cells were incubated for 6h at 37ºC [38,39].

2.2.1 Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. [40] in accordance with general guidelines for use of the comet assay [38,39]. Aliquots of 100µL from whole blood were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Slides were placed in lyses buffer (2.5 NaCl, 100mM EDTA, 10mM Tris, 10% DMSO, pH 10-10,5) to remove cell proteins, leaving DNA as "nucleiods". After treatment with lyses buffer and alkaline buffer solution (300mM NaOH and 1mM EDTA, pH>13), the slides were submitted to a horizontal electrophoresis. This technique was performed for 20 min at 4ºC (25V; 300mA; 0.9V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol [41]. After drying at room temperature overnight, gels were

analyzed using an optical microscope. It were analyzed 100 cells in microscopy and to each cell it was determined a damage class. The damage classes were classified in: 0 = no tail (no damage); 1 = small tail smaller than the diameter of the head; 2 = tail length between one and two times the diameter of the head; 3 = long tail greater than twice the diameter of the head; 4 = long tail and more widespread than class 3 (Figure 1). It was made a multiplication of each damage class by number of cells found in each damage class. The damage index (DI) was determined by the sum of these multiplications. The slides were analyzed under blind conditions at least by two different individuals.

2.3 Statistical Analysis

2.3.1 *In vivo* study:

Data were expressed as mean ± standard deviation. Comparison between means was analyzed by the Student's t test for unpaired samples. Correlations between variables were calculated using the Pearson correlation coefficient. A *p* value lower than 0.05 was considered significant. All the analyses were performed using the Statistical Package for Social Sciences (SPSS) Software in a PC-compatible computer.

2.3.2 *In vitro* study:

Data were expressed as mean ± standard deviation. Comparisons between means were calculated by one-way one-ANOVA followed by the Duncan test when the F value was significant. A *p* value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3. Results

Firstly, we evaluated the *in vivo* oxidative stress parameters and nitrite levels in urine from CBS-deficient patients under treatment. The parameters of lipid (isoprostanes levels) and protein (di-tyrosine levels) oxidative damage, as well as the nitrite levels were compared to those of controls.

The 15-F2t-isoprostanes levels were significantly higher in CBS-deficient patients when compared to controls [$t(15)= 2.458$, $p<0.05$] (Figure 2), indicating oxidative damage to lipids. Similarly, the di-tyrosine levels, a parameter of oxidative damage to proteins, were significantly higher in CBS-deficient patients when compared to controls [$t(15)= 3.711$, $p<0.01$] (Figure 3). Additionally, the nitrite levels were significantly higher in CBS-deficient patients when compared to controls [$t(12)= 2.772$, $p<0.05$] (Figure 4). In order to investigate whether tHcy levels were associated to oxidative stress in urine of CBS-deficient patients, we correlated all parameters investigated with this amino acid. We verified a significant positive correlation between 15-F2t-isoprostanes levels and tHcy levels ($r = 0,780$, $p<0.05$) (Figure 5).

We also verified a possible association between parameters of protein oxidative damage in urine (di-tyrosine) and in plasma (sulphydryl content) of CBS-deficient patients and we observed a significant negative correlation between di-tyrosine levels and sulphydryl groups content ($r = 0,806$, $p<0.05$) (Figure 6).

Posteriorly we investigate the antioxidant activity of catalase enzyme in blood of CBS-deficient patients and we found an increase in catalase activity [$t(16)= 3.320$, $p<0.01$] (Figure 7) in blood of patients, when compared to controls.

Finally, in this work, we evaluated the *in vitro* effect of NAC on DNA damage caused by high levels of Hcy. We demonstrated the higher DNA damage induced by 50

and 200 µM of Hcy and that NAC at concentrations 1 and 5 mM was able to reduce significantly the DNA damage caused by Hcy 50µM (Figure 8) and Hcy 200µM (Figure 9).

4. Discussion

Oxidative stress has been demonstrated to be an underlying pathophysiologic process in various inborn errors of metabolism. Metabolic profiling of oxidative stress may provide a nonspecific measure of disease severity that may further enable physicians to monitor disease [42]. In this work, we investigated two markers of oxidative damage in urinary samples from CBS-deficient patients; the isoprostanes, a measure of lipid peroxidation and di-tyrosine, a measure of protein oxidation. We also determined urinary nitrite levels. Additionally, we evaluated the antioxidant activity of catalase, as well as the *in vitro* effect of NAC on DNA damage induced by Hcy.

Oxidative stress is characterized by an imbalance between increased exposure to reactive species and antioxidant defenses. Free radicals cause direct damage to critical biomolecules including DNA, lipids, and proteins [43]. Isoprostanes are prostaglandin (PG)-like compounds that are produced *in vivo* independently of cyclooxygenase enzymes, primarily by free radical-induced peroxidation of arachidonic acid, a polyunsaturated fatty acid [44,45]. It has now been established that measurement of isoprostanes is the most reliable approach to assess oxidative stress status *in vivo*, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease. In addition, products of the isoprostanes pathway have been found to exert potent biological effects and therefore may be pathophysiological mediators of disease. In this sense, the evaluation of urinary isoprostanes levels becomes interesting, since it is an important marker of oxidative stress evaluated in a fluid easily collected [46].

In this work, we demonstrated that CBS-deficient patients have an increase of 15-F2t-isoprostanes levels, an oxidative damage parameter, in urine. Moreover, we

found a significant positive correlation between 15-F2t-isoprostanes and tHcy levels. It is known that Hcy may lead to oxidative stress by free radical formation through its auto-oxidation. Homocysteine is readily oxidized when added to plasma, principally as consequence of auto-oxidation, leading to the formation of homocystine, homocysteine-mixed disulfides, and homocysteine thiolactone. It has been proposed that during the transition metal ion-catalyzed oxidation of the sulfhydryl group of Hcy, hydrogen peroxide is formed, which promotes oxidative stress and lipid peroxidation through Fenton-type reactions [47-49]. In a previous study, we demonstrated an increase in malondialdehyde (MDA) levels, an end product of membrane fatty acid peroxidation, in plasma of CBS-deficient patients treated and not treated. Additionally, we demonstrated a significant positive correlation between MDA levels and tHcy levels [27]. Interesting, in this study, we found results that complement our earlier work, since it was observed an increase of urinary 15-F2t-isoprostanes levels, a parameter of oxidative damage to lipids, as well as a significant positive correlation between 15-F2t-isoprostanes levels and tHcy levels. Analyzed together, these results suggest that lipid peroxidation, in plasma and urine, probably may be caused by the increase in formation of free radicals induced by Hcy auto-oxidation. The Hcy oxidation occurs in serum in a concentration-dependent manner [47].

In this context, Davì et al. [50] demonstrated that urinary 8-iso-$PGF_{2\alpha}$ excretion was significantly higher in CBS-deficient patients than in healthy subjects. In the same study, a statistically significant correlation was found between plasma tHcy and urinary 8-iso-$PGF_{2\alpha}$. The metabolite 8-iso-$PGF_{2\alpha}$ belongs to F2-isoprostanes, induces vasoconstriction and modulates the function of human platelets. These authors emphasize that hyperhomocysteinemia may cause persistent platelet activation, through free radical-catalyzed peroxidation of arachidonic acid to form the platelet-active F2-

isoprostane 8-iso-$PGF_{2\alpha}$ and possibly other isoeicosanoids. The findings in CBS-deficient patients confirm and extend similar observations made in other clinical settings, such hypercholesterolemia [51], diabetes mellitus [52], and cigarette smoking [53].

Di-tyrosine has been used as an important biomarker for oxidatively modified proteins [54]. In this work, we found an increase of urinary di-tyrosine levels in CBS-deficient patients when compared to controls. The recent findings, demonstrating that protein carbonyl formation and sulfhydryl oxidation were increased in plasma from CBS-deficient patients, further reinforce the view that oxidative damage to proteins may be involved in the pathophysiology of this disease [27]. Furthermore, we verified in this work a negative correlation between di-tyrosine levels and sulphydryl groups content. Sulfhydryl groups can be reversibly oxidized by reactive species, suggesting that they represent a potential antioxidant, acting in cellular defense against oxidative stress [55]. Since two-thirds of sulfhydryl groups are bonded to proteins, a reduction of these groups also characterizes a protein oxidation [56–58]. It is important to emphazise that cystathionine and cysteine are reduced in CBS-deficient patients, whereas the transsulphuration pathway is blocked by the enzymatic defect in CBS. Transsulphuration pathway begins with the condensation of Hcy with serine to form cystathionine, by the action of cystathionine β-synthase (CBS) with pyridoxal phosphate (PLP) acting as a cofactor; after the cystathionine is cleaved to cysteine and α-oxobutyrate, by another PLP–requiring enzyme, the γ- cystathionase [1]. The increase of di-tyrosine levels in urine reinforces protein oxidation found in CBS-deficient patients, since di-tyrosine is formed by the oxidation of adjacent protein tyrosine residues, leading to the formation of a highly stable inter-phenolic bond that does not undergo further metabolism [34]. Protein oxidation by reactive species can lead

enzymes, receptors and transport proteins to malfunction and, eventually, induce alterations of cellular metabolism [43]. Recent studies evidenced an increase of di-tyrosine levels in some inborn error of metabolism [23,59-62], but few studies evaluating this parameter in homocystinuria.

After, in this work we evaluated the nitrite levels in urine of CBS-deficient patients and controls. We found that CBS-deficient patients present nitrite levels significantly higher than controls. Endothelial nitric oxide synthase (NOS III) is the primary endogenous source of nitric oxide (NO) generation in the vascular system [63] and its expression has been reported as directly proportional to plasma nitrite levels [64,65], suggesting nitrite may reflect cardiovascular NO bioavailability. Under basal conditions, NOS III catalyzes the oxidation of L-arginine to NO and L-citrulline, with NADPH and oxygen serving as co-substrates [66]. Nitric oxide has an important vasodilatory activity and acts as molecule regulating vascular inflammation [67], platelet function [68], angiogenesis [69,70], and cellular respiration [71]. Levels of NO in excess can inhibit glycolysis, the mitochondrial respiratory chain and DNA replication [72].

Nitric oxide reacts very rapidly with oxygen radicals; thus NO reacting with $O_2^{-\cdot}$ generates peroxynitrite ($ONOO^-$). Peroxynitrite oxidizes protein thiols forming disulfide, nitrates tyrosyl groups in proteins forming nitrated proteins. One of the consequences of nitrating tyrosyl groups in protein is either a loss or gain of function in the activity (e.g., manganese superoxide dismutase; MnSOD). Nitration can interfere with signal transduction mechanism [73]. Nitric oxide also reacts with lipophilic peroxyl radicals, important propagating species in the biological chain reaction of lipid peroxidation, to generate alkyl peroxynitrates (LOONO). These appear far more stable than $ONOO^-$ [74]. Controversy exists in what concern to the effects of

hyperhomocysteinemia on NO production: it has been shown that hyperhomocysteinemia both increases and suppresses it [75-77]. In this work we found an increase of nitrite levels in urine of CBS-deficient patients, when compared to controls.

The mechanism by which Hcy causes endotelial cell damage, smooth muscle cell proliferation and subsequent atherosclerotic plaque formation has only recently come under investigation. Damage to the vascular endothelium by Hcy is believed to be secondary to H_2O_2 formation accompanying oxidation of the amino acid's sulfhydryl group [47]. Studies demonstrated that H_2O_2 generation from Hcy is prevented by S-nitrosation of Hcy, and that, in contrast to Hcy itself, S-nitroso-homocysteine has potent vasodilator and antiplatelet effects [78]. Upchurch Jr et al. [75] showed that stimulated endothelial cells exposed to increasing concentrations of Hcy produce more NO than control cells, and suggest a potential mechanism by which the cell's ability to produce and secrete NO can be used to minimize toxicity by Hcy and its oxidative by-products. In this work we demonstrated that catalase activity is increased in blood of CBS-deficient patients; this antioxidant enzyme act detoxifying the H_2O_2 formed reducing H_2O_2 to O_2 and H_2O [79]. Analysing all the results together, we can hypothesize that the high tHcy levels found in CBS-deficient patients generate high H_2O_2 levels through auto-oxidation, stimulating a mechanism of antioxidant defense and increasing the catalase activity. Furthermore, in order to prevent the addictional generation of H_2O_2 by high Hcy levels, other compensatory mechanism is activated: stimulation of NO production aiming the detoxification of Hcy through S-nitrosation process [75,79].

Finally in this study, we evaluated the *in vitro* effect of antioxidant NAC on DNA damage caused by Hcy. We found an increase in DI caused by Hcy 50 μM and

200 µM. In presence of NAC 1mM and 5mM, the DI was significantly lower, indicating that NAC was able to prevent or revert the increase of DNA damage Hcy-induced.

Oxidative damage to DNA caused by oxygen-derived species including free radicals is the most frequent type encountered by aerobic cells. Hydroxyl radicals, which are the most toxic oxygen metabolites, are believed to be one of the most potent causes of DNA damage by means of the Fenton reaction [80]. During oxidation of the free thiol group of Hcy, oxidative stress is generated when Hcy binds via a disulphide bridge with plasma proteins — mainly albumin, or with other low-molecular plasma thiols or with a second Hcy molecule. Oxidation of Hcy may induce the subsequent oxidation of proteins, lipids and nucleic acids [81]. Recent works indicate the occurrence of DNA damage secundary to hyperhomocysteinemia: Lin et al. [82] demonstrated the synergic actions of Hcy and *S*-adenosylhomocysteine (SAH) on DNA damage through a mechanism involving ROS; Huang et al. [83] showed that Hcy induces apoptotic DNA damage mediated by increased intracellular generation of H_2O_2. In this study we evidenced an antioxidant effect of NAC on DNA damage caused by Hcy. A number of publications have shown that antioxidants protect the integrity of DNA from genotoxicants and are capable of eliminating ROS generated by oxidative stress [84-86]. Several studies provide evidences that NAC has antigenotoxic and anticarcinogenic properties in a variety of experimentals models [87]. As a source of sulfhydryl groups, which neutralize ROS, NAC plays a critical role in regulating the oxidant/antioxidant balance in cells and directly decreases cell oxidative stress [88]. In this scenario, the supplementation with NAC could be beneficial for CBS-deficient patients, whereas these patients have reduced cysteine levels [1]. According to Ozdemir et al. (2014) [89], supplementation of NAC was able to reduce DNA damage in children with β-thalassemia. Yang et al. (2014) [90] demonstrated by comet assay that

incubation of cells with Ochratoxin A (OTA) for 24 h resulted in DNA strand breaks and an evident comet tail. The pretreatment with NAC significantly inhibited the OTA-induced DNA strand breaks and reduced the formation of comets. Moreover, Hoffman et al. (2015) [91] showed that NAC was able to inhibit the DNA damage caused by phenethyl isothiocyanate (PEITC) through extracellular conjugation, reducing the amount of PEITC available to enter the cells and conjugate with GSH. In this manner, NAC may have preserved the pre-existing levels of GSH. Finally, recent work demonstrated the effect of NAC in X-linked adrenoleukodystrophy (X-ALD), an inborn error of metabolism. These authors showed in an *in vitro* study, that NAC was able to reduce DNA damage in blood of symptomatic X-ALD patients, equaling to control levels, suggesting that the administration of this antioxidant might be considered as an adjuvant therapy for X-ALD [22].

5. Conclusion

Analysing all the results found in this work, we can conclude that oxidative damage to proteins and lipids occurs in urine of CBS-deficient patients, possibly due to high levels to tHcy found in blood of these patients. These results are complementary to that of oxidative damage found in plasma of CBS-deficient patients, previously published by our group.

Additionally, the results allowed to suggest the activation of a possible compensatory antioxidant mechanism in blood of CBS-deficient patients, through the increase of catalase activity, as well as through the prevention of H_2O_2 generation, caused by high NO levels. In this case, NO has the role to minimize toxicity by Hcy and its oxidative by-products, through S-nitrosation process. Finally, it was also

demonstrated an important *in vitro* antioxidant effect of NAC upon DNA damage induced by Hcy.

These results reinforce the role of oxidative stress as possible factor in the pathogenesis of CBS deficiency and allows new perspectives to future studies involving suplementation of NAC to CBS-deficient patients.

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Conflict of interest disclosure

The authors declare that there is no conflict of interest disclosure associated with this manuscript.

6. References

[1] Mudd, S.H., Levy, H.L., Kraus, J.P., 2001. Disorders of transsulfuration, in: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds) The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, pp.2007-2056.

[2] Yap, S., 2003. Classical homocystinuria: Vascular risk and its prevention. J. Inherit. Metab. Dis. 26, 259-265.

[3] Fowler, B., 2008. Homocysteine, S-Adenosylmethionine and S-Adenosylhomocysteine, in: Blau, N., Duran, M., Gibson, K.M. (Eds) Laboratory Guide to the Methods in Biochemical Genetics, Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg, pp.112-135.

[4] Andria, G., Fowler, B., Sebastio, G., 2006. Disorders of Sulfur Amino Acid Metabolism. In: Fernandes, J.; Saudubray, J.M.; Van Den Berghe, G. Walter, J.H. (Eds) Inborn Metabolic Diseases, Diagnosis and Treatment, Würzburg: Springer Medizin Verlag, pp. 273-282.

[5] Vanzin, C.S., Mescka, C.P., Donida, B., Hammerschimidt, T.G., Ribas, G.S., Kolling, J., Scherer, E.B., Vilarinho, L., Nogueira, C., Coitinho, A.S., Wajner, M., Wyse, A.T.S., Vargas, C.R., 2015. Lipid, Oxidative and Inflammatory Profile and Alterations in the Enzymes Paraoxonase and Butyrylcholinesterase in Plasma of Patients with Homocystinuria Due CBS Deficiency: The Vitamin B12 and Folic Acid Importance. Cell. Mol. Neurobiol. 35, 899-911.

[6] Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol. 142, 231–255.

[7] Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging. 18, 685–716.

[8] Butterfield, D.A., 2002. Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review. Free Radic. Res. 36,1307–1313.

[9] Liu, Q., Raina, A.K., Smith, M.A., Sayre, L.M., Perry, G., 2003. Hydroxynonenal, toxic carbonyls, and Alzheimer disease. Mol. Aspects Med. 24, 305–313.

[10] Hagen, T.M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C.M., Ames, B.N., Chisari, F.V., 1994. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. 91, 12808–12812.

[11] Chowienczyk, P.J., Brett, S.E., Gopaul, N.K., Meeking, D., Marchetti, M., Russell-Jones, D.L., Anggard, E.E., Ritter, J.M., 2000. Oral treatment with an antioxidant (raxofelast) reduces oxidative stress and improves endothelial function in men with type II diabetes. Diabetologia. 43, 974–977.

[12] Halliwell, B., 2000. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? Cardiovasc. Res. 47, 410–418.

[13] Das, P.; Biswas, S.; Mukherjee, S.; Bandyopadhyay, S.K. 2016. Association of Oxidative Stress and Obesity with Insulin Resistance in Type 2 Diabetes Mellitus. Mymensingh Med J. 25, 148-52.

[14] Halliwell, B., 2002. Vitamin E and the treatment and prevention of diabetes: a case for a controlled clinical trial. Singapore Med. J. 43, 479–484.

[15] Parthasarathy, S., Santanam, N., Ramachandran, S., Meilhac, O., 2000. Potential role of oxidized lipids and lipoproteins in antioxidant defense. Free Radic. Res. 33, 197–215.

[16] Bridi, R.; Araldi, J.; Sgarbi, M.B.; Testa, C.G.; Durigon, K.; Wajner, M.; Dutra-Filho, C.S. 2003. Induction of oxidative stress in rat brain by the metabolites accumulating in maple syrup urine disease. Int. J. Dev. Neurosci. 21, 327–332.

[17] Vargas, C.R., Wajner, M., Sirtori, L.R., Goulart, L., Chiochetta, M., Coelho, D.M., Latini, A., Llesuy, S., Bello-Klein, A., Giugliani, R., Deon, M., Mello, C.F., 2004. Evidence that oxidative stress is increased in patients with X-linked adrenoleukodistrophy, Biochim. Biophys. Acta. 1688, 26-32.

[18] Barschak, A.G.; Sitta, A.; Deon, M.; de Oliveira, M.H.; Haeser, A.; Dutra-Filho, C.S.; Wajner, M.; Vargas, C.R., 2006. Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease. Metab. Brain Dis. 21, 279–286.

[19] Sitta, A., Barschak, A.G., Deon, M., Terroso, T., Pires, R., Giugliani, R., Dutra-Filho, C.S., Wajner, M., Vargas, C.R., 2006. Investigation of oxidative stress parameters in treated phenylketonuric patients, Metab. Brain Dis. 21, 287-296.

[20] Ribas, G.S., Manfredini, V., de Mari, J.F., Wayhs, C.Y., Vanzin, C.S., Biancini, G.B., Sitta, A., Deon, M., Wajner, M., Vargas, C.R., 2010. Reduction of lipid and protein damage in patients with disorders of propionate metabolism under treatment: a possible protective role of L-carnitine supplementation, Int. J. Devl. Neuroscience 28, 127–132.

[21] Mescka, C.P., Wayhs, C.A.Y., Vanzin, C.S., Biancini, G.B., Guerreiro, G., Manfredini, V., Souza, C., Wajner, M., Dutra-Filho, C.S., Vargas, C.R., 2013. Protein and lipid damage in maple syrup urine disease patients: l-carnitine effect. Int. J. Dev. Neurosci. 31,21-24.

[22] Marchetti, D.P., Donida, B., da Rosa, H.T., Manini, P.R., Moura, D.J., Saffi, J., Deon, M., Mescka, C.P., Coelho, D.M., Jardim, L.B., Vargas, C.R., 2015. Protective effect of antioxidants on DNA damage in leukocytes from X-linked adrenoleukodystrophy patients. Int. J. Dev. Neurosci. 43, 8-15.

[23] Deon, M., Sitta, A., Faverzani, J.L., Guerreiro, G.B., Donida, B., Marchetti, D.P., Mescka, C.P., Ribas, G.S., Coitinho, A.S., Wajner, M., Vargas, C.R., 2015. Urinary biomarkers of oxidative stress and plasmatic inflammatory profile in Phenylketonuric treated patients. Int. J. Dev. Neurosci. 47, 259-265.

[24] Streck, E.L.; Zugno, A.I.; Tagliari, B.; Franzon, R.; Wannmacher, C.M.; Wajner, M.; Wyse, A.T., 2001. Inhibition of rat brain Na^+, K^+-ATPase activity induced by homocysteine is probably mediated by oxidative stress. Neurochem. Res. 26, 1195–1200.

[25] Streck, E.L., Vieira, P.S., Wannmacher, C.M.D., Dutra-Filho, C.S., Wajner, M., and Wyse, A.T.S., 2003. In vitro effect of homocysteine on some parameters of oxidative stress in rat hippocampus. Metab. Brain Dis. 18,147–154.

[26] Matté, C., Mackedanz, V., Stefanello, F.M., Scherer, E.B.S., Andreazza, A.C., Zanotto, C., Moro, A.M., Garcia, S.C., Gonçalves, C.A., Erdtmann, B., Salvador, M., Wyse, A.T.S., 2009. Chronic hyperhomocysteinemia alters antioxidant defenses and increases DNA damage in brain and blood of rats: protective effect of folic acid. Neurochem. Int. 54, 7–13.

[27] Vanzin, C.S., Biancini, G.B., Sitta, A., Wayhs, C.A.Y., Pereira, I.N., Rockenbach, F., Garcia, S.C., Wyse, A.T.S., Schwartz, I.V.D., Wajner, M., Vargas, C.R., 2011. Experimental evidence of oxidative stress in plasma of homocystinuric patients: A possible role for homocysteine, Mol. Genet. Metab. 104, 112-117.

[28] Vanzin, C.S., Manfredini, V., Marinho, A.E., Biancini, G.B., Ribas, G.S., Deon, M., Wyse, A.T.S., Wajner, M., Vargas, C.R., 2014. Homocysteine contribution to DNA damage in cystathionine β-synthase-deficient patients. Gene. 539, 270–274.

[29] Kruman, I.I., Culmsee, C., Chan, S.L., Kruman, Y., Guo, Z., Penix, L., Mattson, M.P., 2000. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J. Neurosci. 20,6920–6926.

[30] Huang, R.F., Huang, S.M., Lin, B.S., Wei, J.S., Liu, T.Z., 2001. Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci. 68,2799–2811.

[31] Fenech, M., Dreosti, I.E., Rinaldi, J.R., 1997. Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. Carcinogenesis 18,1329–1336.

[32] Fenech, M., Aitken, C., Rinaldi, J.R., 1998. Folate, vitamin B12, homocysteine status and chromosome damage in young Australian adults. Carcinogenesis. 19,1163–1171.

[33] De Flora, S., Balansky, R., Bennicelli, C., Camoirano, A., D'Agostini, F., Izzotti, A., Cesarone,C.F., 1995. Mechanisms of anticarcinogenesis: The example of *N*-acetylcysteine, in: Ioannides, C., Lewis, D.F.V. (Eds) Drugs, Diet and Disease, Mechanistic Approaches to Cancer. Ellis Horwood, UK, pp. 151–203.

[34] Kirschbaum, B., 2002. Correlative studies of urine fluorescence and free radical indicators. Clin. Nephrol. 58,344–349.

[35] Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105,121–126.

[36] Magera, M.J., Lacey, J.M., Casetta, B., Rinaldo, P., 1999. Method for the determination of total homocysteine in plasma and urine by stable isotope dilution and electrospray tandem mass spectrometry. Clin. Chem. 45,1517-1522.

[37] Aksenov, M.Y., Markesbery, W.R., 2001. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci. Lett. 302,141-145.

[38] Tice, R.R., Agurell, D., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

[39] Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, G., Speit, G., Thybaud, V., Tice, R.R., 2003. Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis. 18, 45–51.

[40] Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

[41] Nadin, S., Vargas-Roig, L., Ciocca, D., 2001. A silver staining method for single-cell gel assay. J. Histochem. Cytochem. 49, 1183–1186.

[42] Mc Guire, P.J., Parikh, A., Diaz, G.A., 2009. Profiling of oxidative stress in patients with inborn errors of metabolism. Mol. Genet. Metab. 98, 173–180.

[43] Halliwell, B.; Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. fourth ed. Oxford University Press, Oxford.

[44] Roberts, L. J.; Morrow, J. D., 2000. Measurement of F2-isoprostanes as an index of oxidative stress in vivo. Free Radic. Biol. Med. 28,505-513.

[45] Morrow, J.D.; Hill, K.E.; Burk, R.F.; Nammour, T.M.; Badr, K.F.; Roberts, L.J, II., 1990. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc. Natl. Acad. Sci. USA.87,9383-9387.

[46] Montuschi, P.; Barnes, P.; Roberts, L.J., 2007. Insights into oxidative stress: the isoprostanes. Curr. Med. Chem. 14,703–717.

[47] Starkebaum, G.; Harlan, J.M., 1986. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine, J. Clin. Invest. 77,1370–1376.

[48] Loscalzo, J., 1996. The oxidant stress of hyperhomocyst(e)inemia, J. Clin. Invest. 98, 5–7.

[49] Zhang, F.; Slungaard, A.; Vercellotti, G.M.; Iadecola, C., 1998. Superoxide-dependent cerebrovascular effects of homocysteine. Am. J. Physiol. 274, R1704–R1711.

[50] Davì, G.; Di Minno, G.; Coppola, A.; Andria, G.; Cerbone, A.M.; Madonna, P.; Tufano, A.; Falco, A.; Marchesani, P.; Ciabattoni, G.; Patrono, C., 2001. Oxidative stress and platelet activation in homozygous homocystinuria. Circulation. 104, 1124–1128.

[51] Davì, G.; Alessandrini, P.; Mezzetti, A.; Minotti, G.; Bucciarelli, T.; Costantini, F.; Cipollone, F.; Bon, G.B.; Ciabattoni, G.; Patrono, C., 1997. In vivo formation of 8-epiprostaglandin $F_{2\alpha}$ is increased in hypercholesterolemia. Arterioscler. Thromb. Vasc. Biol. 17, 3230 –3235.

[52] Davì, G.; Ciabattoni, G.; Consoli, A.; Mezzetti, A.; Falco, A.; Santarone, S.; Pennese, E.; Vitacolonna, E.; Bucciarelli, T.; Costantini, F.; Capani, F.; Patrono, C., 1999. In vivo formation of 8-iso-prostaglandin $F_{2\alpha}$ and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation. 99, 224 –229.

[53] Morrow, J.D.; Frei, B.; Longmire, A.W.; Gaziano, J.M.; Lynch, S.M.; Shyr, Y.; Strauss, W.E.; Oates, J.A.; Roberts, L.J. 2nd., 1995. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers: smoking as a cause of oxidative damage. N. Engl. J. Med. 332, 1198 –1203.

[54] Giulivi, C.; Traaseth, N.J.; Davies, K.J., 2003. Tyrosine oxidation products: analysis and biological relevance. Amino Acids 25, 227–232.

[55] Bourdon, E.; Blache, D., 2001. The importance of proteins in defense against oxidation. Antioxid. Redox Signal. 3, 293–311.

[56] Thomas, J.A.; Poland, B.; Honzatko, R., 1995. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. 319, 1–9.

[57] Hansen, R.E.; Roth, D.; Winther, J.R., 2009. Quantifying the global cellular thiol-disulfide status. Proc. Natl. Acad. Sci. U.S.A. 106, 422–427.

[58] Requejo, R.; Hurd, T.R.; Costa, N.J.; Murphy, M.P., 2010. Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. FEBS J. 277, 1465–1480.

[59] Ribas, G.S.; Biancini, G.B.; Mescka, C.P.; Wayhs, C.A.Y.; Sitta, A.; Wajner, M.; Vargas, C.R., 2012 Oxidative Stress Parameters in Urine from Patients with Disorders of Propionate Metabolism: a Beneficial Effect of L-Carnitine Supplementation. Cell Mol. Neurobiol. 32, 77–82.

[60] Mello, M.S.; Ribas, G.S.; Wayhs, C.A.Y.; Hammerschmidt, T.; Guerreiro, G.B.; Favenzani, J.L.; Sitta, A.; Coelho, D.M.; Wajner, M.; Vargas, C.R., 2015. Increased oxidative stress in patients with 3-hydroxy-3- methylglutaric aciduria. Mol. Cell Biochem. 402, 149–155.

[61] Guerreiro, G.; Mescka, C.P.; Sitta, A.; Donida, B.; Marchetti, D.; Hammerschmidt, T.; Faverzani, J.; Coelho, D.M.; Wajner, M.; Dutra-Filho, C.S.; Vargas, C.R., 2015. Urinary biomarkers of oxidative damage in Maple syrup urine disease: The l-carnitine role. Int. J. Devl. Neuroscience. 42, 10–14.

[62] Donida, B.; Marchetti, D.P.; Biancini, G.B.; Deon, M.; Manini, P.R.; da Rosa, H.T.; Moura, D.J.; Saffi, J.; Bender, F.; Burin, M.G.; Coitinho, A.S.; Giugliani, R.; Vargas, C.R., 2015. Oxidative stress and inflammation in mucopolysaccharidosis type IVA patients treated with enzyme replacement therapy. Biochim Biophys Acta. 1852, 1012–1019.

[63] Rhodes, P.; Leone, A.M.; Francis, P.L.; Struthers, A.D.; Moncada, S., 1995. The L-arginine: Nitric oxide pathway is the major source of plasma nitrite in fasted humans, Biochem. Biophys. Res. Commun. 209, 590–596.

[64] Kleinbongard, P.; Dejam, A.; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Gödecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G.A.; Bryan, N.S.; Feelisch, M.; Kelm, M., 2003. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radical Biol. Med. 35, 790–796.

[65] Lauer, T.; Preik, M.; Rassaf, T.; Strauer, B.E.; Deussen, A.; Feelisch, M.; Kelm, M., 2001. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. PNAS 98, 12814–12819.

[66] Alderton, W.K.; Cooper, C.E.; Knowles, R.G., 2001. Nitric oxide synthases: Structure, function and inhibition. Biochem. J. 357, 593–615.

[67] Guzik, T.R.; Korbut, T.; Adamek-Guzik, 2003. Nitric oxide and superoxide in inflammation and immune regulation. J. Physiol. Pharmacol. 54, 469–487.

[68] Webb, A.J.; Patel, N.; Loukogeorgakis, S.; Okorie, M.; Aboud, Z.; Misra, S.; Rashid, R.; Miall, P.; Deanfield, J.; Benjamin, N.; MacAllister, R.; Hobbs, A.J.; Ahluwalia, A., 2008. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension 51, 784–790.

[69] Mendoza, M.G.; Robles, H.V.; Romo, E.; Rios, A.; Escalante, B., 2007. Nitric oxide-dependent neovascularization role in the lower extremity disease. Curr. Pharm. Des. 13, 3591–3596.

[70] Kumar, D.; Branch, B.G.; Pattillo, C.B.; Hood, J.; Thoma, S.; Simpson, S.; Illum, S.; Arora, N.; Chidlow, J.H.Jr; Langston, W.; Teng, X.; Lefer, D.J.; Patel, R.P.; Kevil, C.G., 2008. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. Proc. Nat. Acad. Sci. 105, 7540–7545.

[71] Brown, G.; Cooper, C., 1994. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. 356, 295–298.

[72] Darley-Usmar, V.; Wiseman, H.; Halliwell, B., 1995. Nitric oxide and oxygen radicals: a question of balance. FEBS Letters 369, 131-135.

[73] Trostchansky, A.; Bonilla, L.; Gonzalez-Perilli, L.; Rubbo, H., 2013. Nitro-fatty acids: formation, redox signaling, and therapeutic potential. Antioxid. Redox Signal. 19, 1257-1265.

[74] Rubbo, H.; Radi, R.; Trujillo, M.; Telleri, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B.A., 1994. J. Biol. Chem. 269, 26066-26075.

[75] Upchurch Jr, G.R.; Welch, G.N.; Fabian, A.J.; Pigazzi, A.; Keaney Jr, J.F.; Loscalzo, J., 1997. Stimulation of endothelial nitric oxide production by homocyst(e)ine. Atherosclerosis 132, 177–185.

[76] Zhang, X.; Li, H.; Jin, H.; Ebin, Z.; Brodsky, S.; Goligorsky, M.S., 2000. Effects of homocysteine on endothelial nitric oxide production. Am. J. Physiol. Renal Physiol. 279, F671-F678.

[77] Kolling, J.; Scherer, E.B.; da Cunha, A.A.; da Cunha, M.J.; Wyse, A.T.S., 2011. Homocysteine Induces Oxidative–Nitrative Stress in Heart of Rats: Prevention by Folic Acid. Cardiovasc. Toxicol. 11, 67–73.

[78] Stamler, J.S.; Osborne, J.A.; Jaraki, O.; Rabbani, L.E.; Mullins, M.; Singel, D.; Loscalzo, J., 1993. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J. Clin. Invest. 91, 308–318.

[79] Kalyanaraman, B., 2013. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. Redox Biol. 1, 244–257.

[80] Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J.; Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell. Biochem. 266: 37–56.

[81] Zou, C.G.; Banerjee, R., 2005. Homocysteine and redox signaling. Antioxid. Redox Signal. 7, 47–59.

[82] Lin, P.Y.; Yang, T.H.; Lin, H.G.; Hu, M.L., 2007. Synergistic effects of S-adenosylhomocysteine and homocysteine on DNA damage in a murine microglial cell line. Clin. Chim. Acta. 379, 139–144.

[83] Huang, R.F.S.; Huang, S.M.; Lin, B.S.; Wei, J.S.; Liu, T.Z., 2001. Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci. 68, 2799–2811.

[84] Suhail, N.; Bilal, N.; Khan, H.Y.; Hasan, S.; Sharma, S.; Khan, F.; Mansoor, T.; Banu, N., 2012. Effect of vitamins C and E on antioxidant status of breast-cancer patients undergoing chemotherapy. J. Clin. Pharm. Ther. 37, 22–26.

[85] Aydin, S.; Tokac, M.; Taner, G.; Arikok, A.T.; Dundar, H.Z.; Ozkardes, A.B.; et al. 2013. Antioxidant and antigenotoxic effects of lycopene in obstructive jaundice. J. Surg. Res.182, 285–295.

[86] Boyacioglu, M.; Sekkin, S.; Kum, C.; Korkmaz, D.; Kiral, F.; Sultan, H.; Yalinkilinc, MOAk, Akar. F., 2014. The protective effects of vitamin C on the DNA damage, antioxidante defenses and aorta histopathology in chronic hyperhomocysteinemia induced rats. Exp. Toxicol. Pathol. 66, 407–413.

[87] De Flora, S.; Izzotti, A.; D'Agostini, F.; Balansky, R.M., 2001. Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. Carcinogenesis. 22, 999-1013.

[88] Bajt, M.L.; Knight, T.R.; Lemasters, J.J.; Jaeschke, H., 2004. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetylcysteine. Toxicol. Sci. 80, 343–349.

[89] Ozdemir, Z.C.; Koc, A.; Aycicek, A.; Kocyigit, A., 2014. N-Acetylcysteine Supplementation Reduces Oxidative Stress and DNA Damage in Children with β-Thalassemia. Hemoglobin. 38, 359-364.

[90] Yang, Q.; Shi, L.; Huang, K.; Xu, W., 2014. Protective effect of N-acetylcysteine against DNA damage and S-phase arrest induced by ochratoxin A in human embryonic kidney cells (HEK-293). Food Chem. Toxicol. 70, 40–47.

[91] Hoffman, J.D.; Ward, W.M.; Loo, G., 2015. Effect of antioxidants on the genotoxicity of phenethyl isothiocyanate. Mutagenesis. 30, 421-430.

Figure legends:

Figure 1: Evaluation of DNA damage using comet assay (single cell gel electrophoresis) (400×). The cells are assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail.

Figure 2: 15-F2t-isoprostanes levels in urine from CBS-deficient patients and controls. Data represent the mean ± SD (controls: n = 9; patients: n = 9). *$p < 0.05$, compared to controls (Student's t test for unpaired samples).

Figure 3: Di-tyrosine levels in urine from CBS-deficient patients and controls. Data represent the mean ± SD (controls: n = 9; patients: n = 8). **$p < 0.01$, compared to controls (Student's t test for unpaired samples).

Figure 4: Nitrite levels in urine from CBS-deficient patients and controls. Data represent the mean ± SD (controls: n = 5; patients: n = 9). Student's t test for unpaired samples.

Figure 5: Correlation between urinary 15-F2t-isoprostanes levels and plasma tHcy levels from CBS- deficient patients.

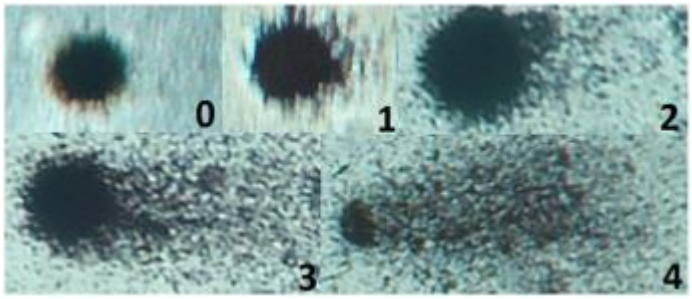
Figure 6: Correlation between urinary di-tyrosine levels and plasma sulphydryl groups content from CBS- deficient patients.

Figure 7: Catalase activity in blood from CBS-deficient patients and controls. Data represent the mean ± SD (controls: n = 9; patients: n = 9). **p < 0.01, compared to controls (Student's t test for unpaired samples).

Figure 8: *In vitro* effect of NAC at concentrations 1 and 5 mM on DNA damage caused by Hcy 50µM. Data represent the mean ± SD of 3 independent experiments. (a) $p<0.05$, compared to control (without Hcy e NAC), (b) $p<0.05$, compared to Hcy 50µM (One-way ANOVA, followed by Duncan test).

Figure 9: *In vitro* effect of NAC at concentrations 1 and 5 mM on DNA damage caused by Hcy 200µM. Data represent the mean ± SD of 3 independent experiments. (a) $p<0.05$, compared to control (without Hcy e NAC), (b) $p<0.05$, compared to Hcy 200µM (One-way ANOVA, followed by Duncan test).

Figure 1:



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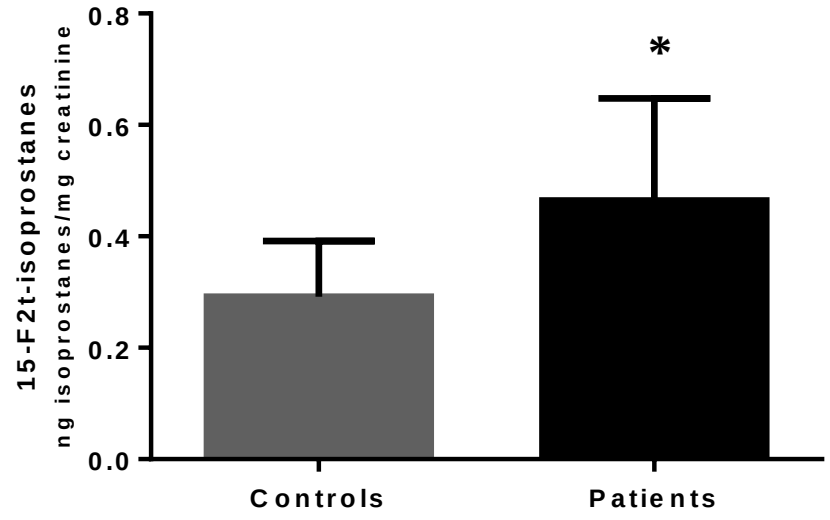

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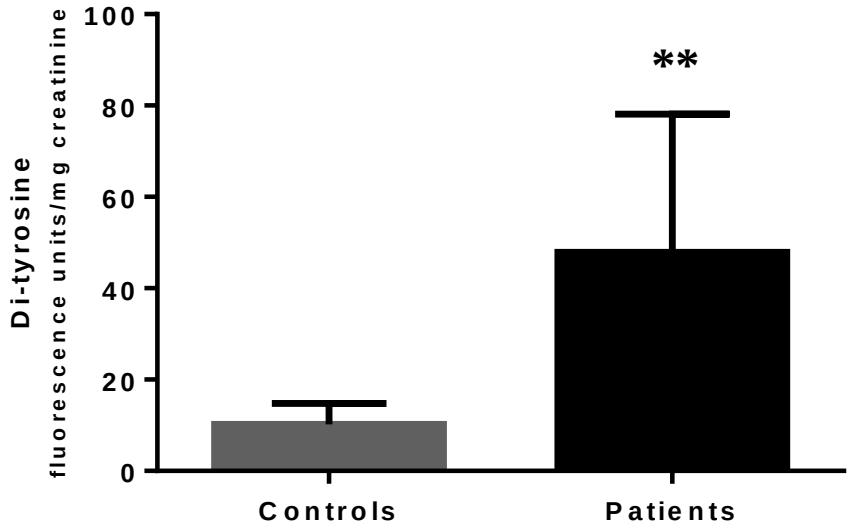

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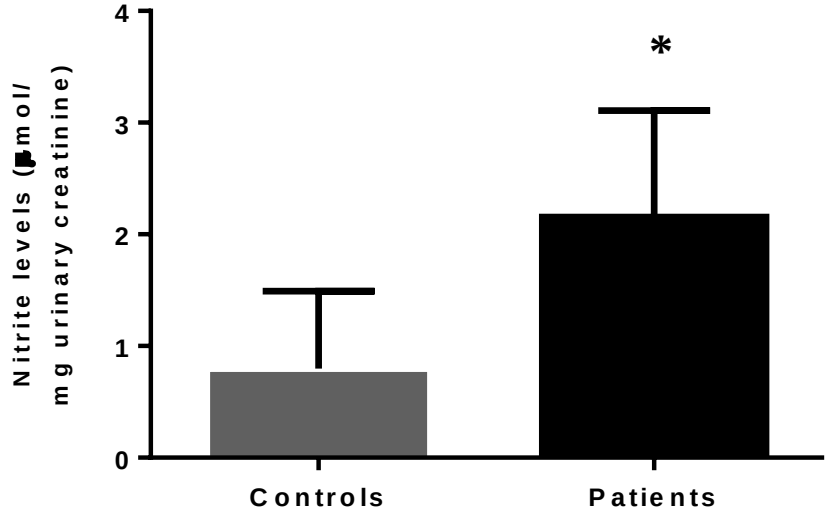

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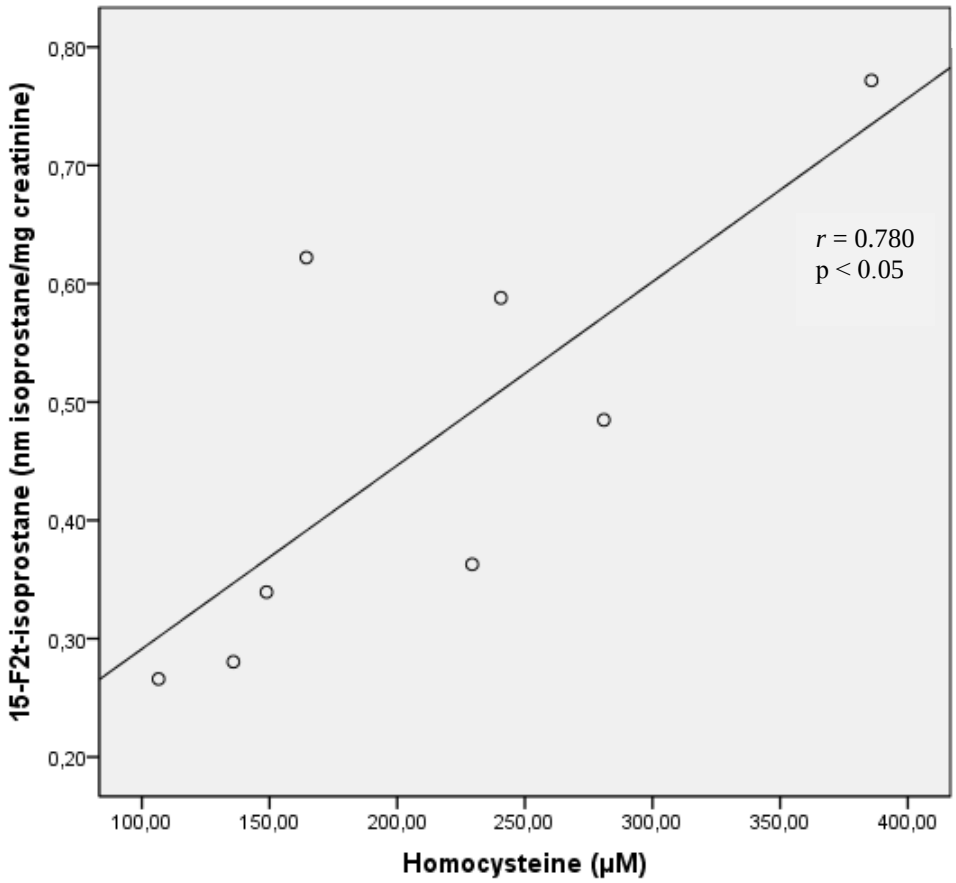

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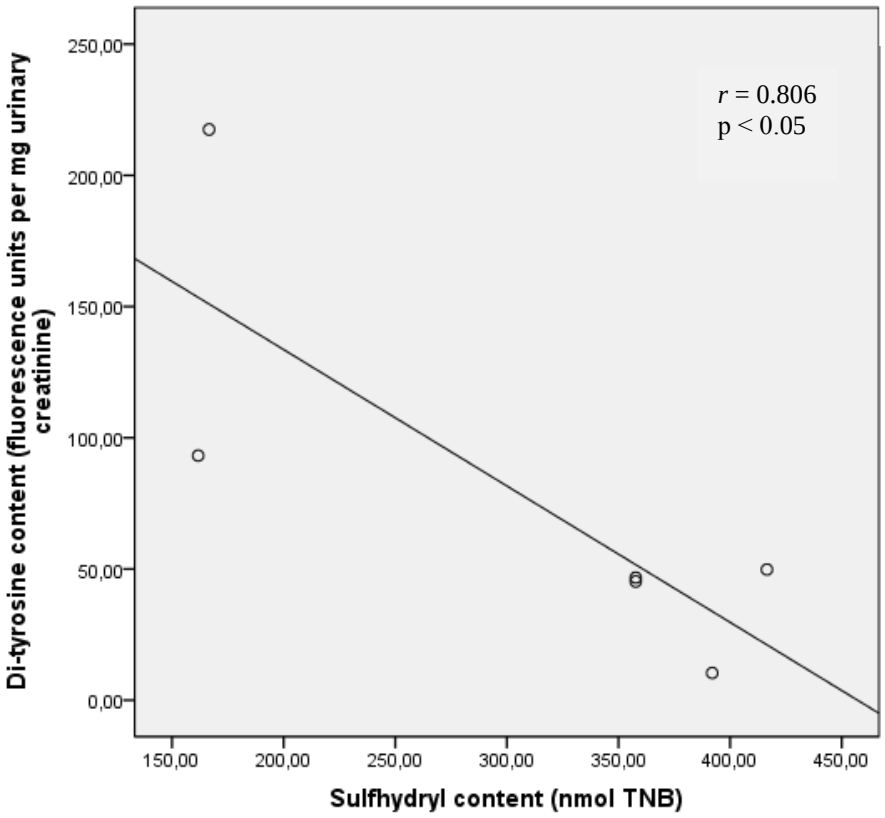

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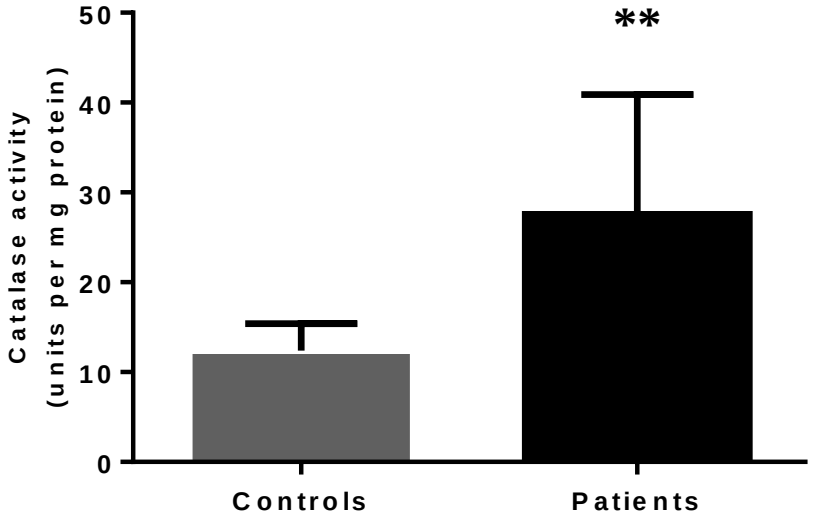

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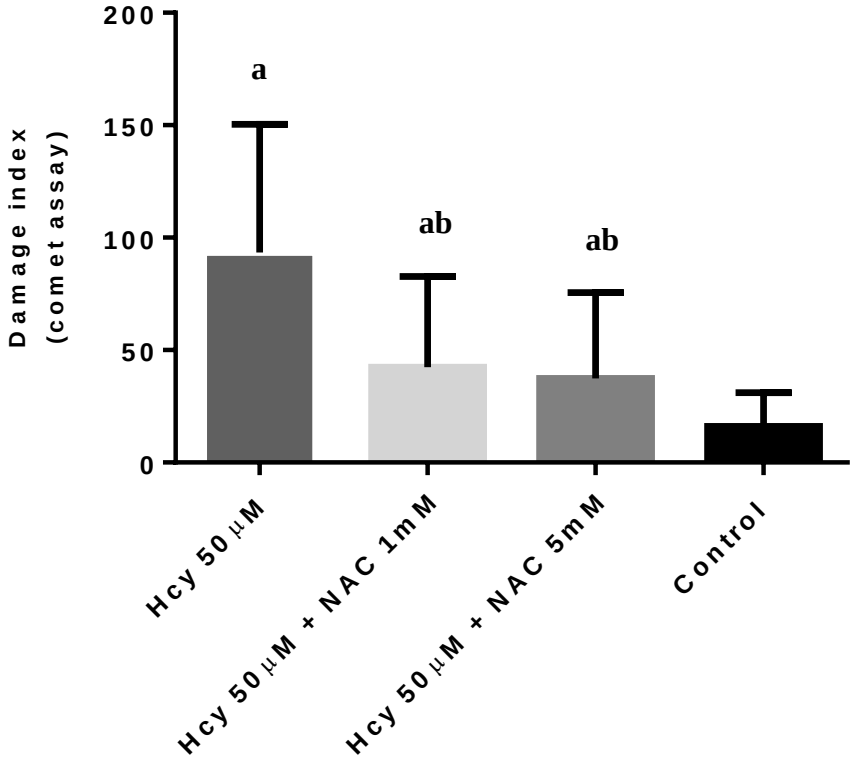
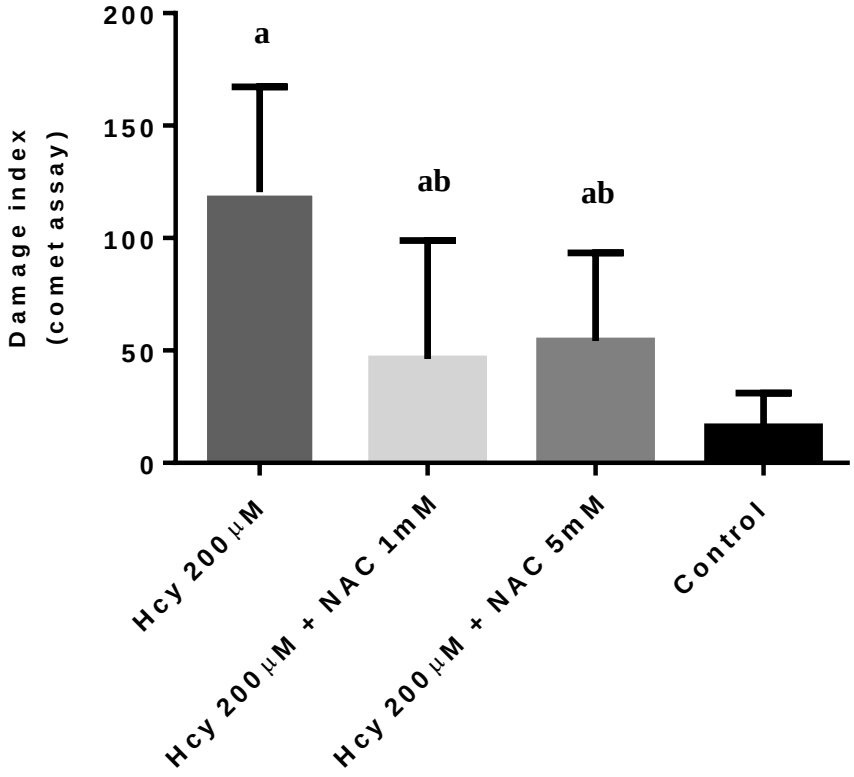


Figure 9:


200
150
100
50
0
Damage index
(comet assay)
a
ab
ab
Hcy 200 µM
Hcy 200 µM + NAC 1mM
Hcy 200 µM + NAC 5mM
Control

3.3 Capítulo III – Artigo 03

Experimental evidence that homocysteine induces chromosomal damage.

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Periódico: Cell Biology and Toxicology

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In vitro experimental evidence that homocysteine induces chromosomal damage.

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Keywords: homocysteine; DNA damage; micronucleus

Abstract

Hyperhomocysteinemia is a condition found in patients affected by deficiency of the enzyme cystathionine β-synthase activity (CBS), as well as in nutritional deficiencies (vitamin B_{12} or folate) and in abnormal renal function. We previously demonstrated evidence of DNA damage in blood of CBS-deficient patients, possibly due to high levels of homocysteine (Hcy). In the present work, we extended these investigations, evaluating chromosomal damage in presence of Hcy, using micronucleus assay. We verified an increase in micronucleus frequency in healthy white blood cells incubated with Hcy at concentrations 50, 100 and 200 μM, when compared to Hcy 10 μM and to controls. Significant positive correlations were observed between micronucleus frequency and Hcy concentrations, as well as between micronucleus frequency and DNA damage (evaluated by comet assay). The present data complements recents studies, showing a mechanism to the DNA damage caused by Hcy.

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is generated from methionine (Met) which concentrations are maintained dynamically by either a transsulfuration or remethylation pathways. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by enzyme cystathionine-β-synthase (CBS). Cystathionine is hydrolyzed by a second enzyme, ɤ-cystathionase, to form cysteine and α-ketobutyrate. In remethylation, Hcy acquires a methyl group from N-5-methyltetrahydrofolate or from betaine to form Met (Selhub, 1999).

High Hcy levels arises from disrupted Hcy metabolism; severe hyperhomocysteinemia occurs due to rare genetic defects resulting in deficiencies in cystathionine beta synthase (CBS), methylenetetrahydrofolate reductase, (MTHFR) or in enzymes involved in Hcy methylation. Mild hyperhomocysteinemia seen in fasting conditions is due to mild impairment in the methylation pathway (i.e. folate or vitamin B_{12} deficiencies or methylenetetrahydrofolate reductase thermolability) (Selhub, 1999). Hyperhomocysteinemia has been identified as an independent risk factor for cerebral, coronary, and peripheral atherosclerosis, although the pathological mechanism of this risk is not fully understood. High plasma levels of Hcy are also associated with an increased risk of neural defects (Wenstrom et al., 2000), Alzheimer's disease, and loss of cognitive functions (Miller, 2000; Breteler, 2000). Interestingly, several studies have also shown that Hcy can induce DNA damage (Kruman et al., 2000; Huang et al., 2001; Vanzin et al., 2014), suggesting a further mechanism by which increased levels of Hcy may contribute to the pathogenesis of the atherosclerosis and neurodegenerative diseases. In addition, observations indicate that plasma Hcy levels are positively correlated with baseline levels of genetic damage in lymphocytes of older men, as measured by the cytokinesis-block micronucleus (MN) assay (Fenech et al., 1997; Fenech et al., 1998).

The observation that chromosomal damage can be caused by exposure to ionising radiation or carcinogenic chemicals was among the first reliable evidence that physical and chemical agents can cause major alterations to the genetic material of eukaryotic cells (Evans, 1977). Evidence suggests that chromosomal abnormalities are a direct consequence and manifestation of damage at DNA level — for example, chromosomal breaks may result from unrepaired double strand breaks in DNA and

chromosomal rearrangements may result from misrepair of strand breaks in DNA (Savage, 1993). An alternative and simpler approach to assess chromosomal damage *in vivo* is the detection of micronucleus, analising cell populations such as the bone-marrow. The micronucleus assay in bone-marrow and peripheral blood cells is now one of the best established *in vivo* cytogenetic assays in the field of genetic toxicology (Fenech, 2000).

In a recent work, we demonstrated experimental evidence that DNA damage occurs in CBS-deficient patients and that this effect may possibly be associated with the high plasma Hcy levels found in these patients. Moreover, the *in vitro* study showed a concentration-dependent effect of Hcy inducing DNA damage (Vanzin et al., 2014). In this work, we extend these investigations analyzing the micronucleus frequency, in order to evaluate whether DNA damage was permanent after cell division. Additionally, we correlate the micronucleus frequency with the Hcy levels and with DNA damage measured by comet assay.

2. Materials and Methods

2.1 *In vitro* study

Venous blood was collected under sterile conditions in heparinized vials from three healthy volunteers individuals. Isolated leukocytes were incubated with various concentrations of Hcy (10, 50, 100 or 200 μM) at 37ºC for 6 h (Tice et al., 2000; Hartmann et al., 2003). This range of Hcy concentrations were based on the normal plasma Hcy levels (10 μM) and the concentrations found in blood from treated and not treated homocystinuric patients (50, 100 or 200 μM).

2.2 Micronucleus assay

Leucocytes obtained from healthy volunteers blood samples by venipuncture were used to perform the MN assay (Schmid, 1975). After the treatment of samples with Hcy, blood samples placed on the surface of the blade to make a smear, and the blood was spread over the surface of the blade. After 24 hours, the samples were fixed in 96% ethanol for 30 min. The samples were stained with Panoptic dye, washed in water and allowed to dry. After drying, the cells analyzed were considered as to have micronuclei if their sizes did not exceed 1/3 of the size of the nuclei in the main core and if they were clearly separated with discernible edges and the same color and refringence as the

core. The preparation of slides and cell counting was performed by double blind procedure and the micronuclei were observed in optical microscope (Leica® DM 500) using an immersion objective (10X100). The results were expressed in micronucleus frequency (%). Figure 1 illustrates the MN analysis.

2.3 Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. (1988) in accordance with general guidelines for use of the comet assay (Tice et al., 2000; Hartmann et al., 2003). Aliquots of 100µL from whole blood were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Slides were placed in lyses buffer (2.5 NaCl, 100mM EDTA, 10mM Tris, 10% DMSO, pH 10-10.5) to remove cell proteins, leaving DNA as "nucleiods". After treatment with lyses buffer and alkaline buffer solution (300mM NaOH and 1mM EDTA, pH>13), the slides were submitted to a horizontal electrophoresis. This technique was performed for 20 min at 4ºC (25V; 300mA; 0.9V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol (Nadin et al., 2001). After drying at room temperature overnight, gels were analyzed using an optical microscope. It were analyzed 100 cells in microscopy and to each cell it was determined a damage class. The damage classes were classified in: 0 = no tail (no damage); 1 = small tail smaller than the diameter of the head; 2 = tail length between one and two times the diameter of the head; 3 = long tail greater than twice the diameter of the head; 4 = long tail and more widespread than class 3. It was made a multiplication of each damage class by number of cells found in each damage class. The damage index (DI) was determined by the sum of these multiplications. The slides were analyzed under blind conditions at least by two different individuals. Figure 2 illustrates comet assay.

2.4 Statistical Analysis

Data were expressed as mean ± standard deviation. Comparisons between means were calculated by one-way one-ANOVA followed by the Tukey test when the F value was significant. A *p* value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3.Results

In this work we investigated the *in vitro* effect of four concentrations of Hcy (10, 50, 100 and 200 µM) on micronucleus frequency. Additionally, we correlate the micronucleus frequency with the Hcy levels and with DNA damage measured by comet assay.

Figure 3 shows the *in vitro* effect of Hcy (10, 50, 100 and 200 µM) on micronucleus frequency. We verified an increase in micronucleus frequency in Hcy at concentrations 50, 100 and 200 µM, when compared to Hcy 10 µM and control [$F(4.10)= 254.56$, $p<0.001$]. We also verified a concentration-dependent effect of Hcy on DNA damage, evaluated by comet assay in white blood cells [$F(4.10)= 639.02$, $p<0.001$], as expected (Table 1).

We also investigated a possible association between micronucleus frequency and Hcy levels, as well as between micronucleus frequency and DNA damage (comet assay). We found a significant positive correlation between micronucleus frequency and Hcy levels ($r = 0.944$, $p<0.001$) (Figure 4), as well as a significant positive correlation between micronucleus frequency and DNA damage evaluated by comet assay ($r = 0.949$, $p<0.001$) (Figure 5).

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4. Discussion

Hyperhomocysteinemia is generated as consequence of a wide range of determinants such as: genetic causes (CBS or MTHFR deficiencies), physiological causes (diet rich in methionine and/or poor in B vitamins), pathological causes (renal disease) or medications (antifolate drugs) (Mudd et al., 2001; Fowler, 2008). Moreover, increased levels of Hcy also appear to be associated with neurodegenerative and vascular disorders, and some types of câncer (Miller, 2000; Breteler, 2000; Mattson and Kruman, 2002; Mattson and Haberman, 2003). Although the pathogenesis of hyperhomocysteinemia is not fully established, Hcy dependent oxidative stress and/or biomolecules structure modifications play an important role in biotoxicity of Hcy (Malinowska et al., 2012). In this sense, we demonstrated in recents studies alterations in parameters of oxidative stress in CBS- deficient patients (Vanzin et al., 2011; Vanzin et al., 2015). Moreover, we showed evidence of DNA damage in blood of CBS-deficient patients, as well as by *in vitro* study, which demonstrated a concentration-dependent effect of Hcy on DNA damage through comet assay (Vanzin et al., 2014). In

this work, we extend these investigations analyzing the micronucleus frequency, in order to evaluate whether DNA damage was permanent after cell division.

We demonstrated by the *in vitro* study an increase in micronucleus frequency in leukocytes incubated with Hcy at concentrations (50, 100 and 200 µM) when compared to control and to Hcy 10 µM. Moreover, we showed significant positives correlations between micronucleus frequency and Hcy levels and between micronucleus frequency and DNA damage evaluated by comet assay.

Chromosomal aberrations and micronucleus are useful biomarkers of DNA lesions and frequently used in genotoxicity testing. Micronucleus are expressed in dividing cells that either contain chromosomal breaks lacking centromeres (acentric fragments) and/or whole chromosomals that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomals and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term "micronucleus". Micronucleus, therefore, provide a convenient and reliable index of both chromosomal breakage and chromosomal loss (Fenech, 2000).

Considering that Hcy undergoes autoxidation, generating reactive species of oxygen (ROS) (Starkebaum and Harlan, 1986; Loscalzo, 1996), a potential mechanism for the generation of genomic damage is the induction of oxidative stress by Hcy, which has been shown in some cell lines (Perez-de-Arce et al., 2005; Sethi et al., 2006). In this sense, our results agree with those of Picerno et al. (2007), who reported that Hcy-treated (at concentration 1mM) human peripheral blood lymphocytes in culture presented increase in DNA damage and in micronucleus frequency, altering immune function. These authors demonstrated an increase of rate of apoptosis in Hcy treated lymphocyte cultures compared to controls. Furthermore, Hcy exposure caused DNA fragmentation as evaluated by single cell gel electrophoresis showing the occurrence of comets, and increased frequency of micronucleated/binucleated cells in Hcy-treated cultures compared to controls, as revealed by cytokinesis block micronucleus assay. Gene mutations and gene silencing are known to play a critical role in the inactivation of genes involved in DNA repair, in cell cycle control, in proper chromosomal segregation during mitosis, and in apoptotic pathways. In this context, Kruman et al. (2002) demonstrated that Hcy impairs DNA repair in hippocampal neurons, and sensitizes them to oxidative stress, since it has been showed that incubation of

hippocampal cultures in the presence homocysteine induced cell death and rendered neurons vulnerable to death induced by amyloid β-peptide, which is toxic and is accumulated in Alzheimer's disease.

Some evidence suggest that different approaches may be useful to ameliorate toxic effects of Hcy, including quenching of oxidative agents and maintenance of transmethylation pathway. In this context, it was demonstrated that folic acid and vitamin B_{12} supplementation significantly reduced the Hcy-increased micronucleated cell frequency in cultured lymphocytes. Folic acid and vitamin B_{12} play an important role in DNA metabolism: folic acid is required for the synthesis of dTMP from dUMP; and both, folic acid and vitamin B_{12} are also required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of methylation patterns in DNA, that determine gene expression and DNA conformation (Fenech, 1999). It is especially interesting since folic acid and vitamin B_{12} are both used in treatment of CBS deficiency, and recently it was demonstrated the importance of these molecules in control of important parameters related with the disease, as reduction in Hcy levels, and increase in apolipoprotein A1 levels and paraoxonase activity (Vanzin et al., 2015).

In conclusion, this *in vitro* study showed that Hcy increases the chromosomal damage, evaluated by micronucleus frequency, and that this damage is correlated with DNA strand breaks (evaluated by comet assay). Further research is needed to verify these prelimilary results and to determine the effects of chronic hyperhomocysteinemia not only in lymphocytes, but also in other cells.

Acknowledgments

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Conflict of interest disclosure

The authors declare that there is no conflict of interest disclosure associated with this manuscript.

5. References

Breteler, M.M. Vascular involvement in cognitive decline and dementia. Epidemiologic evidence from the Rotterdam Study and the Rotterdam Scan Study, Ann. NY Acad. Sci. 2000; 903: 457–465.

Evans, H.J. Molecular mechanisms in the induction of chromosomal aberrations, in: D. Scott, B.A. Bridges, F.H. Sobels (Eds.), Progress in Genetic Toxicology, Elsevier North Holland Biomedical Press, 1977: 57–74.

Fenech, M.; Dreosti, I.E., Rinaldi, J.R. Folate, vitamin B_{12}, homocysteine status and chromosomal damage rate in lymphocytes of older men. Carcinogenesis 1997; 18: 1329–1336.

Fenech, M., Aitken, C., Rinaldi, J.R. Folate, vitamin B_{12}, homocysteine status and chromosomal damage in young Australian adults. Carcinogenesis. 1998; 19:1163–1171.

Fenech, M. Micronucleus frequency in human lymphocytes is related to plasma vitamin B12 and homocysteine. Mutat. Res. 1999; 428: 299–304.

Fenech, M. The *in vitro* micronucleus technique. Mutation Research 2000; 455:81–95.

Fowler, B. Homocysteine, S-adenosylmethionine and S-adenosylhomocysteine, in: Blau, N., Duran, M., Gibson, K.M. (Eds), Laboratory Guide to the Methods in Biochemical Genetics, Springer-Verlag Berlin Heidelberg, Berlin, 2008:112-135.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, G., Speit, G., Thybaud, V., Tice, R.R. Recommendations for conducting the in vivo alkaline comet assay, Mutagenesis 2003; 18: 45–51.

Huang, R.F., Huang, S.M., Lin, B.S., Wei, J.S., Liu, T.Z. Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells, Life Sci. 2001; 68: 2799–2811.

Kruman, I.I., Culmsee, C., Chan, S.L., Kruman, Y., Guo, Z., Penix, L., Mattson, M.P. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity, J. Neurosci. 2000; 20: 6920–6926.

Kruman, I.I., Kumaravel, T.S., Lohani, A., Pedersen, W.A., Cutler, R.G., Kruman, Y., Haughey, N., Lee, J., Evans, M., Mattson, M.P. Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. J. Neurosci. 2002; 22: 1752–1762.

Loscalzo, J. The oxidant stress of hyperhomocyst(e)inemia, J. Clin. Invest. 1996; 98: 5-7.

Malinowska, J., Kolodziejczyk, J., Olas, B. The disturbance of hemostasis induced by hyperhomocysteinemia: the role of antioxidants, Acta Biochim Pol 2012; 59:185–194.

Mattson, M.P., Kruman, I.I., Duan, W. Folic acid and homocysteine in age related disease. Ageing Res. Rev. 2002; 1: 95–111.

Mattson, M.P., Haberman, F. Folate and homocysteine metabolism: therapeutic targets in cardiovascular and neurodegenerative disorders. Curr. Med. Chem. 2003; 10: 1923–1929.

Miller, J.W. Homocysteine, Alzheimer's disease, and cognitive function. Nutrition 2000; 16: 675–677.

Mudd, S.H., Levy, H.L., Kraus, J.P. Disorders of transsulfuration, in: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 2001:.2007-2056.

Nadin, S., Vargas-Roig, L., Ciocca, D. A silver staining method for single-cell gel assay, J. Histochem. Cytochem. 2001; 49: 1183–1186.

Perez-de-Arce, K., Foncea, R., Leighton, F. Reactive oxygen species mediates homocysteine-induced mitochondrial biogenesis in human endothelial cells: modulation by antioxidants. Biochem. Biophys. Res. Commun. 2005; 338: 1103–1109.

Picerno, I., Chirico, C., Condello, S., Visalli, G., Ferlazzo, N., Gorgone, G., Caccamo, D., Ientile, R. Homocysteine induces DNA damage and alterations in proliferative capacity of T-lymphocytes: a model for immunosenescence? Biogerontology. 2007; 8: 111–119.

Savage, J.R.K. Update on target theory as applied to chromosomal aberrations, Env. Mol. Mutagen. 1993; 22:198–207.

Schmid, W. The micronucleus test, Mutat Res. 1975; 31: 9-15.

Selhub, J. Homocysteine metabolismo, Annu. Rev. Nutr. 1999; 19: 217–246.

Sethi, A.S., Lees, D.M., Douthwaite, J.A., Dawnay, A.B., Corder, R. Homocysteine-induced endothelin-1 release is dependent on hyperglycaemia and reactive oxygen species production in bovine aortic endothelial cells. J. Vasc. Res. 2006; 43: 175–183.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 1988; 175: 184–191.

Starkebaum, G., Harlan, JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine, J. Clin. Invest. 1986; 77: 1370-1376.

Tice, R.R., Agurell, D., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 2000; 35:206–221.

Vanzin, C.S., Biancini, G.B., Sitta, A., Wayhs, C.A.Y., Pereira, I.N., Rockenbach, F., Garcia, S.C., Wyse, A.T.S., Schwartz, I.V.D., Wajner, M., Vargas, C.R. Experimental evidence of oxidative stress in plasma of homocystinuric patients: A possible role for homocysteine, Mol. Genet. Metab. 2011; 104: 112-117.

Vanzin, C.S., Mescka, C.P., Donida, B., Hammerschimidt, T.G., Ribas, G.S., Kolling, J., Scherer, E.B., Vilarinho, L., Nogueira, C., Coitinho, A.S., Wajner, M., Wyse, A.T.S., Vargas, C.R. Lipid, oxidative and inflammatory profile and alterations in the enzymes paraoxonase and butyrylcholinesterase in plasma of patients with homocystinuria due cbs deficiency: the vitamin B_{12} and folic acid importance, Cell Mol Neurobiol. 2015; 35: 899-911.

Vanzin, C.S., Manfredini, V., Marinho, A.E., Biancini, G.B., Ribas, G.S., Deon, M., Wyse, A.T.S., Wajner, M., Vargas, C.R. Homocysteine contribution to DNA damage in cystathionine β-synthase-deficient patients. Gene. 2014; 539: 270–274.

Wenstrom, K.D., Johanning, G.L., Owen, J., Johnston, K.E., Acton, S., Tamura, T. Role of amniotic fluid homocysteine level and of fetal 5,10-methylenetetrahydrafolate reductase genotype in the etiology of neural tube defects. Am J Med Genet, 2000; 90: 12–16.

Figure legends:

Figure 1: Micronucleus assay of leukocytes in the presence of Hcy. Cells were incubated for 6h at 37°C in presence of Hcy at concentrations (10, 50, 100 or 200 µM).

Figure 2: Comet assay of leukocytes in the presence of Hcy. Cells were incubated for 6h at 37°C in presence of Hcy at concentrations (10, 50, 100 or 200 µM).

Figure 3: *In vitro* effect of homocysteine on micronucleus frequency in leukocytes. Data represent the mean ± SD of 3 independent experiments. (a) $p<0.001$, compared to the Hcy 10 µmol/L group; (b) $p<0.001$, compared to the Hcy 50 µmol/L group; (c) $p<0.001$, compared to the Hcy 100 µmol/L group (One-way ANOVA, followed by Tukey test).

Figure 4: Correlation between micronucleus frequency and Hcy levels in leukocytes ($r = 0.944$, $p<0.001$).

Figure 5: Correlation between micronucleus frequency and DNA damage evaluated by comet assay ($r = 0.949$, $p<0.001$).

Figure 1:

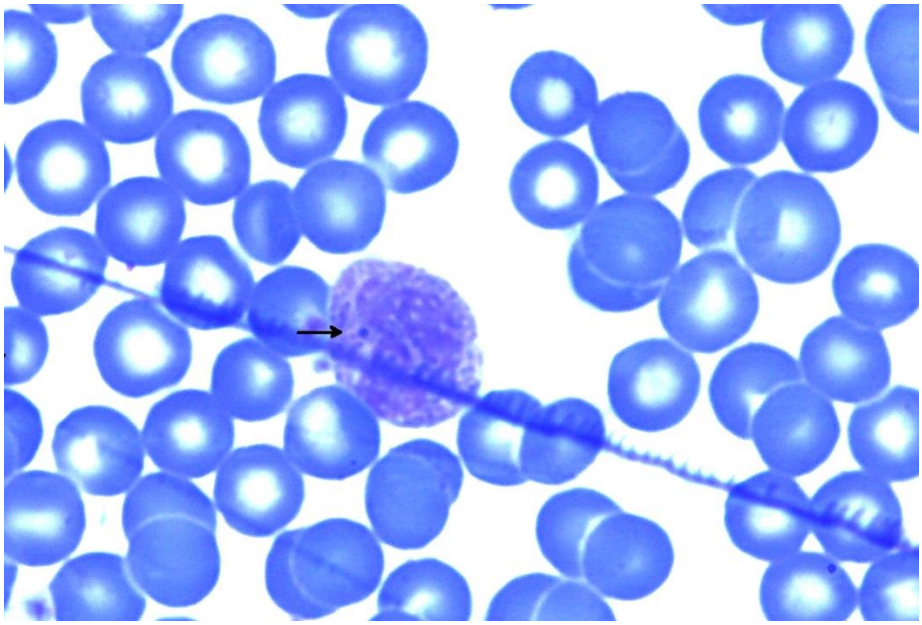
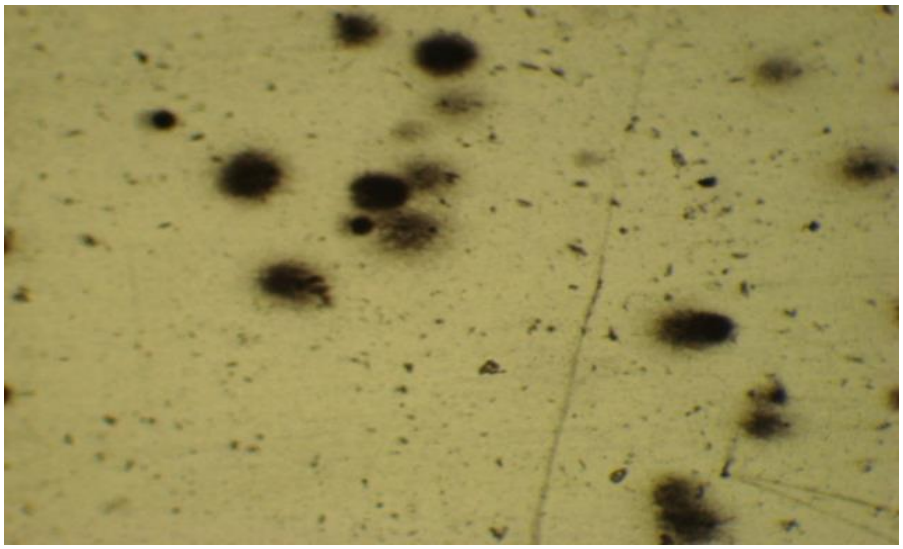


Figure 2:



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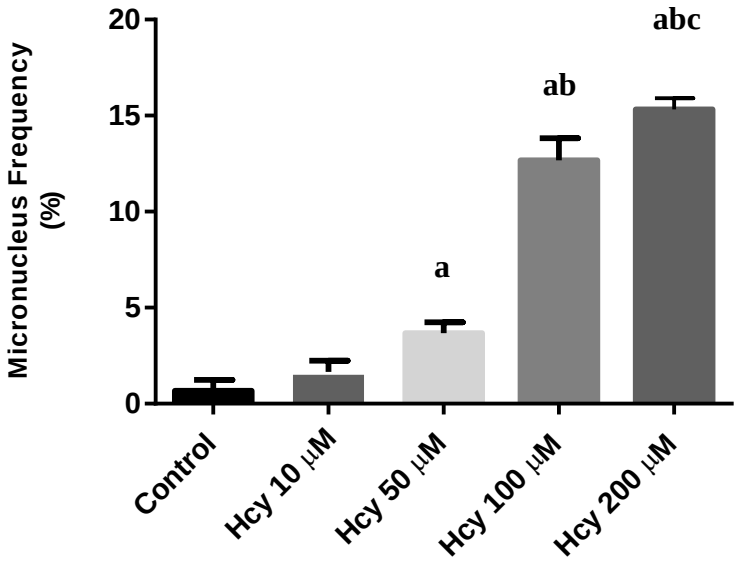

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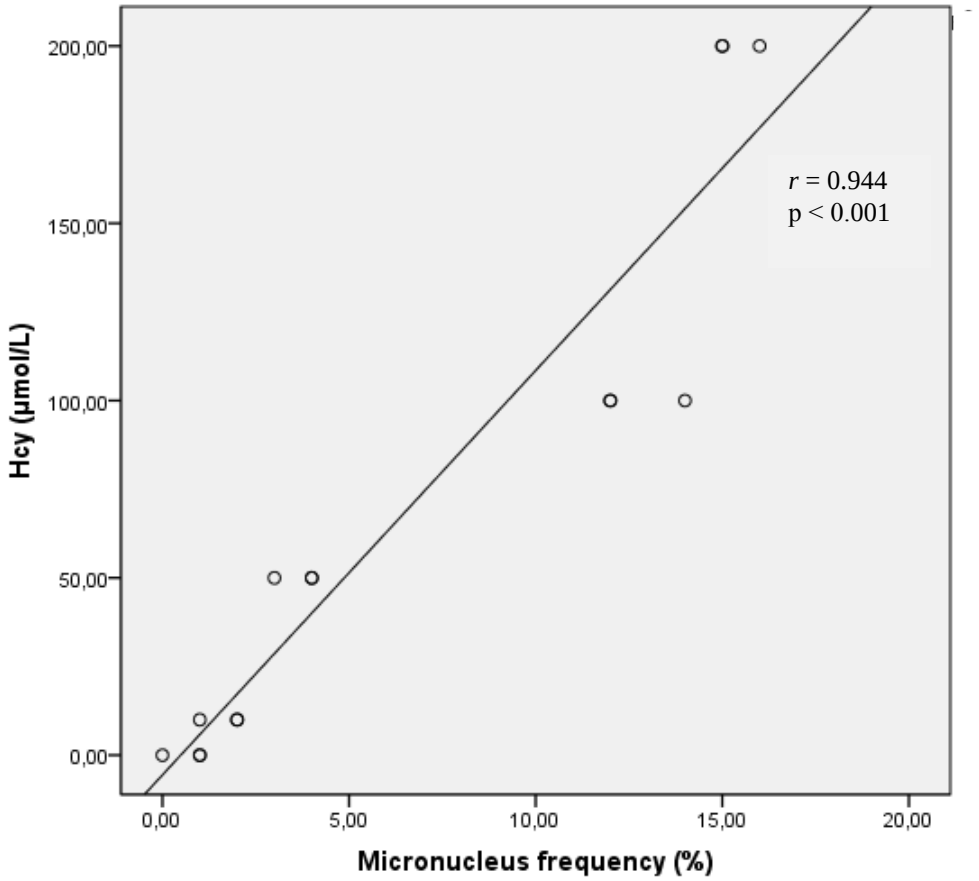

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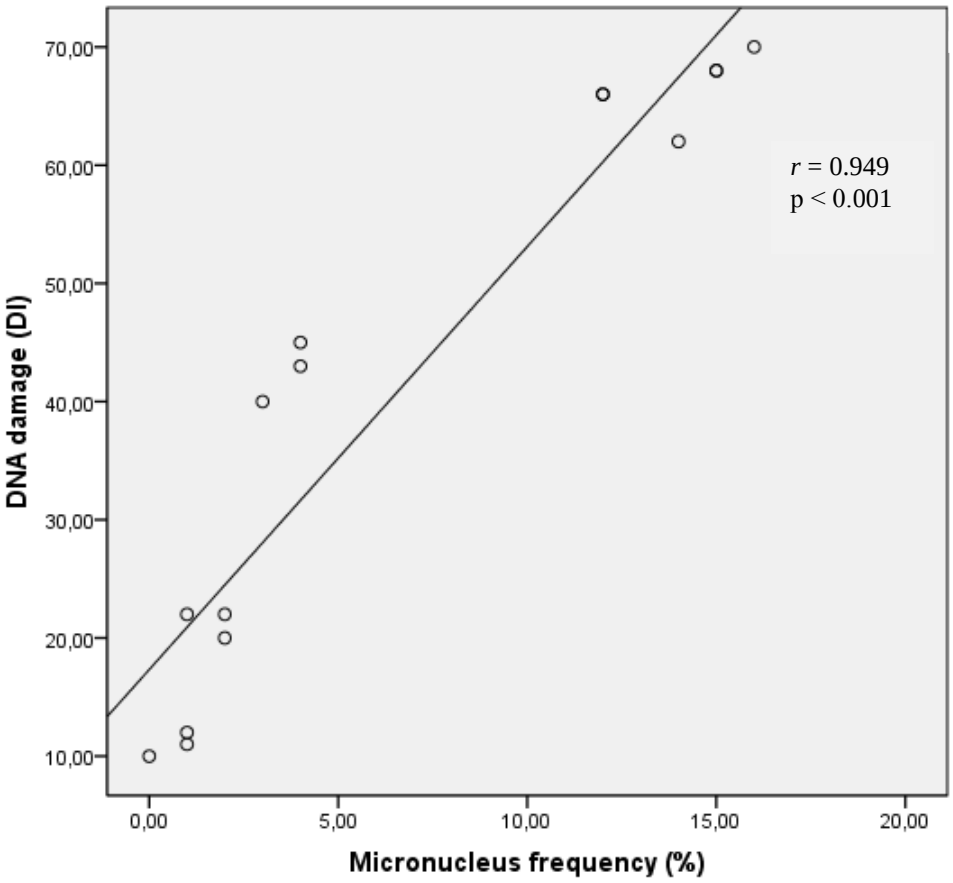


Table 1. *In vitro* effect of Hcy on DNA damage (comet assay).

	Control (without Hcy)	**Hcy 10µM**	**Hcy 50µM**	**Hcy 100µM**	**Hcy 200µM**
Damage index (mean±sd)	11 ± 1,0	21,33 ± 1,15[a]	42,67 ± 2,52[b]	64,67 ± 2,31[c]	68,67 ± 1,15[d]

Data represent the mean ± SD of 3 independent experiments. (a) p<0.001, compared to controls; (b) p<0.001, compared to the Hcy 10 µmol/L group; (c) p<0.001, compared to the Hcy 50 µmol/L group; (d) p<0.001, compared to the Hcy 100 µmol/L group (One-way ANOVA, followed by Tukey test).

4. DISCUSSÃO

A homocistinúria clássica é um distúrbio do metabolismo dos aminoácidos, descrito há mais de 50 anos por Carson et al. (1962). Essa desordem multissistêmica é causada por mutações no gene da enzima cistationina-β-sintase (CBS). As características e sinais fenotípicos da doença incluem retardo mental, *ectopia lentis*, episódios tromboembólicos e osteoporose; enquanto que bioquimicamente a doença se caracteriza pela elevação da tHcy no sangue a qual é quase sempre acompanhada de elevações na Met (Mudd et al., 2001; Skovby et al., 2010). A deficiência de CBS pode ser tratada em pacientes B_6-responsivos com a administração de altas doses de piridoxina (vitamina B_6). Para aqueles pacientes B_6-não responsivos, a terapia inclui a administração de betaína, folato e vitamina B_{12} e, similarmente a outros EIM, também inclui um controle dietético (Wilcken et al., 1985, Melenovská et al., 2015).

Na ausência de programas de triagem neonatal, o diagnóstico da homocistinúria por deficiência de CBS é muitas vezes perdido. Como consequência, se os pacientes não são detectados precocemente, a condição passa a ser associada com significativa morbidade e mortalidade (Cruysberg et al., 1996; Walter et al., 1998; Walter et al., 2011). Uma grande proporção de indivíduos B_6-responsivos (mutação c.833T>C) permanece não diagnosticada (Janosík et al., 2009) e apresenta episódios tromboembólicos na terceira década de vida (Skovby et al., 2010).

Embora ainda não sejam completamente conhecidos os mecanismos moleculares através dos quais a Hcy favorece a aterotrombose, evidências epidemiológicas de uma associação entre hiper-homocisteinemia e doença vascular aterosclerótica são completamente consistentes (Welch e Loscalzo, 1998; den Heijer et al., 1998). Um dos possíveis mecanismos danosos da hiper-homocisteinemia envolve o dano oxidativo. Durante a auto-oxidação da Hcy no plasma, levando a formação de homocistina (o

dímero da Hcy), ERO tais como ânion superóxido e peróxido de hidrogênio (H_2O_2) são geradas; essas moléculas derivadas do oxigênio podem ser potencialmente responsáveis pela disfunção endotelial associada com a hiper-homocisteinemia. Espécies reativas de oxigênio podem iniciar o processo de lipoperoxidação na superfície das células endoteliais; assim, modificações induzidas pela Hcy podem contribuir para a patogênese da aterotrombose em síndromes clínicas de hiper-homocisteinemia (Loscalzo, 1996; Kanani et al., 1999; Davì et al., 2001).

Vários estudos em modelos animais vêm evidenciando um possível papel do estresse oxidativo na patogênese da homocistinúria. Streck et al. (2003) demonstraram, em estudo *in vitro,* que a Hcy nas concentrações de 100 e 500 μmol/L aumenta a lipoperoxidação e diminui a capacidade antioxidante em hipocampo de ratos. Robert et al. (2005) analisaram o dano a proteínas e aos lipídeos em fígado de ratos deficientes da enzima CBS e em ratos que possuíam a atividade normal da enzima. O estudo revelou que os ratos deficientes de CBS apresentaram um aumento nos níveis de proteínas oxidativamente modificadas, bem como um aumento nos níveis de subprodutos da lipoperoxidação, tais como malondialdeído (MDA) e 4-hidroxinonenal (4-HNE). Mais recentemente, da Cunha et al. (2011) demonstraram que a hiper-homocisteinemia crônica aumenta a lipoperoxidação e o dano oxidativo às proteínas, e prejudica as defesas antioxidantes enzimáticas e não enzimáticas em pulmão de ratos. Nesse sentido, nosso grupo de pesquisa tem investigado o efeito do estresse oxidativo na homocistinúria devido a deficiência de CBS, avaliando parâmetros de estresse oxidativo em sangue de pacientes homocistinúricos no momento do diagnóstico (não tratados) e durante o tratamento. Em um estudo recente foi demonstrado um aumento significativo nos níveis de grupamentos carbonilas (parâmetro de dano oxidativo às proteínas) e nos níveis de MDA (parâmetro de dano oxidativo aos lipídeos) no plasma de pacientes

homocistinúricos no momento do diagnóstico. Os pacientes em tratamento tiveram uma redução significativa dos níveis de MDA em relação aos pacientes no diagnóstico, mas não alcançaram os níveis encontrados nos controles. Com relação à capacidade antioxidante não enzimática, essa foi avaliada por dois parâmetros, TAS e sulfidrilas (que também representa um parâmetro inversamente proporcional ao dano a proteínas). Foi demonstrado que tanto pacientes no momento do diagnóstico quanto pacientes em tratamento apresentam uma diminuição significativa de TAS e de grupamentos sulfidrilas no sangue, em comparação com os controles. Esses resultados avaliados em conjunto indicam que existe um aumento no dano oxidativo às proteínas e lipídeos, e uma diminuição nas defesas antioxidantes não enzimáticas em pacientes homocistinúricos, indicando um possível papel do estresse oxidativo na patogênese da doença. Adicionalmente, foi encontrada uma correlação significativa positiva entre os níveis de tHcy e os níveis de MDA, bem como uma correlação significativa negativa entre os níveis de tHcy e os níveis de grupamentos sulfidrilas. Esses resultados indicam um possível papel da Hcy na indução do estresse oxidativo observado (Vanzin et al., 2011). Na sequência, foi avaliado o efeito *in vivo* e *in vitro* da Hcy sobre o dano ao DNA, analisado pelo ensaio cometa em leucócitos. Foi observado que pacientes homocistinúricos tratados apresentam um aumento significativo do dano ao DNA quando comparados aos controles. Além disso, o estudo *in vitro* demonstrou um efeito concentração dependente da Hcy sobre o dano ao DNA (Vanzin et al., 2014).

Considerando que estudos em modelos animais fortemente indicam a influência do estresse oxidativo na patogênese da homocistinúria, e, dando continuidade aos estudos anteriores avaliando o estresse oxidativo em pacientes portadores de homocistinúria, nesse trabalho as investigações foram ampliadas, objetivando um maior conhecimento sobre os mecanismos de dano e o tratamento na homocistinúria. Para tal,

foram avaliados parâmetros de estresse oxidativo, bem como o perfil lipídico e inflamatório em pacientes com homocistinúria clássica no momento do diagnóstico e durante a terapêutica preconizada (dieta hipoproteica, vitamina B_6, ácido fólico, betaína, vitamina B_{12}). Ainda foi objetivo desta tese estudar o efeito *in vitro* da Hcy sobre o dano cromossômico, bem como avaliar o efeito *in vitro* da NAC sobre o dano ao DNA causado pelas altas concentrações de Hcy.

No primeiro momento, foram analisadas amostras de plasma de pacientes homocistinúricos no momento do diagnóstico e durante o tratamento, e de indivíduos saudáveis com idade e sexo comparáveis aos dos pacientes (controles). Foram avaliados os perfis lipídico, inflamatório e oxidativo, bem como a atividade das enzimas paraoxonase-1 (PON1) e butirilcolinesterase (BuChE). Posteriormente, todos os parâmetros foram correlacionados com os níveis de tHcy, bem como com os níveis de ácido fólico e vitamina B_{12}, ambos usados no tratamento da homocistinúria por deficiência de CBS.

O perfil inflamatório foi avaliado pela medida das interleucinas IL-1β, IL-6 e interferon-ɤ. Foi observado um aumento significativo nos níveis de IL-6 em pacientes homocistinúricos no momento do diagnóstico. Os pacientes em tratamento apresentaram uma tendência a redução (27,68%), quando comparados aos pacientes não tratados. As demais interleucinas foram estatisticamente similares em todos os grupos avaliados. Resultados similares foram observados para o perfil oxidativo, avaliado pela medida dos níveis de grupamentos carbonilas, o qual apresentou aumento significativo nos pacientes não tratados, apresentando uma tendência a redução (15,2%) nos pacientes tratados. Ainda, foi observada uma correlação significativa positiva entre os níveis de grupamentos carbonilas e os níveis de IL-6.

Inflamação é fundamentalmente uma resposta protetora do organismo contra um agente, que pode ser um micro-organismo, um estímulo físico ou um agente químico. Alguns dos importantes mediadores inflamatórios incluem: histamina, serotonina, prostaglandinas, leucotrienos, óxido nítrico, citocinas, proteínas de fase aguda e ERO (Das UN, 2006). O balanço relativo entre as citocinas anti-inflamatórias e pró-inflamatórias parece desempenhar um significante papel na etiologia da trombose e da aterogênese (Ross, 1999). Uma das citocinas pró-inflamatórias, IL-6, vem atraindo interesse, uma vez que sua produção causa uma cascata de efeitos que resultam no estímulo de proteínas de fase aguda e ativação do sistema imune inato. Triagens clínicas envolvendo bloqueadores de IL-6 têm demonstrado eficácia em várias condições inflamatórias graves, incluindo artrite reumatóide e artrite juvenil idiopática (Tanaka e Kishimoto, 2012). Nesse trabalho, nós demonstramos que pacientes homocistinúricos não tratados apresentam níveis significantemente aumentados de IL-6, o mesmo foi demonstrado para a medida dos grupamentos carbonilas, um parâmetro de dano oxidativo às proteínas. Curiosamente, foi encontrada uma correlação significativa positiva entre os níveis de IL-6 e os níveis de grupamentos carbonilas, indicando uma possível relação entre inflamação e estresse oxidativo, ambos possivelmente gerados nos pacientes homocistinúricos como resultado dos altos níveis de tHcy e/ou seus metabólitos.

Outro importante resultado encontrado nesse trabalho foi a interrelação entre o estresse oxidativo e o metabolismo lipídico. Foi demonstrada uma correlação significativa positiva entre os níveis de grupamentos sulfidrilas e a atividade da enzima PON1. Ambos os parâmetros foram estatisticamente diminuídos nos dois grupos de pacientes homocistinúricos, tratados e não tratados, em comparação com os controles.

Adicionalmente, os níveis de HDL e de ApoA foram estatisticamente reduzidos em pacientes homocistinúricos tratados e não tratados, quando comparados aos controles.

A PON1 é uma enzima expressa no fígado, que circula no sangue em associação com ApoA1 nas partículas de HDL, contribuindo para as atividades anti-inflamatórias e antioxidantes dessa lipoproteína (Jarvik et al., 2000; Perla-Kaján e Jakubowsky, 2012). Dados adicionais sobre as funções anti-ateroscleróticas da PON1 provêm de estudos com camundongos transgênicos super expressando PON1 humana, uma vez que PON1 humana expressa em camundongos protege contra aterosclerose induzida por dieta rica em gorduras (Tward et al., 2002). Uma proteção aumentada contra o estresse oxidativo induzido por íons cobre também foi observada; PON1 inibe a formação de hidroperóxidos sobre o HDL, protegendo, assim, a função e a integridade da molécula de HDL (Oda et al., 2002). Além disso, a superexpressão do gene da PON1 humana em camundongos reprime a aterogênese e promove a estabilidade da placa aterosclerótica (She et al.,2009). Mais respostas para o mecanismo cardioprotetor da PON1 vieram após a descoberta do seu substrato natural, a Hcy-tiolactona (Jakubowski, 2000). A Hcy-tiolactona é um metabólito reativo da Hcy, que modifica resíduos de lisina em proteínas, através de um processo conhecido como *N*-homocisteinilação (Jakubowski, 1997; Jakubowski, 1999). A *N*-homocisteinilação de proteínas poderia explicar os efeitos danosos da hiper-homocisteinemia. A hipótese da doença vascular mediada pela Hcy-tiolactona (Jakubowski, 1997) explica a toxicidade da Hcy através da sua conversão em Hcy-tiolactona, a qual modifica proteínas para gerar *N*-Hcy-proteínas, levando, por sua vez, a morte celular, inflamação, resposta auto-imune, e como consequência a aterosclerose, trombose e neurotoxicidade (Jakubowski, 2008a; Jakubowski, 2010; Perla-Kaján e Jakubowsky, 2012). Assim, alguns dos efeitos protetores da PON1 se devem ao fato de essa enzima hidrolisar Hcy-tiolactona e

proteger contra o acúmulo de proteínas homocisteinilas *in vitro* e *in vivo* (Perla-Kaján e Jakubowsky, 2010). Em trabalho anterior (Vanzin et al., 2011), foi demonstrado uma correlação significativa negativa entre os níveis de grupamentos sulfidrilas e os níveis de tHcy. Nesse trabalho, os níveis de grupamentos sulfidrilas foram positivamente correlacionados com a atividade da PON1. Assim, é possível que os altos níveis sanguíneos de tHcy estejam sofrendo reações de auto-oxidação e gerando radicais livres capazes de oxidar algum grupamento sulfidrila necessário para a atividade da PON1. Interessantemente, a diminuição nos parâmetros com propriedades ateroprotetoras, a saber, HDL, ApoA e PON1, foi observada tanto em pacientes não tratados como nos pacientes tratados. Essa "falha" do tratamento em reverter as alterações observadas pode ser reflexo da baixa aderência dos pacientes ao tratamento prescrito. Os pacientes homocistinúricos tratados estudados nesse trabalho apresentaram níveis médios de tHcy de 137,8 ± 104,3 μmol/L (média ± desvio padrão), incluindo pacientes responsivos (n=3) e não responsivos (n=7) à terapia. Foi observado nesse trabalho correlações significativas positivas entre os níveis de vitamina B_{12} e PON1 e entre os níveis de vitamina B_{12} e ApoA, e uma correlação significativa negativa entre os níveis de tHcy e ácido fólico. Na homocistinúria, assim como em outras doenças metabólicas, as anormalidades bioquímicas características da doença são responsáveis pelas complicações clínicas. Sendo assim, o tratamento ótimo para a homocistinúria deve objetivar minimizar essas anormalidades bioquímicas (Mudd et al., 2001). Triagem neonatal, instituição precoce do tratamento, boa aderência à dieta e manutenção dos níveis de homocistina livre em níveis menores que 11μmol/L parecem proteger contra as reconhecidas complicações da homocistinúria não tratada (Yap e Naughten, 1998). Nesse trabalho nós sugerimos que a "falha" do tratamento em reverter alterações importantes encontradas nos pacientes não tratados pode ocorrer devido à baixa

aderência ao tratamento. Novas estratégias e abordagens terapêuticas se fazem necessárias para otimizar o tratamento e melhorar os resultados observados em pacientes homocistinúricos tratados.

Ainda no primeiro capítulo desta tese foram avaliados os níveis de colesterol LDL e colesterol LDL-oxidado, os quais foram similares em todos os grupos estudados. Acredita-se que o mecanismo de dano da Hcy nessas moléculas não seja por reações de oxidação, mas possivelmente por reações de homocisteinilação (Jakubowski, 2008b). Finalmente, a avaliação do perfil lipídico incluiu a medida da atividade da enzima BuChE, a qual foi significativamente aumentada nos pacientes homocistinúricos não tratados. Essa enzima está associada com síndrome metabólica e vem sendo positivamente correlacionada com fatores de risco cardiovascular (Santarpia et al., 2013). O aumento da sua atividade no momento do diagnóstico em pacientes com homocistinúria clássica corrobora com o risco cardiovascular a que esses pacientes estão sujeitos.

Na sequência, no segundo capítulo desta tese foram avaliados parâmetros de estresse oxidativo e os níveis de nitritos na urina de pacientes homocistinúricos tratados, cujos níveis médios de tHcy no plasma foram de 191.3 ± 105.3 μmol/L (média ± desvio padrão). Foi demonstrado um aumento dos níveis de 15-F2t-isoprostanos, marcador de dano oxidativo aos lipídeos; um aumento nos níveis de di-tirosina, marcador de dano oxidativo às proteínas; e um aumento nos níveis de nitritos. Essas alterações encontradas na urina parecem se correlacionar com as alterações encontradas no sangue, uma vez que foi demonstrada uma correlação significativa negativa entre os níveis de grupamentos sulfidrilas e os níveis de di-tirosina. Além disso, foi demonstrado uma correlação significativa positiva entre os níveis de 15-F2t-isoprostanos e os níveis

plasmáticos de tHcy, evidenciando mais uma vez o papel da Hcy na indução do estresse oxidativo observado.

Estudos propõem que o H_2O_2 gerado durante a auto-oxidação do grupo sulfidrila da Hcy possa mediar a citotoxicidade endotelial da Hcy através da lipoperoxidação (Starkebaum e Harlan, 1986; Loscalzo, 1996). A lipoperoxidação tem como principais consequências a alteração na fluidez da membrana, o aumento da permeabilidade da membrana e o dano às proteínas de membrana, inativando receptores, enzimas e canais iônicos (Halliwell e Gutteridge, 2007). A quantificação de F2-isoprostanos tem se mostrado um valioso método para avaliação da lipoperoxidação *in vivo*. Com relação a homocistinúria, essa dosagem se torna interessante, visto que quantidades significativas de F2-isoprostanos tem sido encontradas em lesões ateroscleróticas humanas (Gniwotta et al., 1997; Pratico et al., 1997). Além disso, a medida de isoprostanos no sangue e urina de indivíduos tem sido utilizada para estudar o efeito da suplementação de antioxidantes sobre a lipoperoxidação (Halliwell, 2000; Roberts e Morrow, 2002). Isso se torna especialmente interessante no monitoramento do estresse oxidativo na homocistinúria, visto que a suplementação com antioxidantes pode ser sugerida como terapia complementar para essa doença.

O dano oxidativo às proteínas pode ser importante *in vivo* tanto pelo seu efeito direto (afetando a função de receptores, enzimas, proteínas transportadoras), como pelo seu efeito secundário, contribuindo para o dano às outras biomoléculas (por exemplo, inativação de enzimas de reparo do DNA). Di-tirosina, facilmente detectada na urina de humanos, aparentemente não metabolizada e formada por um ataque feito por radicais livres a uma ampla faixa de proteínas, pode ser um biomarcador válido para uso humano (Halliwell e Whiteman, 2004). Pacientes com sepse foram reportados apresentando altos níveis de di-tirosina urinária (Bhattacharjee et al., 2001), assim como

crianças com Kwashiorkor (Manary et al., 2000), uma doença que se acredita estar envolvida com estresse oxidativo (Fechner et al., 2001). Nesse trabalho, os níveis de di-tirosina demonstraram estar aumentados na urina dos pacientes homocistinúricos tratados. Esses resultados são complementares aos encontrados em plasma, visto que demonstrou-se uma correlação significativa negativa entre os níveis de sulfidrilas plasmáticos e os níveis de di-tirosina urinários. Já que dois terços dos grupamentos sulfidrilas estão ligados às proteínas, uma redução desses grupamentos sugere oxidação proteica (Thomas et al., 1995; Hansen et al., 2009; Requejo et al., 2010). Os resultados confirmam a oxidação proteica em pacientes homocistinúricos, visto que di-tirosina é formada pela oxidação de resíduos de tirosina, que leva à formação de uma ligação inter-fenólica altamente estável a qual não sofre metabolismo adicional (Mc Guire et al., 2009).

Ainda no segundo capítulo desta tese, foi demonstrado um aumento dos níveis de nitrito urinário nos pacientes homocistinúricos tratados, em comparação com os controles. Sabe-se que a síntese de óxido nítrico (NO) pode ser avaliada através da medida das concentrações de nitrito e nitrato no plasma e na urina (Tsikas et al. 2006). Em situações fisiológicas, a enzima NOS III (eNOS) catalisa a formação do NO pela incorporação do oxigênio molecular no substrato L-arginina, numa reação que requer NADPH, o ativador alostérico calmodulina e vários co-fatores, tais como tetrahidrobiopterina (BH4) (Verhaar et al., 2002). O NO produzido pelas células endoteliais relaxa as células musculares lisas dos vasos, causa vasodilatação e inibe a agregação plaquetária (Mansoor et al., 2005). Estudos indicam que o folato pode influenciar a atividade enzimática da NOS III, aumentando a síntese de NO (Verhaar et al., 2002; Mansoor et al., 2005); esse fato poderia parcialmente explicar o aumento nos níveis de nitrito observados na urina dos pacientes homocistinúricos, uma vez que todos

os pacientes fazem uso de folato como parte da terapia preconizada para a doença. Além disso, uma hipótese levantada é que o aumento dos níveis de NO poderia ser resultado de um mecanismo compensatório, já que a geração de S-nitroso-Hcy através da nitrosação da Hcy impediria a formação de H_2O_2 pela auto-oxidação, e ainda teria efeito vasodilatador. Assim, o aumento dos níveis de NO poderia diminuir a toxicidade da Hcy. Ainda, nesse trabalho foi demonstrado um aumento da atividade da enzima antioxidante catalase, que reduz H_2O_2 a O_2 e H_2O, e contribui para a redução da toxicidade da Hcy.

As ERO produzidas pela auto-oxidação da Hcy podem além de gerar dano oxidativo às proteínas e lipídeos, também gerar dano ao DNA (Huang et al., 2001; Lin et al., 2007). Matté et al. (2009) demonstraram redução nas defesas antioxidantes e dano ao DNA em ratos submetidos à hiper-homocisteinemia crônica. Em um estudo recente, foi evidenciado aumento do dano ao DNA em sangue de pacientes homocistinúricos tratados. Além disso, observou-se um efeito concentração dependente *in vitro* da Hcy sobre o aumento no dano ao DNA (Vanzin et al., 2014). Nesse trabalho, ampliamos as investigações, avaliando o efeito da NAC sobre o dano ao DNA causado pela Hcy nas concentrações 50µM e 200 µM, as quais são encontradas no sangue de pacientes sob tratamento, e evidenciamos um efeito protetor da NAC nas concentrações 1mM e 5mM.

Evidências sugerem que antioxidantes podem ajudar na manutenção da saúde humana através da redução do dano oxidativo às biomoléculas chaves. Esses biomarcadores de dano oxidativo podem ser úteis no estabelecimento de quais antioxidantes são realmente importantes (Halliwell, 2002). O ensaio cometa pode ser aplicado diretamente nas células e avalia a quebra das fitas do DNA. Estudos usando ensaio cometa parecem ser mais apropriados que outros métodos para mostrar efeitos positivos de intervenções com antioxidantes em voluntários humanos (Halliwell e

Whiteman, 2004). NAC é uma molécula com propriedades antioxidantes que estimula a síntese de glutationa, atua como *scavenger* de radicais livres, e tem demonstrado possuir capacidades neuroprotetoras (Ferrari et al., 1995; Harvey et al., 2008). Estudos recentes mostram os efeitos benéficos da NAC sobre o dano ao DNA: Ozdemir et al. (2014) demonstraram que a suplementação com NAC reduziu o dano da DNA em crianças com β-talassemia; Marchetti et al. (2015) evidenciaram um efeito *in vitro* da NAC na redução do dano ao DNA em sangue de pacientes com X-ALD sintomáticos. Nesse sentido, levando em consideração os trabalhos da literatura e os resultados da NAC na redução do dano ao DNA apresentados nesse estudo, podemos sugerir que a suplementação com NAC para pacientes homocistinúricos poderia ser benéfica e deve ser testada para poder ser considerada terapia adjuvante para a doença.

Além do ensaio cometa, outras técnicas podem ser usadas para avaliar dano ao DNA, como por exemplo o teste de micronúcleos, o qual reflete dano cromossômico (Fenech, 2000). No terceiro capítulo desta tese foi avaliado o efeito *in vitro* da Hcy nas concentrações 10, 50, 100 e 200 µM sobre a frequência de micronúcleos em leucócitos. Foi demonstrado que a Hcy nas concentrações 50, 100 e 200 µM significativamente aumenta a frequência de micronúcleos em leucócitos. Além disso, foram observadas correlações significativas positivas entre a frequência de micronúcleos e a concentração de Hcy, bem como entre a frequência de micronúcleos e o dano ao DNA avaliado pelo ensaio cometa. A presença de micronúcleos fornece um índice conveniente e confiável de quebra e perda cromossômica (Fenech, 2000). Esse efeito danoso da Hcy sobre o DNA pode estar sendo mediado pelo estresse oxidativo secundário a hiper-homocisteinemia (Perez-de-Arce et al., 2005; Sethi et al., 2006). Nossos resultados corroboram com estudos demonstrando a presença de alterações celulares como resposta ao aumento do dano ao DNA, incluindo dano cromossômico, induzido pela

Hcy (Kruman et al, 2002; Picerno et al., 2007). Além disso, tem sido demonstrado um efeito do ácido fólico e da vitamina B_{12} na redução da frequência de micronúcleos, o que é interessante, visto que ambos são usados no tratamento da homocistinúria (Fenech, 1999). O impacto de micronutrientes sobre a estabilidade genômica tem sido intensivamente estudado. O ácido fólico e a vitamina B_{12} demonstram ter um efeito estabilizador sobre os cromossomos, tanto *in vitro* quanto *in vivo* (Fenech, 2001). Especialmente em indivíduos classificados como folato deficientes, um nível elevado de células com micronúcleos foi observado (MacGregor et al., 1997).

Em conclusão, os resultados deste trabalho indicam uma relação entre homocisteína e dano oxidativo à biomoléculas (lipídeos, proteínas e DNA) em pacientes portadores de Homocistinúria Clássica e trazem novas perspectivas para um melhor tratamento destes pacientes.

5. CONCLUSÕES

De acordo com os resultados apresentados nesse trabalho, podemos concluir que o estresse oxidativo parece estar envolvido nos mecanismos de dano observados na homocistinúria, como demonstrado pelas alterações de parâmetros de estresse oxidativo em urina. Além disso, foi demonstrado o efeito *in vitro* da Hcy sobre o dano ao DNA, e mais especificamente sobre o dano cromossômico, bem como um efeito protetor da *N*-acetil-L-cisteína. Finalmente, alterações no metabolismo de lipídeos e inflamação são fatores adicionais que possivelmente podem estar envolvidos na fisiopatologia das alterações vasculares encontradas na doença. Interessantemente, esses eventos parecem estar interconectados, e parecem ser resultado dos altos níveis de tHcy encontrados nos fluidos biológicos dos pacientes com deficiência de CBS. Esse trabalho evidencia a importância da aderência ao tratamento e traz perspectivas para estudos mais detalhados sobre o potencial da *N*-acetil-L-cisteína como um adjuvante na terapia da homocistinúria.

6. PERSPECTIVAS

Pretende-se dar continuidade a esse trabalho, expandindo nossos resultados. Dessa forma, são perspectivas:

a) Avaliar o mecanismo de dano ao DNA na homocistinúria;

b) Avaliar o reparo ao dano ao DNA em sangue de pacientes homocistinúricos clássicos;

c) Avaliar o efeito *in vivo* da suplementação com *N*-acetil-L-cisteína nos parâmetros de estresse oxidativo em pacientes homocistinúricos clássicos;

d) Avaliar o efeito *in vitro* de outros antioxidantes em modelos de hiper-homocisteinemia.

e) Avaliar parâmetros de estresse oxidativo em outras formas de hiper-homocisteinemia.

7. REFERÊNCIAS

Andria, G.; Fowler, B.; Sebastio, G. (2006) Disorders of Sulfur Amino Acid Metabolism. In: Fernandes, J.; Saudubray, J.M.; Van Den Berghe, G. Walter, J.H. (Eds) Inborn Metabolic Diseases, Diagnosis and Treatment. 4th ed. Würzburg: Springer Medizin Verlag, p. 273-282.

Balaban, R.S.; Nemoto, S.; Finkel, T. (2005) Mitochondria, Oxidants, and Aging. Cell. 120: 483-495.

Barschak, A.G., et al. (2006) Evidence that oxidative stress is increased in plasma from patients whit maple syrup urine disease. Metab. Brain Dis. 21:279-286.

Barschak, A.G., et al. (2008) Oxidative stress in plasma from maple syrup urine disease patients during treatment. Metab. Brain Dis. 23:71-80.

Bhattacharjee, S., et al. (2001) NADPH oxidase of neutrophils elevates dityrosine cross-links in proteins and urine during inflammation. Arch. Biochem. Biophys. 395: 69–77.

Bridi, R., et al. (2005) α-keto acids accumulating in maple syrup urine disease stimulate lipid peroxidation and reduce antioxidant defences in cerebral cortex from young rats. Metab. Brain Dis. 20:155-167.

Bydlowski, S.P.; Magnanelli, A.C.; Chamone, D.A.F. (1999) Hiper-Homocisteinemia e Doenças Vaso-Oclusivas. Arq. Bras. Cardiol. 71:69-76.

Carson, N.A.J.; Neill, D.W. (1962) Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland, Arch. Dis. Child. 37:505–513.

Clarke, J.T.R. (2004) A Clinical Guide to Inherited Metabolic Diseases. 2th ed. Cambridge: Cambridge University Press.

Cooke, M.S., et al. (2006) Does measurement of oxidative damage to DNA have clinical significance? Clin. Chim. Acta 365: 30 – 49.

Cruysberg, J.R.M., et al. (1996) Delay in diagnosis of homocystinuria: Retrospective study of consecutive patients. BMJ. 313:1037-1040.

Da Cunha AA, Ferreira AG, Wyse AT. (2010) Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration. Metab. Brain Dis. 25:199–206.

Da Cunha, A.A., et al. (2011) Chronic hyperhomocysteinemia induces oxidative damage in the rat lung. Mol. Cell Biochem. 358:153-160.

Das UN (2006) Clinical laboratory tools to diagnose inflammation. Adv. Clin. Chem. 41:189–229.

Davì, G., et al. (2001) Oxidative Stress and Platelet Activation in Homozygous Homocystinuria. Ciculation. 104:1124-1128.

den Heijer M, et al. (1998) Hyperhomocysteinemia and venous thrombosis: a meta-analysis. Thromb Haemost. 80: 874–877.

Deon, M., et al. (2006) The effect of Lorenzo's oil on oxidative stress in X-linked adrenoleukodistrophy. J. Neurol. Sci. 247:157-164.

Deon, M., et al. (2007) Induction of lipid peroxidation and decrease of antioxidant defenses in symptomatic and asymptomatic patients with X-linked adrenoleukodystrophy. Int. J. Dev. Neurosci. 25:441-444.

Elbini, D.I., et al. (2016) A minireview on N-acetylcysteine: An old drug with new approaches, Life Sciences (2016), doi: 10.1016/j.lfs.2016.03.003

Faraci, F.M.; Lentz, S.R. (2004) Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. Stroke 35:345–347.

Fechner, A., et al. (2001) Antioxidant status and nitric oxide in the malnutrition syndrome kwashiorkor. Pediatr. Res. 49: 237–243.

Fenech, M. (2000) The *in vitro* micronucleus technique. Mutation Research 455: 81–95.

Fenech, M. (2001) The role of folic acid and Vitamin B_{12} in genomic stability of human cells, Mutat. Res. 475: 57–67.

Ferrari, G., Yan, C.Y., Greene, L.A. (1995) N-acetylcysteine (d- and l-stereoisomers) prevents apoptotic death of neuronal cells. J. Neurosci. 15: 2857–2866.

Filippon, L., et al. (2011a) Oxidative stress in patients with mucopolysaccharidosis type II before and during enzyme replacement therapy. Mol. Genet. Metab. 103:121-127.

Filippon, L., et al. (2011b) DNA damage in leukocytes from pretreatment mucopolysaccharidosis type II patients; protective effect of enzyme replacement therapy. Mutat. Res. 721:206-210.

Finkelstein, J.D., et al. (1964) Homocystinuria due to cystathionine synthetase deficiency: The mode of inheritance. Science 146:785-787.

Fontella, F.U., et al. (2000) Proprionic and L-methylmalonic acids induce oxidative stress in brain of young rats. Neuroreport. 11:541-544.

Fowler, B. (2008) Homocysteine, S-Adenosylmethionine and S-Adenosylhomocysteine. In: Blau, N.; Duran, M.; Gibson, K.M. (Eds) Laboratory Guide to the Methods in Biochemical Genetics, Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg, pp.112-135.

Gniwotta, C., et al. (1997) Prostaglandin F2-like compounds, F2-isoprostanes, are present in increased amounts in human atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. 17:3236–3241.

Gori, A.M., et al. (2005) A proinflammatory state is associated with hyperhomocysteinemia in the elderly. Am. J. Clin. Nutr. 82:335–341.

Halliwell, B.; Chirico, S. (1993) Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57:715S-724S.

Halliwell, B. (2000) Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? Cardiovasc. Res. 47, 410–418.

Halliwell, B. (2002) Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Rad. Biol. Med. 32: 968–974.

Halliwell, B.; Whiteman, M. (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol. 142: 231–255.

Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 141:312-322.

Halliwell, B.; Gutteridge, J.M.C. (2007) Free Radicals in Biology and Medicine. 4th ed. Oxford: Oxford University Press.

Hanada, T.; Yoshimura, A. (2002) Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev 13:413-421.

Hansen, R.E.; Roth, D.; Winther, J.R. (2009) Quantifying the global cellular thiol-disulfide status. Proc. Natl. Acad. Sci. U.S.A. 106: 422–427.

Harvey, B.H., et al. (2008) Effect of chronic N-acetyl-l-cysteine administration on oxidative status in the presence and absence of induced oxidative stress in rat striatum. Neurochem. Res. 33: 508–517.

Harpel, P.C.; Chang, V.T.; Borth, W. (1992) Homocysteine and other sulphydryl compounds enhance the binding of lipoprotein(a) to fibrin: A potential biochemical link between thrombosis, atherogenesis, and sulphydryl compound metabolism. Proc. Natl. Acad. Sci. USA 89:10193-10197.

Hong, H.S.; Lee, H.K.; Kwon, K.H. (1997) Homocystinuria presenting with portal vein thrombosis and pancreatic pseudocyst: A case report. Pediatr. Radiol. 27:802-804.

Huang, R.F.S., et al. (2001) Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci. 68:2799–2811.

Jakubowski, H. (1997) Metabolism of homocysteine thiolactone in human cell cultures possible mechanism for pathological consequences of elevated homocysteine levels. J. Biol. Chem. 272:1935–1942.

Jakubowski, H. (1999) Protein homocysteinylation: possible mechanism underlying pathological consequences of elevated homocysteine levels. Faseb J. 13:2277–2283.

Jakubowski, H. (2000) Calcium-dependent human serum homocysteine thiolactone hydrolase a protective mechanism against protein s-homocysteinylation. J. Biol. Chem. 275:3957–3962.

Jakubowski, H. (2008a) Paraoxonase 1 (PON1), a junction between the metabolism of homocysteine and lipids. In: Mackness, B. et al. (Eds.) The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism, pp.87–102.

Jakubowski, H. (2008b) The pathophysiological hypothesis of homocysteine thiolactone-mediated vascular disease. J. Physiol. Pharmacol. 59:155-167.

Jakubowski, H. (2010) The Role of Paraoxonase 1 in the Detoxification of Homocysteine Thiolactone. Paraoxonases Inflamm. Infection. Toxicol. 660:113–127.

Jakubowski, H. (2011) Quality control in tRNA charging editing of homocysteine. Acta Biochim. Pol. 58): 149–163.

Jakubowski, H. (2012) Quality control in tRNA charging. Wiley Interdiscip. Ver. RNA 3: 295–310.

Jakoby, W.B.; Griffith, O.W. (1994) Sulfur and sulfur amino acids. Methods Enzymol. 143: 366-76.

Janosík, M., et al. (2009) Birth prevalence of homocystinuria in Central Europe: frequency and pathogenicity of mutation c.1105C>T (p.R369C) in the cystathionine beta-synthase gene. J. Pediatr. 154: 431–437.

Jarvik, G.P., et al. (2000) Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1(192) or PON1(55) genotype. Arterioscler. Thromb. Vasc. Biol. 20:b2441-2447.

Kanani, P.M.; et al. (1999) Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocysteinemia in humans. Circulation. 100: 1161–1168.

Keating, A.K., (2011) Constitutive induction of pro-inflammatory and chemotactic cytokines in cystathionine beta-synthase deficient homocystinuria. Mol. Genet. Metab. 103: 330–7.

Kerrin, D., et al. (1996) Homocystinuria presenting with sagital sinus thrombosis in infancy. J. Child. Neurol. 11:70-71.

Kienzle Hagen, M.E., et aç. (2002) Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochim. Biophys. Acta. 1586: 344-352.

Kluijtmas, L.A.J., et al. (1999) The molecular basis os cystathionine β-synthase deficiency in Dutch patients with homocystinuria: Effect of CBS genotype on biochemical and clinical phenotype, and on response to treatment. Am. J. Hum. Genet. 65: 59-67.

Kruman, I.I., et al. (2002) Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. J. Neurosci. 22: 1752–1762.

Leandro, P.; Gomes, C.M. (2008) Protein misfolding in conformational disorders: rescue of folding defects and chemical chaperoning. Mini-Rev. Med. Chem. 8: 901–911.

Leonard, J.V.; Morris, A.A. (2000) Inborn errors of metabolism around time of birth. Lancet. 356: 583–587.

Lin, P.Y., et al. (2007) Synergistic effects of S-adenosylhomocysteine and homocysteine on DNA damage in a murine microglial cell line Clin. Chim. Acta. 379: 139–144.

Loscalzo J. (1996) The oxidant stress of hyperhomocyst(e)inemia. J. Clin. Invest. 98: 5–7.

Manary, M.J.; Leeuwenburgh, C.; Heinecke, J.W. (2000) Increased oxidative stress in kwashiorkor. J. Pediatr. 137: 421–424.

Mansoor, M.A., et al. (2005) Relationship between serum folate and plasma nitrate concentrations: possible clinical implications. Clin. Chem. 51: 1266–1268.

Marchetti, D.P., et al. (2015) Protective effect of antioxidants on DNA damage in leukocytes from X-linked adrenoleukodystrophy patients. Int. J. Dev. Neurosci. 43: 8-15.

Matté, C., et al. (2009) Chronic hyperhomocysteinemia alters antioxidant defenses and increases DNA damage in brain and blood of rats: Protective effect of folic acid. Neurochem. Int. 54:7-13.

McCully, K.S. (1983). Homocysteine theory of arteriosclerosis: development and current status. In: Gotto, A.M.Jr., Paoletti, R. (Eds.) Atherosclerosis reviews. 11th ed. New York: Raven Press, pp. 157–246.

McCully, K.S. (2007) Homocysteine, vitamins, and vascular disease prevention. Am. J. Clin. Nutr. 86:1563S–1568S.

MacGregor, J.T., et al., (1997) Spontaneous genetic damage in man: evaluation of interindividual variability, relationship among markers of damage, and influence of nutritional status. Mutat. Res. 377: 125–135.

Mc Guire, P.J.; Parikh, A.; Diaz, G.A. (2009) Profiling of oxidative stress in patients with inborn errors of metabolismo. Mol. Genet. Metab. 98: 173–180.

Melenovská, P., et al. (2015) Chaperone therapy for homocystinuria: the rescue of CBS mutations by heme arginate. J. Inherit. Metab. Dis. 38:287–294.

Mescka, C., et al. (2011) *In vivo* neuroprotective effect of L-carnitine against oxidative stress in maple syrup urine disease. Metab. Brain Dis. 26:21-28.

Mudd, S.H., et al. (1964) Homocystinuria: na enzymatic defect, Science 143: 1443–1445.

Mudd, S.H. et al. (1985) The natural history of homocystinuria due to cystathionine β-synthase deficiency. Am. J. Hum. Genet. 37: 1-31.

Mudd, S.H.; Levy, H.L.; Kraus, J.P. (2001) Disorders of transsulfuration, In: Scriver, C.R.; Beaudet, A.L.; Sly, W.S.; Valle, D. (Eds.) The Metabolic and Molecular Basis of Inherited Disease. 8th ed. New York: McGraw-Hill, pp. 2007–2056.

Muntau, A.C., et al. (2014) Innovative strategies to treat protein misfolding in inborn errors of metabolism: pharmacological chaperones and proteostasis regulators. J. Inherit. Metab. Dis. 37: 505–523.

Naruszewicz, M., et al. (1994) Thiolation of low-density lipoproteins by homocysteine thiolactone causes increased aggregation and interaction with cultured macrophages. Nutr. Metab. Cardiovasc. Dis. 4: 70–77.

Oda, M.N., et al. (2002) Paraoxonase 1 overexpression in mice and its effect on high-density lipoproteins. Biochem. Biophys. Res. Commun. 290: 921-927.

Ozdemir, Z.C., et al. (2014) N-Acetylcysteine Supplementation Reduces Oxidative Stress and DNA Damage in Children with β-Thalassemia. Hemoglobin. 38: 359-364.

Pereira, V.G., et al. (2008) Mutational and oxidative stress analysis in patients with mucopolysaccharidosis type I undergoing enzyme replacement therapy. Clin. Chim. Acta. 387: 75-79.

Perez-de-Arce, K., et al. (2005) Reactive oxygen species mediates homocysteine-induced mitochondrial biogenesis in human endothelial cells: modulation by antioxidants. Biochem. Biophys. Res. Commun. 338: 1103–1109.

Perla-Kaján J, Jakubowsky H. (2010) Paraoxonase 1 protects against protein N-homocysteinylation in humans. FASEB J. 24: 931-6.

Perla-Kaján J, Jakubowsky H. (2012) Paraoxonase 1 and homocysteine metabolismo. Amino Acids 43:1405–1417.

Picerno, I., et al. (2007) Homocysteine induces DNA damage and alterations in proliferative capacity of T-lymphocytes: a model for immunosenescence? Biogerontology. 8: 111–119.

Powers, J.M., et al. (2005) Adreno-leukodystrophy: oxidative stress of mice and men. J. Neuropathol. Exp. Neurol. 64: 1067-1079.

Pratico, D., et al. (1997) Localization of distinct F2-isoprostanes in human atherosclerotic lesions. J. Clin. Invest. 100: 2028 –2034.

Preissler, T., et al. (2015) Phenylalanine induces oxidative stress and decreases the viability of rat astrocytes: possible relevance for the pathophysiology of neurodegeneration in phenylketonuria. Metab Brain Dis. DOI 10.1007/s11011-015-9763-0.

Rao, T.N., et al. (2008) Homocystinuria due to cystathionine-beta-synthase deficiency. Indian J. Dermatol. Venereol. Leprol. 74: 375-378.

Refsum, H., et al. (2004) Birth prevalence of homocystinuria. J. Pediatr. 144: 830-832.

Requejo, R., et al. (2010) Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. FEBS J. 277: 1465–1480.

Ribas, G.S., et al. (2010a) Reduction of lipid and protein damage in patients with disorders of propionate metabolism under treatment: a possible protective role of L-carnitine supplementation. Int. J. Devl. Neurosci. 28: 127–132.

Ribas, G.S., et al. (2010b) Prevention by L-carnitine of DNA damage induced by propionic and L-methylmalonic acids in human peripheral leukocytes in vitro. Mutat. Res. 702: 123–128.

Robert, K., et al. (2005) Cystathionine β synthase deficiency promotes oxidative stress, fibrosis, and steatosis in mice liver. Gastroenterology. 128: 1405-1415.

Roberts II, L.J.; Morrow, J.D. (2002) Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. Cell. Mol. Life Sci. 59: 808–820.

Rosenblatt, D.S.; Fenton, W.A. (2001) Inherited Disorders of Folate and Cobalamin Transport and Metabolism. In: Scriver, C.R., et al. (Eds.) The Metabolic and Molecular Basis of Inherited Disease. 8th ed. New York: McGraw-Hill, pp.3897–3933.

Ross, R. (1999) Atherosclerosis – na inflammatory disease. N. Engl. J. Med. 340: 115-126.

Saudubray, J.M., et al. (2006) A Clinical Approach to Inherited Metabolic Diseases. In: Fernandes, J., et al. (Eds) Inborn Metabolic Diseases, Diagnosis and Treatment. 4th ed. Würzburg: Springer Medizin Verlag, pp.4-48.

Santarpia, L., et al. (2013) Butyrylcholinesterase as a prognostic marker: a review of the literature. J Cachexia Sarcopenia Muscle 4: 31-9.

Scanu, A.M. (1992) Lipoprotein(a): A genetic risk factor for premature coronary heart disease. JAMA 267: 3326-3329.

Schwahn, B.C., et al. (2003) Pharmacokinetics of oral betaine in healthy subjects and patients with homocystinuria. Br. J. Clin. Pharmacol. 55: 6-13.

Scriver, C.R., et al. (2001) The Metabolic and Molecular Basis of Inherited Disease. 8th ed. New York: McGraw-Hill.

Selhub, J. (1999) Homocysteine metabolism. Annu. Rev. Nutr. 19:217–246.

Sethi, A.S., et al., (2006) Homocysteine-induced endothelin-1 release is dependent on hyperglycaemia and reactive oxygen species production in bovine aortic endothelial cells. J. Vasc. Res. 43: 175–183.

She, Z.G., et al. (2009) Human paraoxonase gene cluster transgenic overexpression represses atherogenesis and promotes atherosclerotic plaque stability in ApoE-null mice. Circ Res. 104: 1160-1168.

Skovby, F.; Gaustadnes, M.; Mudd, S.H. (2010) A revisit to the natural history of homocystinuria due to cystathionine b-synthase deficiency. Mol. Genet. Metab. 99: 1–3.

Smith, C.M.; Marks, A.D.; Lieberman, M.A. (2005) Marks' Basic Medical Biochemistry: A Clinical Approach. 2nd ed. Philadelphia: Lippincott Williams & Wilkins.

Starkebaum, G.; Harlan, JM. (1986) Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J. Clin. Invest. 77: 1370-1376.

Thomas, J.A.; Poland, B.; Honzatko, R. (1995) Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. 319: 1–9.

Tward, A., et al. (2002) Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. Circulation. 106: 484-490.

Uhlendorf, B.W.; Mudd, S.H. (1968) Cystathionine synthase activity in human lymphocytes: Induction by phytohemagglutinin. J. Clin. Invest. 51: 1034-1037.

Undas, A., et al. (2004) Autoantibodies against N-homocysteinylated proteins in humans: implications for atherosclerosis. Stroke 35:1299–1304.

Schwartz, I.V.; de Souza, C.F.M.; Giugliani, R. (2008) Tratamento de erros inatos do metabolismo. J. Pediatr. 84: S8-S19.

Sirtori, L.R., et al. (2005) Oxidative stress in patients with phenylketonuria. Biochim. Biophys. Acta 1740: 68–73.

Sitta, A., et al. (2006) Investigation of oxidative stress parameters in treated phenylketonuric patients. Metab. Brain Dis. 21:287-296.

Sitta, A., et al. (2009) L-carnitine blood levels and oxidative stress in treated phenylketonuric patients. Cell Mol. Neurobiol. 29: 211–218.

Sitta, A., et al. (2011) Evidence that L-carnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients. Cell Mol. Neurobiol. 31: 429–436.

Streck, E.L., et al. (2003) In vitro effect of homocysteine on some parameters of oxidative stress in rat hippocampus. Metab. Brain Dis. 18: 147-154.

Tanaka T, Kishimoto T. (2012) Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases. Int. J. Biol. Sci. 8: 1227-1236.

Tsikas, D.; Gutzki, F.M.; Stichtenoth, D.O. (2006) Circulating and excretory nitrite and nitrate as indicators of nitric oxide synthesis in humans: methods of analysis. Eur. J. Clin. Pharmacol. 62: 51–59.

Vanzin, C.S., et al. (2011) Experimental evidence of oxidative stress in plasma of homocystinuric patients: A possible role for homocysteine. Mol. Genet. Metab. 104: 112-7.

Vanzin, C.S., et al. (2014) Homocysteine contribution to DNA damage in cystathionine β-synthase-deficient patients. Gene 539: 270–274.

Vargas, C.R., (2004) Evidence that oxidative stress is increased in patients with X-linked adrenoleukodistrophy. Biochim. Biophys. Acta. 1688: 26-32.

Verhaar, M.C.; Stroes, E.; Rabelink, T.J. (2002) Folates and cardiovascular disease. Arterioscler. Thromb. Vasc. Biol. 22: 6 –13.

Walter, J.H., et al. (1998) Strategies for the treatment of cystathionine β-synthase deficiency: the experience of the Willink Biochemical Genetics Unit over the past 30 years. Eur. J. Pediatr. 157: S71–S76.

Walter, J.H,; Jahnke, N.; Remmington, T. (2011) Newborn screening for homocystinuria. Cochrane Database Syst. Rev. 10: CD008840.

Welch GN, Loscalzo J. (1998) Homocysteine and atherothrombosis. N. Engl. J. Med. 338: 1042–1050.

Wilcken DE, Dudman NPB, Tyrrell PA (1985) Homocystinuria due to cystathionine beta-synthase deficiency-the effects of betaine treatment in pyridoxine-responsive patients. Metabolism. 34: 1115–1121.

Wilcken, D.E.L.; Wilcken, B. (1997) The natural history of vascular disease in homocystinuria and the effects of treatment. J. Inherited Metab. Dis. 20: 295-300.

Yap, S.; Naughten, E. (1998) Homocystinuria due to cystathionine β-synthase deficiency in Ireland: 25 years' experience of a newborn screened and treated population with reference to clinical outcome and biochemical control. J. Inherit. Metab. Dis. 21: 738-747.

Yap, S., et al. (2001) The intelectual abilities of early-treated individuals with pyridoxine-nonresponsive homocystinuria due to cystathionine beta-synthase deficiency. J. Inherit. Metab. Dis. 24: 437-447.

Zakynthinos, E.; Pappa, N. (2009) Inflammatory biomarkers in coronary artery disease. J. Cardiol. 53: 317—333.

ANEXO 1
LISTA DE FIGURAS

ANEXO 2

PARECER DA COMISSÃO CIENTÍFICA E DA COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE DO HCPA

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

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 100290 **Versão do Projeto:** 23/08/2010 **Versão do TCLE:** 23/08/2010

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
CAROLINA FISCHINGER MOURA DE SOUZA
CRISTINA BRINCKMANN OLIVEIRA NETTO
DAIANE GRIGOLO BARDEMAKER RODRIGUES
MOACIR WAJNER
CAMILA SIMIONI VANZIN
IZABELA NETTO PEREIRA
ANGELA SITTA
CARMEN REGLA VARGAS

Título: Investigação de estresse oxidativo em pacientes portadores de Homocistinúria antes e durante o tratamento.

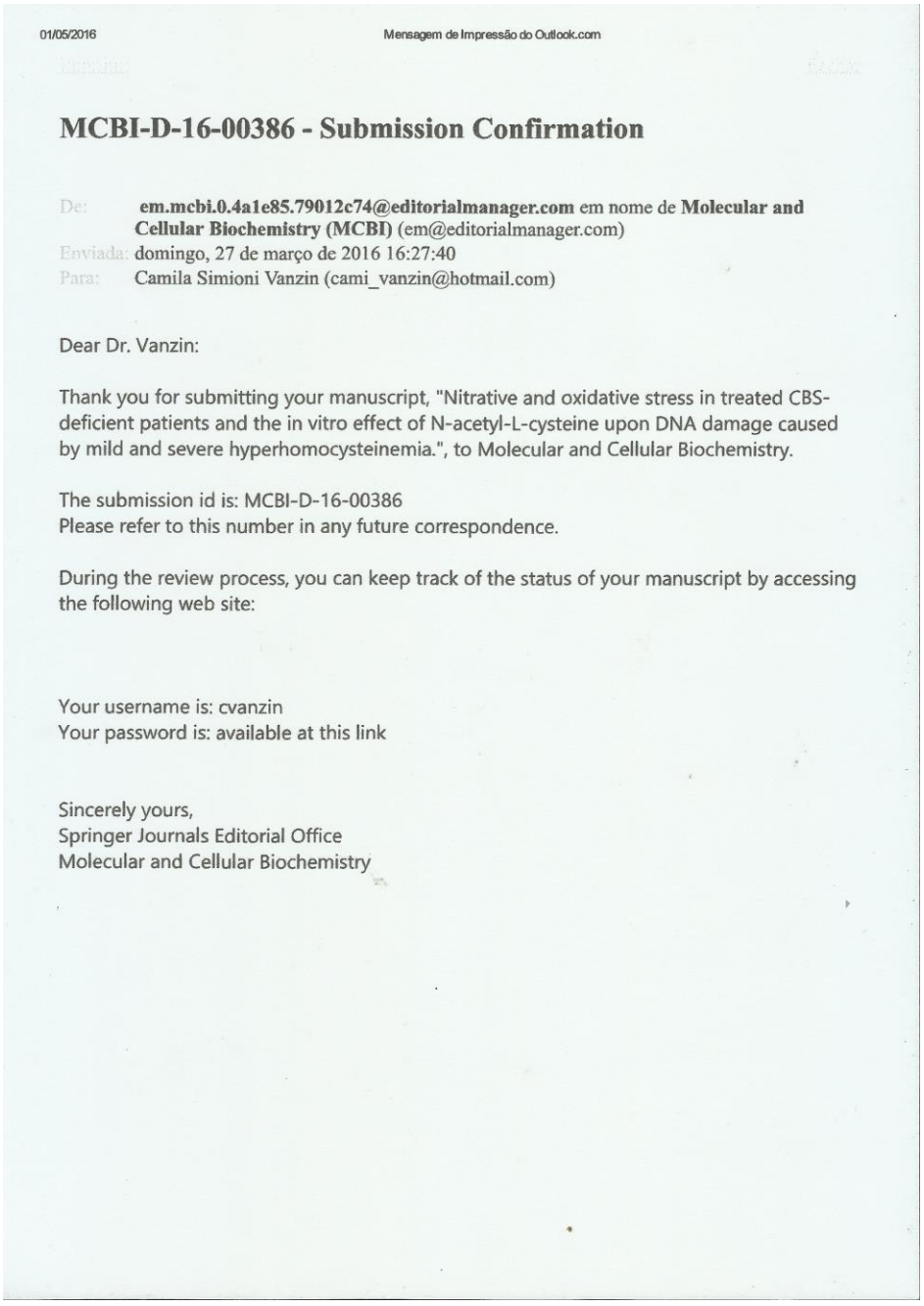
Este projeto foi Aprovado em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto deverá ser comunicada imediatamente ao CEP/HCPA.

Porto Alegre, 26 de agosto de 2010.

Prof. Nadine Clausell
Coordenadora GPPG e CEP/HCPA

ANEXO 3

COMPROVANTE DE ENVIO PARA O PERIÓDICO MOLECULAR AND CELLULAR BIOCHEMISTRY



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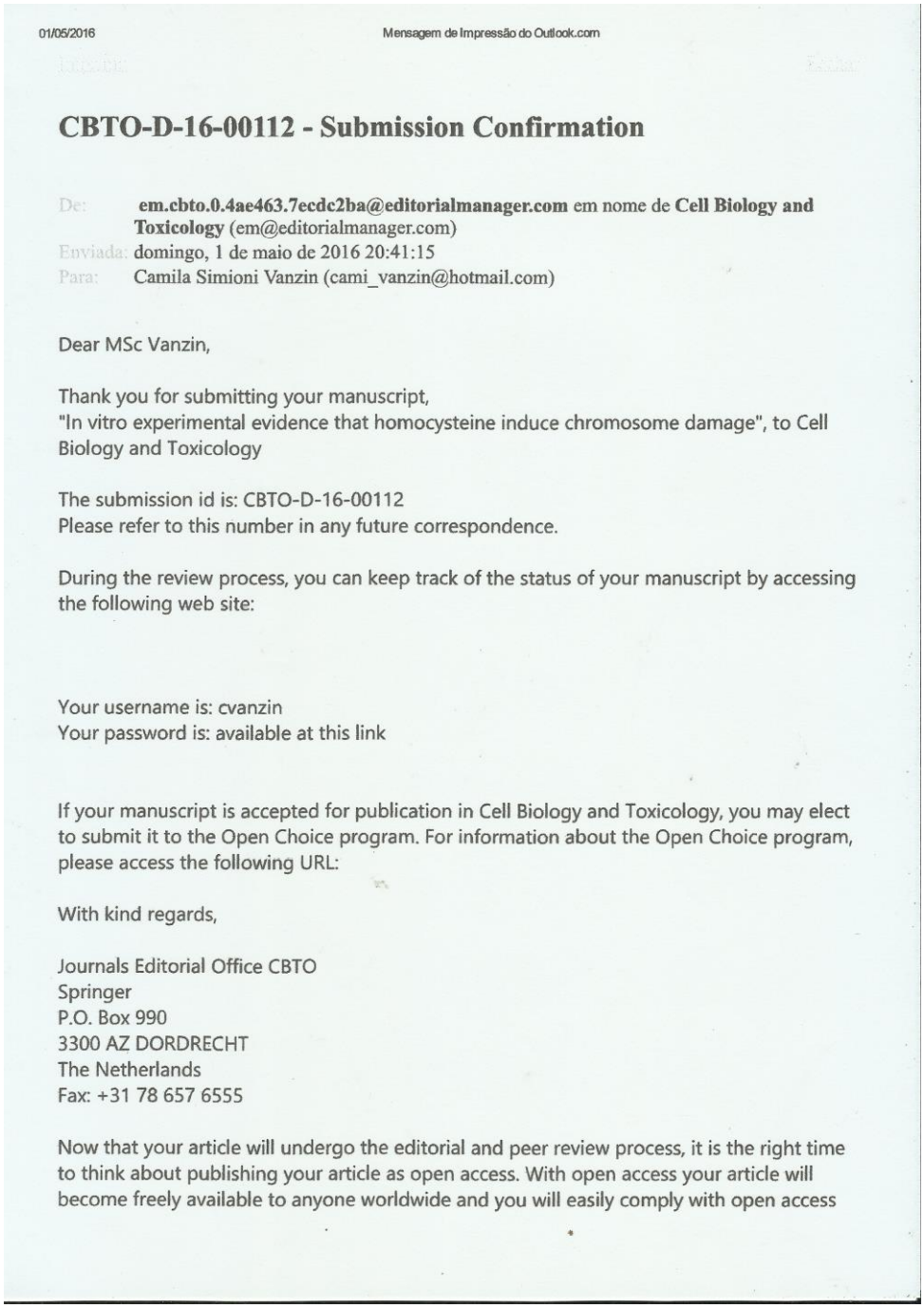
ANEXO 4

COMPROVANTE DE ENVIO PARA O PERIÓDICO CELL BIOLOGY AND TOXICOLOGY



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Enviada: domingo, 1 de maio de 2016 20:41:15
Para: Camila Simioni Vanzin (cami_vanzin@hotmail.com)

Dear MSc Vanzin,

Thank you for submitting your manuscript,
"In vitro experimental evidence that homocysteine induce chromosome damage", to Cell Biology and Toxicology

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