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Danilo Grünig Humberto da Silva

Estresse oxidativo na anemia falciforme: antioxidantes endógenos como possíveis alvos terapêuticos

São José do Rio Preto - SP
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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Profa. Dra. Claudia Regina Bonini Domingos
Coorientador: Prof. Dr. Eduardo Alves de Almeida

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“Dedico este trabalho a minha família”

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A ciência nunca resolve um problema sem criar pelo menos outros dez.

(George Bernard Shaw)

RESUMO

A anemia falciforme (AF) é caracterizada por um curso clínico altamente variável. Essa variabilidade reflete a complexidade de sua fisiopatologia que é afetada por diversos moduladores, como polimorfismos genéticos associados a eventos clínicos e a resposta ao tratamento com hidroxiureia (HU), assim como pelo quadro inflamatório e estresse oxidativo crônico. O presente estudo objetivou avaliar a influência de marcadores genéticos sobre o perfil oxidativo de pessoas com AF, e a sua relação com uso de HU e manifestações fenotípicas; e, verificar possíveis efeitos antioxidantes diretos e indiretos da melatonina (MEL), usando suspensão de células falciformes como modelo experimental. Após consentimento informado, os grupos estudados foram divididos de acordo com o tipo de experimentação a qual foram submetidos. Análises *in vivo*: composto por 95 indivíduos com AF, independente do gênero, com idade variando de 10 a 59 anos, em acompanhamento clínico regular no Hemocentro do Rio de Janeiro (RJ). Todos os pacientes estudados estavam sob tratamento profilático com ácido fólico (5 mg/dia), enquanto 41 (43,2%) deles estavam sob uso de HU (dose média: 22 mg/Kg/dia) por pelo menos 90 dias. Para a confirmação diagnóstica da AF, foram utilizados métodos citológicos, eletroforéticos, cromatográficos e moleculares. Foram investigados polimorfismos genéticos nos genes de enzimas envolvidas em diferentes vias metabólicas: metilenotetrahidrofolato redutase (*MTHFR*; c.677C>T; rs1801133), cistationina beta-sintase (*CBS*; 68ins844) e adenosina deaminase (*ADA*; c.22G>A; rs73598374). Os parâmetros bioquímicos foram avaliados por meio de métodos espectrofotométricos [atividade das enzimas catalase e glutationa *S*-transferase, glutationa peroxidase e glutationa redutase] e cromatográficos [níveis de malondialdeído (MDA) e glutationa reduzida (GSH)]. Análises *ex vivo*: Dez pessoas, previamente diagnosticadas com AF, foram incluídas no estudo - seis mulheres (60%) e quatro homens (40%), dentro da faixa etária de 20 a 40 anos. Todos os participantes estavam sob o mesmo protocolo de acompanhamento clínico no Hemocentro de São José do Rio Preto (SP). Eritrócitos falciformes foram suspensos em solução salina tamponada contendo fosfato em pH 7,4, compondo grupo controle externo. Da mesma forma, outras alíquotas de cada paciente foram suspensas e incubadas a 37ºC, na ausência (grupo controle experimental ou na presença de *N*-acetilcisteína (NAC), MEL e combinação de ambos compostos (NAC+MEL), em concentrações de 100 pM, 100 nM e 100 µM, por uma hora (grupos tratados). Os marcadores bioquímicos foram avaliados de acordo com o exposto para as análises *in vivo*, porém com algumas medidas espectofotométricas adicionais - grau de hemólise e atividade das enzimas glicose-6-fosfato desidrogenase (G6PDH) e superóxido dismutase (SOD). Dentre os 95 pacientes avaliados *in vivo*, 66 (69,5%) eram homozigotos selvagem para *MTHFR*, 27 (28,4%) heterozigotos e dois (2,1%) homozigotos mutante, com freqüências alélicas de 0,84 para o alelo ancestral (677C) e 0,16 para o mutante (677T). A frequência alélica obtida para *CBS*: 0,85 para o alelo ancestral (A) e 0,15 para a inserção (I). Além disso, identificou-se a seguinte distribuição genotípica para o gene *CBS* - 69 (72,6%) homozigotos selvagem, 23 (24,2%) heterozigotos e três (3,2%) homozigotos mutantes. A prevalência da coerança de ambas mutações em pelo menos um cromossomo foi de 8,4%. Enquanto que para o gene *ADA*, não foram encontrados homozigotos mutantes: 80 (84,2%) homozigotos selvagem e 15 (15,8%) heterozigotos; com frequência de 0,92 para o alelo ancestral (22G) e de 0,08 para o alelo mutante (22A). Foram constatados efeitos farmacológicos da HU esperados, como a redução dos níveis de peroxidação lipídica, aumento do conteúdo de hemoglobina fetal (HbF), e melhoria de marcadores hematológicos e hemolíticos, independente da presença de qualquer um dos polimorfismos avaliados. A presença do polimorfismo (677C>T) no gene *MTHFR*

não influenciou a resposta ao tratamento com HU ou os marcadores de estresse oxidativo. Porém, os pacientes com a mutação (677C>T) em pelo menos um cromossomo apresentaram os valores mais baixos de HbF ($p < 0,01$). Para a inserção no gene *CBS*, encontramos atividades elevadas da lactato desidrogenase ($p < 0,01$), aspartato transaminase ($p = 0,01$) e gama-glutamil transferase ($p = 0,04$) nos pacientes com a inserção em pelo menos um cromossomo e em uso de HU. Além disso, observou-se um efeito sinérgico de ambos os polimorfismos na redução dos níveis de GSH, independentemente do tratamento com HU ($p = 0,04$). A presença da mutação no gene *ADA* não exerceu qualquer tipo de influência sobre os marcadores de estresse oxidativo avaliados. Já os resultados *ex vivo* mostraram que o período de incubação foi capaz de causar um aumento de cerca de 64% no grau de hemólise ($p < 0,01$) dos eritrócitos falcêmicos, bem como praticamente dobrou os níveis de peroxidação lipídica ($p < 0,01$). No entanto, quase todos os tratamentos antioxidantes testados foram capazes de neutralizar este efeito do período de incubação, observado sobre níveis de MDA. Dentre os marcadores antioxidantes avaliados, observou-se um efeito modulador do tratamento combinado (NAC+MEL) sobre as atividades da GPx e da SOD ($p <0,01$), culminando na diminuição de aproximadamente 25% em suas atividades. Além disso, verificou-se um efeito antioxidante dose-dependente para MEL sobre os valores de MDA ($r = - 0,29$; $p = 0,03$) e para os tratamentos combinados (NAC+MEL) também sobre os níveis de MDA ($r = - 0,37$; $p = 0,01$) e sobre a atividade da SOD ($r = - 0,54$; $p <0,01$). De forma geral, os resultados obtidos para os polimorfismos nos genes *MTHFR* e *CBS* indicam que seu envolvimento na fisiopatologia da AF, provavelmente, é mais complexo do que o explorado até o momento. Já a presença do polimorfismo no gene *ADA* (22 G> A), aparentemente, não desempenha papel significativo na alteração do metabolismo redox de eritrócitos falciformes. Em contrapartida, os resultados da experimentação *ex vivo* contribuem para a utilização da MEL, individualmente ou em combinação com a NAC, no tratamento da anemia falciforme.

Palavras-chave: hemoglobina S, hidroxiureia, metilenotetrahidrofolato redutase, cistationina beta-sintase, adenosina deaminase, *N*-acetilcisteína, melatonina

ABSTRACT

*Sickle cell anemia (SCA) is characterized by a clinical course highly variable. This feature reflects the complex pathophysiology of SCA which can be affected by a number of modifying factors including polymorphisms associated with clinical aspects and with hydroxyurea (HU) treatment response, and chronic inflammation and oxidative states. Thus, this study aimed to evaluate the influence of genetic markers on oxidative profile of SCA patients. We also correlated these results with hydroxyurea (HU) therapy and with phenotypic manifestations. Moreover we verified a potential direct and indirect antioxidant effects of melatonin (MEL), using a sickle cell suspension as experimental model. After informed consent, the studied groups were divided according to the experimentation manner. Analysis in vivo: the group was composed by 95 SCA patients, regardless gender, and age ranging from 10 - 59 years old. All subjects were regularly in clinical follow-up in the Blood Center of Rio de Janeiro (RJ) and received a prophylactic treatment with folic acid of 5 mg/day since the SCA diagnosis, while 41 (43,2%) of them were under HU treatment (average dose: 22 mg/Kg/day) for at least 90 days. In order to confirm homozygosity of HBB*S gene we used cytological, electrophoretic, chromatographic and molecular methods. We investigated genetic polymorphisms in the genes of enzymes involved in different metabolic pathways: methylenetetrahydrofolate reductase (MTHFR; c.677C>T; rs1801133), cystathionine beta-synthase (CBS; 68ins844) and adenosine deaminase (ADA; c.22G>A; rs73598374). Biochemical parameters were measured using spectrophotometric [catalase, glutathione S-transferase, glutathione peroxidase, glutathione reductase activities] and chromatographic methods [glutathione (GSH) and malondialdehyde (MDA) levels]. Analysis ex vivo: Ten previously diagnosed SCA patients were included in the study - six (60%) women and four (40%) men, within the age range of 20-40 years. All subjects enrolled were under the same clinical follow-up protocol at the Blood Center of São José do Rio Preto (SP). Sickle erythrocytes were suspended in phosphate-buffered saline, pH 7.4, composing external control group. They were also suspended and incubated at 37ºC either in absence (experimental control group) or presence of N-acetylcysteine (NAC), MEL and their combination (NAC+MEL) at concentrations of 100 pM, 100 nM and 100 µM for one hour (treatment groups). Biochemical markers were evaluated in the same manner that in vivo ones, but with some additional spectrophotometric measures - hemolysis degree and activities of the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and superoxide dismutase (SOD). Among the 95 SCA patients evaluated in vivo, we found 66 (69.5%) wild homozygous for MTHFR, 27 (28.4%) heterozygous and two (2.1%) mutant homozygous, leading to an allelic frequency of 0.84 for the ancestral allele (677C) and 0.16 for the mutant one (677T). The allelic frequency obtained for CBS mutation was very similar to the MTHFR one: 0.85 for the ancestral allele (A) and 0.15 for the insertion (I). Moreover, we identified the following genotyping distribution for CBS gene - 69 (72.6%) ancestral homozygous, 23 (24.2%) heterozygous and three (3.2%) insertion homozygous. In addition, the co-heritance prevalence of both mutation in at least one chromosome was 8,4%. While for the ADA gene, we found none homozygous mutant: 80 (84.2%) patients were wild homozygous and 15 (15.8%) were heterozygous; with a frequency of 0.92 for ancestral allele (22G) and 0.08 for mutant one (22A). We observed expected pharmacological effects of HU, such as reduction of the lipid peroxidation levels, increased fetal hemoglobin content (HbF), and improvement of hematological and hemolytic markers, regardless the presence of evaluated polymorphisms. The presence of MTHFR (677C>T) polymorphism did not influence the response to HU treatment or oxidative stress markers. However, patients with the mutation (677C> T) in at*

least one chromosome showed the lowest HbF value (p <0.01). For insertion into the CBS gene, we found high activities of lactate dehydrogenase (p <0.01), aspartate transaminase (p = 0.01), and gamma-glutamyl transferase (p = 0.04) in patients with insertion in at least one chromosome and under HU use. Furthermore, we observed a synergistic effect of both polymorphisms on decreasing GSH levels, regardless of HU treatment. The presence of mutation in ADA gene did not exert any influence on the oxidative stress markers evaluated. Incubation period was able to cause a rise about 64% on hemolysis degree (p < 0.01) as well as practically doubled the lipid peroxidation levels (p < 0.01). However, almost all antioxidants tested treatments neutralized this incubation effect observed in MDA levels. Among the antioxidant biomarkers evaluated, we observed a modulating effect of combined treatment (NAC+MEL) on GPx and SOD activities (p < 0.01), which showed ~25% decrease in their activities. In addition, we found an antioxidant dose-dependent effect for melatonin on lipid peroxidation (r = - 0.29; p = 0.03) and for combined antioxidant treatments also on MDA levels (r = - 0.37; p = 0.01) and on SOD activity (r = - 0.54; p < 0.01). Overall, the results for MTHFR and CBS polymorphisms contribute to highlight that the MTHFR and CBS polymorphisms involvement in SCA pathophysiology is likely to be far more complex than it was explored to date. The presence of the polymorphism in the ADA gene (22 G> A) apparently plays no significant role in disruption of the sickle erythrocyte redox metabolism. On the other hand, ex vivo results contribute with important insight that MEL individually or in combination with NAC may be useful for sickle cell anemia management.

Keywords**:** *hemoglobin S, hydroxyurea, methylenetetrahydrofolate reductase, cystathionine beta-synthase, adenosine deaminase, N-acetylcysteine, melatonin*

SUMÁRIO

Introdução

1. INTRODUÇÃO

1.1. Anemia Falciforme

A anemia falciforme (AF) é uma alteração genética da hemoglobina (Hb), causada pela herança em homozigose da mutação pontual no gene da globina beta (*HBB*; c.20A>T; rs334) (BUNN, 1997; STEINBERG; SEBASTIANI, 2012). O gene *HBB* está localizado no braço curto do cromossomo 11 (11p15.5) e é responsável pela síntese das cadeias globínicas β que compõem a Hb adulta normal (Hb A; $\alpha_2\beta_2$) (BUNN; FORGET, 1986). Entretanto, a troca do vigésimo nucleotídeo (G<u>A</u>G → G<u>T</u>G) no sexto *codon* do *exon* 1 do gene acarreta na substituição do ácido glutâmico (com características hidrofílicas) por uma valina (com características hidrofóbicas) ($HBB^{GLU6VAL}$) e na formação de uma proteína com estrutura e características bioquímicas diferentes, conhecida como Hb S ($\alpha_2\beta^S_2$) (WEATHERALL; CLEGG, 2001; BERTHOLO; MOREIRA, 2006).

O aminoácido substituído na cadeia da Hb S produz regiões hidrofóbicas de contato, no tetrâmero desoxigenado, entre a valina de uma molécula de Hb S e uma alanina, fenilalanina e/ou leucina da molécula de Hb S adjacente que resulta na ligação entre esses aminoácidos, produzindo um polímero que se estende e preenche o eritrócito, rompendo a arquitetura e flexibilidade eritrocitária (WISHNER et al., 1975; FRONTICELLI; GOLD, 1976; SAMUEL; SALMON; BRIEHL, 1990). Essas modificações alteram drasticamente suas propriedades mecânicas e reológicas, prejudicando o fluxo sanguíneo através da microvasculatura (NOGUCHI; SCHECHTER; RODGERS, 1993; BUNN, 1997).

A polimerização da Hb S dentro das hemácias também acarreta alterações celulares: efluxo de íons monovalentes como o potássio, desidratação celular, aumento da densidade dos eritrócitos, oxidação instável da Hb (formação de metahemoglobina e radical superóxido), desnaturação da Hb (formação de hemicromos, heme livre e ferro livre), exposição de epítopos protéicos e lipídicos (banda 3, espectrina, fosfatilserina, CD36, CD47) (Figura 1) e por fim a hemólise (FRENETTE; ATWEH, 2007). Dessa forma, tais alterações celulares e vasculares relacionadas a presença da Hb S culminam em dois importantes mecanismos fisiopatológicos da AF: a vaso-oculsão e, consequentes lesões decorrentes de processos de isquemia-reperfusão e anemia hemolítica (NOGUCHI; SCHECHTER; RODGERS, 1993; BUNN, 1997; REES; WILLIAMS; GLADWIN, 2010). Estes eventos se sobrepõem e se retro-alimentam desencadeando diversas complicações clínicas, as quais apresentam idade e risco de ocorrência, assim como gravidade, altamente variáveis entre as pessoas com AF (Figura 2) (GLADWIN; VICHINSKY, 2008).

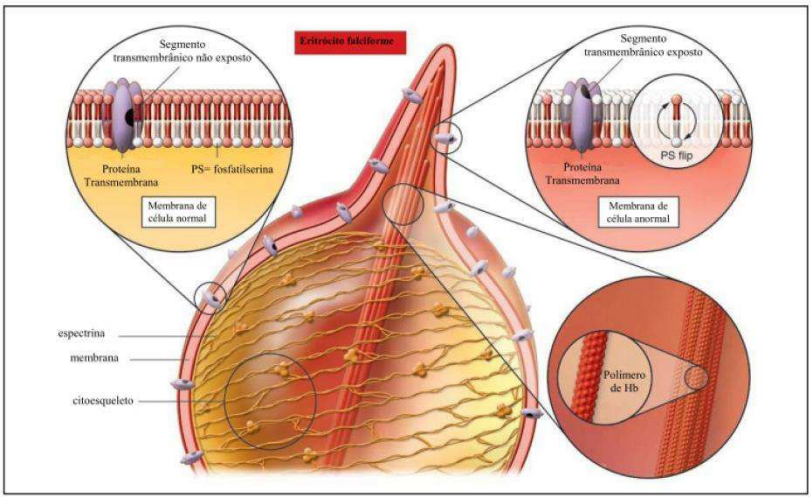

Figura 1. Deformação da membrana eritrocitária pela polimerização da Hb S. A desoxigenação induz a mudança na conformação estrutural da proteína, em que as cadeias globínicas β mutante se ligam ao sítio hidrofóbico complementar, originado pela substituição do ácido glutâmico pela valina, levando à formação do polímero de Hb. Os feixes de polímeros de Hb S alongam-se e rompem a ligação da membrana do eritrócito com proteínas do citoesqueleto, resultando em protrusões na membrana e exposição de proteínas e lipídios (modificado de FRENETE; ATWEH, 2007).

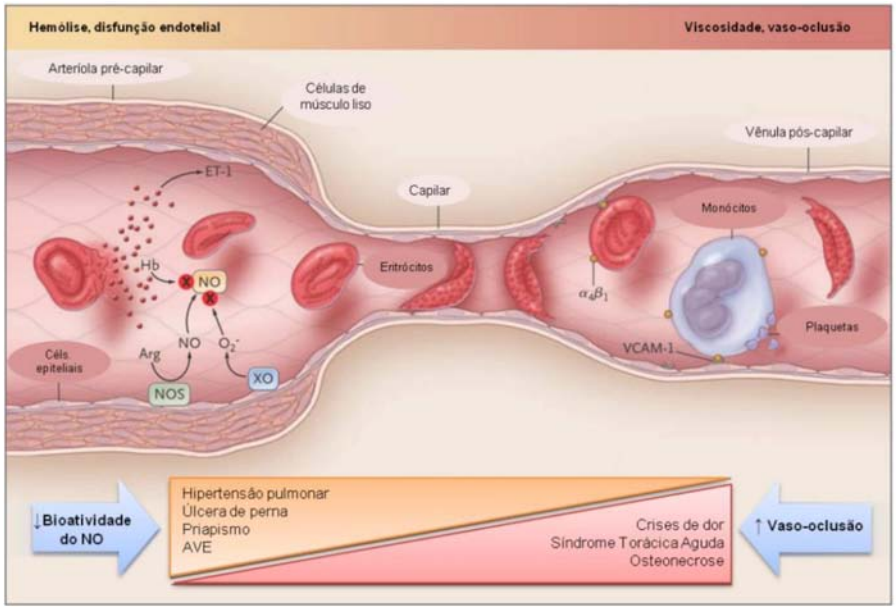

Figura 2. Mecanismos hipotéticos de subfenótipos clínicos da AF. Supõe-se que muitas das complicações da AF podem ser divididas em dois subfenótipos de sobreposição, cada um acionado por mecanismos distintos. Úlceras de perna, priapismo, hipertensão pulmonar, morte súbita e acidente vascular encefálico (AVE) estão associados com níveis baixos de Hb durante o período estacionário da doença e com aumento da taxa de hemólise intravascular, mostrado do lado esquerdo da figura. Estas complicações vasculares resultam de disfunção endotelial, mediada pela inativação de óxido nítrico (NO) pela Hb livre e liberação e formação de espécies reativas de oxigênio, catabolismo da arginina (Arg) promovido pela arginase livre no plasma. Este processo de hemólise acompanhado de disfunção endotelial causa a ativação hemostática e proliferação neointimal e do músculo liso. Tais complicações clínicas como crises de dor vaso-oclusivas, a síndrome torácica aguda, necrose avascular dos ossos estão associadas com o número elevado de leucócitos durante período estacionário e níveis elevados de Hb. Estas complicações resultam da obstrução dos capilares e vênulas pós-capilares por eritrócitos falcizados e por leucócitos (representados pelo monócito), como mostrado no lado direito da figura. ET-1: endotelina-1; NOS: óxido nítrico sintetas; O_2^-: superóxido, VCAM-1: proteína de adesão vascular-1; XO: xantina oxidase (GLADWIN; VICHINSKY, 2008).

Apesar da base molecular para a formação da HbS ser bem conhecida, e, da descrição da polimerização da HbS como o evento primário indispensável na patogênese molecular da doença, estes mecanismos não são suficientes para explicar a alta heterogeneidade fenotípica encontrada nos indivíduos com AF (REES; WILLIAMS; GLADWIN, 2010; BALLAS et al., 2012). Dentre os diversos biomarcadores estudados, os níveis de HbF e coerança da α-talassemia são principais modulares da gravidade clínica de pessoas com AF, amplamente relatados na literatura (HIGGS et al., 1982; KAR et al., 1986; STEINBERG, 2009; REES; GIBSON, 2012; DAMANHOURI et al., 2015). No entanto, desde a primeira descrição de hemácias falciformes feita por Herrick, em 1910, o entendimento da fisiopatologia da AF tem aumentado gradualmente e, entre as novas evidências, destacam-se a coerança de diferentes marcadores genéticos associados aos eventos clínicos da AF e a resposta ao tratamento com hidroxiureia (HU), e o estresse oxidativo crônico e sistêmico (STEINBERG, 2005; STEINBERG; ADEWOYE, 2006; FRENETTE; ATWEH, 2007; GLADWIN; VICHINSKY, 2008; FERTRIN; COSTA, 2010; SILVA et al., 2013b).

1.2. Estresse oxidativo

A produção de espécies reativas de oxigênio (ERO), espécies reativas de nitrogênio (ERN), entre outras espécies reativas, é parte integrante do metabolismo humano, observada em diversas condições fisiológicas (FINKEL; HOLBROOK, 2000). Por exemplo, é estimado que 1 - 2% dos elétrons que se deslocam ao longo da cadeia respiratória "escapam" da via e reduzem o oxigênio (O_2) a superóxido ($O_2^{\bullet-}$), que por sua vez pode sofrer dismutação, gerando peróxido de hidrogênio (H_2O_2) (FREI; STOCKER; AMES, 1988; THOMAS; DAVIES; STOCKER, 1998). O sistema do citocromo P450 e oxidases, tais como o nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase das células fagocíticas (por exemplo, neutrófilos e monócitos), são outras fontes endógenas de produção de $O_2^{\bullet-}$ (FINKEL; HOLBROOK, 2000). O excedente de ERO é, em grande parte, neutralizado por um sistema de defesa antioxidante, formado por compostos enzimáticos e não enzimáticos, a fim de controlar e restabelecer o equilíbrio (HALLIWELL, 1997; FINKEL; HOLBROOK, 2000; SCHAFER; BUETTNER, 2001). Tal equilíbrio entre a produção de ERO e as defesas antioxidantes determina o grau de estresse oxidativo, que pode acarretar em danos/modificações em proteínas celulares, lipídios e DNA (Figura 3) (FINKEL; HOLBROOK, 2000).

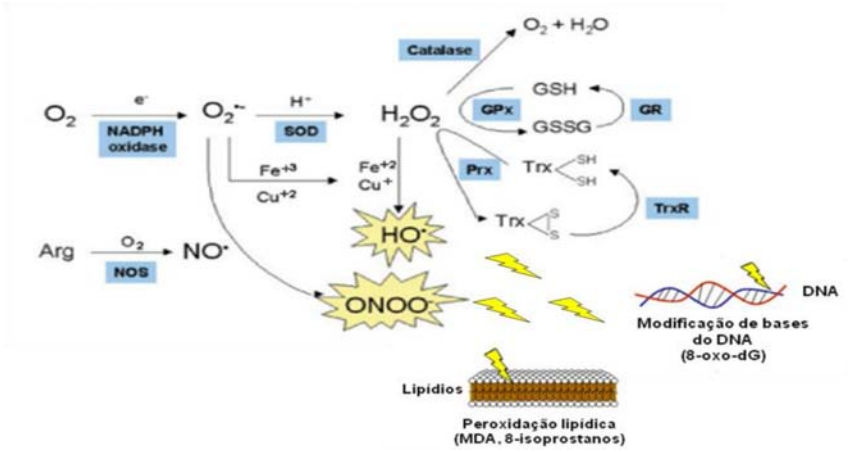

Figura 3. Esquema simplificado de sistemas oxidativos e antioxidantes. O metabolismo aeróbio apresenta uma série de reações que podem formar ERO e ERN, como pela ação da enzima NADPH oxidase, presente em diversas células. A oxidação não-enzimática de lipídios de membrana por ERO produz MDA ou F8-isoprostano como subprodutos, enquanto a oxidação do DNA pode originar o mutagênico 8-oxo-dG, por exemplo. NOS: óxido nítrico sintase; SOD: superóxido dismutase; GPx: glutationa peroxidase; Prx: peroxirredoxinas; GR: glutationa redutase; TrxR: tiorredoxina redutase; Trx:tiorredoxina; GSH: glutaiona reduzida; GSSG: glutationa dissulfeto; MDA: malondialdeído; 8-oxo-dG: 8-oxo-2'-desoxiguanosina (BARBOSA; DE MEDEIROS; AUGUSTO, 2006).

Nos eritrócitos, a auto-oxidação da Hb com consequente formação de meta-hemoglobina e $O_2^{\bullet-}$ pode representar uma fonte adicional e quantitativamente significativa de geração $O_2^{\bullet-}$ em sistemas biológicos (CARRELL; WINTERBOURN; RACHMILEWITZ, 1975; ASLAN; THORNLEY-BROWN; FREEMAN, 2000). Entretanto, existe uma taxa "fisiológica" normal de formação de meta-hemoglobina nos glóbulos vermelhos que proporciona uma fonte contínua de $O_2^{\bullet-}$ que, por sua vez gera H_2O_2 e de O_2 como subprodutos de dismutação enzimática (FREEMAN; CRAPO, 1982). Dessa forma, perturbações na função e estrutura dos eritrócitos podem desencadear aumento no fluxo de geração de pró-oxidantes com consequente estabelecimento de estresse oxidativo (SILVA et al., 2013b). Com intuito de neutralizar o estresse oxidativo, os eritrócitos possuem um sistema de defesa antioxidante auto-sustentável, incluindo as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR), além de antioxidantes de baixo peso molecular , tais como a glutationa (GSH) e vitaminas E e C, proporcionando uma proteção antioxidante não apenas para si, mas também para outros tecidos e órgãos (SIEMS; SOMMERBURG; GRUNE, 2000; PERRONE et al., 2012).

Porém, as hemácias falciformes apresentam uma auto-oxidação instável da HbS e altas exigências metabólicas devido aos ciclos recorrentes de polimerização e despolimerização da HbS que exacerbam a geração de ERO (HEBBEL et al., 1988; BANERJEE; KUYPERS, 2004). Dessa forma, as hemácias falciformes geram pelo menos duas vezes mais $O_2^{\bullet-}$, H_2O_2,

radicais hidroxil ($HO^{\bullet}$) e produtos de oxidação lipídica em relação a eritrócitos contendo Hb A (RICE-EVANS; OMORPHOS; BAYSAL, 1986; HEBBEL et al., 1988; HEBBEL, 1991; SHENG; SHARIFF; HEBBEL, 1998; ASLAN; THORNLEY-BROWN; FREEMAN, 2000; KLINGS; FARBER, 2001). A geração incessante de agentes pró-oxidantes na AF culminam no consumo das defesas antioxidante e, estabelecimento do estresse oxidativo (BANERJEE; KUYPERS, 2004; REID et al., 2006; CHAVES; LEONART; DO NASCIMENTO, 2008).

A glutationa (GSH) é um tripeptídeo, γ-L-glutamil-L-cisteinilglicina, presente em concentrações elevadas (cerca de 2 mM) em eritrócitos saudáveis como o tiol não-proteico mais abundante, atuando como antioxidante não enzimático e co-fator de enzimas antioxidantes (LU, 2013). Entretanto, eritrócitos de pacientes com AF podem apresentar redução de até 50% dos seus níveis de GSH, mesmo com taxa de reciclagem e biossíntese mais elevadas em relação a eritrócitos de indivíduos saudáveis (TATUM; CHOW, 1996; REID et al., 2006; MORRIS et al., 2008). A biossíntese ocorre no meio intracelular (exceto em células epiteliais), pela ação consecutiva de duas enzimas e com consumo de ATP: formação da γ-L-glutamil-L-cisteína a partir do ácido glutâmico e cisteína, catalisada pela glutamato-cisteína ligase (GCL); e a ligação deste dipeptídeo à glicina pela ação da glutationa sintetase (GS) (Figura 4) (LU, 2013).

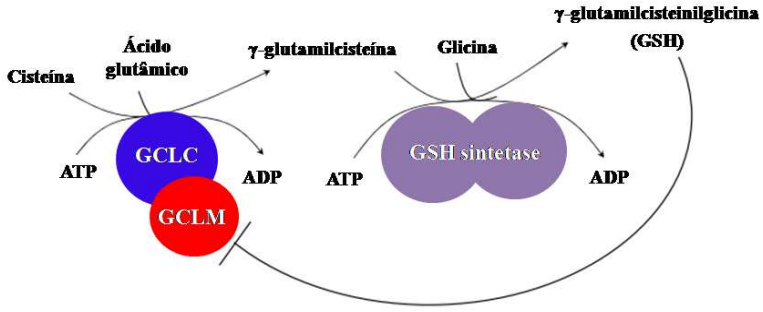

Figura 4. Biossíntese da glutationa. Primeiramente é catalisada a formação da γ-glutamilcisteína pela GCL, que é composta por duas subunidades catalítica (GCLC) e modificadora (GCLM). Posteriormente, a síntese da γ-glutamilcisteinilglicina (GSH) pela ação da GSH sintetase. A GCL sofre regulação pela GSH através de um *feedback* negativo, o que previne a produção excessiva desta ou o acúmulo do intermediário γ-glutamilcisteína. GCL: glutamato-cisteína ligase; GS: glutationa sintetase; ATP: adenosina trifosfato; ADP: adenosina difosfato (adaptado de LU, 2013).

Sob condições fisiológicas normais, a taxa de síntese de GSH é em grande parte determinada por dois fatores, a biodisponibilidade da cisteína e da atividade da GCL (LU, 2013; VAN ZWIETEN R.; VERHOEVEN; ROOS, 2014). A cisteína é normalmente derivada a partir da dieta, da degradação de proteínas e principalmente no fígado, por meio da via de

transulfuração da homocisteína (FINKELSTEIN, 1990; LU, 2013). Além disso, o estresse oxidativo é um regulador positivo da via de transulfuração, pois ativa a enzima cistationina beta-sintase (CBS), promovendo assim a conversão de metionina à cisteína, com consequente aumento da síntese de GSH. Entretanto, mecanismo exato não é completamente compreendido (MCBEAN, 2012).

1.3. Metabolismo da homocisteína dependente de folato

A CBS é a enzima fundamental na via de transulfuração, pois catalisa a condensação irreversível da homocisteína (Hmc) com serina para formar cistationina numa reação dependente de vitamina B6. Em seguida, a cistationina é clivada por outra enzima dependente de vitamina B6, a γ-cistationase, liberando cisteína para a síntese de GSH (Figura 5) (FINKELSTEIN, 1990). A via de transulfuração desempenha papel significativo no complexo metabolismo da Hmc dependente de folato, em conjunto com a via de remetilação (dependente de folato e vitamina B12), cuja enzima principal é a metilenotetrahidrofolato redutase (MTHFR) (SELHUB, 1999). A MTHFR catalisa a redução irreversível do 5,10-metilenotetrahidrofolato a 5-metiltetrahidrofolato (a forma circulante do folato), que por sua vez atua como co-substrato da enzima metionina sintetase, na remetilação da metionina a partir de Hmc (Figura 5) (SELHUB, 1999). Dessa forma, polimorfismos genéticos nos genes que codificam as enzimas MTHFR e CBS são importantes determinantes dos níveis de Hmc e, podem estar envolvidos na biodisponibilidade da cisteína.

Entre os polimorfismos descritos para estas enzimas, os mais estudados são mutação de ponto no gene *MTHFR* (c.677C> T;. rs1801133), localizado no braço curto do cromossomo 1 (1p36.3) (FROSST et al., 1995) e uma inserção de 68 pb na região codificadora do exon 8 do gene *CBS* (844ins68; 21q22.3) (SEBASTIO et al., 1995). O alelo mutante *MTHFR* 677T está associado à redução da atividade enzimática, a diminuição das concentrações de folato no plasma e nas células vermelhas do sangue, aumento dos níveis de Hcm plasmáticos (FROSST et al., 1995; JACQUES et al., 1996), e é um fator de risco para complicações vasculares (TRABETTI, 2008), inclusive em pessoas com AF (MOREIRA et al., 2006; ALVES JACOB; DA CUNHA; BONINI-DOMINGOS, 2011; NISHANK; SINGH; YADAV, 2013). Já para a inserção no gene *CBS*, não há consenso sobre seu efeito sobre a atividade da CBS e sobre os níveis de Hmc (SEBASTIO et al., 1995; TSAI et al., 1996; TSAI et al., 1999). Apesar disso, Alves-Jacob et al. (2011) demonstraram que a presença da mutação é um fator de risco para episódios vaso-oclusivos em pacientes SCA. Além disso,

estudos recentes têm demonstrado que alterações nas vias de transsulfuração e remetilação estão associadas ao aumento da geração de ERO (DRAGANI et al., 2012; RAHIMI et al., 2013; RICHARD et al., 2013), o que poderia agravar a perfil oxidativo de pacientes com AF.

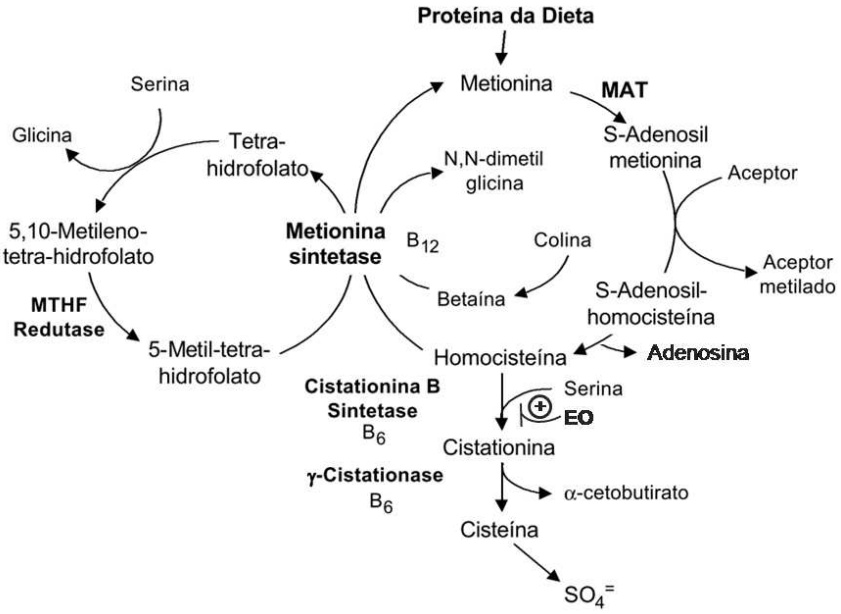

Figura 5. Metabolismo da homocisteína. A fim de manter o fornecimento de cisteína para a síntese de glutationa, estresse oxidativo (EO) atua como regulador positivo da via de transulfuração. MAT: metionina adenosiltransferase; MTHFR: metilenotetrahidrofolato redutase (modificado de FINKELSTEIN, 1990).

Também é parte integrante do catabolismo da metionina, a geração de S-adenosilmetionina (SAM), o principal doador biológico de grupamentos metil, por meio da reação catalisada pela metionina adenosiltransferase (MAT) (LU; MATO, 2012). Posteriormente, SAM doa seu grupo metil a uma variedade de moléculas receptoras em reações catalisadas por metiltransferases (MTs), gerando S-adenosil-homocisteina (SAH), que por sua vez é hidrolisada reversivelmente pela SAH-hidrolase, formando Hmc e adenosina (Figura 5) (FINKELSTEIN, 1990). A adenosina funciona como molécula sinalizadora que induz múltiplas respostas fisiológicas associadas a fisiopatologia da AF (ZHANG; XIA, 2012).

1.4. Adenosina

A adenosina é um nucleosídeo de purina endógeno envolvido na sinalização da vasodilatação, redução da inflamação e na citoproteção durante processos de isquemia e reperfusão em diversos órgãos, por meio de quatro subtipos de receptores de adenosina A_1, A_{2A}, A_{2B} e A_3, (MAHAFFEY et al., 1999; HASKO et al., 2008). Dentro das células, a

adenosina é formada, predominantemente, por meio da desfosforilação do monofosfato de adenosina (AMP), catalisada pela 5'-nucleotidase citosólica (Cito 5'-NT), enquanto que no meio extracelular, ectonucleotidases (CD39 e CD 73) desempenham papel fundamental na formação de adenosina (ZHANG; XIA, 2012). E, tanto intra quanto extracelularmente, a adenosina deaminase (ADA) catalisa a conversão irreversível da adenosina a inosina, promovendo a manutenção de seus níveis (ZHANG; XIA, 2012). O complexo metabolismo da adenosina encontra-se resumido na Figura 6.

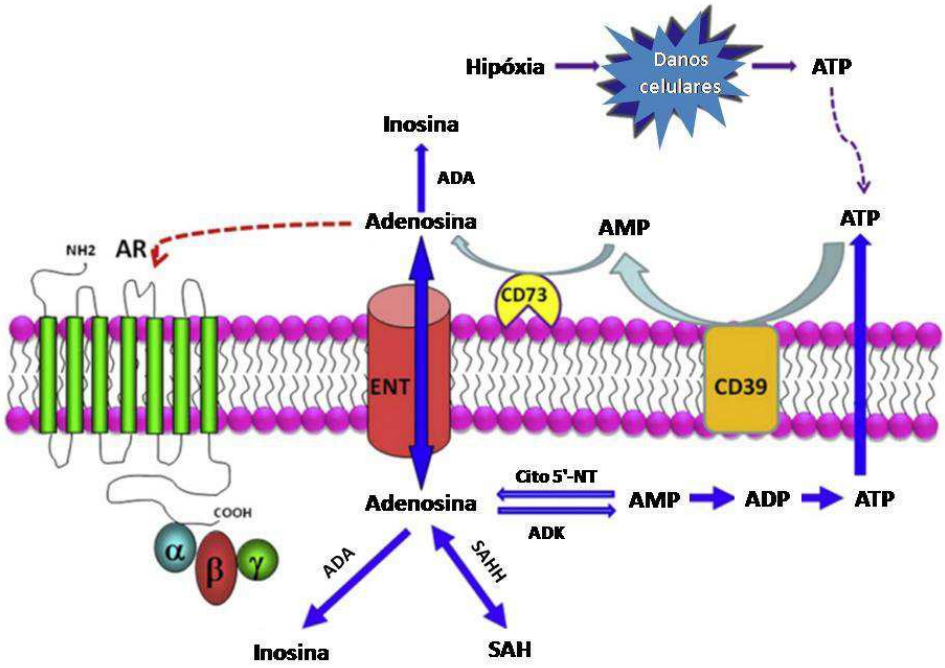

Figura 6. Metabolismo e sinalização da adenosina. As células liberam ATP em resposta à hipóxia e danos celulares. A ATP extracelular é convertida a adenosina pela ação consecutiva de duas ectonucleotidases, CD39 e CD73. A adenosina resultante pode ativar os receptores de adenosina (AR), ser convertida a inosina pela ação da adenosina desaminase (ADA) ou ainda, reingressar na célula por meio de transportadores de nucleosídeos (ENT). Dentro das células a adenosina tem vários destinos: conversão em inosina através de desaminação; conversão em S-adenosilhomocisteína (SAH) por intermédio da S-adenosilhomocisteína hidrolase (SAHH); ou a conversão em AMP pela adenosina-quinase (ADK). A adenosina também pode ser derivada do AMP por ação de 5'-nucleotidase citosólica (Cito 5'-NT) (modificado de ZHANG; XIA, 2012).

O metabolismo da adenosina pode ser prejudicado pela presença de mutações no gene *ADA* (20q13.11). Por exemplo, o gene ADA apresenta uma mutação pontual no seu primeiro exon (c.22G>A; rs73598374), promovendo a troca de um ácido aspártico por asparagina na posição 8 da proteína ($ADA^{ASP8ASN}$). A presença do polimorfismo (22A) confere perda de aproximadamente 35% da atividade da enzima, consequentemente portadores apresentam níveis mais elevados de adenosina circulante e intracelular, e possível comprometimento de respostas fisiológicas (BATTISTUZZI et al., 1981; RIKSEN et al., 2008), principalmente em pessoas com AF, que já possuem níveis elevados de adenosina (GLADWIN, 2011).

Neste contexto, a coerança de *HBB*S* e *ADA* (22G>A) poderia agravar o efeito prejudicial causado pela adenosina em eritrócitos falciformes, observado por Zhang et al. (2011). Resumidamente, os autores demonstraram em eritrócitos normais que a adenosina pode aumentar a síntese de 2,3-bisfosfoglicerato (2,3-BPG) através da ativação do receptor A_{2B} (via proteína quinase dependente de cAMP). Este mecanismo sugere que o aumento dos níveis de adenosina tem um papel fundamental em eritrócitos normais, de promover a liberação do O_2 e evitar lesão tecidual isquêmica aguda. No entanto, em eritrócitos falciformes, este papel benéfico da adenosina torna-se prejudicial, pois ao promover a desoxigenação, favorece a polimerização da Hb S (ZHANG et al., 2011) e, consequentemente, desencadeamento dos processos fisiopatológicos previamente descritos.

1.5. Terapia antioxidante

A compreensão acerca dos processos oxidativos que ocorrem na AF resultou em novos percepções sobre os mecanismos de ação de algumas terapias atualmente aceitas, como a HU, e também em novas abordagens terapêuticas fundamentadas nas propriedades antioxidantes de diferentes compostos (GIZI et al., 2011; SILVA et al., 2013b), com intuito de prevenir ou retardar o desenvolvimento de complicações clínicas nos indivíduos com AF (NUR et al., 2011).

Estudos *in vitro* e em animais experimentais demonstraram que o tratamento com *N*-acetilcisteína (NAC) de células sanguíneas de pessoas com AF aumentou a concentração intracelular de GSH e diminuiu parâmetros do estresse oxidativo (AMER et al., 2006). Posteriormente, Nur et al (2012) demonstraram que o tratamento oral com NAC (2400 mg) de indivíduos com AF promoveu a redução da exposição de fosfatidilserina no exterior da membrana de eritrócitos, dos níveis plasmáticos de produtos finais de glicação avançada (AGEs), e dos níveis de Hb livre. Porém, o grupo carboxil da NAC é carregado negativamente em pH fisiológico, limitando a capacidade do agente de atravessar membranas celulares (GRINBERG et al., 2005) e, consequentemente seu efeito farmacológico esperado.

Ao contrário da NAC, a melatonina (*N*-acetil-5-metoxitriptamina) possui capacidade de atravessar qualquer barreira morfofisiológica (REITER et al., 2013) e exibe propriedades antioxidantes importantes na neutralização de efeitos deletérios promovidos pelas ERO (REITER et al., 2003). De grande interesse e importância são as observações de que não só a melatonina (MEL) é um antioxidante altamente eficaz contra uma variedade de ERO, mas também os metabolitos que resultam da sua interação com as mesmas (GALANO; TAN;

REITER, 2013). Dessa forma, a redução dos níveis de MEL tem sido observada em muitas doenças inflamatórias e/ou com quadro oxidativo crônico (REITER et al., 2003), inclusive na AF (SHIMAUTI et al., 2010).

A administração de MEL em ratos espontaneamente hipertensos reverteu, parcialmente, a hipertensão estabelecida; e, o seu efeito anti-hipertensivo foi mais pronunciado do que o efeito observado nos ratos tratados com NAC nas mesmas condições experimentais (PECHANOVA et al., 2007). Miller et al (2011) demonstraram que a administração de MEL (10 mg) em pacientes com esclerose múltipla aumentou significativamente as atividades das enzimas antioxidantes SOD e catalase. As propriedades antioxidantes da MEL, especialmente em doses farmacológicas parecem ser evidentes, pois a MEL foi capaz de reduzir a peroxidação lipídica de forma mais eficaz que a vitamina C ou E em homogenatos de fígado de ratos. Além disso, *in vivo,* foi demonstrada sua capacidade de reduzir os danos causados por isquemia-reperfusão em vários órgãos, incluindo o coração (TAN et al., 1998), rim (SAHNA et al., 2003), cérebro (CHO et al., 1997) e fígado (SEWERYNEK et al., 1996) de ratos. Ou seja, tais implicações terapêuticas promissoras da MEL sugerem seu uso como um adjuvante na redução do estresse oxidativo em pessoas com AF.

Os processos fisiopatológicos complexos e intimamente relacionados com estresse oxidativo crônico e sistêmico fundamentam a hipótese do presente estudo de que polimorfismos genéticos em enzimas de importantes vias metabólicas podem agravar o quadro oxidativo e, modular manifestações fenotípicas da AF. Dessa forma, a MEL pode ser um alvo terapêutico promissor nessa afecção genética, de maneira mais eficaz que o antioxidante NAC, atuando como adjuvante na redução do estresse oxidativo.

Objetivos

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a influência de marcadores genéticos sobre o perfil oxidativo de pessoas com anemia falciforme, e a sua relação com uso de medicação específica e manifestações fenotípicas. Ainda, verificar possíveis efeitos antioxidantes diretos e indiretos da melatonina, usando uma suspensão de células falciformes como modelo experimental.

2.2 Objetivos específicos

- Investigar as frequências de polimorfismos nos genes de enzimas da via metabólica da homocisteína dependente de folato (metilenotetrahidrofolato redutase e cistationina-β-sintetase) e do metabolismo da adenosina (adenosina desaminase) em indivíduos com anemia falciforme;

- Avaliar marcadores do estresse oxidativo e capacidade antioxidante, correlacionando-os com a presença dos polimorfismos supracitados;

- Relacionar os resultados obtidos com o uso de medicação específica e marcadores do acompanhamento clínico dos pacientes;

- Avaliar o efeito antioxidante da *N*-acetilcisteína, da melatonina e da combinação destes compostos sobre marcadores de hemólise e do estresse oxidativo, por meio do tratamento *ex vivo* de células sanguíneas de pessoas com anemia falciforme.

Material e métodos

3. MATERIAL E MÉTODOS

3.1. Considerações éticas

O presente projeto foi registrado sob o nº 0023.0.229.000-11 CAAE (Certificado de Apresentação para Apreciação Ética) e aprovado pelo Comitê de Ética em Pesquisa (CEP) da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto (UNESP/IBILCE) sob o protocolo número 071/11.

3.2. Casuística

O grupo amostral geral constituiu-se de 105 indivíduos com AF, independente do sexo e da etnia e, provenientes da região sudeste do Brasil. Todas as amostras de sangue foram coletadas após obtenção de assinatura no Termo de Consentimento Livre e Esclarecido, conforme a Resolução do Conselho Nacional de Saúde (CNS) Nº 466/12.

Todos os indivíduos foram avaliados por meio de questionário, quanto ao atendimento dos critérios de inclusão/exclusão do estudo. Mulheres grávidas, fumantes e pessoas que haviam ingerido álcool dentro de 24 horas anteriores à coleta das amostras biológicas foram excluídos do estudo. Dentro deste mesmo período, aqueles sujeitos que estavam sob uso de medicamentos, tais como ácido acetilsalicílico, antibióticos, vitaminas ou quelantes de ferro, conhecidos por afetarem os parâmetros avaliados, também foram excluídos do estudo. Aqueles pacientes que estivessem em crise de dor ou hemolítica no dia da coleta; e que tiveram acidentes vasculares encefálicos ou que tinham recebido transfusão sanguínea dentro do período de 120 dias anteriores a data da coleta, também foram excluídos do estudo. Histórico de episódios de crise de dor, necessidade de internação e diagnóstico de complicações agudas nos últimos 12 meses também foram abordados no questionário.

Todas essas informações foram confirmadas por meio de consulta aos prontuários médicos, aos bancos de dados dos hemocentros envolvidos; assim como a obtenção dos dados referentes ao uso de medicação específica e manifestações fenotípicas. Toda a consulta aos prontuários banco de dados foi supervisionada pelos clínicos responsáveis pelos pacientes. Todos os marcadores de acompanhamento clínico dos pacientes, por exemplo, hemograma completo, marcadores hemolíticos e de função hepática e renal, foram realizados por laboratórios especializados nos respectivos hemocentros envolvidos neste estudo.

3.2.1. Experimentação in vivo

O grupo de estudo foi composto por 95 indivíduos com AF; 53 (55,8%) mulheres e 42 (44,2%) homens, com média de idade de 24,4 anos, variando de 10 a 59 anos. Todos os pacientes estavam em acompanhamento clínico regular no Hemocentro do Rio de Janeiro (RJ) - HEMORIO. Todos os pacientes estudados estavam sob tratamento profilático com ácido fólico (5 mg/dia) desde o diagnóstico de doença, enquanto 41 (43,2%) deles estavam sob uso de HU (dose média: 22 mg/Kg/dia) por pelo menos 90 dias (média de dias: 1151,1; variação: 90 - 2897 dias).

3.2.2. Experimentação ex vivo

Dez indivíduos, previamente diagnosticados com AF, foram incluídos no estudo: seis mulheres (60%) e quatro homens (40%), com média de idade de 27,4 anos, e, dentro da faixa etária de 20 a 40 anos, pois a idade é um fator limitante da síntese e secreção de melatonina. Todos os pacientes estavam em acompanhamento clínico regular no Hemocentro de São José do Rio Preto (SP). A Tabela 1 apresenta a caracterização do grupo de estudo quanto a dados demográficos, perfil haplotípico da globina beta, uso de medicação específica e históricos transfusional e clínico.

Tabela 1. Caracterização do grupo de estudo

Característica	**n = 10**
Idade (anos: $\overline{X} \pm DP$)	27,4 ± 7,6
Gênero [n (%)]	
Feminino	6 (60,0)
Masculino	4 (40,0)
Peso (Kg: $\overline{X} \pm DP$)	55,3 ± 7,0
Perfil haplotípico da globina beta [n (%)]	
Bantu/Bantu	3 (30,0)
Bantu/Benin	3 (30,0)
Bantu/Atípico	1 (10,0)
Benin/Atípico	2 (20,0)
Atípico/atípico	1 (10,0)
*Uso de medicação**	
HU^+ (dose média: 23,7 ± 3,3)	3 (30,0)
HU^-	7 (70,0)
Histórico transfusional[#] [n (%)]	
0 - 10	0 (0,0)
11 - 20	0 (0,0)
21 - 30	3 (30,0)
31 - 40	0 (0,0)
> 40	7 (70,0)
Histórico clínico	
Nº de crises de dor no último ano [n (%)]	
0 - 2	7 (70,0)
3 - 5	3 (30,0)
> 5	0 (0,0)
Nº de infecções no último ano [n (%)]	
0 - 2	10 (100,0)
3 - 5	0 (0,0)
> 5	0 (0,0)
Nº de internações no último ano [n (%)]	
0 - 2	10 (100,0)
3 - 5	0 (0,0)
> 5	0 (0,0)

$\overline{X} \pm DP$: média ± desvio padrão.
*Todos os pacientes estavam sob uso de ácido fólico (5 mg/dia).
[#]Número de transfusões recebidas até a data da coleta.

3.3. Metodologia

3.3.1. Técnicas citológicas, eletroforéticas e cromatográfica para identificação da Hb S

Análise, a fresco, da morfologia eritrocitária (BONINI-DOMINGOS, 2006)

Os esfregaços sanguíneos foram analisados ao microscopia de luz, com objetivas de 40x, quanto ao tamanho, forma e quantidade de Hb nos eritrócitos.

Resistência globular osmótica em solução de NaCl a 0,36% (SILVESTRONI; BIANCO, 1975)

Técnica seletiva, baseada no aumento resistência globular osmótica promovido, principalmente, pela presença de eritrócitos microcíticos. A microcitose é característica de algumas hemoglobinopatias, porém mais comum em casos de talassemias do tipo beta, principalmente na forma heterozigota, e também em anemias carências e hemoglobinopatia do tipo Hb C.

Eletroforese de hemoglobinas em pH alcalino (MARENGO-ROWE, 1965)

As amostras de sangue, previamente hemolisadas segundo Naoum (1990) (1990), foram submetidas à eletroforese de Hb em pH 8,6 em acetato de celulose. Técnica qualitativa utilizada para detecção perfil hemoglobínico normal e de Hb variantes, baseada nas diferentes cargas líquida dessas proteínas que acarretam mobilidades eletroforéticas específicas.

Eletroforese de hemoglobinas em pH ácido (VELLA, 1968)

Técnica utilizada para diferenciação de alguns tipos de Hb variantes que migram em posições muito semelhantes na eletroforese em pH alcalino. Os hemolisados, previamente preparados, são submetidos a uma corrida eletroforética em gel de ágar-fosfato, pH 6,2.

Cromatografia líquida de alta performance de frações hemoglobínicas (INSTRUCTION MANUAL OF BIO-RAD, 2006)

Cromatografia de troca iônica acoplada a detecção UV/Vis (415 nm). O sistema utilizado foi o VARIANT I (BIO-RAD) com Kit de análise Beta Talassemia Heterozigota, que permitiu a separação e identificação presumível de Hb variantes por meio de "janelas" específicas de tempo de retenção, e a quantificação percentual relativa das frações hemoglobínicas, baseada em calibradores previamente injetados no sistema.

3.3.2. Análises moleculares

Extração de DNA (SAMBROOK; FRITSCH; MANATIS, 1989)

O DNA genômico foi extraído de leucócitos do sangue periférico, segundo a técnica de extração por fenol-clorofórmio e precipitação por etanol.

*Confirmação da homozigose para o polimorfismo no gene HBB*S* (c.20A>T; rs334) *por PCR-RFLP (SAIKI et al., 1985)*

Para a realização da PCR foram utilizados os seguintes oligonucleotídeos iniciadores: P 277 (*sense*) 5' GGC AGA GCC ATC TAT TGC TTA 3' e P 278 (*antisense*) 5' ACC TTA GGG TTG CCC ATA AC 3'. Após a amplificação, o fragmento gerado de 382 pb foi submetido à restrição enzimática a 37 ºC por 5 minutos pela enzima *FastDigest Dde*I. A mutação elimina um sítio de restrição; assim após a digestão o alelo normal gerou 3 fragmentos de 201 pb, 88 pb e 87 pb o alelo mutante gerou dois, um de 288 pb e outro de 88 pb

*Análise dos haplótipos HBB*S (SUTTON; BOUHASSIRA; NAGEL, 1989)*

A determinação dos haplótipos foi realizada por PCR – RFLP, por meio da análise de seis sítios polimórficos (Figura 7).

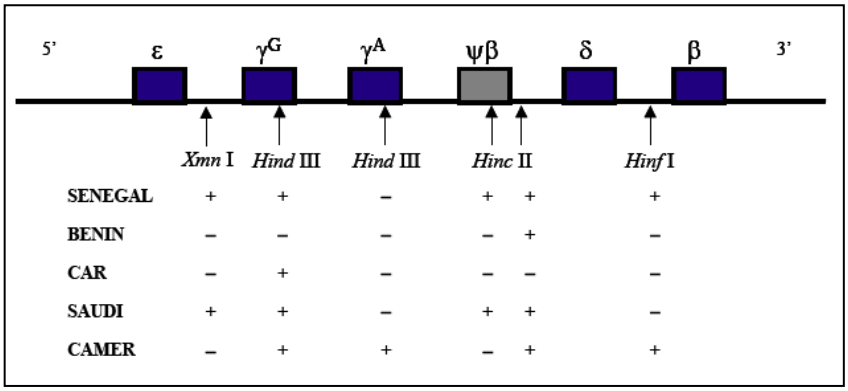

Figura 7. Representação esquemática dos sítios polimórficos estudados na determinação dos haplótipos β^S (*SUTTON; BOUHASSIRA; NAGEL, 1989*).

As sequências dos oligonucleotídeos iniciadores para análise dos polimorfismos do *cluster* da globina β estão listadas na Tabela 2.

Tabela 2. Oligonucleotídeos iniciadores utilizados para amplificação de regiões do *cluster* β.

Oligonucleotídeo iniciador	Sequência do oligonucleotídeo iniciador (5' – 3')	Direção	Região
H0	AACTGTTGCTTTATAGGATTTT	→	5'γ^G
H1	AGGAGCTTATTGATAACTCAGAC	←	
H2	AAGTGTGGAGTGTGCACATGA	←	γ^G
H3	TGCTGCTAATGCTTCATTACAA	→	
H3	TGCTGCTAATGCTTCATTACAA	→	γ^A
H4	TAAATGAGGAGCATGCACACAC	←	
H5	GAACAGAAGTTGAGATAGAGA	→	ψβ
H6	ACTCAGTGGTCTTGTGGGCT	←	
H7	TCTGCATTTGACTCTGTTAGC	→	3'ψβ
H8	GGACCCTAACTGATATAACTA	←	
H9	CTACGCTGACCTCATAAATG	→	5' β
H10	CTAATCTGCAAGAGTGTCT	←	

→: sense ; ← anti-sense

O produto da PCR foi digerido, a 37ºC durante 3 horas, para a região 5'γ^G e para as demais foi digerido por 5 minutos a 37ºC com endonucleases de restrição apropriadas para cada sítio polimórfico. O tamanho dos produtos de amplificação e após clivagem, podem ser observados na Tabela 3.

Tabela 3. Tamanho dos produtos amplificados e após a clivagem com as endonucleases específicas.

Oligonucleotídeo iniciador	Enzima	Região	Tamanho do fragmento amplificado	Tamanho dos fragmentos após a clivagem
H0 e H1	*Xmn* I	5'γ^{G}	657 pb	450 pb + 200 pb
H2 e H3	*Hind* III	γ^{G}	780 pb	430 pb + 340 pb + 10 pb
H3 e H4	*Hind* III	γ^{A}	760 pb	400 pb + 360 pb
H5 e H6	*Hinc* II	ψβ	701 pb	360 pb + 340 pb + 1 pb
H7 e H8	*Hinc* II	3'ψβ	590 pb	470 pb + 120 pb
H9 e H10	*Hinf* I	5' β	380 pb	240 pb + 140 pb

Detecção do SNP (c.677C>T; rs1801133) no gene MTHFR por PCR-RFLP (FROSST et al., 1995)

A detecção da mutação do gene *MTHFR* foi realizada por PCR seguido de análise de restrição enzimática. Os oligonucleotídeos iniciadores utilizados foram: MTHFR 1 (*sense*) 5´ AGG ACG GTG CGG TGA GAG TG 3' e o MTHFR 2 (*antisense*) 5´TGA AGG AGA AGG TGT CTG CGG 3´. Após a amplificação, o fragmento de 198 pb foi digerido a 37°C por 5 minutos pela enzima *FastDigest Hinf*I. A mutação gera um sítio de restrição para a enzima, culminando na obtenção de dois fragmentos para o alelo mutante: 175 pb e 23 pb.

Investigação da inserção de 68 pb (844ins68) no gene CBS por PCR (TSAI et al., 1996)

A detecção da inserção de 68 pb (844ins68) no gene da *CBS* foi realizada por PCR com a utilização dos seguintes oligonucleotídeos iniciadores: Cys A (*sense*) 5'CTG GCC TTG AGC CCT GAA3' e Cys B (*antisense*) 5' GGC CGG GCT CTG GAC TC 3'. Assim, após a amplificação o alelo ancestral gera um fragmento 184 pb, enquanto que o alelo mutante gera um fragmento de 252 pb.

Identificação do SNP (c.22G>A; rs73598374) no gene ADA por PCR (HotStart)-RFLP (SAFRANOW et al., 2007)

A detecção da mutação foi realizada por *PCR HotStart* seguido de análise de restrição. Os oligonucleotídeos iniciadores utilizados foram: ADA*1 (*sense*) 5´GCC CGG CCC GTT AAG AAG AGC3' e ADA*2 (*antisense*) 5'GGT CAA GTC AGG GGC AGA AGC AGA3'. Após a amplificação, o fragmento de 397 pb foi submetido a restrição enzimática a 65 ºC por 10 minutos pela enzima *FastDigest Taq*I. A mutação elimina o sítio de restrição para a enzima, portanto o alelo ancestral gera dois fragmentos - 245 e 152 pb.

3.3.3. Análises bioquímicas

Experimentação ex vivo (adaptado de GRINBERG et al., 2005)

As amostras de sangue total (cerca de 8 mL) foram coletadas em tubos contendo EDTA , entre às 7:00h e às 9:00h. Em seguida, foram imediatamente centrifugadas a 800 *g* por 10 minutos a 4°C. O plasma e a camada de leucócitos foram removidos e a fração celular sanguínea lavada três vezes com tampão PBS-1 (136 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7,4).

Os eritrócitos lavados foram ressuspensos em tampão PBS-2 (100 mM NaCl; 0,5 mM EDTA, 50 mM Na_2HPO_4/ NaH_2PO_4, pH 7,4) em um hematócrito final de 10%. A suspensão eritrocitária foi submetida a 11 condições experimentais diferentes:

- Grupo 1 (G1 - controle externo): suspensão de eritrócitos falciformes (hematócrito final de 10%) não foi tratada com quaisquer antioxidantes e processadas imediatamente para posterior análise;

- Grupo 2 (G2 - controle experimental): suspensão de eritrócitos falciformes (hematócrito final de 10%) não foi tratada com quaisquer antioxidantes, mas foi incubada durante uma hora a 37 ° C;

- Grupos de 3 a 5 (G3-G5 - tratamentos com NAC): suspensões de eritrócitos falciformes (hematócrito final de 10%) foram tratadas com NAC nas concentrações finais de 100 pM, 100 nM e 100 uM, respectivamente, e incubadas durante uma hora a 37 ° C;

- Grupo 6 a 8 (G6-G8 - tratamentos com MEL): suspensões de eritrócitos falciformes (hematócrito final de 10%) foram tratadas com MEL nas concentrações finais de 100 pM, 100 nM e 100 uM, respectivamente, e incubou-se durante uma hora a 37 ° C;

- Grupo 9 a 11 (G9-G11 - tratamentos combinados): suspensões de eritrócitos falciformes (hematócrito final de 10%) foram tratadas com NAC e MEL nas mesmas concentrações finais de 100 pM, 100 nM e 100 uM, respectivamente, e foram incubadas durante uma hora a 37°C. Ao final do período de exposição, cada suspensão celular foi centrifugada a 800 *g* por 10 minutos a temperatura ambiente, o sobrenadante foi coletado para as análises de grau de hemólise e níveis de peroxidação lipídica. Enquanto que com a fração celular foram preparados hemolisados com água ultra pura para as demais análises dos marcadores de capacidade antioxidante.

Análise do grau de hemólise eritrocitária (LI et al., 2006)

O grau de hemólise da suspensão eritrocitária foi determinado pela absorbância da Hb a 540 nm, comparada a absorbância do padrão100% de hemólise, obtido pela adição de 10 µL de Triton X-100 (10% V/V) a 1 mL da suspensão de eritrócitos.

Avaliação dos níveis de peroxidação lipídica (MDA) (ESTERBAUER; ZOLLNER, 1989; DE ALMEIDA et al., 2003; DE ALMEIDA et al., 2004)

Os níveis de peroxidação lipídica foram avaliados por meio do produto formado a partir de malondialdeído (MDA) e ácido tiobarbitúrico (TBA), de acordo com Esterbauer e Zollner (1989), com algumas modificações. O produto foi detectado por meio de cromatografia líquida de alto desempenho acoplada a um detector UV/Vis (UV/Vis-HPLC). Resumidamente, 0,5 mL do sobrenadante da suspensão celular foi misturado em uma proporção de 1:1 (V/V) com uma solução contendo 40% de ácido tricloroacético. Em seguida, esta mistura foi centrifugada a 1500 g durante 5 minutos a 4°C. Ao sobrenadante obtido foi adicionada 0,3 mL de uma solução de TBA (40 mg em 10 mL de HCl 0,2 M) e ambos foram homogeneizados. As misturas resultantes foram aquecidas a 90°C durante 40 minutos. Em seguida, 1 ml de n-butanol foi adicionado e as amostras foram vigorosamente agitadas e centrifugadas a 1500 *x*g durante 5 minutos para extrair os derivados de MDA-TBA. Os extratos de MDA-TBA foram coletados e injetados diretamente no sistema do HPLC.

O sistema HPLC consistia de bomba ESA584 e um detector UV/Vis ESA526 fixado em 532 nm. Os extratos foram separados por uma coluna Shimadzu C18 (150 x 4,6 mm, 5 µm) munida de uma pré-coluna com a mesma composição. A fase móvel (fosfato de potássio 0,05 M; pH 7,0; com 40% de metanol) foi bombeada a um fluxo isocrático de 1 mL min^{-1}. O

monitoramento dos cromatogramas e identificação e quantificação dos pico foram realizados utilizando o *software* EZ Chrom Elite (Agilent Technologies). Os cálculos basearam-se em uma curva de calibração previamente construída e preparada de acordo com o mesmo procedimento utilizado para as amostras a partir de padrões autênticos.

Determinação da atividade enzimática da catalase (BEUTLER, 1975)

Os hemolisados foram submetidos a análise da atividade catalase. A quantificação se baseia na velocidade de decomposição do peróxido de hidrogênio pela enzima, por meio do decréscimo de absorbância em 240 nm.

Determinação da atividade enzimática da GST (KEEN; HABIG; JAKOBY, 1976)

A atividade da GST foi feita nos hemolisados preparados previamente. A amostra foi adicionada em um meio de reação contendo 1 mM de 1-cloro-2,4-dinitrobenzeno (CDNB), 1 mM de GSH, e o aumento de absorbância foi acompanhado a 340 nm.

Análise da atividade enzimática da glutationa peroxidase (GPx) (SIES et al., 1979)

A análise da GPx foi feita pela medida do decréscimo de absorbância a 340 nm, promovido durante a redução da glutationa oxidada (GSSG). A redução é catalisada pela glutationa redutase (GR) em presença de NADPH.

Análise da atividade enzimática da glutationa redutase (GR) (BEUTLER, 1969)

A amostra lisada foi adicionada em um meio de reação contendo 125 mM de GSSG e 6 mM de NADPH, e o decréscimo de absorbância é acompanhado a 340 nm.

Determinação da atividade enzimática da G6PDH (BEUTLER, 1994)

O teste mediu a taxa de redução de $NADP^+$ a NADPH durante a incubação do hemolisado com glicose-6-fosfato, acompanhando a absorbância a 340 nm.

Análise da atividade enzimática da superóxido dismutase (SOD) (MCCORD; FRIDOVICH, 1969)

O método baseia-se em um sistema de geração de superóxido (xantina/xantina oxidase) acoplado à redução do citocromo c pelo radical ânion superóxido, provocando aumento de absorbância em 550 nm. A adição da amostra contendo a SOD promove uma inibição na velocidade de redução do citocromo c, uma vez que a SOD compete com o citocromo c pelo radical superóxido.

Determinação dos níveis plasmáticos e eritrocitários da GSH (RODRIGUEZ-ARIZA; TORIBIO; LOPEZBAREA, 1994)

A concentração de GSH foi analisada por cromatografia líquida de alta performance (HPLC) acoplada a detector eletroquímico coulométrico ajustado com potencial de 650 mV para a GSH. Cada amostra de hemolisado foi foi filtrada em unidades filtradoras Millex (0,22 μm) acopladas a seringa de insulina sem agulha e, posteriormente injetadas diretamente no sistema de HPLC.

A coluna foi uma ESA C18 (250 x 4,6 mm, 5 μm) igualmente munida de uma pré-coluna com a mesma composição. A fase móvel consistiu em fosfato de sódio a 0,05 M, ácido octanossulfónico a 0,025 M; pH 2,5, com acetonitrila a 2% (V/V) e foi bombeada a um fluxo de isocrático de 0,9 mL min^{-1}. O monitoramento dos cromatogramas e identificação e quantificação dos pico foram realizados por meio do mesmo *software* utilizado no ensaio da peroxidação lipídica. Os cálculos basearam-se uma curva de calibração previamente construída através da injeção de padrões autênticos de GSH no sistema de HPLC.

Análises estatísticas

As análises estatísticas foram realizadas nos grupos que continham pelo menos três indivíduos, utilizando-se o software Statistica 9.0 (Statsoft Inc., Tulsa, OK, USA). Os dados foram testados quanto à normalidade e homogeneidade das variâncias, por meio dos testes de Shapiro Wilk e Levene, respectivamente. Os grupos que atenderam às premissas (dados paramétricos) foram comparados por meio da aplicação do teste t ou *one-way* ANOVA seguido pelo *post hoc* de Tukey. Aqueles grupos que não cumpriram os pressupostos (dados não paramétricos) foram comparados pelo teste de Mann-Whitney ou Kruskal-Wallis seguido pelo *post hoc* de Dunn.

A comparação dos tratamentos da experimentação *ex vivo*, por se tratarem de dados pareados, foi feita por meio do método de ANOVA de medidas repetidas seguido pelo *post hoc* de Tukey para dados paramétricos, ou teste de Friedman pelo *post hoc* de Dunnet para dados não paramétricos. Para avaliar o grau de associação entre as variáveis estudadas, utilizou-se a correlação de Pearson para dados paramétricos e de Spearman para dados não-paramétricos. O nível de significância foi fixado em $p < 0,05$.

Resultados

4. RESULTADOS

Os resultados estão apresentados sob o formato de artigos científicos, de acordo com as normas das revistas selecionadas para posterior submissão, em cumprimento às normas para a obtenção do título de Doutor em Genética, pelo Programa de Pós-Graduação em Genética, Instituto de Biociências, Letras e Ciências Exatas, da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP.

Os dados obtidos a partir das análises *in vivo*, de 95 pessoas com AF, para os polimorfismos nos genes *MTHFR* e *CBS* foram reunidos no artigo intitulado *"Impact of genetic polymorphisms in key enzymes of homocysteine metabolism on the pathophysiology of sickle cell anemia"* (Capítulo I) que será submetido à revista Molecular Genetics and Metabolism (FI = 2,827). Trata-se de uma abordagem da influência da coerança destes polimorfismos e AF sobre marcadores do estresse oxidativo, parâmetros de rotina do acompanhamento clínico dos pacientes e resposta diferencial do tratamento com HU.

O Capítulo II compreende os três primeiros objetivos específicos propostos, com o artigo *"Relationship between adenosine deaminase polymorphism (22G>A) and oxidative stress in sickle cell anemia"*que será submetido à revista *Blood Cells Molecules and Disease* (FI = 2,331). Este artigo apresenta, pela primeira vez na literatura, a frequência do polimorfismo 22G>A no gene *ADA* em indivíduos com AF e sua relação com perfil oxidativo de pacientes tratados ou não com HU.

Os resultados obtidos, de acordo com o último objetivo específico proposto neste estudo, foram compilados no artigo científico intitulado *"Potential utility of melatonin as an antioxidant therapy in the management of sickle cell anemia"* (capítulo III), publicado na revista *Journal of Pineal Reaserch* (FI = 7,812). O trabalho abordou efeitos antioxidantes diretos e indiretos da melatonina sob uma suspensão de células falciformes, como modelo experimental.

É importante ressaltar que parte da fundamentação teórica deste projeto também resultou em um artigo de revisão, publicado na revista *Free Radical Biology and Medicine* (FI = 5,710) sob o título de *"Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies"* (ANEXO D).

Capítulo I

Influence of genetic polymorphisms of methylenetetrahydrofolate reductase and cystathionine β-synthase on the pathophysiology of sickle cell anemia

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Abstract: This work aimed at studying a possible influence of *methylenetetrahydrofolate reductase* (*MTHFR*; c. 677C>T) and *cystathionine β-synthase* (*CBS*; 844ins68) polymorphisms on overall oxidative status of SCA patients and on routine markers, correlating them with hydroxyurea (HU) treatment. We evaluated 95 unrelated Brazilian sickle cell anemia (SCA) patients. All patients received a prophylactic treatment with folic acid of 5 mg/day, while 41 (43.2%) of them were under HU treatment (average dose: 22 mg/Kg/day) for at least 90 days. *MTHFR* and *CBS* polymorphisms were identified by PCR-RFLP. Biochemical parameters were measured using spectrophotometric [catalase, glutathione *S*-transferase, glutathione peroxidase, glutathione reductase activities] and chromatographic methods [fetal hemoglobin (HbF), glutathione (GSH) and malondialdehyde (MDA) levels]. Routine markers were developed by specialized laboratory. We did not find any differential response to HU treatment or influence on oxidative markers caused by *MTHFR* 677T mutation, but it was related to the lowest HbF levels in the patients untreated with HU. For *CBS* polymorphism, we found high activities of lactate dehydrogenase, aspartate transaminase and gamma-glutamyl transferase in those patients under HU use. Moreover, we observed a synergic effect of both polymorphisms on decreasing GSH levels, regardless HU treatment. These findings may contribute to highlight that the *MTHFR* and *CBS* polymorphisms involvement in SCA pathophysiology is likely to be far more complex than it was explored to date.

Key words:
Hemoglobin S
Methylenetetrahydrofolate reductase
Cystathionine β-synthase
Hydroxyurea

1. Introduction

Sickle hemoglobin (HbS) is caused by single point mutation in *beta-globin* gene (*HBB*; c.20A>T; rs334), and its homozygosity is termed sickle cell anemia (SCA) which is a chronic and progressively debilitating medical condition common worldwide [1,2]. SCA is an hemolytic anemia characterized by a complex pathophysiology that is affected by a number of modifying factors, including coinheritance of polymorphisms associated to clinical aspects [3,4] and to treatment response [5], as well as a chronic and systemic oxidative stress [6], culminating in antioxidant deficiency [7,8].

Glutathione (GSH) is a tripeptide (γ-L-glutamyl-L-cysteinylglycine), present at high concentrations (about 2 mM) in healthy erythrocytes as the most abundant non-protein thiol that protects against oxidative stress [9]. However, GSH concentration is significantly reduced in SCA patients [7,10], with some studies noting a 50% decrease in sickle erythrocytes compared with healthy ones [11], even though its de novo synthesis and recycling rate have been shown to be higher as compared to healthy erythrocytes [7]. GSH is synthesized from cysteine, glutamic acid, and glycine in a two ATP-requiring enzymatic steps, being the availability of cysteine a rate limiting of GSH synthesis [9,12]. Under physiological conditions, cysteine is derived from the diet, protein breakdown and transsulfuration pathway that allows the utilization of methionine for GSH synthesis [9,13].

Transsulfuration pathway plays a significant role in the complex folate-dependent homocysteine (Hcy) metabolism along to the remethylation pathway [14]. Methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) are key enzymes that play a role in the balance of Hcy concentrations by converting it to methionine or cystathionine, respectively [15,16,17]. Genetic defects occur in either genes encoding for MTHFR and CBS enzymes, leading to an alteration of Hcy levels that may be involved in the cysteine availability. Among the polymorphisms described for these enzymes, the most studied ones are single point mutation in *MTHFR* gene (c. 677C>T; rs1801133) [18] and an 68-bp insertion in the exon 8 coding region of *CBS* (844ins68) [19].

The *MTHFR 677T* allele is associated with reduced enzymatic activity, decreased folate concentrations in serum and red blood cells, increased plasma Hcy levels [18,20], and it is a risk factor for vascular complications [21], including SCA patients [22-24]. Otherwise, there is no consensus about the 68-bp insertion effect on CBS activity and on Hcy levels [19,25,26]. In spite that, Alves-Jacob [23] showed that the presence of the 844ins68 mutation in the *CBS* gene is a risk factor for vaso-occlusive episodes in SCA patients. Furthermore,

recent studies have demonstrated that defects in either transsulfuration and remethylation pathways are related to increased generation of reactive oxygen species (ROS) [27-29] which could exacerbate the oxidative condition in SCA patients.

Some studies have been carried out to determine the association of the *MTHFR* (677C>T) or *CBS* (844ins68) polymorphisms with higher risk to develop vascular complications in SCA patients. However there is no report regarding the functional impacts of these polymorphisms on GSH concentration and consequently on oxidative stress and routine markers in SCA patients. For this reason, this work aimed at studying a possible influence of the mentioned polymorphisms on overall oxidative status of SCA patients and on routine markers, correlating them with hydroxyurea (HU) treatment.

2. Subjects and Methods

2.1. Subjects

It were analyzed 95 unrelated Brazilian SCA patients (53 women and 42 men; mean age: 24.4 years old; range: 10 – 59 years old). All subjects were regularly in clinical follow-up in the Blood Center of Rio de Janeiro (RJ, Brazil) and had access to the same medication protocol established and regulated by the Brazilian Ministry of Health for the entire national territory. All the patients studied received a prophylactic treatment with folic acid of 5 mg/day since the SCA diagnosis, while 41 (43,2%) of them were under HU treatment (average dose: 22 mg/Kg/day) for at least 90 days. The study was approved by the Data Safety Monitoring Board (DSMB) according to Brazilian Regulations.

After they gave their informed consent, all patients have answered a questionnaire in order to screen them according to the exclusion criteria listed below. Patients were asked specifically whether they were taking any nutritional supplements, nonsteroidal anti-inflammatory drugs (NSAID), opioids, or iron chelating agents. Further exclusion criteria were pregnant women, smokers or drinkers and patients who had had a stroke, pain and/or hemolytic crisis, or received blood transfusion within the 120 days prior to the study start. The medications used were accessed and those ones taking medication known to affect the analyzed parameters (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection were also excluded.

Patient routine markers, such as complete blood count, hemolytic, hepatic and renal markers, were developed by the Blood Center of Rio de Janeiro, Brazil. These data were

obtained by reviewing the medical records from the Blood Center database, where the informations given in questionaries were also confirmed for each patient, under supervision of clinicians responsible for the patients.

2.2. Biological samples

Blood samples (about 8 mL) were collected through venipuncture in ethylenediamine tetra acetic acid (EDTA) tubes. Four milliliters of whole blood were used for cytological, electrophoretic and chromatographic hemoglobin identification tests, as well as for DNA extraction from leukocytes for further molecular analysis. An aliquot of the other 4 mL were firstly used to prepare hemolysate for CAT activity analysis - whole blood diluted in ultrapure water (1:50, v/v). Then the remaining whole blood samples were centrifuged in a refrigerated centrifuge at 800 ×g for 15 min at 4°C in order to obtain plasma for lipid peroxidation assay while buffy coat were carefully removed. The erythrocytes obtained were washed three times with cold phosphate-buffered saline (PBS - 136 mM NaCl, 3 mM KCl, 10 mM Na2HPO4 / KH2PO4, pH 7.4). The supernatant and buffy coat were carefully removed after each wash. Finally, the erythrocytes were diluted in a 3.5 µM 2-mercaptoethanol 10 µM NADP 2.7 mM EDTA hemolyzing solution (1:20, v/v) for GST, GPx and GR activities and GSH content measurement. All hemolysates and plasma samples were immediately frozen at -80°C until the analysis.

2.3. Hemoglobin identification tests

Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald-Giemsa at photonic microscopy using 40× objective. Hb phenotype identification was performed using electrophoresis on cellulose acetate at pH 8.6 [30], and agar electrophoresis at pH 6.2 [31]. The Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANTTM equipment (Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer's manual.

2.4. Molecular analysis

DNA was extracted from peripheral blood leucocytes and isolated by phenol-chloroform methodology, according to Sambrook et al. [32]. *HBB*S* homozigous genotype

was confirmed by molecular analysis by PCR-RFLP using DdeI as restriction endonuclease (New England BioLabs, Ipswich, MA, USA) [33]. *MTHFR* (c.677C>T) polymorphism was assessed through PCR-RFLP using *FastDigest Hinf*I (Thermo Fisher Scientific Inc., Waltham, MA, USA) as restriction endonuclease, according to Frosst et al. [18]. Briefly, the PCR product (198-bp) remained uncleaved for ancestral allele, whereas mutant one produced 175- and 23-bp fragments. *CBS* (844ins68) mutation was detected using PCR as previously described by Tsai et al. [25]. After PCR, we obtained a 184-bp fragment for ancestral allele and a 252-bp for mutant one.

2.4. Biochemical measurements

Lipid peroxidation levels were assessed in the plasma samples by the product formed from malondialdehyde (MDA) and thiobarbituric acid (TBA), according to Silva et al. [34]. The product was detected by high performance liquid chromatography coupled to UV/Vis detector (UV/Vis-HPLC) [35,36]. The HPLC system consisted of ESA584 pump and an ESA526 UV/Vis detector set in 532 nm. The MDA–TBA derivative extracts were separated by a Shimadzu (Kyoto, japan) C18 column (150 x 4.6 mm, 5 μm) that was fitted with a guard column cartridge of the same composition. The mobile phase (potassium phosphate 0.05 M, pH 7.0, with 40% methanol) was pumped at an isocratic flow of 1 mL min-1. Chromatogram monitoring and peak identification and quantification were performed using the EZ Chrom Elite software (Agilent Technologies, Santa Clara, CA, USA). The calculations were based on a calibration curve previously constructed and prepared according to same procedure used for the samples from authentic standards. The results were expressed in ng/mL.

Catalase (CAT) [37], glutathione S-transferase (GST) [38], glutathione peroxidase (GPx) [39], and glutathione reductase (GR) [40], activities were determined spectrophotometrically, while GSH concentration was determined HPLC coupled to a coulometric electrochemical detection (ECD-HPLC) [41]. A hemolysate aliquot was filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC system, consisted of ESA584 pump and an electrochemical coulometric detector (ESA Coulochem III, Bedford, MA, USA) with potential set at 650 mV. The column was an ESA C18 (250 x 4.6 mm, 5 μm) also fitted with a guard column cartridge of the same composition. The mobile phase consisted of sodium phosphate 0.05 M, octanesulfonic acid 0.025 M, pH 2.5, with acetonitrile (2%; v/v) was pumped at an isocratic flow of 0.9 mL min-1.

Chromatogram monitoring and peak identification and quantification were performed using the same software used in the lipid peroxidation assay. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into the HPLC system. The antioxidant enzymes were expressed in U/mL, while GSH levels were in μM.

2.5. Statistical Analysis

Statistical analysis was performed in groups with at least three individuals using the Statistica 9.0 software (Statsoft Inc., Tulsa, OK, USA). Data were firstly tested regarding normality and homogeneity of variances assumptions according to Shapiro-Wilk test and Levene's test, respectively. Groups that met the assumptions (parametric data) were compared by applying one-way ANOVA followed by Tukey's *post hoc* test. Those groups that did not meet the assumptions (non-parametric data) were compared by Kruskal-Wallis followed by Dunn's *post hoc* test.

Data were expressed as mean ± standard error and we considered $p < 0.05$ as statistically significant.

3. Results

Among the 95 SCA patients evaluated, we found 66 (69.5%) wild homozygous for *MTHFR* (677CC), 27 (28.4%) heterozygous (677CT) and two (2.1%) mutant homozygous (677TT), leading to an allelic frequency of 0.84 for the ancestral allele (677C) and 0.16 for the mutant one (677T) in the studied group. The allelic frequency obtained for *CBS* (844ins68) mutation was very similar to the *MTHFR* one: 0.85 for the ancestral allele (A) and 0.15 for the insertion (I). Moreover, we identified the following genotyping distribution for *CBS* gene - 69 (72.6%) ancestral homozygous (AA), 23 (24.2%) heterozygous (AI) and three (3.2%) insertion homozygous (II). In addition, the co-heritance prevalence of both mutation in at least one chromosome (T_/I_) was 8.4%.

In order to better address *MTHFR* 677T polymorphism influence on HU response, and on oxidative stress and routine markers, we formed four sample subgroups - wild homozygous treated [CC (HU+)] or not treated with HU [CC (HU-)] and the association of heterozygous and mutant homozygous under HU use [T_ (HU+)] or not [T_ (HU-)]. We did not find any differential response to HU treatment caused by 677T mutation, since no statistical difference was found between CC (HU+) and T_ (HU+) subgroups for all the

markers evaluated. HU presented its well-documented pharmacological effects such as reduction of lipid peroxidation levels, HbF content increase, and improvement of hematological and hemolytic markers (Table 1).

The presence of *MTHFR* 677T allele did not influence any of the oxidative stress parameters studied. Unexpectedly, this mutation was related to the lowest HbF levels in the patients not treated with HU [T_ (HU-)]. This subgroup presented less than a half of HbF amount found in the CC (HU-) subgroup ($p < 0.01$). We did not find any other *MTHFR* 677T influence on routine markers (Table 1).

Table 1. *MTHFR* 677T polymorphism influence on HU response, and on oxidative stress and routine markers

Markers	Subgroups MTHFR (677C>T)				*P* value	Reference values
	CC (HU^+) n = 27	T_ (HU^+) n = 14	CC (HU^-) n = 39	T_ (HU^-) n = 15		
MDA (ng/mL)	307.5 ± 30.7^a	328.3 ± 46.7^a	532.9 ± 33.2^b	509.3 ± 64.3^b	< 0.01*	-----
CAT (U/mL)	3416.1 ± 189.8^a	3544.8 ± 303.9^a	3876.6 ± 234.4^a	2936.1 ± 289.7^a	0.09*	-----
GST (U/mL)	1.1 ± 0.1^a	0.9 ± 0.1^a	1.1 ± 0.1^a	1.1 ± 0.1^a	0.65*	-----
GPx (U/mL)	1.1 ± 0.1^a	1.3 ± 0.2^a	1.0 ± 0.1^a	0.9 ± 0.1^a	0.28*	-----
GR (U/mL)	0.5 ± 0.1^a	0.4 ± 0.1^a	0.5 ± 0.0^a	0.6 ± 0.1^a	$0.27^\#$	-----
GSH (µM)	770.1 ± 94.7^a	747.1 ± 137.2^a	813.7 ± 67.1^a	502.1 ± 56.3^a	$0.10^\#$	-----
Hb F levels (%)	13.9 ± 1.7^a	12.2 ± 1.8^a	8.1 ± 0.9^b	3.7 ± 0.7^c	$< 0.01^\#$	0.0 - 1.0
Erythrocytes (k/µL)	2.4 ± 0.1^a	2.5 ± 0.2^a	2.6 ± 0.1^a	2.7 ± 0.1^a	0.25*	4.5 - 6.0
Hemoglobin (g/dL)	8.6 ± 0.3^a	8.5 ± 0.5^a	8.0 ± 0.2^a	8.3 ± 0.2^a	$0.50^\#$	11.5 - 17.0
Hematocrit (%)	24.2 ± 0.8^a	24.3 ± 1.4^a	22.9 ± 0.5^a	23.4 ± 0.7^a	$0.63^\#$	35.0 - 43.0
MCV (fL)	101.7 ± 2.6^a	99.6 ± 3.9^a	88.0 ± 1.1^b	85.7 ± 1.4^b	$< 0.01^\#$	77.0 - 100.0
MCH (pg)	36.2 ± 1.1^a	35.1 ± 1.5^a	31.3 ± 0.5^b	30.5 ± 1.0^b	$< 0.01^\#$	25.0 - 34.0
MCHC (g/dL)	35.6 ± 0.2^a	35.2 ± 0.3^a	35.4 ± 0.2^a	35.5 ± 0.3^a	0.66*	31.0 - 37.0
Leucocytes (k/µL)	9.1 ± 0.6^a	8.4 ± 0.7^a	10.6 ± 0.5^a	10.2 ± 0.5^a	0.05*	5.0 - 13.0
Neutrophils (k/µL)	$4.4 \pm 0.4^{a,b}$	3.3 ± 0.2^b	5.0 ± 0.3^a	$4.8 \pm 0.3^{a,b}$	$0.03^\#$	1.5 - 10.0
Lymphocytes (k/µL)	$3.5 \pm 0.3^{a,b}$	3.0 ± 0.4^b	4.2 ± 0.2^a	$4.1 \pm 0.4^{a,b}$	0.01*	1.0 - 6.0
Monocytes (k/µL)	$0.7 \pm 0.1^{a,b}$	0.5 ± 0.04^b	0.8 ± 0.04^a	0.7 ± 0.1^a	$< 0.01^\#$	0.08 - 1.3
Eosinophils (k/µL)	0.2 ± 0.03^a	$0.2 \pm 0.05^{a,b}$	$0.3 \pm 0.04^{a,b}$	0.4 ± 0.1^b	0.01*	0.0 - 0.6
Basophils (k/µL)	0.1 ± 0.01^a	0.1 ± 0.02^a	0.2 ± 0.01^a	0.2 ± 0.03^a	$0.13^\#$	0.0 - 0.2
Platelets (k/µL)	410.0 ± 24.6^a	453.4 ± 42.1^a	425.4 ± 21.1^a	457.3 ± 26.2^a	0.62*	140.0 - 400.0
Reticulocytes (%)	8.7 ± 0.7^a	8.8 ± 0.9^a	10.0 ± 0.5^a	10.7 ± 0.7^a	0.18*	0.8 - 2.
Reticulocytes (k/µL)	207.1 ± 17.5^a	221.2 ± 18.7^a	$244.6 \pm 6.7^{a,b}$	276.1 ± 13.5^b	$< 0.01^\#$	60.0 - 150.0
LDH (U/L)	895.3 ± 85.5^a	799.0 ± 60.5^a	1153.5 ± 61.6^b	1206.5 ± 125.2^b	< 0.01*	< 480.0
AST (U/L)	48.6 ± 3.6^a	46.9 ± 4.1^a	55.7 ± 2.9^a	58.5 ± 4.7^a	0.13*	< 35.0
ALT (U/L)	24.6 ± 2.0^a	21.8 ± 2.3^a	19.9 ± 1.2^a	23.8 ± 2.8^a	0.20*	< 41.0
TB (mg/dL)	2.4 ± 0.2^a	2.2 ± 0.4^a	3.7 ± 0.3^b	$3.7 \pm 0.4^{a,b}$	< 0.01*	< 1.0
DB (mg/dL)	0.6 ± 0.04^a	0.5 ± 0.1^a	0.6 ± 0.02^a	0.6 ± 0.04^a	0.08^*	< 0.2
IB (mg/dL)	1.8 ± 0.2^a	1.8 ± 0.4^a	3.1 ± 0.3^b	$2.7 \pm 0.3^{a,b}$	$< 0.01^\#$	< 0.8
ALP (U/L)	137.9 ± 10.5^a	121.6 ± 11.3^a	137.7 ± 9.9^a	138.6 ± 19.3^a	0.82*	53.0 - 141.0
GGT (U/L)	63.0 ± 8.4^a	50.6 ± 9.3^a	33.7 ± 2.5^a	44.7 ± 1.6^a	$0.05^\#$	8.0 - 73.0
Creatinine (mg/dL)	0.7 ± 0.03^a	0.7 ± 0.1^a	0.7 ± 0.03^a	0.7 ± 0.04^a	0.52*	0.6 - 1.3

CC: wild homozygous; T_: heterozygous and mutant homozygous; (HU+): patients under hydroxyurea use; (HU-): patients not treated with hydroxyurea; MDA: malondialdehyde; CAT: catalase; GST: glutathione S-transferase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; Hb F: fetal hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase; TB: total bilirubin; DB: direct bilirubin; IB: indirect bilirubin; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase

*Comparisons were made by one-way ANOVA followed by Tukey's post hoc test when applicable

#Comparisons were made by Kruskal-Wallis followed by Dunn's post hoc test when applicable

Different letters indicate statistical differences among the subgroups

We investigated *CBS* 844ins68 insertion influence in the same manner as *MTHFR* gene, through subgroups formation: ancestral homozygous under HU use [AA (HU+)] or not [AA (HU-)] and the association of heterozygous and insertion homozygous treated [I_ (HU+)] or not with HU [T_ (HU-)]. Among these *CBS* subgroups, all biomarkers evaluated presented average values very similar to the *MTHFR* subgroups. Furthermore, HU treatment showed the same *MTHFR* gene pattern of beneficial effects on lipid peroxidation and HbF levels and hematological markers (Fig. 1) but slightly different on some hemolytic parameters.

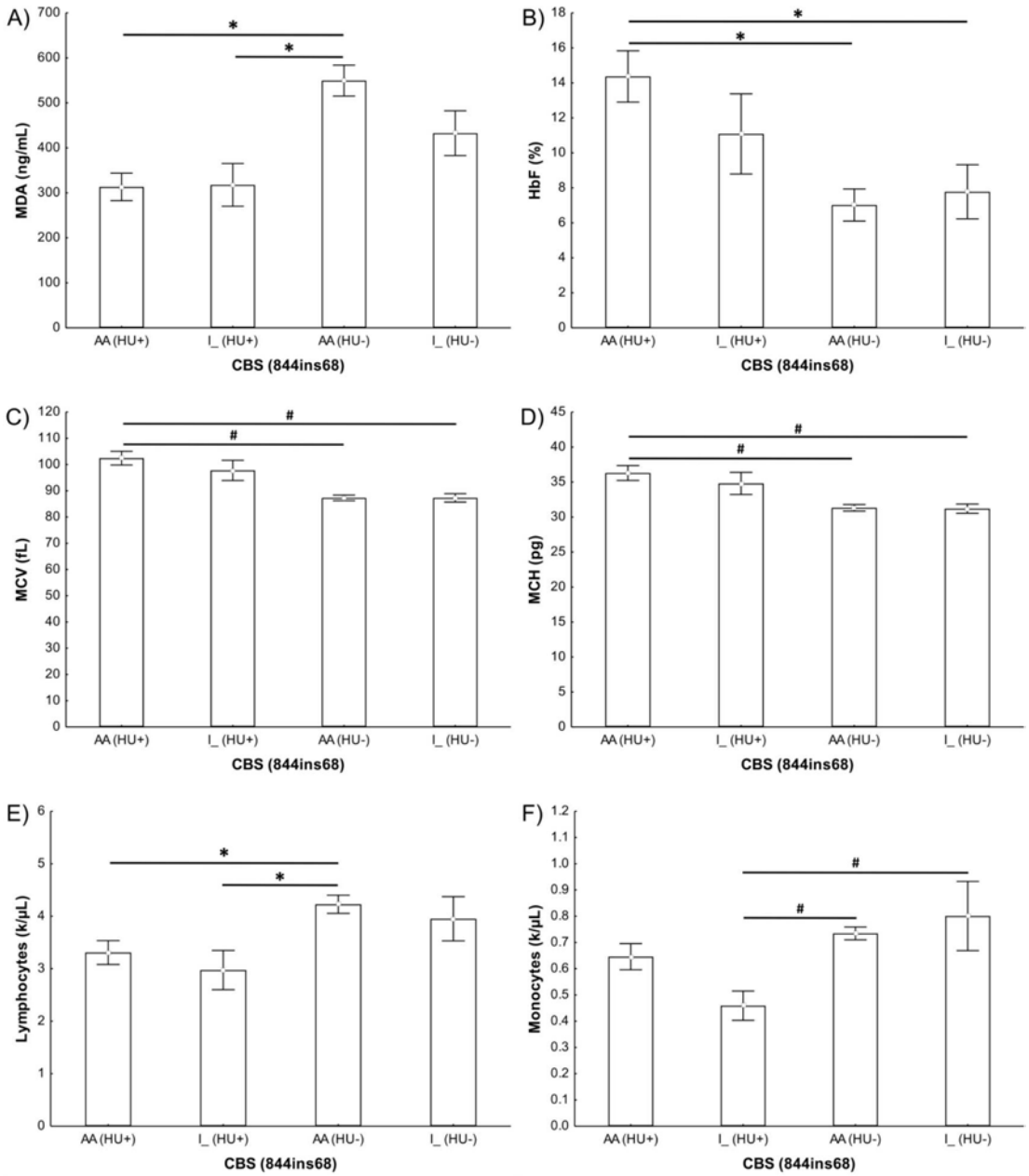

Fig. 1. HU beneficial effects according to *CBS* (844ins68) subgroups studied. Lower lipid peroxidation levels (A) in the patients under HU use than in the patients without the insertion not treated with HU, regardless polymorphism presence. HbF (B), MCV (C) and MCH (D) increment in the AA (HU+) subgroup when compared to AA (HU-) and I_ (HU-) ones. Lymphocyte (E) and monocyte (F) count decrease due to HU use in SCA patients, regardless polymorphism presence.
AA (HU+): ancestral homozygous under HU use (N = 28); I_ (HU+): association of heterozygous and insertion homozygous under HU use (N = 13); AA (HU-): ancestral homozygous not treated with HU (N = 41); I_ (HU-): association of heterozygous and insertion homozygous not treated with HU (N = 13).
*Indicates statistical differences ($p < 0.01$; one-way ANOVA followed by Tukey's post hoc test)
#Indicates statistical differences ($p < 0.01$; Kruskal-Wallis followed by Dunn's post hoc test)

Similarly to the results obtained for *MTHFR* subgroups, we observed both expected HU response and no significant *CBS* (844ins68) influence on reticulocyte and bilirubin levels (Figure 2). On the other hand, the presence of the insertion in at least one chromosome seemed to cause differential HU treatment response, since we found higher LDH ($p < 0.01$)

and AST (p = 0.01) activities in the I_ (HU+) subgroup when compared to the AA (HU+) subgroup. Furthermore, I_ (HU+) showed threshold reference value for GGT activity and higher than both subgroups untreated with HU (p = 0.04) (Figure 3). The other routine and antioxidant markers analyzed did not differ statistically among the *CBS* subgroups (data not shown).

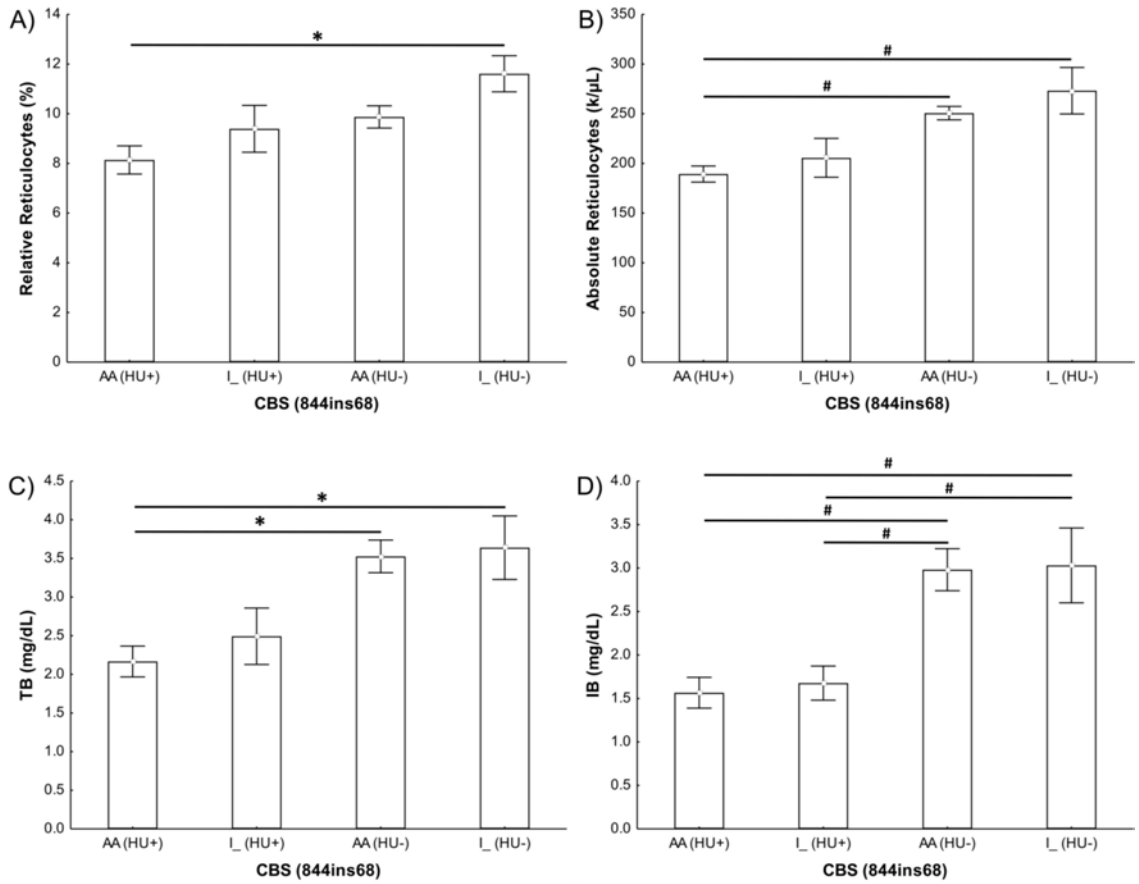

Fig. 2. HU improvement of some hemolytic markers evaluated. A) AA (HU+) subgroup showed decreased relative reticulocyte count in relation to I_ (HU-) one. Lower absolute reticulocyte amount (B) and total bilirubin (TB) levels (C) in the AA (HU+) subgroup compared to both subgroups not treated with HU. D) Effective decrease of indirect bilirubin (IB) levels due HU use, regardless *CBS* polymorphism presence.
AA (HU+): ancestral homozygous under HU use (N = 28); I_ (HU+): association of heterozygous and insertion homozygous under HU use (N = 13); AA (HU-): ancestral homozygous not treated with HU (N = 41); I_ (HU-): association of heterozygous and insertion homozygous not treated with HU (N = 13).
*Indicates statistical differences (p < 0.01; one-way ANOVA followed by Tukey's post hoc test)
#Indicates statistical differences (p < 0.01; Kruskal-Wallis followed by Dunn's post hoc test)

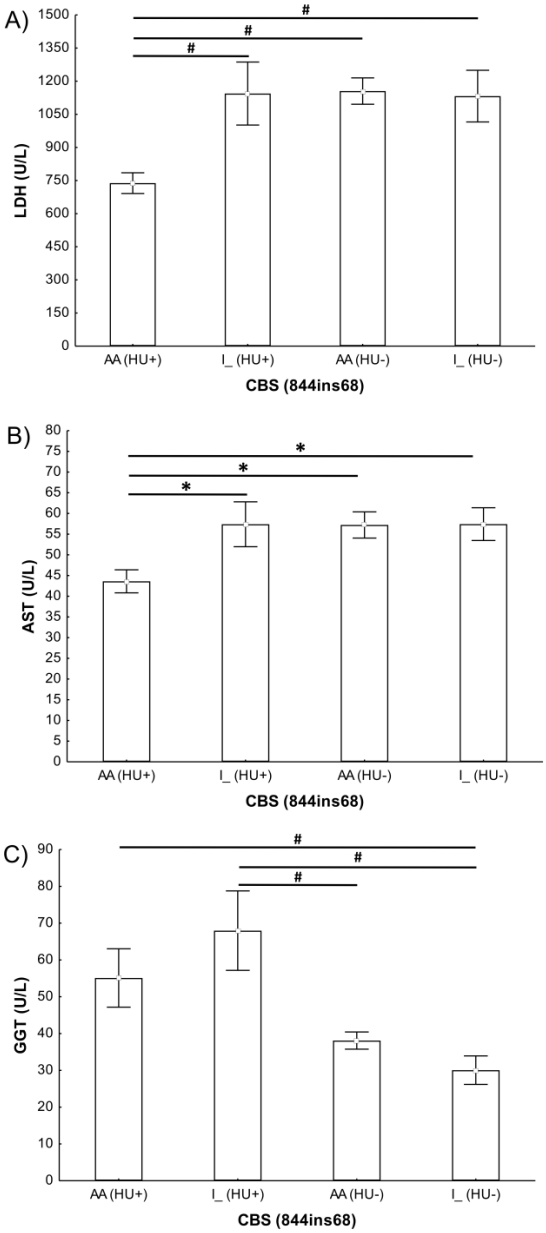

Fig. 3. Influence of *CBS* (844ins68) polymorphism on HU treatment responses. Decreased LDH (A) and AST (B) activities in the AA (HU^+) subgroup in relation to other ones. C) Higher GGT activity in the I_ (HU^+) subgroup when compared to AA (HU^-) and I_ (HU^-) ones. AA (HU^+) also showed higher GGT activity than I_ (HU^-) subgroup.
AA (HU^+): ancestral homozygous under HU use (N = 28); I_ (HU^+): association of heterozygous and insertion homozygous under HU use (N = 13); AA (HU^-): ancestral homozygous not treated with HU (N = 41); I_ (HU^-): association of heterozygous and insertion homozygous not treated with HU (N = 13).
*Indicates statistical differences ($p < 0.05$; one-way ANOVA followed by Tukey's *post hoc* test)
#Indicates statistical differences ($p < 0.05$; Kruskal-Wallis followed by Dunn's *post hoc* test)

We also assessed a possible gene-gene interaction effect on HU response and on biomarkers evaluated. In this way, the statistical comparisons were performed among the following subgroups: ancestral homozygous for both *MTHFR* (677C>T) and *CBS* (844ins68) polymorphisms treated [CC/AA (HU+)] or not treated with HU [CC/AA (HU-)], and at least heterozygous for both polymorphisms studied, under HU use [T_/I_ (HU+)] or not [T_/I_ (HU-)]. We obtained mostly the same results for the association gene analysis that those ones for each gene individually (data not shown). Thus, HU influence on patient hematological and hemolytic profile, regardless the presence of the polymorphism association.

Notwithstanding, we found only one synergic effect of 677T and "I" alleles on GSH levels. The co-inheritance of the polymorphisms investigated was related to about 35% reduction of GSH levels in SCA patients under HU use or untreated with HU (Fig. 4).

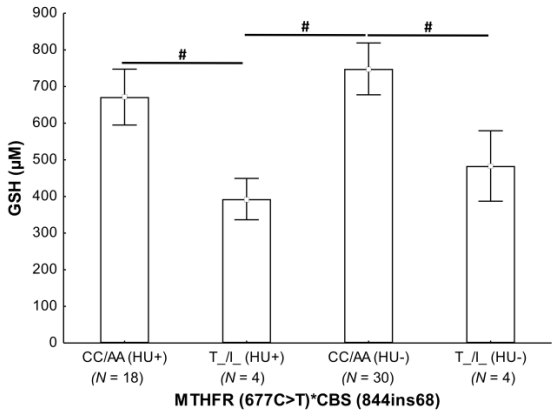

Fig. 4. Influence of 677T and "I" alleles on GSH levels. T_/I_ (HU^+) subgroup showed about 1.7-fold decrease in the GSH amount compared to CC/AA (HU^+) one. The same pattern of allelic synergism occurred in the untreated subgroups. CC/AA: wild homozygous for both MTHFR (677C>T) and CBS (844ins68); T_/I_: at least heterozygous for both polymorphisms studied; (HU^+): SCA patients treated with HU; (HU^-): untreated patients.
#Indicates statistical differences (p = 0.04; Kruskal-Wallis followed by Dunn's *post hoc* test).

4. Discussion

Although SCA was one of the first disorders clearly defined at molecular level, genetic understanding of the basis for disease expression variability is still unclear [42]. In this way, despite HbS presence is indispensable for the disease establishment, several other phenomena affected by a multitude of genes other than the one directly involved (HBB*S) play an important role [43]. Thus, to our knowledge, this is the first report focusing on the possible influence of the polymorphisms in *MTHFR* and *CBS* genes and their association on oxidative stress markers and routine measurements, and on HU treatment in SCA patients.

The prevalence of *MTHFR* (677C>T) and *CBS* (844ins68) mutations reveals an extensive ethnic and geographic variability [44,45]. For instance, the prevalence of MTHFR homozygosity (677TT) may vary between 1 and 30% in the general population [46], while *CBS* homozygosity shows a much lower variance, ranging from absent to 4% [45]. However, the genotypic frequency data obtained for these polymorphisms in our study is in agreement to previous studies in Brazilian SCA patients [22,23,47].

HU administration is the only currently available disease-modifying therapy for SCA [48,49,50]. HU is an antineoplastic drug which its main pharmacological action is to increase Hb F levels [48]. It has other potentially beneficial effects including improved nitric oxide (NO) metabolism, reduced red cell-endothelial interaction, decreased erythrocyte density [48], improvement of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) [51,52], and reduction of white cell counts [53], lipid peroxidation levels [54,55] and

hemolysis [56]. We observed these expected pharmacological effects, regardless the presence of both polymorphisms investigated, except on some hemolytic markers (LDH and AST activities) in those SCA patients at least heterozygous for 68-bp insertion in *CBS* [I_ (HU+)].

The polymorphism (844ins68) in the *CBS* gene was initially thought to mandate the use of an insertion-associated premature stop codon in the *CBS* mRNA and result in the translation of an inactive truncated enzyme [19]. Subsequently, Tsai et al. [25] showed that the 68-bp insertion generates an alternative splice site that permits the elimination of the entire inserted region, thereby allowing the formation of a normal mRNA transcript and a fully functional CBS enzyme. Moreover, it was speculated that this insertion is associated with somewhat higher CBS enzyme activity [26]. Assuming that the *CBS* (844ins68) mutation causes higher CBS activity, and taking into consideration: i) transsulfuration pathway is particularly active in hepatocytes [13]; ii) HU is mostly metabolized in the liver [57]; iii) LDH and AST are also hepatic enzymes [58,59]; and iv) threshold reference value obtained for GGT activity, we hypothesized that these are signal of initial establishment of hepatic dysfunction in the SCA patients from the I_ (HU+) subgroup. Therefore, our results leave perspectives for further studies to better address this hypothesis.

The single nucleotide polymorphism at position 677 in the *MTHFR* gene had the functional influence on 30 and 65% reduced enzyme activity in heterozygous (CT) and homozygous (TT) variants, respectively [60]. Thus, it is associated with elevated Hcy levels [61,62], which in turn are responsible for an increased ROS production in several tissues such as bone and vascular endothelium [63,64], and correlated positively with MDA levels [65]. In this way, we expected that the co-inheritance of *HBB*S* and *MTHFR* 677T would worsen the oxidative status in SCA patients. However, none of the polymorphisms influenced individually any of the oxidative stress parameters assessed. We believe that the lack of *MTHFR* and *CBS* mutations on oxidative stress markers can be attributed to daily folic acid treatment administered in the SCA patients evaluated. According to Dragani et al [27], a regimen of folate intake (5 mg daily) is capable to produce a 58% decrease in Hcy levels, improving vascular function and oxidative stress in patients with coronary heart disease.

Despite the lack of *MTHFR* 677T polymorphism influence on oxidative status and on HU response, this mutation may be related to ineffective erythropoiesis in SCA patients, that was *in vivo* demonstrated to contribute with SCA pathophysiology [66]. MTHFR reduced activity caused by the polymorphism studied promotes a significantly reduction in the production of 5-methyl-THF (the primary form of plasma folate), culminating in lower plasma and erythrocyte folate concentrations [67]. In addition, SCA patients normally present

lower serum cobalamin (Vitamin B12) levels, without macrocytosis or hypersegmented neutrophils [68-70]. Deficiency in any one of these micronutrient can cause increased erythroid progenitor cell death through impaired DNA synthesis and its consequences during erythropoiesis [71]. Moreover, according to Moestrup [72], during increased hemolysis (one characteristic pathophysiological event of SCA), erythropoiesis is accelerated by erythropoietin (EPO), making this process extremely sensitive to deficiency of cobalamin and folate. Altogether, this hypothesis is plausible and worthy of further investigation.

The "ineffective erythropoiesis" hypothesis may partially explain the lowest HbF levels we found in the SCA patients at least heterozygous for *MTHFR* untreated with HU [T_ (HU-)]. According to Wiles and Howard [73], erythroid progenitors that divide faster tend to be those with a high HbS level, while the red cells with a high HbF tend to arise from progenitors that divide less rapidly. In this way, if we assume that extra- and intramedullar hemolysis is occurring due to *HBB*S* and *MTHFR* 677T co-inheritance, the hyper-hemolysis state might be triggering erythroid hyperplasia through enhanced EPO-stimulus, favoring production of red cells with a low Hb F amount. This idea is corroborated by the highest reticulocyte count found in the same SCA patient subgroup [T_ (HU-)]. One might speculate that folic acid supplementation would mitigate or blunt the processes in the above mentioned hypothesis. However, even though SCA patients routinely using folic acid to improve erythropoiesis, folate status can be found at sub-clinical levels when compared to healthy control serum folate levels [74].

Finally, we demonstrated a synergic effect of 677T and "I" alleles on decreasing GSH levels. This observation may be a result of a "Hcy-trapping effect", since from 10 - 20% of Hcy is present as homocysteine-cysteine in the circulation [21], depleting cysteine availability before its utilization for GSH synthesis. Either, a genetic or functional defect in the γ - cystathionase, the second enzyme of the transsulfuration pathway [21]. However, this reflection should be carefully interpreted due to the small sample size in the affected subgroups, and it leaves new perspectives for further studies.

5. Conclusions

A number of limitations of the present study need to be acknowledged, including no functional analysis of MTHFR and CBS enzymes, neither measurements of folate, cobalamin, and even Hcy, and the small sample size in some subgroups evaluated.

This work was an outstanding hypothesis-generating study, suggesting that *MTHFR* (677C>T) and *CBS* (844ins68) polymorphisms involvement in SCA pathophysiology is likely to be far more complex than it was explored to date. Briefly, we provided evidence that: i) the polymorphisms investigated had minor impact on modifying oxidative status in SCA patients, only a synergic effect on GSH concentration; ii) *HBB*S* and *MTHFR* 677T co-inheritance showed a detrimental effect on HbF levels in SCA patients untreated with HU; iii) *HBB*S* and *CBS* (844ins68) co-inheritance may be related to hepatic dysfunction in SCA patients under HU use. Furthermore, we suggest the need for carefully designed dose-finding studies as a basis for further clinical trials of acid folic supplementation.

Conflict of interest

The authors declare no competing financial or other relationship with any people or organization interests.

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References

1. D. Weatherall, K. Hofman, G. Rodgers et al., A case for developing North-South partnerships for research in sickle cell disease, Blood 105 (2005) 921-923.
2. M.H. Steinberg, P. Sebastiani, Genetic modifiers of sickle cell disease, Am J Hematol 87 (2012) 795-803.
3. M.H. Steinberg, Predicting clinical severity in sickle cell anaemia, Br J Haematol 129 (2005) 465-481.
4. M.H. Steinberg, A.H. Adewoye, Modifier genes and sickle cell anemia, Curr Opin Hematol 13 (2006) 131-136.
5. K.Y. Fertrin, F.F. Costa, Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment, Expert Rev Hematol 3 (2010) 443-458.

6. D.G.H. Silva, E. Belini-Junior, E.A. de Almeida et al., Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies, Free Radic Biol Med 65 (2013) 1101-1109.

7. M. Reid, A. Badaloo, T. Forrester et al., In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease, Am J Physiol Endocrinol Metab 291 (2006) E73-E79.

8. M.A. Chaves, M.S. Leonart, A.J. do Nascimento, Oxidative process in erythrocytes of individuals with hemoglobin S, Hematology 13 (2008) 187-192.

9. S.C. Lu, Glutathione synthesis, Biochim Biophys Acta 1830 (2013) 3143-3153.

10. C.R. Morris, J.H. Suh, W. Hagar et al., Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease, Blood 111 (2008) 402-410.

11. V.L. Tatum, C.K. Chow, Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress, Free Radic Res 25 (1996) 133-139.

12. van Zwieten R., A.J. Verhoeven, D. Roos, Inborn defects in the antioxidant systems of human red blood cells, Free Radic Biol Med 67 (2014) 377-386.

13. J.D. Finkelstein, Methionine metabolism in mammals, J Nutr Biochem 1 (1990) 228-237.

14. J. Selhub, Homocysteine metabolism, Annu Rev Nutr 19:217-46. (1999) 217-246.

15. L.J. Hoffer, Homocysteine remethylation and trans-sulfuration, Metabolism 53 (2004) 1480-1483.

16. P.R. Mandaviya, L. Stolk, S.G. Heil, Homocysteine and DNA methylation: A review of animal and human literature, Mol Genet Metab 113 (2014) 243-252.

17. F.H. Nazki, A.S. Sameer, B.A. Ganaie, Folate: metabolism, genes, polymorphisms and the associated diseases, Gene 533 (2014) 11-20.

18. P. Frosst, H.J. Blom, R. Milos et al., A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase, Nat Genet 10 (1995) 111-113.

19. G. Sebastio, M.P. Sperandeo, M. Panico et al., The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations, Am J Hum Genet 56 (1995) 1324-1333.

20. P.F. Jacques, A.G. Bostom, R.R. Williams et al., Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations, Circulation 93 (1996) 7-9.

21. E. Trabetti, Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk, J Appl Genet 49 (2008) 267-282.

22. N.F. Moreira, D.M. Lourenco, M.A. Noguti et al., The clinical impact of MTHFR polymorphism on the vascular complications of sickle cell disease, Braz J Med Biol Res 39 (2006) 1291-1295.

23. M. Alves Jacob, C.C. Bastos, C.R. Bonini-Domingos, The 844ins68 cystathionine beta-synthase and C677T MTHFR gene polymorphism and the vaso-occlusive event risk in sickle cell disease, Arch Med Sci 7 (2011) 97-101.

24. S.S. Nishank, M.P. Singh, R. Yadav, Clinical impact of factor V Leiden, prothrombin G20210A, and MTHFR C677T mutations among sickle cell disease patients of Central India, Eur J Haematol 91 (2013) 462-466.

25. M.Y. Tsai, M. Bignell, K. Schwichtenberg et al., High prevalence of a mutation in the cystathionine beta-synthase gene, Am J Hum Genet 59 (1996) 1262-1267.

26. M.Y. Tsai, F. Yang, M. Bignell et al., Relation between plasma homocysteine concentration, the 844ins68 variant of the cystathionine beta-synthase gene, and pyridoxal-5'-phosphate concentration, Mol Genet Metab 67 (1999) 352-356.

27. A. Dragani, A. Falco, F. Santilli et al., Oxidative stress and platelet activation in subjects with moderate hyperhomocysteinaemia due to MTHFR 677 C-->T polymorphism, Thromb Haemost 108 (2012) 533-542.

28. Z. Rahimi, S. Malek-Khosravi, Z. Rahimi et al., MTHFR C677T and eNOS G894T variants in preeclamptic women: Contribution to lipid peroxidation and oxidative stress, Clin Biochem 46 (2013) 143-147.

29. E. Richard, L.R. Desviat, M. Ugarte et al., Oxidative stress and apoptosis in homocystinuria patients with genetic remethylation defects, J Cell Biochem 114 (2013) 183-191.

30. A.J. Marengo-Rowe, Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate, J Clin Pathol 18 (1965) 790-792.

31. F. Vella, Acid-agar gel electrophoresis of human hemoglobins, Am J Clin Pathol 49 (1968) 440-442.

32. J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Laboratory Press (1989) pp. 10.51-10.67.

33. R.K. Saiki, S. Scharf, F. Faloona et al., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230 (1985) 1350-1354.

34. D.G.H. Silva, O. Ricci-Junior, E.A. de Almeida et al., Potential utility of melatonin as an antioxidant therapy in the management of sickle cell anemia, J Pineal Res (2014) (In Press)-

35. E.A. de Almeida, S.A. Marques, C.F. Klitzke et al., DNA damage in digestive gland and mantle tissue of the mussel Perna perna, Comp Biochem Physiol C Toxicol Pharmacol 135C (2003) 295-303.

36. E.A. de Almeida, S. Miyamoto, A.C. Bainy et al., Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels Perna perna exposed to different metals, Mar Pollut Bull 49 (2004) 386-392.

37. E. Beutler, Red cell metabolism: A Manual of Biochemical Methods, 83 (1975) 919-

38. J.H. Keen, W.H. Habig, W.B. Jakoby, Mechanism for the several activities of the glutathione S-transferases, J Biol Chem 251 (1976) 6183-6188.

39. H. Sies, O.R. Koch, E. Martino et al., Increased biliary glutathione disulfide release in chronically ethanol-treated rats, FEBS Lett 103 (1979) 287-290.

40. E. Beutler, Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies, J Clin Invest 48 (1969) 1957-1966.

41. A. Rodriguez-Ariza, F. Toribio, J. Lopez-Barea, Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection, J Chromatogr B Biomed Appl 656 (1994) 311-318.

42. A. Inati, A. Taher, A.W. Bou et al., Beta-globin gene cluster haplotypes and HbF levels are not the only modulators of sickle cell disease in Lebanon, Eur J Haematol 70 (2003) 79-83.

43. R.L. Nagel, Severity, pathobiology, epistatic effects, and genetic markers in sickle cell anemia, Semin Hematol 28 (1991) 180-201.

44. S. Liew, E.D. Gupta, Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: Epidemiology, metabolism and the associated diseases, Eur J Med Genet 58 (2015) 1-10.

45. R.F. Franco, J. Elion, J. Lavinha et al., Heterogeneous ethnic distribution of the 844ins68 in the cystathionine beta-synthase gene, Hum Hered 48 (1998) 338-342.

46. P. Durand, M. Prost, N. Loreau et al., Impaired homocysteine metabolism and atherothrombotic disease, Lab Invest 81 (2001) 645-672.

47. F.D. Couto, W.V. Boas, I. Lyra et al., A C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism and G20210A mutation in the prothrombin gene of sickle cell anemia patients from Northeast Brazil, Hemoglobin 28 (2004) 237-241.

48. R.E. Ware, B. Aygun, Advances in the use of hydroxyurea, Hematology Am Soc Hematol Educ Program (2009) 62-69.

49. R.E. Ware, How I use hydroxyurea to treat young patients with sickle cell anemia, Blood 115 (2010) 5300-5311.

50. R.E. Ware, Hydroxycarbamide: clinical aspects, C R Biol 336 (2013) 177-182.

51. D.L. Jain, M. Apte, R. Colah et al., Efficacy of fixed low dose hydroxyurea in Indian children with sickle cell anemia: a single centre experience, Indian Pediatr 50 (2013) 929-933.

52. A.M.M. Queiroz, C.L.C. Lobo, E.M. Nascimento et al., The Mean Corpuscular Volume and Hydroxyurea in Brazilian Patients with Sickle Cell Anemia: A Surrogate Marker of Compliance, J Blood Disorders Transf 4 (2013) 1-4.

53. S. Charache, Mechanism of action of hydroxyurea in the management of sickle cell anemia in adults, Semin Hematol 34 (1997) 15-21.

54. A. Agil, S.M. Sadrzadeh, Hydroxy-urea protects erythrocytes against oxidative damage, Redox Rep 5 (2000) 29-34.

55. D.G.H. Silva, E. Belini-Junior, L.S. Torres et al., Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia, Blood Cells Mol Dis 47 (2011) 23-28.

56. G.P. Rodgers, G.J. Dover, C.T. Noguchi et al., Hematologic responses of patients with sickle cell disease to treatment with hydroxyurea, N Engl J Med 322 (1990) 1037-1045.

57. J. Huang, M. Yakubu, D.B. Kim-Shapiro et al., Rat liver-mediated metabolism of hydroxyurea to nitric oxide, Free Radic Biol Med 40 (2006) 1675-1681.

58. S.K. Ballas, Lactate dehydrogenase and hemolysis in sickle cell disease, Blood 121 (2013) 243-244.

59. C. Koh, T. Turner, X. Zhao et al., Liver stiffness increases acutely during sickle cell vaso-occlusive crisis, Am J Hematol 88 (2013) E250-E254.

60. P. Goyette, J.S. Sumner, R. Milos et al., Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification, Nat Genet 7 (1994) 195-200.

61. L.B. Bailey, J.F. Gregory, III, Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement, J Nutr 129 (1999) 919-922.

62. M. Yakub, N. Moti, S. Parveen et al., Polymorphisms in MTHFR, MS and CBS genes and homocysteine levels in a Pakistani population, PLoS One 7 (2012) e33222-

63. A.F. Perna, D. Ingrosso, N.G. De Santo, Homocysteine and oxidative stress, Amino Acids 25 (2003) 409-417.

64. J.M. Koh, Y.S. Lee, Y.S. Kim et al., Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation, J Bone Miner Res 21 (2006) 1003-1011.

65. C.S. Vanzin, G.B. Biancini, A. Sitta et al., Experimental evidence of oxidative stress in plasma of homocystinuric patients: a possible role for homocysteine, Mol Genet Metab 104 (2011) 112-117.

66. C.J. Wu, L. Krishnamurti, J.L. Kutok et al., Evidence for ineffective erythropoiesis in severe sickle cell disease, Blood 106 (2005) 3639-3645.

67. P.J. Bagley, J. Selhub, A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells, Proc Natl Acad Sci U S A 95 (1998) 13217-13220.

68. A.K. al-Momen, Diminished vitamin B12 levels in patients with severe sickle cell disease, J Intern Med 237 (1995) 551-555.

69. P. Kamineni, S. Chirla, K. Dinh et al., Low cobalamin levels in African Americans with and without sickle cell disease, J Natl Med Assoc 98 (2006) 352-356.

70. O.I. Ajayi, S. Bwayo-Weaver, S. Chirla et al., Cobalamin status in sickle cell disease, Int J Lab Hematol 35 (2013) 31-37.

71. M.J. Koury, P. Ponka, New insights into erythropoiesis: the roles of folate, vitamin B12, and iron, Annu Rev Nutr 24:105-31. (2004) 105-131.

72. S.K. Moestrup, New insights into carrier binding and epithelial uptake of the erythropoietic nutrients cobalamin and folate, Curr Opin Hematol 13 (2006) 119-123.

73. N. Wiles, J. Howard, Role of hydroxycarbamide in prevention of complications in patients with sickle cell disease, Ther Clin Risk Manag 5 (2009) 745-755.

74. F.P. van der Dijs, J.J. Schnog, D.A. Brouwer et al., Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients, Am J Hematol 59 (1998) 192-198.

Capítulo II

Relationship between adenosine deaminase polymorphism (c.22G>A) and oxidative stress in sickle cell anemia

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Abstract

The aim of this study was to identify, in people with sickle cell anemia (SCA), adenosine deaminase (*ADA*; c. 22G>A; rs73598374) polymorphism, and correlating it with oxidative stress and antioxidant capacity markers. We evaluated 95 unrelated and diagnosed Brazilian sickle cell anemia (SCA) patients. All patients received a prophylactic treatment with folic acid of 5 mg/day, while 41 (43.2%) of them were under HU treatment (average dose: 22 mg/Kg/day) for at least 90 days. *ADA* polymorphism was identified by PCR-RFLP. Biochemical parameters were measured using spectrophotometric [catalase, glutathione *S*-transferase, glutathione peroxidase, glutathione reductase activities] and chromatographic methods [fetal hemoglobin (HbF), glutathione (GSH) and malondialdehyde (MDA) levels]. Among the 95 SCA patients evaluated, we identified 80 (84.2%) wild homozygous for *ADA* (22GG), 15 (15.8%) heterozygous (22GA) and none mutant homozygous (22AA), leading to an allelic frequency of 0.92 for the ancestral allele (22G) and 0.08 for the mutant one (22A). Unexpectedly, we did not observe any influence of *ADA* polymorphism on oxidative stress markers evaluated. However, we confirmed a well-described protective effect of HU treatment on decreasing MDA levels ($p < 0.01$), regardless the presence of *ADA* polymorphism. Thus, we concluded that *ADA* (22G>A) polymorphism does not play significant role in the disruption of sickle erythrocyte redox metabolism.

Key words:
Hemoglobin S
Adenosine
Fetal hemoglobin
Hydroxyurea

Introduction

Sickle cell anemia (SCA) is a devastating genetic hemolytic disorder associated with a high morbidity and mortality [1-3]. The underlying abnormality is a single nucleotide substitution (c.20A>T; rs334) in the gene that encodes the β-globin chain [4]. The mutated globin chain will form the abnormal hemoglobin S (HbS), due to a substitution of valine for glutamic acid at position six of the protein [5]. This substitution creates a hydrophobic patch in the HbS tetramer that results in a propensity to polymerize in its deoxygenated state, forming long polymers that distort the shape of the red blood cells (RBCs) [6]. However, HbS polymerization is reversible; fibers "melt" as oxygen is taken up by the HbS and the normal discoid shape returns [1]. The higher energy expenditure due to the increased metabolic turnover upon polymerization and depolymerization results in higher reactive oxygen species (ROS) production in sickle erythrocytes [7,8].

Despite our precise knowledge of the molecular defect that is associated with HbS in RBCs [9-11] and recent progress in understanding the molecular events that control polymerization of HbS and sickling of erythrocytes [12,13], the specific factors and signaling pathways that are involved in this process are unclear. In this way, Zhang et al. [14] demonstrated that adenosine can enhance 2,3-bisphosphoglycerate (2,3-BPG) production via A_{2B} receptor activation, suggesting that elevated adenosine had an unrecognized role in normal RBCs to promote oxygen (O_2) release and prevent acute ischemic tissue injury. However, in sickle erythrocytes, the beneficial role of excessive adenosine-mediated 2,3-BPG induction becomes detrimental by promoting deoxygenation, HbS polymerization and subsequent sickling.

Adenosine displays a complex metabolism in which it is generated intracellularly and extracellularly by degradation of adenine nucleotides [15]. In both media, the enzyme adenosine deaminase, encoded by *ADA* gene (20q13.11), catalyzes the hydrolytic deamination of adenosine or 2'-deoxyadenosine to inosine or 2'-deoxyinosine and ammonia, contributing to the regulation of adenosine levels [16,17]. Furthermore, a common functional variant of the *ADA* gene has been described as a guanine to adenine transition (c.22G>A; rs73598374) which leads to the substitution of asparagine for aspartic acid at the eightieth codon of the gene [16]. It has been found that this functional polymorphism leads to a decrease in ADA activity in erythrocytes and lymphocytes [18].

Taken the above observations into consideration, we hypothesized that the co-inheritance of SCA and *ADA* (22G> A) polymorphism would enhance adenosine levels due to *ADA* (22G>A) mutation, and through adenosine-mediated 2,3-BPG induction mechanism, exacerbate ROS generation inside the sickle erythrocytes, culminating in the worsening of the SCA patient oxidative condition. Thus, the aim of this study was to identify, in people with SCA, *ADA* (c. 22G>A; rs73598374) polymorphism, and correlating it with oxidative stress and antioxidant capacity markers.

Methods

Subjects

We evaluated 95 unrelated Brazilian SCA patients (53 women and 42 men; mean age: 24.4 years old; range: 10 – 59 years old). All subjects were regularly in clinical follow-up in the Blood Center of Rio de Janeiro (RJ, Brazil) and had access to the same medication protocol established and regulated by the Brazilian Ministry of Health for the entire national territory. All the patients studied received a prophylactic treatment with folic acid of 5 mg/day since the SCA diagnosis, while 41 (43,2%) of them were under HU treatment (average dose: 22 mg/Kg/day) for at least 90 days. The study was approved by the Data Safety Monitoring Board (DSMB) according to Brazilian Regulations.

After they gave their informed consent, all patients have answered a questionnaire in order to screen them according to the exclusion criteria listed below. Patients were asked specifically whether they were taking any nutritional supplements, nonsteroidal anti-inflammatory drugs (NSAID), opioids, or iron chelating agents, e.g. Further exclusion criteria were pregnant women, smokers or drinkers and patients who had had a stroke, pain and/or hemolytic crisis, or received blood transfusion within the 120 days prior to the study start. The medications used were accessed and those ones taking medication known to affect the analyzed parameters (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection were also excluded. The informations given in questionaries were also confirmed for each patient, by reviewing the medical records from the Blood Center database under supervision of clinicians responsible for the patients.

Biological samples

Blood samples (about 8 mL) were collected through venipuncture in ethylenediamine tetra acetic acid (EDTA) tubes. Four milliliters (mL) of whole blood were used for cytological, electrophoretic and chromatographic hemoglobin identification tests, as well as for DNA extraction from leukocytes for further molecular analysis. The other 4 mL were firstly used to prepare hemolysate for CAT activity analysis - whole blood diluted in ultrapure water (1:50, v/v). Then the blood samples were centrifuged in a refrigerated centrifuge at 800 ×g for 15 min at 4°C in order to obtain plasma for lipid peroxidation assay while buffy coat were carefully removed. The erythrocytes obtained were washed three times with cold phosphate-buffered saline (PBS - 136 mM NaCl, 3 mM KCl, 10 mM Na2HPO4 / KH2PO4, pH 7.4). The supernatant and buffy coat were carefully removed after each wash. Finally, the erythrocytes were diluted in a 3.5 μM 2-mercaptoethanol 10 μM NADP 2.7 mM EDTA hemolyzing solution (1:20, v/v) for GST, GPx and GR activities and GSH content measurement. All hemolysates and plasma samples were immediately frozen at -80°C until the analysis.

Hemoglobin identification tests

Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald-Giemsa at photonic microscopy using 40x objective [19]. Hb phenotype identification was performed using electrophoresis on cellulose acetate pH 8.6 [20], and agar electrophoresis at pH 6.2 [21]. The Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANT^{TM} equipment (Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer's manual.

Molecular analysis

DNA was extracted from peripheral blood leucocytes and isolated by phenol-chloroform methodology, according to Sambrook et al. [22]. *HBB*S* homozigous genotype was confirmed by molecular analysis by PCR-RFLP using *Dde*I as restriction endonuclease (New England BioLabs, Ipswich, MA, USA) [23]. ADA (c.22G>A) polymorphism was assessed through PCR-RFLP using *FastDigest Taq*I (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to Safranow et al. [24].

Biochemical measurements

Lipid peroxidation levels were assessed in the plasma samples by the product formed from malondialdehyde (MDA) and thiobarbituric acid (TBA), according to Silva et al. [25]. The product was detected by high performance liquid chromatography coupled to UV/Vis detector (UV/Vis-HPLC) [26,27]. The HPLC system consisted of ESA584 pump and an ESA526 UV/Vis detector set in 532 nm. The MDA–TBA derivative extracts were separated by a Shimadzu (Kyoto, Japan) C18 column (150 x 4.6 mm, 5 μm) that was fitted with a guard column cartridge of the same composition. The mobile phase (potassium phosphate 0.05 M, pH 7.0, with 40% methanol) was pumped at an isocratic flow of 1 mL min-1. Chromatogram monitoring and peak identification and quantification were performed using the EZ Chrom Elite software (Agilent Technologies, Santa Clara, CA, USA). The calculations were based on a calibration curve previously constructed and prepared according to same procedure used for the samples from authentic standards. The results were expressed in ng/mL.

Catalase (CAT) [28], glutathione S-transferase (GST) [29], glutathione peroxidase (GPx) [30], and glutathione reductase (GR) [31], activities were determined spectrophotometrically, while GSH concentration was determined HPLC coupled to a coulometric electrochemical detection (ECD-HPLC) [32]. A hemolysate aliquot was filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC system, consisted of ESA584 pump and an electrochemical coulometric detector (ESA Coulochem III, Bedford, MA, USA) with potential set at 650 mV. The column was an ESA C18 (250 x 4.6 mm, 5 μm) also fitted with a guard column cartridge of the same composition. The mobile phase consisted of sodium phosphate 0.05 M, octanesulfonic acid 0.025 M, pH 2.5, with acetonitrile (2%; v/v) was pumped at an isocratic flow of 0.9 mL min-1.

Chromatogram monitoring and peak identification and quantification were performed using the same software used in the lipid peroxidation assay. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into the HPLC system. The antioxidant enzymes were expressed in U/mL, while GSH levels were in μM.

Statistical Analysis

Statistical analysis was performed in groups with at least three individuals using the Statistica 9.0 software (Statsoft Inc., Tulsa, OK, USA). Data were firstly tested regarding normality and homogeneity of variances assumptions according to Shapiro-Wilk test and

Levene's test, respectively. Groups that met the assumptions (parametric data) were compared by applying one-way ANOVA followed by Tukey's *post hoc* test. Those groups that did not meet the assumptions (non-parametric data) were compared by Kruskal-Wallis followed by Dunn's *post hoc* test.

In order to assess association degree between the studied variables, we used Pearson's correlation for parametric data and Spearman's rank correlation for non-parametric data. Data were expressed as mean ± standard error and we considered $p < 0.05$ as statistically significant.

Results

Among the 95 SCA patients evaluated, we identified 80 (84,2%) wild homozygous for *ADA* (22GG), 15 (15.8%) heterozygous (22GA) and none mutant homozygous (22AA), leading to an allelic frequency of 0.92 for the ancestral allele (22G) and 0.08 for the mutant one (22A). Due to the lack of mutant homozygous, and as HU is an important modulator of SCA oxidative status, we did all the comparisons among the following subgroups: wild homozygous treated [GG (HU+)] or not treated with HU [GG (HU-)] and patients heterozygous under HU use [GA (HU+)] or not [GA (HU-)].

Unexpectedly, the presence of *ADA* 22A allele did not influence any of the oxidative stress parameters assessed (Fig. 1). We only observed an well-documented HU effect on lipid peroxidation levels. Those patients treated with HU showed lower MDA levels than the patients untreated, regardless the presence of mutation ($p < 0.01$) (Fig. 1-A).

In order to better understand the non-participation of *ADA* 22A polymorphism in the expression of oxidative stress markers, further investigation was carried out according to HbF amount of SCA patients, because HbF is capable of changing the intracellular concentrations of HbS that dictate the pace and extent of polymerization [33]. Thus, we analyzed the association degree between HbF levels and biochemical markers studied in SCA patients heterozygous for *ADA* polymorphism, since they showed high HbF levels variation (mean value: 8.6%; range: 0.5 - 23%). However, we found no correlation between HbF amount and each variable measured (Fig. 2).

Discussion

Even with improved knowledge of the human genome, development of new genomic tools and identification of single nucleotide polymorphisms (SNPs) associated with subphenotypes of SCD by genome-wide association studies (GWAS) [28], there is still a major challenge to combine all these variables and establish potential predictors of the SCD severity [34]. In addition, the understanding of SCA pathophysiology has gradually increased [35], and among the new evidences, oxidative stress has been increasingly related to both cause and consequence of inflammation, hemolysis, vasculopathy, vaso-occlusion, infection, injury by ischemia/reperfusion, e.g. [2]. However, studies associating genetic and biochemichal markers are scarce, thus this study, to our knowledge, yields a unique opportunity in which both genetic factor (*ADA* polymorphism) and oxidative stress markers were simultaneously measured and correlated with HbF levels and HU use in persons with SCA.

The frequency of mutated allele in *ADA* gene (22A) is estimated at 0.06 in Western populations, lower among individuals of African descents and higher in Southeast Asian populations [16,36,37]. Previous studies from Brazilian population showed different allele frequencies. For instance, Dutra et al. [38] obtained a 0.11 allele (22A) frequency for individuals from Rio Grande do Sul State. While, Nunes et al [39] and Mazzotti et al. [40] found a frequency about 0.05 for people from Sao Paulo State. These data are in accordance to studies developed in European populations [24,41,42]. This way, we found a frequency lower than those reported for Caucasians and higher than that expected for African descents that might to be only the reflection of ethnic and racial admixture of our population. Moreover, to our knowledge, this is the first report about *ADA* (22G>A) polymorphism in people with SCA from Rio de Janeiro State.

During periods of cellular hypoxia or stress, adenosine is released from cells along with the adenine nucleotides, ATP, ADP, and AMP, which are converted to adenosine by ectonucleotidases [43,44]. This response is even more pronounced in SCA patients due to increased amounts of ATP in the circulation derived from chronic sickle red cell hemolysis and tissue damage from vasoocclusion [45]. This observation causes the postulated hypothesis of this study is more plausible, but we did not observe any influence of *ADA* polymorphism on oxidative stress markers evaluated. Firstly, we thought that it could be the result of a known protective effect of HbF levels shown by Dasgupta et al. [46] in SCA transgenic mice, and further confirmed in SCA patients by Silva et al [47], in which Hb F protective effect is

primarily mediated by decreased intravascular sickling, resulting in a decreased oxidative stress. However, we found no association between HbF levels and each biochemical parameters assessed.

Although, the enzyme encoded by the A22 allele has about 35% less catalytic activity than enzyme encoded by G22 allele, individuals carrying one copy of mutated allele display 15–20% lower activity compared to ancestral homozygotes [18]. This way, we demonstrated that enzyme inefficiency caused by mutation is not sufficient to trigger a worsening in oxidative outcome of people with SCA. Nevertheless, Safranow et al. [24] demonstrated an association of *ADA* 22A allele with decreased risk of coronary artery disease. While Mi et al. [48] showed that mice lacking ADA developed priapic activity and penile vascular fibrosis. Thus, further studies with larger sample size and association with clinical manifestations can predict the relative risk generated by co-inheritance of *ADA* (22G>A) polymorphism and SCA in disease progression.

In conclusion, we showed that *ADA* (22 G>A) polymorphism does not play significant role in the disruption of sickle erythrocyte redox metabolism. However, taking into consideration the studies summarized above, we suggest that this polymorphism might be involved in the broad spectrum of SCA phenotypic expression.

Conflict of interest

The authors declare no competing financial or other relationship with any people or organization interests.

Acknowledgments

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References

[1] M.J. Stuart, R.L. Nagel, Sickle-cell disease, Lancet. 364 (2004) 1343-1360.

[2] D.C. Rees, T.N. Williams, M.T. Gladwin, Sickle-cell disease, Lancet. 376 (2010) 2018-2031.

[3] F.B. Piel, S.I. Hay, S. Gupta et al., Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions, PLoS. Med. 10 (2013) e1001484-

[4] M.H. Steinberg, P. Sebastiani, Genetic modifiers of sickle cell disease, Am. J Hematol. 87 (2012) 795-803.

[5] H.F. Bunn, Pathogenesis and treatment of sickle cell disease, N. Engl. J Med. 337 (1997) 762-769.

[6] R.P. Hebbel, Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology, Blood. 77 (1991) 214-237.

[7] T. Banerjee, F.A. Kuypers, Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells, Br. J Haematol. 124 (2004) 391-402.

[8] S.A. Akohoue, S. Shankar, G.L. Milne et al., Energy expenditure, inflammation, and oxidative stress in steady-state adolescents with sickle cell anemia, Pediatr. Res. 61 (2007) 233-238.

[9] V.M. Ingram, A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin, Nature. 178 (1956) 792-794.

[10] C. Madigan, P. Malik, Pathophysiology and therapy for haemoglobinopathies. Part I: sickle cell disease, Expert Rev. Mol. Med. 8 (2006) 1-23.

[11] G.W. Christoph, J. Hofrichter, W.A. Eaton, Understanding the shape of sickled red cells, Biophys. J. 88 (2005) 1371-1376.

[12] F.A. Ferrone, Polymerization and sickle cell disease: a molecular view, Microcirculation. 11 (2004) 115-128.

[13] M.M. de, Management of sickle cell disease, BMJ. 337:a1397. doi: 10.1136/bmj.a1397. (2008) a1397-

[14] Y. Zhang, Y. Dai, J. Wen et al., Detrimental effects of adenosine signaling in sickle cell disease, Nat. Med. 17 (2011) 79-86.

[15] Y. Zhang, Y. Xia, Adenosine signaling in normal and sickle erythrocytes and beyond, Microbes Infect. 14 (2012) 863-873.

[16] R. Hirschhorn, D.R. Yang, A. Israni, An Asp8Asn substitution results in the adenosine deaminase (ADA) genetic polymorphism (ADA 2 allozyme): occurrence on different chromosomal backgrounds and apparent intragenic crossover, Ann. Hum. Genet. 58 (1994) 1-9.

[17] B.B. Fredholm, J.F. Chen, R.A. Cunha et al., Adenosine and brain function, Int. Rev. Neurobiol. 63:191-270. (2005) 191-270.

[18] G. Battistuzzi, P. Iudicone, P. Santolamazza et al., Activity of adenosine deaminase allelic forms in intact erythrocytes and in lymphocytes, Ann. Hum. Genet. 45 (1981) 15-19.

[19] C.R. Bonini-Domingos, Metodologias laboratoriais para o diagnóstico de hemoglobinopatias e talassemias., (2006) 121 p.

[20] A.J. Marengo-Rowe, Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate, J. Clin. Pathol. 18 (1965) 790-792.

[21] F. Vella, Acid-agar gel electrophoresis of human hemoglobins, Am. J. Clin. Pathol. 49 (1968) 440-442.

[22] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Laboratory Press. (1989) pp. 10.51-10.67.

[23] R.K. Saiki, S. Scharf, F. Faloona et al., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science. 230 (1985) 1350-1354.

[24] K. Safranow, R. Rzeuski, A. Binczak-Kuleta et al., ADA*2 allele of the adenosine deaminase gene may protect against coronary artery disease, Cardiology. 108 (2007) 275-281.

[25] D.G.H. Silva, O. Ricci-Junior, E.A. de Almeida et al., Potential utility of melatonin as an antioxidant therapy in the management of sickle cell anemia, J. Pineal Res. (2014) (In Press)-

[26] E.A. de Almeida, S.A. Marques, C.F. Klitzke et al., DNA damage in digestive gland and mantle tissue of the mussel Perna perna, Comp Biochem. Physiol C. Toxicol. Pharmacol. 135C (2003) 295-303.

[27] E.A. de Almeida, S. Miyamoto, A.C. Bainy et al., Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels Perna perna exposed to different metals, Mar. Pollut. Bull. 49 (2004) 386-392.

[28] E. Beutler, Red cell metabolism: A Manual of Biochemical Methods, 83 (1975) 919-

[29] J.H. Keen, W.H. Habig, W.B. Jakoby, Mechanism for the several activities of the glutathione S-transferases, J. Biol. Chem. 251 (1976) 6183-6188.

[30] H. Sies, O.R. Koch, E. Martino et al., Increased biliary glutathione disulfide release in chronically ethanol-treated rats, FEBS Lett. 103 (1979) 287-290.

[31] E. Beutler, Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies, J. Clin. Invest. 48 (1969) 1957-1966.

[32] A. Rodriguez-Ariza, F. Toribio, J. Lopez-Barea, Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection, J. Chromatogr. B Biomed. Appl. 656 (1994) 311-318.

[33] G.A. Damanhouri, J. Jarullah, S. Marouf et al., Clinical biomarkers in sickle cell disease, Saudi J Biol. Sci. 22 (2015) 24-31.

[34] G. Lettre, The search for genetic modifiers of disease severity in the beta-hemoglobinopathies, Cold Spring Harb. Perspect. Med. 2 (2012) a015032-

[35] D.C. Rees, J.S. Gibson, Biomarkers in sickle cell disease, Br. J Haematol. 156 (2012) 433-445.

[36] D.A. Hopkinson, P.J. Cook, H. Harris, Further data on the adenosine deaminase (ADA) polymprphism and a report of a new phenotype, Ann. Hum. Genet. 32 (1969) 361-367.

[37] J. Weissmann, M. Vollmer, O. Pribilla, Survey of the distribution of adenosine deaminase and superoxide dismutase markers in different populations, Hum. Hered. 32 (1982) 344-356.

[38] G.P. Dutra, G.L. Ottoni, D.R. Lara et al., Lower frequency of the low activity adenosine deaminase allelic variant (ADA1*2) in schizophrenic patients, Rev. Bras. Psiquiatr. 32 (2010) 275-278.

[39] D.P. Nunes, L.C. Spegiorin, C.C. Mattos et al., The ADA*2 allele of the adenosine deaminase gene (20q13.11) and recurrent spontaneous abortions: an age-dependent association, Clinics (Sao Paulo). 66 (2011) 1929-1933.

[40] D.R. Mazzotti, C. Guindalini, A.A. de Souza et al., Adenosine deaminase polymorphism affects sleep EEG spectral power in a large epidemiological sample, PLoS. One. 7 (2012) e44154-

[41] A.M. Persico, R. Militerni, C. Bravaccio et al., Adenosine deaminase alleles and autistic disorder: case-control and family-based association studies, Am. J Med. Genet. 96 (2000) 784-790.

[42] V. Bachmann, F. Klaus, S. Bodenmann et al., Functional ADA polymorphism increases sleep depth and reduces vigilant attention in humans, Cereb. Cortex. 22 (2012) 962-970.

[43] K.A. Jacobson, Z.G. Gao, Adenosine receptors as therapeutic targets, Nat. Rev. Drug Discov. 5 (2006) 247-264.

[44] H.K. Eltzschig, P. Carmeliet, Hypoxia and inflammation, N. Engl. J Med. 364 (2011) 656-665.

[45] G. Hasko, J. Linden, B. Cronstein et al., Adenosine receptors: therapeutic aspects for inflammatory and immune diseases, Nat. Rev. Drug Discov. 7 (2008) 759-770.

[46] T. Dasgupta, M.E. Fabry, D.K. Kaul, Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in transgenic-knockout sickle mice, Am. J Physiol Regul. Integr. Comp Physiol. 298 (2010) R394-R402.

[47] D.G.H. Silva, E. Belini-Junior, L.S. Torres et al., Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia, Blood Cells Mol. Dis. 47 (2011) 23-28.

[48] T. Mi, S. Abbasi, H. Zhang et al., Excess adenosine in murine penile erectile tissues contributes to priapism via A2B adenosine receptor signaling, J Clin. Invest. 118 (2008) 1491-1501.

Figures

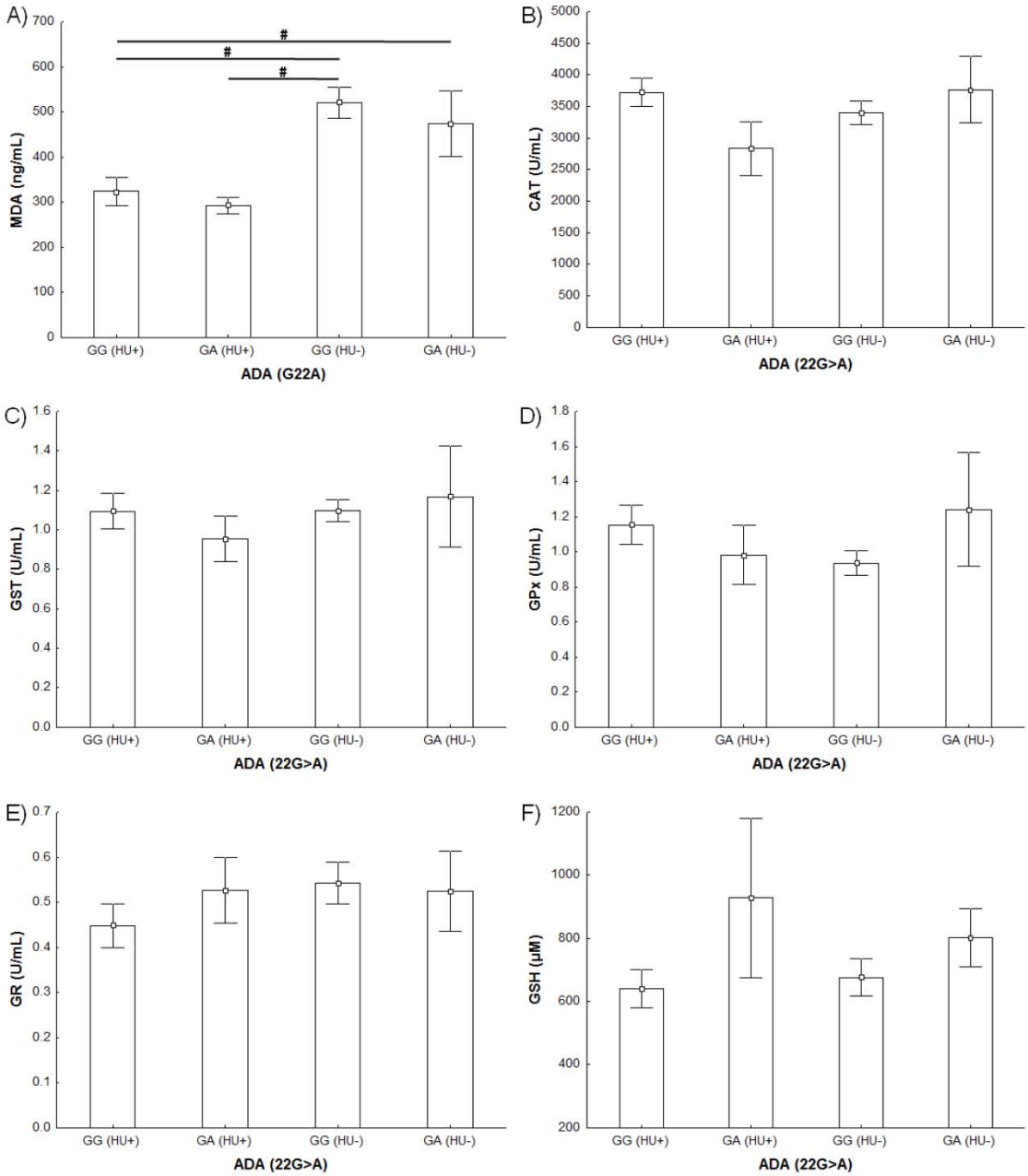

Fig. 1. Influence of ADA (22G>A) polymorphism on oxidative stress markers. A) Lower lipid peroxidation levels in the patient subgroups under HU use when compared to patients not treated with HU, regardless polymorphism presence ($p < 0.01$; Kruskal-Wallis follewd by Dunn's post hoc test). Absence of differential expression of the evaluated antioxidants among the patient subgroups: B) catalase activity ($p = 0.32$; one-way ANOVA); C) glutathione S-transferase activity ($p = 0.83$; one-way ANOVA); D) glutathione peroxidase activity ($p = 0.29$; one- way ANOVA); E) glutathine reductase activity ($p = 0.53$; one- way ANOVA); F) glutathione concentration ($p = 0.55$; Kruskal-Wallis).
GG (HU+): ancestral homozygous under HU use (N = 34); GA (HU+): heterozygous patients under HU use (N = 8); GG (HU-): ancestral homozygous not treated with HU (N = 46); GA (HU-): heterozygous patients untreated with HU (N = 7).
[#]Indicates statistical differences.

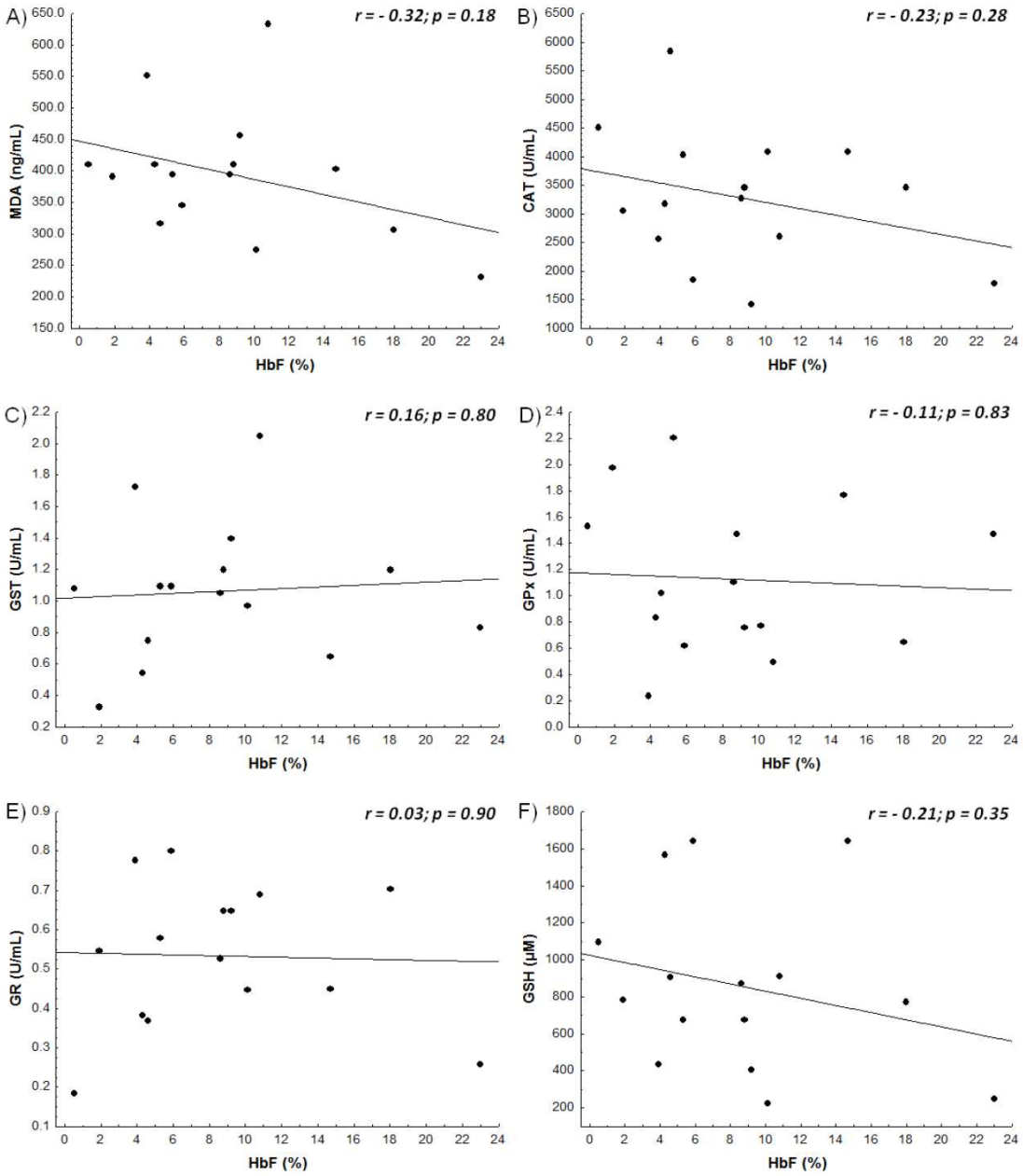

Fig. 2. No association between HbF amount and each oxidative stress parameters evaluated in SCA patients heterozygous for ADA (22G>A) polymorphims. A) Lipid peroxidation measured through malondialdehyde levels. Activities of antioxidant enzymes (B) catalase; (C) glutathione *S*-transferase; (D) glutathione peroxidase; (E) glutathione reductase. F) Glutathione levels.
All correlation analysis were made by Spearman's rank test.

Capítulo III

Potential utility of melatonin as an antioxidant therapy in the management of sickle cell anemia

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Running title: Melatonin: a promising treatment for SCA

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Key words: hemoglobin S; oxidative stress; alternative therapy; antioxidant capacity; melatonin

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Abstract: This study aimed to assess antioxidant effects of melatonin treatment compared to *N*-acetylcysteine (NAC) and to their combination in a sickle cell suspension. Sickle erythrocytes were suspended in phosphate-buffered saline, pH 7.4, composing external control group. They were also suspended and incubated at 37ºC either in absence (experimental control group) or presence of NAC, melatonin and their combination at concentrations of 100 pM, 100 nM and 100 μM for one hour (treatment groups). The melatonin influences were evaluated by spectrophotometric [hemolysis degree, catalase (CAT), glutathione *S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and superoxide dismutase (SOD) activities] and chromatographic methods [glutathione (GSH) and malondialdehyde (MDA) levels]. Incubation period was able to cause a rise about 64% on hemolysis degree as well as practically doubled the lipid peroxidation levels ($p < 0.01$). However, almost all antioxidants tested treatments neutralized this incubation effect observed in MDA levels. Among the antioxidant biomarkers evaluated, we observed a modulating effect of combined treatment on GPx and SOD activities ($p < 0.01$), which showed ~25% decrease in their activities. In addition, we found an antioxidant dose-dependent effect for melatonin on lipid peroxidation ($r = - 0.29$; $p = 0.03$) and for combined antioxidant treatments also on MDA levels ($r = - 0.37$; $p = 0.01$) and on SOD activity ($r = - 0.54$; $p < 0.01$). Hence, these findings contribute with important insight that melatonin individually or in combination with NAC may be useful for sickle cell anemia management.

Introduction

Sickle cell anemia (SCA) encompasses inherited hemolytic anemia due to a single point mutation in beta globin gene ($HBB^{glu6val}$) that result in the formation of sickle hemoglobin (HbS) [1], which drives to a complex physiopathology. Oxidative stress plays a major role in SCA pathophysiology, contributing significantly to hemolysis, hypercoagulability, endothelial activation, decreased nitric oxide (NO) bioavailability and organ damage [2-7], leading to devastating clinical manifestations [8]. One of the most important prooxidant sources in SCA are sickle erythrocytes [9,10]. Sheng et al. [11] have shown excessive biologic oxidation of HbS because the cytoplasm of sickle red blood cells, unlike that of normal cells, are exposed to abnormal amounts of oxidants. Moreover, the high metabolic turnover during polymerisation and depolymerisation suffered by HbS molecules after deoxygenation and reoxygenation respectively is a potentially important source of reactive oxygen species (ROS) production [12,13]. Thus, the repeated polymerization/depolymerization process can lead to a vicious cycle inciting blood cell adhesion, hemolysis, vaso-occlusion, and ischemia-reperfusion injury [14], in other words multiple sources of prooxidant processes with consequent overwhelming, chronic and systemic oxidative stress.

An improved understanding of the abnormal oxidative processes that occur in SCA has led to new insights into the action mechanisms of some currently accepted therapies, e.g. hydroxycarbamide (HC), and it has also suggested new therapies for this disease [15,16]. For this reason, antioxidant therapy is being a worthy, promising and increasing goal for SCA treatment. In this way, Udupi and Rice-Evans [17] showed the efficacious and non toxic effects of *N*-acetylcysteine (NAC) in sickle erythrocyte suspension, as experimental model. Afterwards, other *in vitro* [18,19] and *in vivo* [20] studies showed that NAC could block formation of dense and irreversibly sickled cells, and inhibit sickle erythrocyte dehydration due to its ability to maintain an appropriate level of reduced glutathione (GSH) and its direct antioxidant activity. More recently, Nur et al. [21] concluded that oral NAC treatment of SCA patients seems to reduce erythrocyte outer membrane phosphatidylserine exposure, plasma levels of advanced glycation end-products (AGEs), and cell-free hemoglobin levels.

However, NAC has shown a limited clinically application, mainly because it failed to provide significant antioxidant effects *in vivo*, presumably due to its low lipid solubility and tissue distribution [22,23]. Conversely, melatonin (*N*-acetyl-5-methoxytryptamine) is an amphiphilic molecule, able to cross all morphophysiological barriers [24,25]. Moreover,

melatonin protective effects against deleterious effects caused by oxidative stress are well documented [26-31], thus reducing oxidative damage in both lipid and aqueous cell environments. In addition, one of the most appealing properties of melatonin, which distinguishes it from most antioxidants, is that its metabolites also have the ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) [32]. The continuous protection exerted by melatonin and its metabolites, referred as the free radical scavenging cascade [33-35], makes melatonin highly effective in protecting organisms from oxidative stress, even at low concentrations [32]. These melatonin properties should be beneficial for the treatment of SCA patients, since they have shown a ~2.5-fold reduction of melatonin levels compared to a control group of individuals without hemoglobinopathies, suggesting an involvement of melatonin in the antioxidant defense system [36].

Taken together, SCA pathophysiologic complexity intimately related with a hyperoxidative status, the well-established action of NAC as an antioxidant therapy for SCA patients and the promising therapeutical implications of melatonin, this study aimed to assess antioxidant effects of melatonin treatment compared to NAC and the combination of both using a sickle cell suspension as experimental model. Other cellular antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione *S*-transferase, glucose-6-phosphate dehydrogenase and reduced glutathione levels) were also measured to verify a possible indirect action of melatonin in ameliorating oxidative stress due to modulation of antioxidant defenses.

Methods

Subjects

Ten previously diagnosed Brazilian SCA patients were included in the study (six women and four men; mean age: 27.4 years old; range: 20 – 40 years old). All subjects were regularly in clinical follow-up in the Blood Center of Sao Jose do Rio Preto (SP, Brazil) and had access to the same medication protocol established and regulated by the Brazilian Ministry of Health for the entire national territory. The study was approved by the Data Safety Monitoring Board (DSMB) according to Brazilian Regulations.

After they gave their informed consent, all patients have answered a questionnaire in order to screen them according to the exclusion criteria listed below. Patients were asked specifically whether they were taking any nutritional supplements, nonsteroidal anti-

inflammatory drugs (NSAID), opioids, or hydroxycarbamide (HC). History of pain crisis episodes, need of hospitalization and diagnosis of acute complications in the last 12 months were particularly emphasized. Pregnant, smokers or drinkers were excluded from the study, as well as anyone who had had a stroke, pain and/or hemolytic crisis or had received blood transfusion within the 120 days prior to the study start. The medications used were accessed and those ones taking medication known to affect the analyzed parameters (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection were also excluded.

All patient routine markers, e.g. complete blood count, hemolytic and hepatic and renal markers, were developed by specialized laboratory in the Blood Center of Sao Jose do Rio Preto. These data were obtained by reviewing the medical records from the Blood Center database, where the informations given in questionaries were also confirmed for each patient, under supervision of clinicians responsible for the patients.

Hemoglobin phenotypes, genotypes and β^S -globin haplotypes

Hb phenotype identification was performed using electrophoresis on cellulose acetate pH 8.4, and agar electrophoresis at pH 6.2. The Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANT^{TM} equipment (Bio-Rad Laboratories, CA, USA) [37]. Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald-Giemsa at photonic microscopy using 40x objective. In all patient samples, Hb genotype was developed by molecular analysis using PCR-RFLP. The segment amplification that encodes the β^S gene was accomplished by specific primers and the amplicon was cleaved by the *Dde*I restriction endonuclease (New England BioLabs, MA, USA) [38]. Beta globin haplotypes were determined through the PCR-RFLP analysis of the following polymorphic restriction sites: γG (Hind III), γA (Hind III), ψβ (Hinc II), 3′ψβ (Hinc II) and 5′β (Hinf I), as previously described [39].

Erythrocyte suspension and experimental design

Blood samples (about 8 mL) were collected through venipuncture in EDTA tubes from each of the ten SCA patient, between 7:00 a.m. to 9:00 a.m. Then each blood sample was individually and immediately centrifuged in a refrigerated centrifuge at 800 ×*g* for 10 min at 4°C. Plasma and buffy coat were carefully removed, and sickle erythrocytes were washed

three times with cold phosphate-buffered saline (PBS-1 - 136 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 / KH_2PO_4, pH 7.4). The supernatant and buffy coat were carefully removed after each wash.

The sickle erythrocytes obtained from each patient were separately suspended in PBS-2 (100 mM NaCl, 0.5 mM EDTA, 50 mM Na_2HPO_4 / NaH_2PO_4, pH 7.4) to obtain a hematocrit of 10%, composing the external control group. The higher buffer capacity of PBS-2 improved pH stability and the presence of EDTA prevented free iron from entering the cells [23]. They were also suspended in the same manner and then separately incubated at 37ºC either in absence (experimental control group) or presence of NAC, melatonin and their combination at concentrations of 100 pM, 100 nM and 100 μM for one hour (treatment groups). Thus, in order to verify melatonin antioxidant effects, the RBC suspensions were submitted to evaluation through 11 different experimental groups, each one composed by 10 samples, as follow:

- Group 1 (G1) as external control: sickle erythrocyte suspension (final hematocrit of 10%) was not treated with any antioxidants and immediately processed for further analysis;
- Group 2 (G2) as experimental control: sickle erythrocyte suspension (final hematocrit of 10%) was not treated with any antioxidants, but incubated for one hour at 37°C;
- Group 3 to 5 (G3-G5) as NAC treatments: sickle erythrocyte suspensions (final hematocrit of 10%) were treated with NAC at final concentration of 100 pM, 100 nM and 100 μM, respectively, and incubated for one hour at 37°C;
- Group 6 to 8 (G6-G8) as melatonin treatments: sickle erythrocyte suspensions (final hematocrit of 10%) were treated with melatonin at final concentration of 100 pM, 100 nM and 100 μM, respectively, and incubated for one hour at 37°C;
- Group 9 to 11 (G9-G11) as combined treatments: sickle erythrocyte suspensions (final hematocrit of 10%) were treated with NAC and melatonin at same final concentration of 100 pM, 100 nM and 100 μM, respectively, and incubated for one hour at 37°C.

Measurement of hemolysis degree and lipid peroxidation assay

Sickle erythrocyte suspension was centrifuged at 800 ×*g* for 10 min at room temperature. Then hemolysis degree was determined by the absorbance of hemoglobin at 540

nm in the supernatant. Absorbance at 100% hemolysis was determined by adding 10 µl of Triton X-100 (10%, V/V) to 1 mL of the erythrocyte suspension, according to Li et al [40].

Lipid peroxidation levels were assessed by the product formed from malondialdehyde (MDA) and thiobarbituric acid (TBA), according to Esterbauer and Zollner [41] with some modifications. The product was detected by high performance liquid chromatography coupled to UV/Vis detector (UV/Vis-HPLC) [42,43]. Briefly, 0.5 mL of cellular suspension supernatant was mixed in a ratio of 1:1 (v/v) with a solution containing 40% of trichloroacetic acid. Then these mixtures were centrifuged at 1500 x*g* for 5 min at 4°C. The supernatants were combined to 0.3 mL of a TBA solution (40 mg in 10 mL of HCl 0.2 M) and homogenized. The resulted mixtures were heated at 90°C for 40 min. Next, 1 mL of n-butanol was added and samples were vigorously stirring and centrifuged at 1500 x*g* for 5 min to extract the MDA–TBA derivatives. The MDA–TBA derivative extracts were collected and directly injected into HPLC system.

The HPLC system consisted of ESA584 pump and an ESA526 UV/Vis detector set in 532 nm. The extracts were separated by a Shimadzu C18 column (150 x 4.6 mm, 5 µm) that was fitted with a guard column cartridge of the same composition. The mobile phase (potassium phosphate 0.05 M, pH 7.0, with 40% methanol) was pumped at an isocratic flow of 1 mL min^{-1}. Chromatogram monitoring and peak identification and quantification were performed using the EZ Chrom Elite software (Agilent Technologies). The calculations were based on a calibration curve previously constructed and prepared according to same procedure used for the samples from authentic standards. The results were expressed in ng/mL.

Measurement of enzymatic and nonenzymatic antioxidants

After centrifugation of sickle erythrocyte suspension, packed RBCs were diluted in ultrapure water (1:20, v/v), in order to obtain a hemolysate solution which was aliquoted and stocked at -80°C for further use in the assays of antioxidant markers.

Catalase (CAT) [44], glutathione *S*-transferase (GST) [45], glutathione peroxidase (GPx) [46], glutathione reductase (GR) [47], glucose-6-phosphate dehydrogenase (G6PDH) [48] and superoxide dismutase (SOD) [49] activities were determined spectrophotometrically, while GSH concentration was determined HPLC coupled to a coulometric electrochemical detection (ECD-HPLC) [50]. A hemolysate aliquot was filtered through Millex syringe filter units (0.22 µm) and directly injected into the HPLC system, consisted of ESA584 pump and an electrochemical coulometric detector (ESA Coulochem III, Bedford, MA, USA) with

potential set at 650 mV. The column was an ESA C18 (250 x 4.6 mm, 5 μm) also fitted with a guard column cartridge of the same composition. The mobile phase consisted of sodium phosphate 0.05 M, octanesulfonic acid 0.025 M, pH 2.5, with acetonitrile (2%; v/v) was pumped at an isocratic flow of 0.9 mL min^{-1}.

Chromatogram monitoring and peak identification and quantification were performed using the same software used in the lipid peroxidation assay. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into the HPLC system. The antioxidant enzymes were expressed in U/mL, while GSH levels were in μM.

Statistical analysis

Statistical analysis was performed using Statistica 9.0 software (Statsoft Inc.). Data were firstly tested regarding normality and homogeneity of variances assumptions according to Shapiro-Wilk test and Levene's test, respectively. For patients' characterization analysis, groups that met the assumptions (parametric data) were compared by applying *t* test. While those groups that did not meet the assumptions (non-parametric data) were compared by Mann-Whitney test. For the *in vitro* experimentation analysis, due to the dependency among the groups, we used repeated measures ANOVA followed by Tukey's *post hoc* test for parametric data, or Friedman test followed by Dunnett's *post hoc* test for non-parametric data. In order to assess association degree between the studied variables, we used Pearson's correlation for parametric data and Spearman's rank correlation for non-parametric data. We considered $p < 0.05$ as statistically significant.

Results

Patients' characterization

In order to ensure a homogeneous study group, we characterized the baseline outcomes from the patients, regarding demographic data, beta globin haplotype profile, specific medication use, and transfusional and clinical history in the last 12 months (Table 1).

Despite all patients in clinical follow-up in the Blood Center of Sao Jose do Rio Preto have access to the same medication protocol, among those ones enrolled in our study only three were under HC treatment. As the HC is an important modulator of SCA phenotypic

expression [51,52], we verified its influence on routine markers available and on the proposed biochemical parameters (Table 2).

Differences in the patient baseline outcomes could alter the antioxidant responses during *in vitro* experimentation. However, we did not find any significant differences in the hematological and biochemical markers that might have affected the results obtained. For this reason, all patient samples were considered as only one studied group for the further analysis.

Antioxidant treatment effects on hemolysis degree and on lipid peroxidation levels

The oxidative markers evaluated showed different responses among the studied groups. The incubation period was able to cause a rise about 64% on hemolysis degree, when we compared external control (G1) to experimental one (G2). Unexpectedly, high hemolysis rates were also found in combined treatment (NAC+melatonin) groups at the extreme doses administrated, 100 pM and 100 μM (G9 and G11), respectively, in relation to external control. But these rates were not worse than the experimental control. Only the lowest melatonin dose (G6) showed a protective effect against hemolysis when compared to G11 ($p < 0.01$) (Fig. 1A). The incubation period practically doubled the lipid peroxidation levels, however almost all the tested antioxidant treatments were able to blunt MDA levels, except by the extreme doses of NAC treatment, G3 and G5 ones ($p < 0.01$) (Fig. 1B).

The results obtained for G1 and G2 suggested a possible relationship between MDA levels and hemolysis degree, since high lipid peroxidation rates may compromise the lipid membrane structure, commonly resulting in cell lysis. However, no association was found between these biochemical parameters ($r = 0.23$, $p = 0.16$; Spearman's rank correlation), suggesting that hemolysis degree observed was due to events unrelated to oxidative stress generated in the sickle erythrocyte suspension.

Antioxidant treatment modulations on antioxidant capacity biomarkers

As hormone-mediated modulations of different erythrocyte antioxidant systems were previously demonstrated, including by melatonin itself [53,54], we measured different cellular antioxidant defenses in order to verify a possible melatonin-mediated modulation on sickle erythrocyte responses to oxidative stress, thus allowing a better attribution of direct melatonin antioxidant effects. Among the antioxidant biomarkers evaluated, we observed a modulating effect of the combined treatment only on GPx and on SOD activities. Although almost all

tested treatments have apparently shown a protective effect, we found merely one significant difference that was a less GPx activity in the combined treatment group at the lowest dose (G9) than in the G2 ($p < 0.01$) (Fig. 2A). In relation to SOD, we found decreased activities in the intermediate (G10) and highest (G11) doses of combined treatment when compared to G2, G5, G6 and G7 ($p < 0.01$) (Fig. 2B). In addition, Table 3 summarizes the results obtained for other antioxidants evaluated, which did not present any statistically significant differences.

Dose-dependent antioxidant response investigation

We investigated possible occurrence of dose-dependent response in the hemolysis rates, lipid peroxidation levels and antioxidant biomarkers through association degree analysis. For this analysis, we firstly transformed the pM concentrations values of the tested treatments into a identical measure unit: $\log_{10}$[pM]+1. Then, we correlated them to the biochemical parameters evaluated in this study.

We confirmed an antioxidant dose-dependent effect for melatonin and combined antioxidant treatments on lipid peroxidation, once MDA levels decreased according to increased treatment doses (Fig. 3A and 3B). We also found a negative correlation between combined treatment concentrations and SOD activity (Fig. 3C). We did not find any association among the other biochemical markers evaluated. Considering that no positive modulation of antioxidant defenses were observed due to melatonin and/or NAC treatment, these results supporting our hypothesis of a direct antioxidant effect of melatonin and of the combined treatment tested on the sickle erythrocyte suspension.

Discussion

Many recent studies, both *in vitro* and *in vivo*, have investigated different strategies as antioxidant therapy for SCA treatment [16,55,56], but to the authors' knowledge, this study provides the first evidence to support the beneficial effects of melatonin for this purpose. We adopted rigorous exclusion criteria and well-characterized baseline outcomes of the SCA patients, for the study group composition, as well as a consolidated and well-described experimental model. This model provided a self-sustaining environment of oxidative stress, according to the rise in lipid peroxidation obtained, allowing an effective assessment of the antioxidant effects of melatonin on sickle erythrocyte suspension.

Oxidative stress may not be the primary etiology of different hemolytic anemias, but it mediates several of their pathological processes, including hemolysis itself [57], which plays a important role in the pathophysiological scheme of SCA [58]. For this reason, we firstly investigated hemolysis inhibition capacity of NAC, melatonin or their combination *in vitro*. Unexpectedly, we did not find any evident antioxidant effect of the tested treatments, thus one might speculate that the lack of hemolytic state improvement was, in fact, a consequence of low doses administered. However melatonin treatment at concentration of 100 pM was the only one that seemed to decrease the hemolysis rate. Furthermore, we did not observe any relationship between hemolysis and lipid peroxidation, suggesting that the hemolysis observed was a result of mechanical disruption of erythrocyte membrane structure, probably caused by HbS polimerization. This process of self-assembly or polymerization/sickling generates rigid HbS fibers that distort and damage RBC membrane and cytoskeleton and alters RBC biochemical properties [59].

Amer et al. [60] demonstrated high lysis inhibition capacity of NAC in a dose-dependent manner (concentration range from 0.2 mM to 2 mM), through β-thalassemic erythrocyte suspension incubated overnight. Furthermore, Krokosz et al. [61] observed in normal erythrocyte incubated up to 96 hrs, a decrease in hemolysis rate in the presence of melatonin at 0.6 mM, however the membrane proteins of incubated erythrocytes were subjected to aggregation which took place mainly by disulfide bridges. The presence of melatonin during the erythrocyte incubation did not influence the level of aggregates, which showed that melatonin did not protect thiol protein groups. This way, our hypothesis of a mechanical disruption of sickle erythrocyte membrane structure due to HbS polymer formation corroborate with the results of both studies.

Sickle erythrocytes are deeply related to high lipid peroxidation levels, due to an increased endogenous extent of oxidized lipid and consequent increase of susceptibility for further lipid peroxidation, when compared with HbA-containing erythrocytes [62-65]. Thus, mitigating this process may show beneficial outcomes during clinical follow-up of SCA patient. To date, HC is the only disease-modifying therapy approved for SCA, which had demonstrated ability to reduce lipid peroxidation levels [66,67]. For the first time, we showed that melatonin and its associated treatment with NAC were able to blunt MDA formation in a sickle erythrocyte system in a dose-dependent manner. Our results for melatonin treatments were in accordance to Chakravarty and Rizvi [68] who had previously demonstrated a dose-dependent antioxidant effect of melatonin on MDA content after *in vitro* oxidative insult by incubating normal erythrocytes with tert-butyl hydroperoxide (t-BHP). Later, Krokosz et al.

[61] also showed that melatonin at 0.02 mM prevented lipid peroxidation in normal erythrocyte suspension incubated for 24 hours.

Redox metabolism maintenance in sickle cell erythrocytes is constantly challenged, because they are a significant source of ROS [9,10], and are also exposed to prooxidative actions of ROS produced in the circulation [69]. In order to counteract oxidative stress, erythrocytes contain an efficient endogenous antioxidant system, including SOD, CAT and glutathione-related enzymes, as well as low molecular weight antioxidants, such as GSH itself, and vitamins E and C [69,70]. Furthermore, red blood cells (RBCs) have a plasma membrane redox system (PMRS) that transfers electrons from intracellular substrates to extracellular electron acceptors which may be NAD^+ or/and vitamin C [71]. Unexpectedly, the *in vitro* system used was not able to cause significant alterations in the antioxidant markers evaluated. We found only a synergistic effect of the tested antioxidant on GPx and SOD activities, suggesting that this treatment counteracted or prevented the formation of pro-oxidant species needed for the activation of these enzymes, in agreement to Imaga et al. [72]. The authors observed reduced enzyme activities (SOD, CAT, and GST) after blood samples had been incubated with extracts of *Carica papaya* leaves, indicating that these extracts may quickly mop up free radicals produced under the experimental conditions tested. In addition, Daak et al. [73] showed that SCA patients under omega-3 fatty acid treatment had lower SOD and GPx activities compared with their placebo counterparts, after six months of intervention period. The authors suggested that these decreases in the activities could not be explained by an increase in oxidative overload, since there were no similar reduction or depletion of the enzyme co-factors and non-enzymatic antioxidants, in accordance to our results.

Despite the nucleus lack of erythrocytes, hormone-mediated modulation of SOD has previously been demonstrated by Unfer et al. [54]. In this case, natural and synthetic steroid hormones caused a stimulatory effect on the SOD activity from human erythrocytes. In this sense, Chakravarty and Rizvi have determined *in vitro* melatonin-mediated modulations of different antioxidant systems in human erythrocytes, as activities of PMRS, ascorbate free radical (AFR) reductase [74], and membrane-associated Na^+/K^+-ATPase and Na^+/H^+ exchanger [53]. These last systems play an important role in maintaining cytosolic pH, ionic homeostasis, cell osmolarity and in the regulation of transmembrane ion movement [53]. Thus, these systems could be involved in the response of GPx and SOD activities that we obtained in our study, however the exact mechanisms to explain our findings and their clinical significance remain to be determined.

Sickle erythrocytes are characterized by decreased GSH concentrations with some studies noting a 50% decrease compared with normal erythrocytes [6,10,75-77], even though its *de novo* synthesis and recycling rate have been shown to be higher as compared to healthy erythrocytes [77]. Following GSH depletion, its synthesis is limited by the supply of cysteine and it has been assumed that deacetylation of NAC within erythrocytes provides cysteine to accelerate GSH production [78], but to achieve maximum rates of GSH synthesis by this process *in vivo*, plasma NAC concentrations would have to exceed 1.0 mM, which is therapeutically unattainable [79]. For this reason, we investigated only its direct antioxidant capacity, in order to compare with that one of melatonin. We did not find any significant difference between them, suggesting that both molecules have similar antioxidant properties at least at the conditions utilized in this study. However, Chakravarty and Rizvi [68] showed a dose-dependent increase in GSH content in the presence of melatonin in erythrocytes subjected to oxidative stress by incubating with t-BHP. Moreover, Pechánová et al. [80] have demonstrated *in vivo* that antioxidant activity of melatonin measured by TEAC (trolox equivalent antioxidant capacity) assay was 2.27 times higher than that of NAC, using adult spontaneously hypertensive rats as experimental model. Indeed, a multitude of studies have shown melatonin to have a higher antioxidant capacity compared to other antioxidants under the specific conditions of the experiments, in terms of its protective actions against oxidative stress [81].

The discovery of alternative and synergistic therapy for the treatment of SCA due to increasing concerns regarding its effect on the health of individuals worldwide is gaining attention [82]. In this context, McCarty [83] suggested the use of comprehensive nutraceutical strategy for mitigating the contribution of oxidative stress to SCA pathogenesis, dubbed as ‘‘full-spectrum antioxidant therapy”. Full-spectrum antioxidant therapy (FSAT) has been defined as a supplementation program that incorporates phycocyanobilin (a phytonutrient of spirulina), high-dose folic acid, a clinically effective phase 2 inducer such as lipoic acid, and NAC. However, paradoxical observations with regard of certain prooxidant effects of antioxidant compounds have been reported under some experimental conditions, indicating the complex interdependency among the pool of physiological relevant cellular antioxidants [84]. Interestingly, melatonin seems to escape to this paradoxical hypothesis because its endogenous levels are so low that they would not compete with antioxidants that are present in higher concentrations [85], even more in SCA patients who have a decreased melatonin levels [36].

Furthermore, melatonin displays an exceptional multiplicity of actions. Briefly, melatonin is involved in sleep initiation, vasomotor control, adrenal function, antiexcitatory actions, immunomodulation including antiinflammatory properties, direct and indirect antioxidant actions, and energy metabolism [28,74,86,87]. Additionally, Chakravarty and Rizvi also showed *in vivo* a circadian modulation of human erythrocyte MDA and GSH levels [68], as well as of PMRS and AFR reductase activities [74], which emphasize the role of melatonin as an antioxidant and its function against oxidative stress in RBCs. Thus, many of melatonin actions are directly involved in the SCA pathophysiological process, configuring another argument that should be taken into consideration to better address the hypothesis of pharmacological use of melatonin for SCA patient treatment. Notwithstanding a couple of reports that may indicate otherwise [85], and taking into consideration our results and the studies summarized above, we conclude that melatonin should be looked at as a key element in further investigations of antioxidant therapy for SCA patient treatment.

Competing interests

The authors declare no competing financial or other relationship with any people or organization interests.

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Author Contributions

D.G.H.S.: study concept and design, data design, data acquisition, data analysis, statistical analysis, data interpretation and drafting of the manuscript. **O.R.J.:** provision of samples and of routine marker data and critical review of manuscript. **E.A.A.:** study concept and design, guidance on standardization of the biochemical methods, critical review of the

manuscript and approval of the article..**C.R.B.D.:** study concept and design, critical revision of the manuscript and approval of the article.

References

1. STEINBERG MH, SEBASTIANI P. Genetic modifiers of sickle cell disease. Am J Hematol 2012; **87**:795-803.

2. ASLAN M, RYAN TM, ADLER B et al. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. Proc Natl Acad Sci U S A 2001; **98**:15215-15220.

3. FIBACH E, RACHMILEWITZ E. The role of oxidative stress in hemolytic anemia. Curr Mol Med 2008; **8**:609-619.

4. HEBBEL RP, OSAROGIAGBON R, KAUL D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. Microcirculation 2004; **11**:129-151.

5. KLINGS ES, FARBER HW. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. Respir Res 2001; **2**:280-285.

6. MORRIS CR, SUH JH, HAGAR W et al. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. Blood 2008; **111**:402-410.

7. NUR E, BRANDJES DP, SCHNOG JJ et al. Plasma levels of advanced glycation end products are associated with haemolysis-related organ complications in sickle cell patients. Br J Haematol 2010; **151**:62-69.

8. BUNN HF. Pathogenesis and treatment of sickle cell disease. N Engl J Med 1997; **337**:762-769.

9. HEBBEL RP, MORGAN WT, EATON JW et al. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. Proc Natl Acad Sci U S A 1988; **85**:237-241.

10. BANERJEE T, KUYPERS FA. Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells. Br J Haematol 2004; **124**:391-402.

11. SHENG K, SHARIFF M, HEBBEL RP. Comparative oxidation of hemoglobins A and S. Blood 1998; **91**:3467-3470.

12. COLETTA M, HOFRICHTER J, FERRONE FA et al. Kinetics of sickle haemoglobin polymerization in single red cells. Nature 1982; **300**:194-197.

13. AKOHOUE SA, SHANKAR S, MILNE GL et al. Energy expenditure, inflammation, and oxidative stress in steady-state adolescents with sickle cell anemia. Pediatr Res 2007; **61**:233-238.

14. CHIRICO EN, PIALOUX V. Role of oxidative stress in the pathogenesis of sickle cell disease. IUBMB Life 2012; **64**:72-80.

15. GIZI A, PAPASSOTIRIOU I, APOSTOLAKOU F et al. Assessment of oxidative stress in patients with sickle cell disease: The glutathione system and the oxidant-antioxidant status. Blood Cells Mol Dis 2011; **46**:220-225.

16. SILVA DG, BELINI JE, DE ALMEIDA EA et al. Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies. Free Radic Biol Med 2013; **65**:1101-1109.

17. UDUPI V, RICE-EVANS C. Thiol compounds as protective agents in erythrocytes under oxidative stress. Free Radic Res Commun 1992; **16**:315-323.

18. GIBSON XA, SHARTAVA A, MCINTYRE J et al. The efficacy of reducing agents or antioxidants in blocking the formation of dense cells and irreversibly sickled cells in vitro. Blood 1998; **91**:4373-4378.

19. SHARTAVA A, SHAH AK, GOODMAN SR. N-acetylcysteine and clotrimazole inhibit sickle erythrocyte dehydration induced by 1-chloro-2,4-dinitrobenzene. Am J Hematol 1999; **62**:19-24.

20. PACE BS, SHARTAVA A, PACK-MABIEN A et al. Effects of N-acetylcysteine on dense cell formation in sickle cell disease. Am J Hematol 2003; **73**:26-32.

21. NUR E, BRANDJES DP, TEERLINK T et al. N-acetylcysteine reduces oxidative stress in sickle cell patients. Ann Hematol 2012; **91**:1097-1105.

22. COTGREAVE IA. N-acetylcysteine: pharmacological considerations and experimental and clinical applications. Adv Pharmacol 1997; **38**:205-227.

23. GRINBERG L, FIBACH E, AMER J et al. N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. Free Radic Biol Med 2005; **38**:136-145.

24. BONNEFONT-ROUSSELOT D, COLLIN F, JORE D et al. Reaction mechanism of melatonin oxidation by reactive oxygen species in vitro. J Pineal Res 2011; **50**:328-335.

25. REITER RJ, TAN DX, ROSALES-CORRAL S et al. The universal nature, unequal distribution and antioxidant functions of melatonin and its derivatives. Mini Rev Med Chem 2013; **13**:373-384.

26. BONNEFONT-ROUSSELOT D, COLLIN F, JORE D et al. Reaction mechanism of melatonin oxidation by reactive oxygen species in vitro. J Pineal Res 2011; **50**:328-335.

27. WANG H, LI L, ZHAO M et al. Melatonin alleviates lipopolysaccharide-induced placental cellular stress response in mice. J Pineal Res 2011; **50**:418-426.

28. HARDELAND R. Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms. J Pineal Res 2013; **55**:325-356.

29. ROMERO A, RAMOS E, DE LOS RC et al. A review of metal-catalyzed molecular damage: protection by melatonin. J Pineal Res 2014; **56**:343-370.

30. ZHANG HM, ZHANG Y. Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. J Pineal Res 2014; **57**:131-146.

31. GALLEY HF, LOWES DA, ALLEN L et al. Melatonin as a potential therapy for sepsis: a phase I dose escalation study and an ex vivo whole blood model under conditions of sepsis. J Pineal Res 2014; **56**:427-438.

32. GALANO A, TAN DX, REITER RJ. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J Pineal Res 2013; **54**:245-257.

33. TAN DX, MANCHESTER LC, REITER RJ et al. Significance of melatonin in antioxidative defense system: reactions and products. Biol Signals Recept 2000; **9**:137-159.

34. ROSEN J, THAN NN, KOCH D et al. Interactions of melatonin and its metabolites with the ABTS cation radical: extension of the radical scavenger cascade and formation of a novel class of oxidation products, C2-substituted 3-indolinones. J Pineal Res 2006; **41**:374-381.

35. TAN DX, MANCHESTER LC, TERRON MP et al. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007; **42**:28-42.

36. SHIMAUTI EL, SILVA DG, DE ALMEIDA EA et al. Serum melatonin level and oxidative stress in sickle cell anemia. Blood Cells Mol Dis 2010; **45**:297-301.

37. BONINI-DOMINGOS CR. Metodologias laboratoriais para o diagnóstico de hemoglobinopatias e talassemias. 2006; 121 p.

38. SAIKI RK, SCHARF S, FALOONA F et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985; **230**:1350-1354.

39. SUTTON M, BOUHASSIRA EE, NAGEL RL. Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes. Am J Hematol 1989; **32**:66-69.

40. LI SD, SU YD, LI M et al. Hemin-mediated hemolysis in erythrocytes: effects of ascorbic acid and glutathione. Acta Biochim Biophys Sin (Shanghai) 2006; **38**:63-69.

41. ESTERBAUER H, ZOLLNER H. Methods for determination of aldehydic lipid peroxidation products. Free Radic Biol Med 1989; **7**:197-203.

42. DE ALMEIDA EA, MARQUES SA, KLITZKE CF et al. DNA damage in digestive gland and mantle tissue of the mussel Perna perna. Comp Biochem Physiol C Toxicol Pharmacol 2003; **135C**:295-303.

43. DE ALMEIDA EA, MIYAMOTO S, BAINY AC et al. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels Perna perna exposed to different metals. Mar Pollut Bull 2004; **49**:386-392.

44. BEUTLER E. Red Cell Metabolism: A Manual of Biochemical Methods. Ann Intern Med 1975; **83**:919.

45. KEEN JH, HABIG WH, JAKOBY WB. Mechanism for the several activities of the glutathione S-transferases. J Biol Chem 1976; **251**:6183-6188.

46. SIES H, KOCH OR, MARTINO E et al. Increased biliary glutathione disulfide release in chronically ethanol-treated rats. FEBS Lett 1979; **103**:287-290.

47. BEUTLER E. Effects of flavin compounds on glutathione reductase activity: in vivo and in vitro studies. J Clin Invest 1969; **48**:1957-1966.

48. BEUTLER E. G6PD deficiency. Blood 1994; **84**:3613-3636.

49. MCCORD JM, FRIDOVICH I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; **244**:6049-6055.

50. RODRIGUEZ-ARIZA A, TORIBIO F, LOPEZ-BAREA J. Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection. J Chromatogr B Biomed Appl 1994; **656**:311-318.

51. SHETH S, LICURSI M, BHATIA M. Sickle cell disease: time for a closer look at treatment options? Br J Haematol 2013; **162**:455-464.

52. WARE RE. Hydroxycarbamide: clinical aspects. C R Biol 2013; **336**:177-182.

53. CHAKRAVARTY S, RIZVI SI. Circadian modulation of sodium-potassium ATPase and sodium - proton exchanger in human erythrocytes: in vitro effect of melatonin. Cell Mol Biol (Noisy -le-grand) 2011; **57**:80-86.

54. UNFER TC, MAURER LH, KEMERICH DM et al. Non-genomic, direct modulatory effect of 17beta-estradiol, progesterone and their synthetic derivatives on the activity of human erythrocyte CuZn superoxide dismutase. Free Radic Res 2013; **47**:219-232.

55. VICHINSKY E. Emerging 'A' therapies in hemoglobinopathies: agonists, antagonists, antioxidants, and arginine. Hematology Am Soc Hematol Educ Program 2012; **2012**:271-275.

56. IMAGA NA. Phytomedicines and nutraceuticals: alternative therapeutics for sickle cell anemia. ScientificWorldJournal 2013; **2013**:1-12.

57. AMER J, ZELIG O, FIBACH E. Oxidative status of red blood cells, neutrophils, and platelets in paroxysmal nocturnal hemoglobinuria. Exp Hematol 2008; **36**:369-377.

58. CONNES P, LAMARRE Y, WALTZ X et al. Haemolysis and abnormal haemorheology in sickle cell anaemia. Br J Haematol 2014; **165**:564-572.

59. STUART MJ, NAGEL RL. Sickle-cell disease. Lancet 2004; **364**:1343-1360.

60. AMER J, ATLAS D, FIBACH E. N-acetylcysteine amide (AD4) attenuates oxidative stress in beta-thalassemia blood cells. Biochim Biophys Acta 2008; **1780**:249-255.

61. KROKOSZ A, GREBOWSKI J, SZWEDA-LEWANDOWSKA Z et al. Can melatonin delay oxidative damage of human erythrocytes during prolonged incubation? Adv Med Sci 2013; **58**:134-142.

62. DAS SK, NAIR RC. Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation of normal and sickled erythrocytes. Br J Haematol 1980; **44**:87-92.

63. JAIN SK, SHOHET SB. A novel phospholipid in irreversibly sickled cells: evidence for in vivo peroxidative membrane damage in sickle cell disease. Blood 1984; **63**:362-367.

64. RICE-EVANS C, OMORPHOS SC, BAYSAL E. Sickle cell membranes and oxidative damage. Biochem J 1986; **237**:265-269.

65. ESSIEN EU. Increased susceptibility of erythrocyte membrane lipids to peroxidation in sickle cell disease. Cent Afr J Med 1994; **40**:217-220.

66. DASGUPTA T, FABRY ME, KAUL DK. Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in

transgenic-knockout sickle mice. Am J Physiol Regul Integr Comp Physiol 2010; **298**:R394-R402.

67. SILVA DG, BELINI JE, TORRES LS et al. Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia. Blood Cells Mol Dis 2011; **47**:23-28.

68. CHAKRAVARTY S, RIZVI SI. Day and Night GSH and MDA Levels in Healthy Adults and Effects of Different Doses of Melatonin on These Parameters. Int J Cell Biol 2011; **2011**:404591-

69. PERRONE S, TATARANNO ML, STAZZONI G et al. Oxidative injury in neonatal erythrocytes. J Matern Fetal Neonatal Med 2012; **25**:104-108.

70. CHISTE RC, FREITAS M, MERCADANTE AZ et al. Carotenoids inhibit lipid peroxidation and hemoglobin oxidation, but not the depletion of glutathione induced by ROS in human erythrocytes. Life Sci 2014; **99**:52-60.

71. RIZVI SI, JHA R, MAURYA PK. Erythrocyte plasma membrane redox system in human aging. Rejuvenation Res 2006; **9**:470-474.

72. IMAGA NOA, SHAIRE EA, OGBEIDE S et al. In vitro biochemical investigations of the effects of Carica papaya and Fagara zanthoxyloides on antioxidant status and sickle erythrocytes. Afr J Biochem Res 2011; **5**:226-236.

73. DAAK AA, GHEBREMESKEL K, MARINIELLO K et al. Docosahexaenoic and eicosapentaenoic acid supplementation does not exacerbate oxidative stress or intravascular haemolysis in homozygous sickle cell patients. Prostaglandins Leukot Essent Fatty Acids 2013; **89**:305-311.

74. CHAKRAVARTY S, RIZVI SI. Circadian modulation of human erythrocyte plasma membrane redox system by melatonin. Neurosci Lett 2012; **518**:32-35.

75. TATUM VL, CHOW CK. Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress. Free Radic Res 1996; **25**:133-139.

76. SOMJEE SS, WARRIER RP, THOMSON JL et al. Advanced glycation end-products in sickle cell anaemia. Br J Haematol 2005; **128**:112-118.

77. REID M, BADALOO A, FORRESTER T et al. In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. Am J Physiol Endocrinol Metab 2006; **291**:E73-E79.

78. RAFTOS JE, WHILLIER S, CHAPMAN BE et al. Kinetics of uptake and deacetylation of N-acetylcysteine by human erythrocytes. Int J Biochem Cell Biol 2007; **39**:1698-1706.

79. WHILLIER S, RAFTOS JE, CHAPMAN B et al. Role of N-acetylcysteine and cystine in glutathione synthesis in human erythrocytes. Redox Rep 2009; **14**:115-124.

80. PECHANOVA O, ZICHA J, PAULIS L et al. The effect of N-acetylcysteine and melatonin in adult spontaneously hypertensive rats with established hypertension. Eur J Pharmacol 2007; **561**:129-136.

81. REITER RJ, PAREDES SD, MANCHESTER LC et al. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit Rev Biochem Mol Biol 2009; **44**:175-200.

82. AFOLABI IS, OSIKOYA IO, FAJIMI OD et al. Solenostemon monostachyus, Ipomoea involucrata and Carica papaya seed oil versus Glutathione, or Vernonia amygdalina: methanolic extracts of novel plants for the management of sickle cell anemia disease. BMC Complement Altern Med 2012; **12**:1-11.

83. MCCARTY MF. Potential utility of full-spectrum antioxidant therapy, citrulline, and dietary nitrate in the management of sickle cell disease. Med Hypotheses 2010; **74**:1055-1058.

84. CHAN AC, CHOW CK, CHIU D. Interaction of antioxidants and their implication in genetic anemia. Proc Soc Exp Biol Med 1999; **222**:274-282.

85. REITER RJ, TAN DX, SAINZ RM et al. Melatonin: reducing the toxicity and increasing the efficacy of drugs. J Pharm Pharmacol 2002; **54**:1299-1321.

86. HARDELAND R, PANDI-PERUMAL SR, CARDINALI DP. Melatonin. Int J Biochem Cell Biol 2006; **38**:313-316.

87. MAURIZ JL, COLLADO PS, VENEROSO C et al. A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and new perspectives. J Pineal Res 2013; **54**:1-14.

Tables

Table 1. Characterization of study group.

Characteristics	n = 10
Age (years: $\overline{X} \pm SD$)	27.4 ± 7.6
Gender [n (%)]	
Female	6 (60.0)
Male	4 (40.0)
Weight (kilograms: $\overline{X} \pm SD$)	55.3 ± 7.0
Beta globin haplotype profile [n (%)]	
Bantu/Bantu	3 (30.0)
Bantu/Benin	3 (30.0)
Bantu/Atipic	1 (10.0)
Benin/Atipic	2 (20.0)
Atipic/Atipic	1 (10.0)
*Medication use**	
HC^+ (mean dose: 23.7 ± 3.3)	3 (30.0)
HC^-	7 (70.0)
*Transfusional history** [n (%)]*	
0 - 10	0 (0.0)
11 - 20	0 (0.0)
21 - 30	3 (30.0)
31 - 40	0 (0.0)
> 40	7 (70.0)
Clinical history	
Number of pain crisis episodes per person in the last 12 months [n (%)]	
0 - 2	7 (70.0)
3 - 5	3 (30.0)
> 5	0 (0.0)
Number of infections [n (%)]	
0 - 2	10 (100.0)
3 - 5	0 (0.0)
> 5	0 (0.0)
Number of need of hospitalization per person in the last 12 months [n (%)]	
0 - 2	10 (100.0)
3 - 5	0 (0.0)
> 5	0 (0.0)

$\overline{X} \pm$ SD: mean ± standard deviation; n: number of patients; HC^+: patients under HC treatment; HC^-: patients not treated with hydroxycarbamide.
*All patients were under use of folic acid (5 mg/day).
**Transfused packed red blood cells units received to date

Table 2. Hydroxycarbamide influence on routine markers and on proposed biochemical parameters

Markers	HC^+ (n = 3)	HC^- (n = 7)	*P* value	Reference values
Age (years)	22.8 ± 4.7	30.3 ± 6.8	0.07#	-----
Weight (kilograms)	57.2 ± 7.7	55.6 ± 3.0	0.76*	-----
Hb F levels (%)	17.8 ± 8.4	8.1 ± 4.3	0.10*	0.0 - 1.0
Erythrocytes (million/mm^3)	2.4 ± 0.4	2.4 ± 0.5	0.96*	4.0 - 5.0
Hematocrit (%)	27.8 ± 2.4	24.5 ± 2.8	0.10*	36.0 - 47.0
Hemoglobin (g/dL)	9.5 ± 1.0	8.3 ± 0.7	0.11*	12.0 - 16.0
MCV (fl)	117.1 ± 15.3	100.6 ± 0.7	0.25#	80.0 - 100.0
MCH (pg)	40.1 ± 5.9	34.4 ± 1.9	0.16*	27.0 - 31.0
MCHC (g/dL)	34.1 ± 1.2	34.2 ± 1.7	0.98*	32.0 - 36.0
RDW (%)	16.3 ± 2.4	19.4 ± 5.7	0.60#	12.0 - 16.0
Leucocytes (million /mm^3)	9078.3 ± 4959.2	10100.0 ± 4504.4	0.60#	4000.0 - 11000.0
Platelets (million /mm^3)	335.5 ± 137.7	432.3 ± 108.9	0.32*	150.0 - 450.0
Reticulocytes (%)	6.1 ± 2.7	17.7 ± 9.3	0.01*	0.5 - 1.5
LDH (U/L)	387.0 ± 115.8	707.0 ± 186.5	0.01*	1.0 - 250.0
Ferritin (ng/mL)	369.1 ± 27.0	266.0 ± 226.2	0.43#	15.0 - 400.0
TB (mg/dL)	1.9 ± 0.9	5.6 ± 5.3	0.19#	< 1.0
DB (mg/dL)	0.5 ± 0.1	0.5 ± 0.2	0.91*	< 0.2
IB (mg/dL)	1.4 ± 0.8	5.0 ± 5.2	0.20#	< 0.8
AST (U/mL)	35.0 ± 6.7	54.6 ± 8.5	<0.01*	0.0 -40.0
ALT (U/mL)	24.4 ± 10.8	21.6 ± 6.0	0.70*	0.0 - 41.0
ALP (U/mL)	77.0 ± 16.5	40.0 ± 10.0	0.01*	40.0 - 130.0
Creatinin (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.51#	0.5 - 0.9
Potassium (mM)	4.5 ± 0.2	4.9 ± 0.2	0.07#	3.5 - 5.1
Hemolysis degree (%)	1.4 ± 0.6	1.5 ± 0.7	0.84*	-----
MDA (ng/mL)	7.6 ± 5.3	5.8 ± 3.8	0.63*	-----
CAT (U/mL)	12181.3 ± 2147.4	13011.7 ± 3909.2	0.68*	-----
GST (U/mL)	2.7 ± 0.4	2.5 ± 1.5	0.80*	-----
GPx (U/mL)	3.8 ± 1.6	2.2 ± 0.5	0.14*	-----
GR (U/mL)	1.1 ± 0.1	1.2 ± 0.5	0.79#	-----
G6PDH (U/mL)	2.1 ± 0.4	2.4 ± 1.1	0.98#	-----
SOD (U/mL)	2339.2 ± 556.6	1983.5 ± 709.4	0.43*	-----
GSH (µM)	854.3 ± 343.5	958.6 ± 237.3	0.65*	-----

Hb F: fetal hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; RDW: Red cell distribution width; LDH: lactate dehydrogenase; TB: total bilirubin; DB: direct bilirubin; IB: indirect bilirubin; AST: aspartate transaminase; ALT: alanine transaminase; AF: alkaline phosphatase; MDA: malondialdehyde; CAT: catalase; GST: glutathione *S*-transferase; GPx: glutathione peroxidase; GR: glutathione reductase; G6PDH: glucose-6-phosphate dehydrogenase; SOD: superoxide dismutase; GSH: glutathione

Data were expressed as mean ± standard deviation

*Comparisons were made by *t* test

#Comparisons were made by Mann-Whitney's test

Table 3. Results of unchanged antioxidant capacity markers among the studied groups

	CAT (U/mL)	GST (U/mL)	GR (U/mL)	G6PDH (U/mL)	GSH (µM)
G1	12750.6 ± 2860.9	2.7 ± 0.8	1.1 ± 0.3	1.9 ± 0.9	890.8 ± 301.4
G2	11204.8 ± 1993.8	2.8 ± 1.0	1.2 ± 0.4	1.9 ± 1.1	989.3 ± 222.0
G3	11415.4 ± 1635.8	2.7 ± 0.6	1.0 ± 0.1	1.7 ± 0.6	929.3 ± 320.0
G4	11180.4 ± 1058.7	2.9 ± 0.3	1.3 ± 0.3	2.2 ± 0.9	998.4 ± 122.9
G5	11624.2 ± 2679.7	2.9 ± 0.8	1.2 ± 0.4	1.9 ± 0.9	892.1 ± 318.0
G6	12157.4 ± 2300.7	2.9 ± 0.8	1.1 ± 0.3	1.9 ± 1.0	915.1 ± 287.8
G7	11788.7 ± 2656.7	3.0 ± 0.6	1.3 ± 0.4	2.0 ± 0.6	860.2 ± 178.9
G8	11177.9 ± 3346.4	2.9 ± 0.8	1.1 ± 0.4	2.0 ± 1.3	837.7 ± 316.2
G9	11499.6 ± 2241.1	3.1 ± 0.8	1.0 ± 0.2	2.2 ± 0.8	914.5 ± 355.0
G10	12537.5 ± 2816.4	3.0 ± 0.7	1.1 ± 0.2	2.2 ± 0.9	742.3 ± 102.0
G11	12669.7 ± 3028.1	3.1 ± 0.8	1.1 ± 0.3	1.8 ± 0.7	909.0 ± 27.5
	p = 0.30[#]	*p = 0.24*[#]	*p = 0.11*[#]	*p = 0.25**	*p = 0.10**

CAT: catalase; GST: glutathione *S*-transferase; GR: glutathione reductase; G6PDH: glucose-6-phosphate dehydrogenase; GSH: glutathione

G1: external control; G2: experimental control; G3: NAC treatment at 100 pM; G4: NAC treatment at 100 nM; G5: NAC treatment at 100 µM; G6: melatonin treatment at 100 pM; G7: melatonin treatment at 100 nM; G8: melatonin treatment at 100 µM; G9: NAC+melatonin treatment both at 100 pM; G10: NAC+melatonin treatment both at 100 nM; G11: NAC+melatonin treatment both at 100 µM. Each group was composed by N = 10 samples.

*Comparisons were made by Friedman's test

[#]Comparisons were made by repeated measures ANOVA

Figures

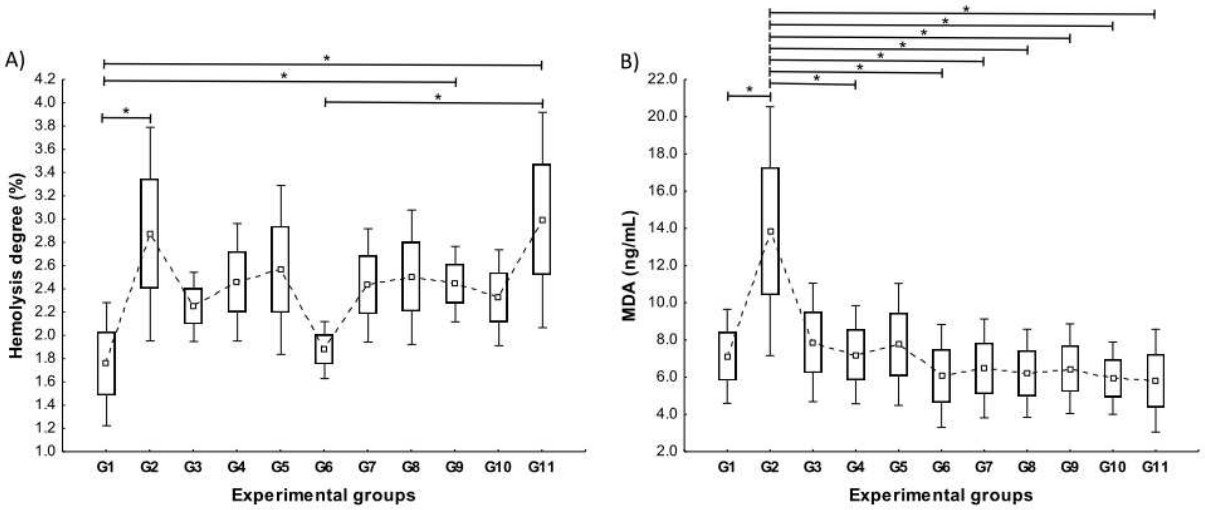

Fig. 1. Antioxidant treatment effects on hemolysis degree and lipid peroxidation. A) Increase of hemolysis degree due to incubation period and unexpected antioxidant treatment responses. B) MDA levels increment through incubation period, but effective antioxidant responses.
G1: external control; G2: experimental control; G3: NAC treatment at 100 pM; G4: NAC treatment at 100 nM; G5: NAC treatment at 100 μM; G6: melatonin treatment at 100 pM; G7: melatonin treatment at 100 nM; G8: melatonin treatment at 100 μM; G9: NAC+melatonin treatment both at 100 pM; G10: NAC+melatonin treatment both at 100 nM; G11: NAC+melatonin treatment both at 100 μM. Each group was composed by $N = 10$ samples. Data were expressed as mean ± standard error
*Indicates statistical differences ($p < 0.01$; Friedman's test, followed by Dunnett's test)

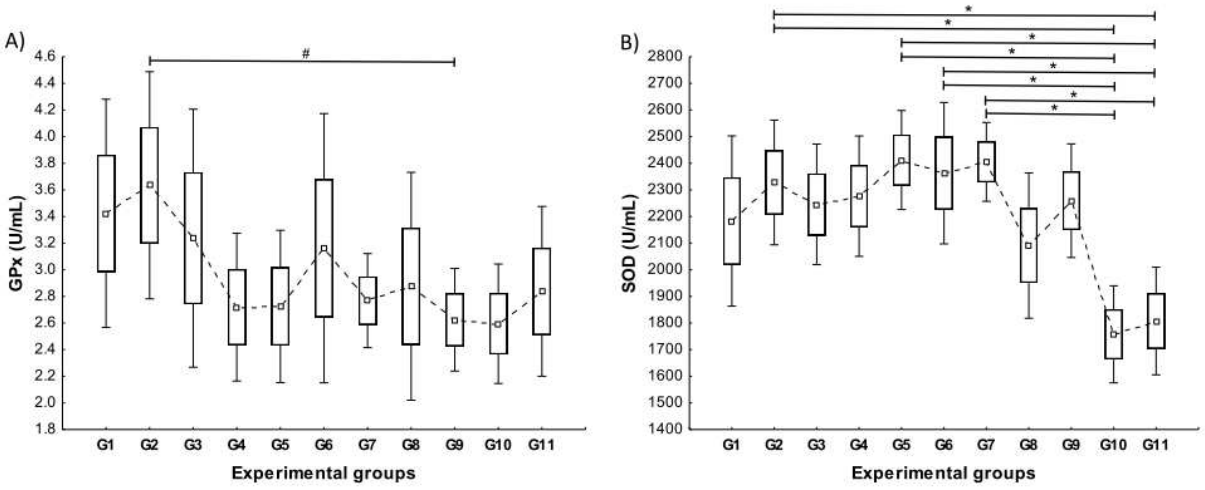

Fig. 2. Modulating effect on antioxidant enzymes. A) Decreased GPx activity in G9 compared to G2. B) G10 and G11 showed lower request of SOD activation in relation to G2, G5, G6 and G7.
G1: external control; G2: experimental control; G3: NAC treatment at 100 pM; G4: NAC treatment at 100 nM; G5: NAC treatment at 100 μM; G6: melatonin treatment at 100 pM; G7: melatonin treatment at 100 nM; G8: melatonin treatment at 100 μM; G9: NAC+melatonin treatment both at 100 pM; G10: NAC+melatonin treatment both at 100 nM; G11: NAC+melatonin treatment both at 100 μM. Each group was composed by $N = 10$ samples. Data were expressed as mean ± standard errors
#Indicates statistical differences ($p < 0.01$; repeated measures ANOVA followed by Tukey's test)
*Indicates statistical differences ($p < 0.01$; Friedman's test followed by Dunnett's test)

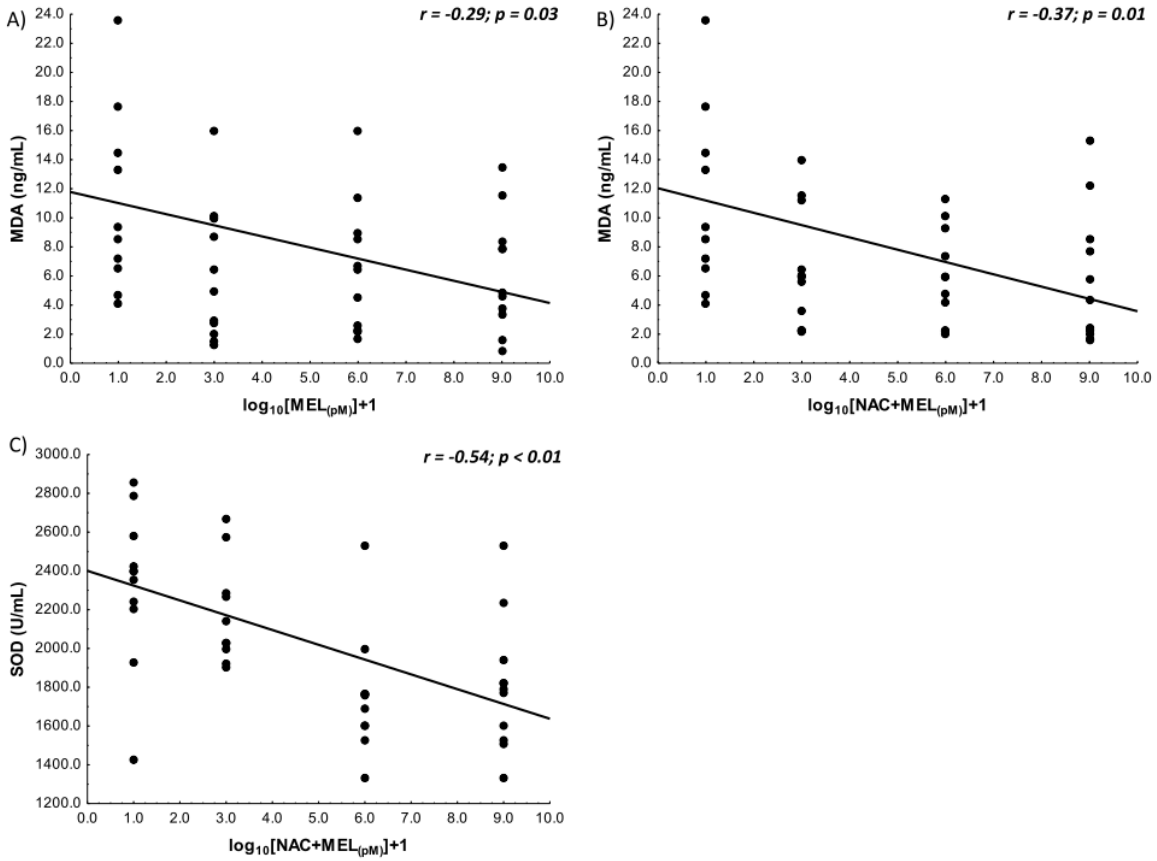

Fig. 3. Dose-dependent effects of antioxidant therapy. Lipid peroxidation level reductions according to MEL treatment (A) or NAC and MEL combination (B) doses. C) Negative correlation between combined antioxidant treatment (NAC+MEL) concentration and SOD activity.
All statistical analysis were made by Spearman's rank correlation test

Discussão

5. DISCUSSÃO

A AF tem significativa importância epidemiológica em virtude da prevalência, morbidade e mortalidade, sendo considerada um problema de saúde pública no Brasil. Os processos fisiopatológicos complexos e diversificados que caracterizam essa afecção genética desencadeiam manifestações clínicas variadas, desde anemia até recorrência de acidentes vasculares encefálicos, por exemplo, e estão intimamente ligados ao quadro de estresse oxidativo crônico e podem influenciar a resposta ao tratamento (STUART; NAGEL, 2004; FRENETTE; ATWEH, 2007; BANDEIRA et al., 2007; REES; WILLIAMS; GLADWIN, 2010). Dessa forma, estudos ligados a esta afecção que permitam o estabelecimento de relações entre o perfil genético do portador com fatores associados à fisiopatologia da doença, tanto genéticos quanto bioquímicos e a resposta ao tratamento, possibilitam melhor entendimento dos eventos clínicos e, podem direcionar ao tratamento individualizado aos pacientes. Visto que estes fatores são potenciais moduladores da expressão fenotípica na AF, neste trabalho foram avaliados, em homozigotos para Hb S, marcadores genéticos associados aos processos fisiopatológicos da AF, marcadores bioquímicos relacionados ao estresse oxidativo e a evolução clínica dos pacientes, correlacionando-os com uso de HU.

Diante do exposto acima, foi investigada a frequência de diferentes polimorfismos genéticos que alteram a expressão de enzimas envolvidas no metabolismo da homocisteína dependente de folato (*MTHFR* e *CBS*) e metabolismo da adenosina (*ADA*). A frequência desses polimorfismos tem sido caracterizada como étnico-dependente e amplamente divergente entre as populações em todo o mundo (FRANCO et al., 1998; NUNES et al., 2011; LIEW; GUPTA, 2015). Por exemplo, a prevalência da homozigose para *MTHFR* (677TT) pode variar entre 1 e 30% na população geral (DURAND et al., 2001), enquanto que a homozigose da inserção no gene *CBS* (68 pb-II) apresenta uma variação muito mais baixa, variando de ausente a 4% (FRANCO et al., 1998). No entanto, os dados de frequência dos genótipos obtidos para esses polimorfismos em nosso estudo está de acordo com estudos anteriores em brasileiros com AF (COUTO et al., 2004; MOREIRA et al., 2006; ALVES JACOB; DA CUNHA; BONINI-DOMINGOS, 2011). No caso do polimorfismo no gene *ADA*, a homozigose é rara e, este é o primeiro trabalho que relata a frequência do mesmo em pacientes com AF. Entretanto, a frequência alelo mutante (22A) é estimada em 0,06 para populações do Ocidente (CONCETTI et al., 2015), valor pouco menor ao encontrado no grupo de pessoas com AF estudado (0,08).

Segundo Fertrin e Costa (2010) o desenvolvimento da biologia molecular permitiu, de forma significativa, a identificação de vários polimorfismos genéticos responsáveis pela diversidade clínica observada na AF. Consequentemente, vários estudos investigaram a associação entre polimorfismos de nucleotídeo único (SNPs) com risco de desenvolvimento de diferentes manifestações clínicas. Além disso, a compreensão dos processos fisiopatológicos da AF tem crescido gradativamente e, entre as novas evidências, estresse oxidativo tem sido cada vez relacionado a tais processos (REES; GIBSON, 2012), tanto como causa quanto como consequência da inflamação, hemólise, vasculopatia, vaso-oclusão, infecção, lesões devido a processos de isquemia/reperfusão, entre outros (REES; WILLIAMS; GLADWIN, 2010). Porém, estudos que relacionam tais marcadores genéticos e bioquímicos são escassos e restritos aos haplótipos da β^S-globina (RUSANOVA et al., 2010; CARVALHO-DOS SANTOS et al., 2012; SILVA et al., 2013a), mutações no gene *HFE* (VIANA-BARACIOLI et al., 2011) e nos genes responsáveis pela síntese em diferentes classes da enzima glutationa *S*-transferase (SILVA et al., 2011).

Dessa forma, o presente estudo proporciona oportunidade única em que ambos, fatores genéticos e marcadores do estresse oxidativo, foram simultaneamente avaliados em um grupo de pessoas com AF. Entretanto, o único efeito observado foi o sinergismo entre os alelos mutantes *677T* e *"I"* (inserção de 68 pb) sobre decréscimo dos níveis de GSH, mecanismo o qual necessita de mais estudos para melhor entendimento. A relação destes polimorfismos com parâmetros clínicos e uso de HU também foi investigada. Foram constatados efeitos prejudicial da coerança de *HBB*S* com *MTHFR* sobre os níveis de HbF de indivíduos não tratados com HU; e com *CBS* sobre valores de LDH, AST e GGT naqueles pacientes sob uso de HU. Estas respostas relatadas pela primeira vez na literatura e, sugerem um envolvimento complexo das vias metabólicas afetadas pelos polimorfismos na fisiopatologia da AF.

Apesar de bem fundamentada a hipótese de que o aumento dos níveis de adenosina causado pela presença do polimorfismo no gene *ADA* acarretaria maior geração de ERO intraeritrocitária, baseado no mecanismo descrito por Zhang et al. (2011), não foi encontrada qualquer influência da coerança de *HBB*S* e *ADA* (22A) sobre os marcadores do estresse oxidativo avaliados. No entanto, este é o primeiro estudo relatando a frequência deste polimorfismo em indivíduos com AF, dessa forma estudos posteriores com número amostral maior e associação com manifestações clínicas poderão predizer o risco relativo gerado pela coerança desse polimorfismo e a homozigose para HbS na progressão clínica da doença.

Muitas pesquisas priorizam a identificação de variações genéticas inter-individuais, subjacentes às diferentes respostas farmacológicas ao uso de fármacos (COLLINS et al.,

2003). Na AF, esse paradigma está sendo aplicado na tentativa de desenvolver terapias individualizadas (STEINBERG, 2005). Porém, com o aumento das evidências acerca da importância do estresse oxidativo na patogênese da AF, o desenvolvimento de pesquisas com enfoque em possíveis terapias antioxidantes está sendo um objetivo valioso, promissor e crescente para o tratamento da AF (MCCARTY, 2010; VICHINSKY, 2012; IMAGA, 2013; SILVA et al., 2013b). Neste contexto, este estudo fornece a primeira evidência para apoiar os efeitos favoráveis da MEL para esta finalidade.

Conclusões

6. CONCLUSÕES

Os resultados obtidos permitiram as seguintes conclusões:

- As frequências obtidas para os polimorfismos nos genes mitelenotetrahidrofolato redutase (*MTHFR*) e cistationina beta-sintase (*CBS*) foram similares às relatadas em estudos com pessoas com anemia falciforme (AF). Para a mutação avaliada no gene *ADA*, foi moderadamente maior que a estimativa para populações do Ocidente;
- Foi encontrado efeito sinérgico da herança concomitante dos polimorfismos nos genes *MTHFR* e *CBS* sobre o decréscimo dos níveis de glutationa reduzida (GSH), enquanto o defeito genético avaliado no gene adenosina deaminase (*ADA*) não influenciou significativamente os parâmetros do estresse oxidativo estudados;
- Foi confirmado efeito favorável do uso de hidroxiureia (HU) sobre níveis de peroxidação lipídica, quantidade de HbF, índices hematológicos e marcadores de hemólise na maioria dos subgrupos avaliados, exceto para os pacientes com pelo menos um cromossomo mutante para *MTHFR* (677T). Estes apresentaram atividades aumentadas das enzimas lactato desidrogenase, aspartato transaminase e gama-glutamil transferase quando comparados aos homozigotos selvagem (CC) também sob uso do fármaco. Já nos indivíduos não tratados com HU, foi constatado um efeito deletério causado pela inserção no gene *CBS* sobre os níveis de hemoglobina Fetal (HbF);
- Foi demonstrado pela primeira vez na literatura o efeito antioxidante individual da melatonina ou associada à *N*-acetilcisteína, usando suspensão celular de hemácias falcêmicas como modelo experimental.

Referências

REFERÊNCIAS

ALVES JACOB, M.; DA CUNHA, B.C.; BONINI-DOMINGOS, C.R. The 844ins68 cystathionine beta-synthase and C677T MTHFR gene polymorphism and the vaso-occlusive event risk in sickle cell disease. **Archives of Medical Science**, v. 7, p. 97-101, 2011.

AMER, J.; GHOTI, H.; RACHMILEWITZ, E. et al. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. **British Journal of Haematology**, v. 132, p. 108-113, 2006.

ASLAN, M.; THORNLEY-BROWN, D.; FREEMAN, B.A. Reactive species in sickle cell disease. **Annals of the New York Academy of Sciences**, v. 899:375-91., p. 375-391, 2000.

BALLAS, S. K.; KESEN, M.R.; GOLDBERG, M.F. et al. Beyond the definitions of the phenotypic complications of sickle cell disease: an update on management. **The Scientific World Journal**, v. 2012, p. 9495352012.

BANDEIRA, F. M. G. C.; BEZERRA, M.A.C.; SANTOS, M.N.N. et al. Importância dos programas de triagem para o gene da hemoglobina S. **Revista Brasileira De Hematologia e Hemoterapia**, v. 29, p. 179-184, 2007.

BANERJEE, T.; KUYPERS, F.A. Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells. **British Journal of Haematology**, v. 124, p. 391-402, 2004.

BARBOSA, L. F.; DE MEDEIROS, M.H.G.; AUGUSTO, O. Danos oxidativos e neurodegeneração: o quê aprendemos com animais transgênicos e nocautes? **Química Nova**, v. 29, p. 1352-1360, 2006.

BATTISTUZZI, G.; IUDICONE, P.; SANTOLAMAZZA, P. et al. Activity of adenosine deaminase allelic forms in intact erythrocytes and in lymphocytes. **Annals of Human Genetics**, v. 45, p. 15-19, 1981.

BERTHOLO, L. C.; MOREIRA, H.W. Focalização isoelétrica na identificação das hemoglobinas. **Jornal Brasileiro De Patologia e Medicina Laboratorial**, v. 42, p. 163-168, 2006.

BEUTLER, E. Red Cell Metabolism: A Manual of Biochemical Methods. **Annals of Internal Medicine**, v. 83, -919, 1975.

BEUTLER, E. Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies. **Journal of Clinical Investigation**, v. 48, p. 1957-1966, 1969.

BEUTLER, E. G6PD deficiency. **Blood**, v. 84, p. 3613-3636, 1994.

BONINI-DOMINGOS, C.R. **Metodologias Laboratoriais para o diagnóstico de Hemoglobinopatias e Talassemias,** São José do Rio Preto: HN Editora, 2006.

BUNN, H. F. Pathogenesis and treatment of sickle cell disease. **New England Journal of Medicine**, v. 337, p. 762-769, 1997.

BUNN, H.F.;Forget, B. G. **Hemoglobin: Molecular Genetics and Clinical Aspects,** Philadelphia, Saunders, 1986.

CARRELL, R. W.; WINTERBOURN, C.C.; RACHMILEWITZ, E.A. Activated oxygen and haemolysis. **British Journal of Haematology**, v. 30, p. 259-264, 1975.

CARVALHO-DOS SANTOS, B. S.; AS-ELIAS, D.B.; DA SILVA-ROCHA, L.B. et al. Impact of beta(S)-globin haplotypes on oxidative stress in patients with sickle cell anemia in steady state. **Archives of Medical Research**, v. 43, p. 536-540, 2012.

CHAVES, M. A.; LEONART, M.S.; DO NASCIMENTO, A.J. Oxidative process in erythrocytes of individuals with hemoglobin S. **Hematology**, v. 13, p. 187-192, 2008.

CHO, S.; JOH, T.H.; BAIK, H.H. et al. Melatonin administration protects CA1 hippocampal neurons after transient forebrain ischemia in rats. **Brain Research**, v. 755, p. 335-338, 1997.

COLLINS, F. S.; GREEN, E.D.; GUTTMACHER, A.E. et al. A vision for the future of genomics research. **Nature**, v. 422, p. 835-847, 2003.

CONCETTI, F.; CARPI, F.M.; NABISSI, M. et al. The functional polymorphism rs73598374:G>A (p.Asp8Asn) of the ADA gene is associated with telomerase activity and leukocyte telomere length. **European Journal of Human Genetics**, v. 23, p. 267-270, 2015.

COUTO, F. D.; BOAS, W.V.; LYRA, I. et al. A C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism and G20210A mutation in the prothrombin gene of sickle cell anemia patients from Northeast Brazil. **Hemoglobin**, v. 28, p. 237-241, 2004.

DAMANHOURI, G. A.; JARULLAH, J.; MAROUF, S. et al. Clinical biomarkers in sickle cell disease. **Saudi Journal of Biological Sciences**, v. 22, p. 24-31, 2015.

DE ALMEIDA, E. A.; MARQUES, S.A.; KLITZKE, C.F. et al. DNA damage in digestive gland and mantle tissue of the mussel Perna perna. **Comparative Biochemistry and Physiology Part C: Toxicology**, v. 135C, p. 295-303, 2003.

DE ALMEIDA, E. A.; MIYAMOTO, S.; BAINY, A.C. et al. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels Perna perna exposed to different metals. **Marine Pollution Bulletin**, v. 49, p. 386-392, 2004.

DRAGANI, A.; FALCO, A.; SANTILLI, F. et al. Oxidative stress and platelet activation in subjects with moderate hyperhomocysteinaemia due to MTHFR 677 C-->T polymorphism. **Thrombosis and Haemostasis**, v. 108, p. 533-542, 2012.

DURAND, P.; PROST, M.; LOREAU, N. et al. Impaired homocysteine metabolism and atherothrombotic disease. **Laboratory Investigation**, v. 81, p. 645-672, 2001.

ESTERBAUER, H.; ZOLLNER, H. Methods for determination of aldehydic lipid peroxidation products. **Free Radical Biology and Medicine**, v. 7, p. 197-203, 1989.

FERTRIN, K. Y.; COSTA, F.F. Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment. **Expert Review of Hematology**, v. 3, p. 443-458, 2010.

FINKEL, T.; HOLBROOK, N.J. Oxidants, oxidative stress and the biology of ageing. **Nature**, v. 408, p. 239-247, 2000.

FINKELSTEIN, J. D. Methionine metabolism in mammals. **The Journal of Nutritional Biochemistry**, v. 1, p. 228-237, 1990.

FRANCO, R. F.; ELION, J.; LAVINHA, J. et al. Heterogeneous ethnic distribution of the 844ins68 in the cystathionine beta-synthase gene. **Human Heredity**, v. 48, p. 338-342, 1998.

FREEMAN, B. A.; CRAPO, J.D. Biology of disease: free radicals and tissue injury. **Laboratory Investigation**, v. 47, p. 412-426, 1982.

FREI, B.; STOCKER, R.; AMES, B.N. Antioxidant defenses and lipid peroxidation in human blood plasma. **Proceedings of the National Academy of Sciences of the United States of America**, v. 85, p. 9748-9752, 1988.

FRENETTE, P. S.; ATWEH, G.F. Sickle cell disease: old discoveries, new concepts, and future promise. **Journal of Clinical Investigation**, v. 117, p. 850-858, 2007.

FRONTICELLI, C.; GOLD, R. Conformational relevance of the beta6Glu replaced by Val mutation in the beta subunits and in the beta(1-55) and beta(1-30) peptides of hemoglobin S. **Journal of Biological Chemistry**, v. 251, p. 4968-4972, 1976.

FROSST, P.; BLOM, H.J.; MILOS, R. et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. **Nature Genetics**, v. 10, p. 111-113, 1995.

GALANO, A.; TAN, D.X.; REITER, R.J. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. **Journal of Pineal Research**, v. 54, p. 245-257, 2013.

GIZI, A.; PAPASSOTIRIOU, I.; APOSTOLAKOU, F. et al. Assessment of oxidative stress in patients with sickle cell disease: The glutathione system and the oxidant-antioxidant status. **Blood Cells Molecules and Diseases**, v. 46, p. 220-225, 2011.

GLADWIN, M. T. Adenosine receptor crossroads in sickle cell disease. **Nature Medicine**, v. 17, p. 38-40, 2011.

GLADWIN, M. T.; VICHINSKY, E. Pulmonary complications of sickle cell disease. **New England Journal of Medicine**, v. 359, p. 2254-2265, 2008.

GRINBERG, L.; FIBACH, E.; AMER, J. et al. N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. **Free Radical Biology and Medicine**, v. 38, p. 136-145, 2005.

HALLIWELL, B. Antioxidants and human disease: a general introduction. **Nutrition Reviews**, v. 55, p. S44-S49, 1997.

HASKO, G.; LINDEN, J.; CRONSTEIN, B. et al. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. **Nature Reviews Drug Discovery**, v. 7, p. 759-770, 2008.

HEBBEL, R. P. Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology. **Blood**, v. 77, p. 214-237, 1991.

HEBBEL, R. P.; MORGAN, W.T.; EATON, J.W. et al. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. **Proceedings of the National Academy of Sciences of the United States of America**, v. 85, p. 237-241, 1988.

HIGGS, D. R.; ALDRIDGE, B.E.; LAMB, J. et al. The interaction of alpha-thalassemia and homozygous sickle-cell disease. **New England Journal of Medicine**, v. 306, p. 1441-1446, 1982.

IMAGA, N. A. Phytomedicines and nutraceuticals: alternative therapeutics for sickle cell anemia. **Scientific World Journal**, v. 2013:269659. doi: 10.1155/2013/269659. Epub;%2013 Feb 14., p. 2696592013.

INSTRUCTION MANUAL OF BIO-RAD. Variante ß-Thalassemia Short Program, Instruction Manual. 2006.

JACQUES, P. F.; BOSTOM, A.G.; WILLIAMS, R.R. et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. **Circulation**, v. 93, p. 7-9, 1996.

KAR, B. C.; SATAPATHY, R.K.; KULOZIK, A.E. et al. Sickle cell disease in Orissa State, India. **Lancet**, v. 2, p. 1198-1201, 1986.

KEEN, J. H.; HABIG, W.H.; JAKOBY, W.B. Mechanism for the several activities of the glutathione S-transferases. **Journal of Biological Chemistry**, v. 251, p. 6183-6188, 1976.

KLINGS, E. S.; FARBER, H.W. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. **Respiratory Research**, v. 2, p. 280-285, 2001.

LI, S. D.; SU, Y.D.; LI, M. et al. Hemin-mediated hemolysis in erythrocytes: effects of ascorbic acid and glutathione. **Acta Biochimica Et Biophysica Sinica**, v. 38, p. 63-69, 2006.

LIEW, S.; GUPTA, E.D. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: Epidemiology, metabolism and the associated diseases. **European Journal of Medical Genetics**, v. 58, p. 1-10, 2015.

LU, S. C. Glutathione synthesis. **Biochimica Et Biophysica Acta**, v. 1830, p. 3143-3153, 2013.

LU, S. C.; MATO, J.M. S-adenosylmethionine in liver health, injury, and cancer. **Physiological Reviews**, v. 92, p. 1515-1542, 2012.

MAHAFFEY, K. W.; PUMA, J.A.; BARBAGELATA, N.A. et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. **Journal of the American College of Cardiology**, v. 34, p. 1711-1720, 1999.

MARENGO-ROWE, A. J. Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. **Journal of Clinical Pathology**, v. 18, p. 790-792, 1965.

MCBEAN, G. J. The transsulfuration pathway: a source of cysteine for glutathione in astrocytes. **Amino Acids**, v. 42, p. 199-205, 2012.

MCCARTY, M. F. Potential utility of full-spectrum antioxidant therapy, citrulline, and dietary nitrate in the management of sickle cell disease. **Medical Hypotheses**, v. 74, p. 1055-1058, 2010.

MCCORD, J. M.; FRIDOVICH, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). **Journal of Biological Chemistry**, v. 244, p. 6049-6055, 1969.

MILLER, E.; MROWICKA, M.; MALINOWSKA, K. et al. The effects of whole-body cryotherapy and melatonin supplementation on total antioxidative status and some antioxidative enzymes in multiple sclerosis patients. **Polski Merkuriusz Lekarski**, v. 31, p. 150-153, 2011.

MOREIRA, N. F.; LOURENCO, D.M.; NOGUTI, M.A. et al. The clinical impact of MTHFR polymorphism on the vascular complications of sickle cell disease. **Brazilian Journal Of Medical and Biological Research**, v. 39, p. 1291-1295, 2006.

MORRIS, C. R.; SUH, J.H.; HAGAR, W. et al. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. **Blood**, v. 111, p. 402-410, 2008.

NAOUM, P.C. **Eletroforese, técnicas e diagnósticos,** São Paulo: Santos, 1990.

NISHANK, S. S.; SINGH, M.P.; YADAV, R. Clinical impact of factor V Leiden, prothrombin G20210A, and MTHFR C677T mutations among sickle cell disease patients of Central India. **European Journal of Haematology**, v. 91, p. 462-466, 2013.

NOGUCHI, C. T.; SCHECHTER, A.N.; RODGERS, G.P. Sickle cell disease pathophysiology. **Baillière's Clinical Haematology**, v. 6, p. 57-91, 1993.

NUNES, D. P.; SPEGIORIN, L.C.; MATTOS, C.C. et al. The ADA*2 allele of the adenosine deaminase gene (20q13.11) and recurrent spontaneous abortions: an age-dependent association. **Clinics (Sao Paulo)**, v. 66, p. 1929-1933, 2011.

NUR, E.; BIEMOND, B.J.; OTTEN, H.M. et al. Oxidative stress in sickle cell disease; pathophysiology and potential implications for disease management. **American Journal of Hematology**, v. 86, p. 484-489, 2011.

NUR, E.; BRANDJES, D.P.; TEERLINK, T. et al. N-acetylcysteine reduces oxidative stress in sickle cell patients. **Annals of Hematology**, v. 91, p. 1097-1105, 2012.

PECHANOVA, O.; ZICHA, J.; PAULIS, L. et al. The effect of N-acetylcysteine and melatonin in adult spontaneously hypertensive rats with established hypertension. **European Journal of Pharmacology**, v. 561, p. 129-136, 2007.

PERRONE, S.; TATARANNO, M.L.; STAZZONI, G. et al. Oxidative injury in neonatal erythrocytes. **Journal of Maternal-Fetal and Neonatal Medicine**, v. 25, p. 104-108, 2012.

RAHIMI, Z.; MALEK-KHOSRAVI, S.; RAHIMI, Z. et al. MTHFR C677T and eNOS G894T variants in preeclamptic women: Contribution to lipid peroxidation and oxidative stress. **Clinical Biochemistry**, v. 46, p. 143-147, 2013.

REES, D. C.; GIBSON, J.S. Biomarkers in sickle cell disease. **British Journal of Haematology**, v. 156, p. 433-445, 2012.

REES, D. C.; WILLIAMS, T.N.; GLADWIN, M.T. Sickle-cell disease. **Lancet**, v. 376, p. 2018-2031, 2010.

REID, M.; BADALOO, A.; FORRESTER, T. et al. In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. **American Journal of Physiology - Endocrinology and Metabolism**, v. 291, p. E73-E79, 2006.

REITER, R. J.; TAN, D.X.; MAYO, J.C. et al. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. **Acta Biochimica Polonica**, v. 50, p. 1129-1146, 2003.

REITER, R. J.; TAN, D.X.; ROSALES-CORRAL, S. et al. The universal nature, unequal distribution and antioxidant functions of melatonin and its derivatives. **Mini-Reviews in Medicinal Chemistry**, v. 13, p. 373-384, 2013.

RICE-EVANS, C.; OMORPHOS, S.C.; BAYSAL, E. Sickle cell membranes and oxidative damage. **Biochemical Journal**, v. 237, p. 265-269, 1986.

RICHARD, E.; DESVIAT, L.R.; UGARTE, M. et al. Oxidative stress and apoptosis in homocystinuria patients with genetic remethylation defects. **Journal of Cellular Biochemistry**, v. 114, p. 183-191, 2013.

RIKSEN, N. P.; FRANKE, B.; VAN DEN, B.P. et al. The 22G>A polymorphism in the adenosine deaminase gene impairs catalytic function but does not affect reactive hyperaemia in humans in vivo. **Pharmacogenetics and Genomics**, v. 18, p. 843-846, 2008.

RODRIGUEZ-ARIZA, A.; TORIBIO, F.; LOPEZBAREA, J. Rapid-Determination of Glutathione Status in Fish Liver Using High-Performance Liquid-Chromatography and Electrochemical Detection. **Journal of Chromatography B-Biomedical Applications**, v. 656, p. 311-318, 1994.

RUSANOVA, I.; ESCAMES, G.; COSSIO, G. et al. Oxidative stress status, clinical outcome, and beta-globin gene cluster haplotypes in pediatric patients with sickle cell disease. **European Journal of Haematology**, v. 85, p. 529-537, 2010.

SAFRANOW, K.; RZEUSKI, R.; BINCZAK-KULETA, A. et al. ADA*2 allele of the adenosine deaminase gene may protect against coronary artery disease. **Cardiology**, v. 108, p. 275-281, 2007.

SAHNA, E.; PARLAKPINAR, H.; OZTURK, F. et al. The protective effects of physiological and pharmacological concentrations of melatonin on renal ischemia-reperfusion injury in rats. **Urological Research**, v. 31, p. 188-193, 2003.

SAIKI, R. K.; SCHARF, S.; FALOONA, F. et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. **Science**, v. 230, p. 1350-1354, 1985.

SAMBROOK, J.; Fritsch, E. F.;Manatis, T. **Molecular Cloning: A Laboratory Manual,** ed.2, Cold Spring: Harbor Laboratory Press, 1989.

SAMUEL, R. E.; SALMON, E.D.; BRIEHL, R.W. Nucleation and growth of fibres and gel formation in sickle cell haemoglobin. **Nature**, v. 345, p. 833-835, 1990.

SCHAFER, F. Q.; BUETTNER, G.R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. **Free Radical Biology and Medicine**, v. 30, p. 1191-1212, 2001.

SEBASTIO, G.; SPERANDEO, M.P.; PANICO, M. et al. The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations. **American Journal of Human Genetics**, v. 56, p. 1324-1333, 1995.

SELHUB, J. Homocysteine metabolism. **Annual Review of Nutrition**, v. 19:217-46., p. 217-246, 1999.

SEWERYNEK, E.; REITER, R.J.; MELCHIORRI, D. et al. Oxidative damage in the liver induced by ischemia-reperfusion: protection by melatonin. **Hepatogastroenterology**, v. 43, p. 898-905, 1996.

SHENG, K.; SHARIFF, M.; HEBBEL, R.P. Comparative oxidation of hemoglobins A and S. **Blood**, v. 91, p. 3467-3470, 1998.

SHIMAUTI, E. L.; SILVA, D.G.; DE ALMEIDA, E.A. et al. Serum melatonin level and oxidative stress in sickle cell anemia. **Blood Cells Molecules and Diseases**, v. 45, p. 297-301, 2010.

SIEMS, W. G.; SOMMERBURG, O.; GRUNE, T. Erythrocyte free radical and energy metabolism. **Clinical Nephrology**, v. 53, p. S9-17, 2000.

SIES, H.; KOCH, O.R.; MARTINO, E. et al. Increased biliary glutathione disulfide release in chronically ethanol-treated rats. **FEBS Letter**, v. 103, p. 287-290, 1979.

SILVA, D. G.; BELINI, J.E.; CARROCINI, G.C. et al. Genetic and biochemical markers of hydroxyurea therapeutic response in sickle cell anemia. **BMC Medical Genetics**, v. 14:108. doi: 10.1186/1471-2350-14-108., p. 108-114, 2013a.

SILVA, D. G.; BELINI, J.E.; TORRES, L.S. et al. Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia. **Blood Cells Molecules and Diseases**, v. 47, p. 23-28, 2011.

SILVA, D. G.; BELINI-JUNIOR E.; DE ALMEIDA, E.A. et al. Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies. **Free Radical Biology and Medicine**, v. 65, p. 1101-1109, 2013b.

SILVESTRONI, E.; BIANCO, I. Screening for microcytemia in Italy: analysis of data collected in the past 30 years. **American Journal of Human Genetics**, v. 27, p. 198-212, 1975.

STEINBERG, M. H. Predicting clinical severity in sickle cell anaemia. **British Journal of Haematology**, v. 129, p. 465-481, 2005.

STEINBERG, M. H. Genetic etiologies for phenotypic diversity in sickle cell anemia. **The Scientific World Journal**, v. 9, p. 46-67, 2009.

STEINBERG, M. H.; ADEWOYE, A.H. Modifier genes and sickle cell anemia. **Current Opinion in Hematology**, v. 13, p. 131-136, 2006.

STEINBERG, M. H.; SEBASTIANI, P. Genetic modifiers of sickle cell disease. **American Journal of Hematology**, v. 87, p. 795-803, 2012.

STUART, M. J.; NAGEL, R.L. Sickle-cell disease. **Lancet**, v. 364, p. 1343-1360, 2004.

SUTTON, M.; BOUHASSIRA, E.E.; NAGEL, R.L. Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes. **American Journal of Hematology**, v. 32, p. 66-69, 1989.

TAN, D. X.; MANCHESTER, L.C.; REITER, R.J. et al. Ischemia/reperfusion-induced arrhythmias in the isolated rat heart: prevention by melatonin. **Journal of Pineal Research**, v. 25, p. 184-191, 1998.

TATUM, V. L.; CHOW, C.K. Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress. **Free Radical Research**, v. 25, p. 133-139, 1996.

THOMAS, S. R.; DAVIES, M.J.; STOCKER, R. Oxidation and antioxidation of human low-density lipoprotein and plasma exposed to 3-morpholinosydnonimine and reagent peroxynitrite. **Chemical Research in Toxicology**, v. 11, p. 484-494, 1998.

TRABETTI, E. Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk. **Journal of Applied Genetics**, v. 49, p. 267-282, 2008.

TSAI, M. Y.; BIGNELL, M.; SCHWICHTENBERG, K. et al. High prevalence of a mutation in the cystathionine beta-synthase gene. **American Journal of Human Genetics**, v. 59, p. 1262-1267, 1996.

TSAI, M. Y.; YANG, F.; BIGNELL, M. et al. Relation between plasma homocysteine concentration, the 844ins68 variant of the cystathionine beta-synthase gene, and pyridoxal-5'-phosphate concentration. **Molecular Genetics and Metabolism**, v. 67, p. 352-356, 1999.

VAN ZWIETEN R.; VERHOEVEN, A.J.; ROOS, D. Inborn defects in the antioxidant systems of human red blood cells. **Free Radical Biology and Medicine**, v. 67, p. 377-386, 2014.

VELLA, F. Acid-agar gel electrophoresis of human hemoglobins. **American Journal of Clinical Pathology**, v. 49, p. 440-442, 1968.

VIANA-BARACIOLI, L. M.; TUKAMOTO JUNIOR, N.C.; RICCI, J.O. et al. Comparison of oxidative stress and the frequency of polymorphisms in the HFE gene between hemoglobin S trait blood donors and sickle cell disease patients. **Genetics and Molecular Research**, v. 10, p. 3446-3454, 2011.

VICHINSKY, E. Emerging 'A' therapies in hemoglobinopathies: agonists, antagonists, antioxidants, and arginine. **Hematology American Society of Hematology Education Program**, v. 2012:271-5. doi: 10.1182/asheducation-2012.1.271., p. 271-275, 2012.

WEATHERALL, D. J.; CLEGG, J.B. Inherited haemoglobin disorders: an increasing global health problem. **Bulletin of the World Health Organization**, v. 79, p. 704-712, 2001.

WISHNER, B. C.; WARD, K.B.; LATTMAN, E.E. et al. Crystal structure of sickle-cell deoxyhemoglobin at 5 A resolution. **Journal of Molecular Biology**, v. 98, p. 179-194, 1975.

ZHANG, Y.; DAI, Y.; WEN, J. et al. Detrimental effects of adenosine signaling in sickle cell disease. **Nature Medicine**, v. 17, p. 79-86, 2011.

ZHANG, Y.; XIA, Y. Adenosine signaling in normal and sickle erythrocytes and beyond. **Microbes and Infection**, v. 14, p. 863-873, 2012.

Apêndices

APÊNCIDES

APÊNDICE A - Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Comitê de Ética em Pesquisa
IBILCE-UNESP

Termo de Consentimento Livre e Esclarecido - TCLE

(Conselho Nacional de Saúde, Resolução 196/96)

Você está sendo convidado a participar como voluntário do projeto de pesquisa "Estresse oxidativo na anemia falciforme: antioxidantes endógenos como possíveis alvos terapêuticos" sob responsabilidade do pesquisador M.Sc. Danilo Grünig Humberto da Silva. O estudo será realizado com amostras de sangue periférico para avaliar, em portadores da anemia falciforme, marcadores genéticos e do estresse oxidativo e da capacidade antioxidante, no intuito de validar a atuação da melatonina como importante antioxidante e seu uso, assim como de outros antioxidantes endógenos, como possíveis alvos terapêuticos. O risco é considerado mínimo, caracterizado pela possibilidade de mancha roxa no local da picada da agulha durante coleta da amostra de sangue. Você poderá consultar o pesquisador responsável em qualquer época, pessoalmente ou pelo telefone da instituição, para esclarecimento de qualquer dúvida. Você está livre para, a qualquer momento, deixar de participar da pesquisa. Todas as informações por você fornecidas e os resultados obtidos serão mantidos em sigilo e, estes últimos só serão utilizados para divulgação em reuniões e revistas científicas. Você será informado de todos os resultados obtidos, independentemente do fato destes poderem mudar seu consentimento em participar da pesquisa. Você não terá quaisquer benefícios ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa. Este estudo é importante porque seus resultados fornecerão informações para o possível desenvolvimento de novos tratamentos e/ou de terapia individualizada na anemia falciforme. O material biológico cedido será armazenado e você poderá ser chamado para dar a sua autorização para novo(s) projeto(s). Caso isso seja impossível, seu material biológico somente será utilizado mediante aprovação pelo CEP ou pela CONEP, em cumprimento à Resolução CNS 347/2005.

Diante das explicações, se você concorda em participar deste projeto, coloque sua assinatura a seguir e forneça os dados solicitados.

Nome:_____R.G._____

Endereço:_____Fone:_____

_____,_____de_____de 20

_____ _____

Usuário ou responsável legal Pesquisador responsável

OBS.: Termo apresenta duas vias, uma destinada ao usuário ou seu representante e a outra ao pesquisador

Nome do Pesquisador: M.Sc. Danilo Grünig Humberto da Silva **Cargo/Função:** Biólogo/Doutorando

Instituição: Universidade Estadual Paulista/Instituto de Biociências, Letras e Ciências Exatas

Endereço: Rua Cristóvão Colombo, 2265 – Jardim Nazareth – CEP: 15054-000

Projeto submetido ao Comitê de Ética em Pesquisa do IBILCE/UNESP

São José do Rio Preto – fone 17-3221.2456/2545 e 3221.2384

APÊNDICE B - Questionário

Questionário do projeto de Pesquisa “Estresse oxidativo na anemia falciforme: antioxidantes endógenos como possíveis alvos terapêuticos”.

Data: _____/_____/_____ Matrícula:_____

Nome completo: _____

Acomp./to clínico: ( ) Não ( ) Sim Intervalo:_____ Data da última consulta:_____

História Clínica: Complicações Agudas ou Crônicas

Nº de crises de dor por ano no último ano: ( ) 0 a 2 ( ) 3 a 5 ( ) maior ou igual a 6

Nº de infecções por ano no último ano: ( ) 0 a 2 ( ) 3 a 5 ( ) maior ou igual a 6

Nº de internações por ano no último ano: ( ) 0 a 2 ( ) 3 a 5 ( ) maior ou igual a 6

Histórico Transfusional

-Nº de unidades de concentrado de hemácias transfundidas até o início do estudo?

( ) menor 10 ( ) 10 a 20 ( ) 21 a 30 ( ) 31 a 10 ( ) maior que 40

- Em regime regular de transfusão de hemácias?

( ) Não ( ) Sim Intervalo:_____ Data da última transfusão:_____

Medicamentos em uso

- Hidroxiuréia: ( ) Não ( ) Sim Início (data):_____ Dose diária:_____
- Desferroxamina (Desferal): ( ) Não ( ) Sim Início (data):_____ Dose diária:_____
- Deferiprona (Ferriprox): ( ) Não ( ) Sim Início (data):_____ Dose diária:_____
- Deferasirox (Exjade): ( ) Não ( ) Sim Início (data):_____ Dose diária:_____
- Ácido fólico: ( ) Não ( ) Sim Início (data):_____ Dose diária:_____
- Vitaminas: ( ) Não ( ) Sim Quais?_____
- Analgésicos: ( ) Não ( ) Sim Quais?_____
- Outros: ( ) Não ( ) Sim Quais?_____

OBS:

_____

_____

_____

_____

Anexos

ANEXOS

Anexos I - IX - Artigos científicos publicados durante o período de desenvolvimento do projeto de doutorado (Fev/2011 a Fev/2015) e que estão, de alguma forma, relacionados ao objeto de estudo.

As artigos a seguir são trabalhos independentes dos objetivos específicos propostos neste projeto, porém denotam toda a fundamentação teórico-científica da linha de pesquisa desenvolvida. Os artigos estão apresentados em ordem cronológica de publicação e/ou aceite:

A. Relationship between oxidative stress, glutathione *S*-transferase polymorphisms and hydroxyurea treatment in sickle cell;
B. The influence of hydroxyurea on oxidative stress in sickle cell anemia;
C. Oxidative stress and antioxidant capacity in sickle cell anaemia patients receiving different treatments and medications for different periods of time;
D. Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies;
E. Influence of β^S allele in the lipid peroxidation and antioxidant capacity parameters;
F. Genetic and biochemical markers of hydroxyurea therapeutic response in sickle cell anemia;
G. Frequencies of -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms in sickle cell anemia patients from Brazil;
H. Hemoglobin D-Punjab: Origin, distribution and laboratory diagnosis;
I. Severity of Brazilian sickle cell disease patients: severity scores and feasibility of the Bayesian Network Model use.

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Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia

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ABSTRACT

This study evaluated the oxidative stress and antioxidant capacity markers in sickle cell anemia (SCA) patients with and without treatment with hydroxyurea. We assessed *GSTT1*, *GSTM1* and *GSTP1* polymorphisms in patients and a control group. The study groups were composed of 48 subjects without hemoglobinopathies and 28 SCA patients, 13 treated with HU [SCA (+HU)], and 15 SCA patients not treated with HU [SCA (−HU)]. We observed a significant difference for *GSTP1* polymorphisms in SCA patients with the V/V genotype that showed higher glutathione (GSH) and Trolox equivalent antioxidant capacity (TEAC) ($p = 0.0445$ and $p = 0.0360$), respectively, compared with the I/I genotype. HU use was associated with a 35.2% decrease in the lipid peroxidation levels of the SCA (+HU) group ($p < 0.0001$). Moreover, the SCA (+HU) group showed higher TEAC as compared to the control group ($p = 0.002$). We did not find any significant difference in glutathione-S-transferase (GST) activity between the groups ($p = 0.76$), but the catalase (CAT) activity was about 17% and 30% decreased in the SCA (+HU) and SCA (−HU) groups, respectively ($p < 0.00001$). Whereas the plasma GSH levels were ~2 times higher in the SCA patients than the control group ($p = 0.0005$). HU use has contributed to higher CAT activity and TEAC, and lower lipid peroxidation in patients under treatment. These findings may explain the influence of HU in ameliorating oxidative stress on SCA subjects.

Introduction

Sickle cell anemia (SCA), an inherited monogenic disease, is caused by a transversion in the codon of the sixth amino acid of the beta globin chain, on chromosome 11 [1], leading to formation of a defective hemoglobin form, hemoglobin S (Hb S). In the deoxygenated state, the Hb S tends to aggregate into rodlike polymers, resulting in the deformed sickle shape and rigidity of red blood cells (RBCs) characteristic of this condition [2].

Normal RBCs are usually subjected to oxidative stress as a result of continuous reactive oxygen species (ROS) production that accompanies Hb autoxidation, a condition that increases in SCA [3–5], leading to a continuous inflammatory response and oxidative stress [6]. Among the SCA-derived oxidative stress consequences are an increase in membrane lipid peroxidation levels and alterations in antioxidant defense systems. However, the few studies that have examined antioxidant enzymes in SCA patients have found contradictory results [7].

Glutathione S-transferases (GSTs) constitute multifunctional enzymes that are coded by at least eight distinct loci: α (*GSTA*), μ (*GSTM*), θ (*GSTT*), π (*GSTP*), σ (*GSTS*), κ (*GSTK*), ω (*GSTO*), and ζ (*GSTZ*), each one composed by one or more homodimeric or heterodimeric isoforms [8,9]. These enzymes are involved in the conjugation reactions during phase II of the xenobiotic metabolism, catalyzing reactions between glutathione (GSH) and a variety of potentially toxic and carcinogenic electrophilic compounds [10,11], besides, GSTs also display peroxidase activity and can thus protect from oxidative damage [12]. The deficiency in the activity of this enzyme can be derived from the inherited GSTs polymorphisms, e.g., *GSTT1* (22q11.23), *GSTM1* (1q13.3) and *GSTP1* (11q13) [13].

Hydroxyurea (HU) administration is one of the most important therapies for SCA patients [14,15]. The pharmacologic effects that may contribute to the efficacy of HU in SCA treatment includes the induction of Hb F production and increase in RBC water content (which reduces the polymerization of Hb S). HU also increases microvascular

Abbreviations: CAT, catalase; GSH, glutathione; GST, glutathione-S-transferase; HU, hydroxyurea; HPLC, high performance liquid chromatography; RBC, red blood cells; ROS, reactive oxygen species; SCA, sickle cell anemia; TBARS, thiobarbituric-acid-reacting substances; TEAC, Trolox equivalent antioxidant capacity.

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navigation by sickled cells, and decreases RBC adhesion to endothelium by diminishing the expression of endothelial adhesion molecules [15,16].

There are no reports about the influences of *GSTT1*, *GSTM1* and *GSTP1* polymorphisms on oxidative stress parameters in SCA patients. Therefore, the aim of this study was to evaluate, in SCA patients, the oxidative stress and antioxidant capacity markers, correlating them with HU treatment and *GSTT1*, *GSTM1* and *GSTP1* polymorphisms compared with a control group.

Methods

Subjects

Seventy-six Brazilian subjects were included in the study (30 males and 46 females; mean age: 23.8 years old; range: 9–65 years old). They were from the northwest region of Sao Paulo state and southwest region of Rio de Janeiro state in Brazil. The test group was composed by 28 SCA patients (11 males and 17 females; mean age: 27.7 years old) in a clinical follow-up in São Jose do Rio Preto (SP) and Rio de Janeiro (RJ). The control group was composed of 48 subjects without hemoglobinopathies living also in the northwest region of Sao Paulo state (19 males and 29 females; mean age: 21.9 years old).

All the patients were screened using a questionnaire, and were excluded if they were smokers or drinkers. They were also asked about pain crisis, hemolytic crisis, and if they received blood transfusion or had had stroke in the last two months. The medications used were also accessed, and those taking medication known to affect the analyzed parameters were excluded from the study. All subjects gave their informed consent, and the study was approved by the Data Safety Monitoring Board (DSMB) according to Brazilian regulations.

All SCA patients had access to the same medication protocol and then were separated into two groups: SCA patients treated with HU [SCA (+HU); $n=13$] and SCA patients not treated with HU [SCA (−HU); $n=15$].

Biological samples

Blood samples (11 mL) were collected through venipuncture in heparinized and EDTA tubes. The heparinized blood (7 mL) was incubated for 20 min at 37 °C and then centrifuged at 206*g* for 20 min to separate the plasma for the thiobarbituric-acid-reacting substances (TBARS) and TEAC. The EDTA sample fraction (4 mL) was aliquoted, being 2 mL for the hemoglobinopathies tests, genotypic determination, and enzymatic activities analysis. The other 2 mL was submitted to centrifugation at 825*g* for 10 min to obtain the plasma and then were frozen at −80 °C for GSH determination.

Hemoglobin phenotypes and genotypes

Hb identification was performed using electrophoresis on cellulose acetate pH 8.4, and agar electrophoresis at pH 6.2. The Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANT™ equipment (Bio-Rad Laboratories, CA, USA) [17]. Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald–Giemsa. In all patient samples, the Hb genotype was developed by molecular analysis using PCR–RFLP. The segment amplification that encodes Hb S was accomplished by specific primers, and the amplicon was cleaved by the DdeI restriction endonuclease (New England BioLabs, MA, USA) [18].

Glutathione S-transferase polymorphism genotyping

The polymorphism analysis for the *GSTM1* and the *GSTT1* genes was determined simultaneously in a single assay using a multiplex PCR approach with a housekeeping *CYP1A* gene co-amplification as an internal control [19].

GSTP1 polymorphism was performed using PCR–RFLP and the specific primers for exon 5 (Ile105Val). Following PCR, the product was digested with BsmA1 restriction endonuclease (New England BioLabs, MA, USA). The digest was submitted to an electrophoresis on a 3.5% agarose gel containing ethidium bromide [20].

Biochemical analysis

Lipid peroxidation levels were assessed in heparinized plasma using the thiobarbituric-acid-reactive substances assay [21]. Antioxidant capacity was also determined in heparinized plasma samples according to their equivalence to Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) [22]. For the total GST activity, the blood samples were diluted in a 3.5 μM 2-mercaptoethanol, 10 μM NADP, and 2.7 mM EDTA hemolyzing solution (1:20, v/v) and then assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm. The assay was carried out in 0.2 M K-phosphate buffer, pH 6.5, 1 mM CDNB, 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) [23]. For the analysis of CAT activity, blood samples were diluted in ultrapure water (1:50, v/v) and then 10 uL was used to measure CAT activity, by the decrease in absorbance at 240 nm ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of H_2O_2 (10 mM H_2O_2 in 1 M Tris–HCl buffer pH 8.0 containing 5 mM EDTA) [24].

GSH concentration was determined in EDTA plasma samples using HPLC coupled to a coulometric electrochemical detector (Coulochem III ESA, Bedford, MA) [25]. Under these conditions, GSH clearly eluted at ~6 min. GSH was extracted from the plasma samples by adding perchloric acid to the plasma sample (10% final concentration). After vigorous stirring and 10 min on ice, the mixture was centrifuged at 825 g for 10 min at 4 °C. The extract was then filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC system. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into the HPLC system.

Statistical analysis

Statistical analysis was performed using the Statistica 9.0 software (Statsoft Inc.). Data were expressed as mean ± standard deviation and were tested for normal distribution using the Shapiro Wilk test. Data were also tested for homogeneity of variances using the Levene test. Means between the groups were compared by applying the *t* test or one-way ANOVA followed by the post hoc Tukey test for parametric data, and the Mann–Whitney test or Kruskal–Wallis followed by the post hoc Dunn test for non-parametric data. To assess the degree of association between the studied variables, we used the Pearson correlation for parametric data and the Spearman correlation for non-parametric data and linear regression. To compare the GST genotypes between the studied groups Pearson Chi-square test supplemented by Fisher's exact test was used. The level of significance was set at $p<0.05$.

Table 1
Hemoglobin fraction concentration in SCA patients and control group.

	CG ($n=48$)	SCA (+HU) ($n=13$)	SCA (−HU) ($n=15$)
Hb A (%)*	94.88 ± 2.12^a	12.58 ± 16.39^b	8.06 ± 14.2^b
Hb A_2 (%)*	2.75 ± 0.39^a	3.99 ± 0.66^b	3.75 ± 1.02^b
Hb F (%)*	1.08 ± 1.13^a	10.93 ± 7.07^b	6.16 ± 4.54^b
Hb S (%)#	0.0	70.40 ± 16.07^a	80.39 ± 16.08^b

CG: control group; SCA (+HU): patients treated with HU; SCA (−HU): patients not treated with HU. Data were expressed as mean ± standard deviation.

* Different letters indicate statistical difference (Kruskal–Wallis test followed by post hoc Dunn test, $p<0.05$).

\# Different letters indicate statistical difference (Mann–Whitney test, $p<0.05$).

Table 2
Prevalence of glutathione S-transferase polymorphisms in the control group and SCA patients.

	Control group *N* (%)	SCA patients *N* (%)	*p* value[a]
GSTM1			
[+]	21 (43.7)	20 (71.4)	0.0195
[−]	27 (56.3)	8 (21.6)	0.0195
GSTT1			
[+]	27 (56.3)	9 (32.2)	0.0423
[−]	21 (43.7)	19 (67.8)	0.0423
GSTM1/T1			
[+]/[−]	13 (27.0)	14 (50.0)	0.0440
[+]/[+]	8 (16.7)	6 (21.4)	0.6054
[−]/[+]	19 (39.6)	3 (10.7)	0.0074
[−]/[−]	8 (16.7)	5 (17.9)	0.8994
GSTP1			
I/I	34 (70.8)	5 (17.9)	0.0001
I/V	12 (25.0)	16 (57.1)	0.0050
V/V	2 (4.2)	7 (25.0)	0.0066

Wild [+] and null [−] genotypes, I/I wild homozygous; I/V heterozygate; V/V mutant homozygous.
[a] Comparisons were made by Pearson Chi square test supplemented by Fisher's exact test.

Results and discussion

It has been shown that HU treatment improves SCA patient's clinical conditions. In our study, all hemoglobin parameters were significantly different between the groups (Table 1), as expected for the SCA, and the levels of Hb F was increased in SCA patients treated with HU, confirming the previously well-described HU effect [15]. It is interesting to note the high Hb F levels in the SCA (−HU) group which may have been influenced by non-target factors in this study, such as the three major loci – Xmn1-HBG2 single nucleotide polymorphism, HBS1L-MYB intergenic region on chromosome 6q, and BCL11A – that contribute 20%–50% of the trait Hb F variance in SCA patients [26].

It should be mentioned that the Hb A_2 levels were slightly higher than expected for SCA, and this is due to the automated HPLC characteristics in which Hb S acetylated subfractions elute at the Hb A_2 window increasing Hb A_2 levels [27], and the high Hb A levels observed in both SCA groups are transfusion remnants, because the blood transfusions are indispensable and very frequent in SCA treatment [28]. The transfusion remnant Hb A levels could have masked the well-characterized chronic oxidative stress in SCA widely reported in the literature [4–7], since the increased Hb A levels foster a dilution of erythrocyte Hb S levels and a subsequent decline in the Hb S polymerization [29] that is a primary pathophysiologic event in this genetic disorder [30]. However, these biased results were not observed in the obtained data.

The co-inheritance of polymorphisms associated to the SCA patients may modulate phenotypic expression [31], therefore we investigated the frequency of GST polymorphisms (*GSTM1*, *GSTT1* and *GSTP1*) and its relationship with oxidative stress markers in SCA and in the control group. Human GSTs have been well characterized as ethnic-dependent polymorphism frequencies [32] and largely divergent among populations around the world [33]. The gene distribution of GST polymorphisms in this study is summarized in Table 2.

The frequency data obtained for the control group are similar to those described in the literature, whereas the SCA patients group presented lower frequencies for the null *GSTM1* (21.6%) and I/I (17.9%) genotypes when compared with the frequencies obtained for Brazilian population [32,34–37]. However, this is the first study reporting the *GSTM1*, *GSTT1* and *GSTP1* polymorphisms' frequency in SCA patients and the significant difference when compared with the control group.

The association of *GSTM1*, *GSTT1* and *GSTP1* polymorphisms with various diseases such as cancer and inflammatory diseases, and responses in the metabolism, efficacy and toxicity of certain drugs have been widely investigated [38–40] but few studies have investigated their relationship with oxidative stress parameters. Bessa et al. [41] showed that subjects with null *GSTM1* and *GSTT1* or null genotype for both genes had significantly lower erythrocyte GST activity than those with *GSTM1*+/*GSTT1*+, and also higher lipid peroxidation levels, lower erythrocyte GSH levels and decreased activities of CAT, superoxide dismutase (SOD) and GPx, whereas there was no significant difference in these oxidative stress-related parameters between the *GSTM1* and *GSTT1* genotypes.

The influences of *GSTM1*, *GSTT1* and *GSTP1* polymorphisms on the oxidative stress parameters (TBARS, TEAC and GSH plasma levels, and activities of CAT and GST) in the control group and SCA patients are shown in Table 3. Our results showed that the GST polymorphisms did not influence significantly on any of the biochemical markers in the control group, whereas in SCA patients, we observed significant difference only for *GSTP1* polymorphisms. SCA patients with the *V/V* genotype showed higher GSH and TEAC levels ($p = 0.0445$ and $p = 0.0360$, respectively) compared with the *I/I* genotype, indicating a possible correlation between these polymorphisms with oxidative stress in SCA patients. However, further researches with larger sample size and association with SCA clinical manifestations are needed, to

Table 3
Mean values and standard desviation of measured biochemical parameters in the control group and patients in the presence of different GST polymorphisms found.

	GSTM1/T1				*p* value*	*GSTP1*			*p* value*
	[+]/[−]	[+]/[+]	[−]/[+]	[−]/[−]		I/I	I/V	V/V	
TBARS (ng/mL)									
Control group	290.03 ± 209.15	220.12 ± 65.95	191.77 ± 139.21	252.87 ± 187.29	0.4166	218.20 ± 158.26	245.91 ± 164.59	331.00 ± 169.70	0.5916
SCA patients	1501.00 ± 472.72	1532.40 ± 775.47	1466.78 ± 558.57	1634.19 ± 837.66	0.9867	1624.80 ± 535.62	1535.28 ± 629.53	1478.89 ± 590.81	0.8505
TEAC (mM)									
Control group	1.93 ± 0.15	1.91 ± 0.14	1.87 ± 0.13	1.87 ± 0.14	0.4166	1.88 ± 0.02	1.93 ± 0.12	1.98 ± 0.12	0.3811
SCA patients	2.00 ± 0.18	2.02 ± 0.14	2.13 ± 0.08	1.96 ± 0.21	0.5733	1.86 ± 0.11[a]	2.02 ± 0.18	2.08 ± 0.11[b]	0.0445[#]
GST (U/mL)									
Control group	1.72 ± 0.81	1.26 ± 0.54	1.63 ± 0.70	1.32 ± 0.76	0.4166	1.51 ± 0.64	1.51 ± 0.92	1.78 ± 0.61	0.7015
SCA patients	1.62 ± 0.51	1.97 ± 0.96	1.13 ± 0.33	1.52 ± 0.52	0.3454	1.58 ± 0.61	1.54 ± 0.57	1.70 ± 0.92	0.9576
CAT (U/mL)									
Control group	2339.65 ± 466.35	2332.74 ± 298.25	2427.23 ± 314.53	2301.05 ± 167.25	0.5623	2387.81 ± 388.69	2439.55 ± 256.80	2334.50 ± 219.10	0.8751
SCA patients	1804.98 ± 445.88	1828.63 ± 587.26	1685.44 ± 763.56	1921.12 ± 691.05	0.9600	1823.23 ± 689.74	1769.58 ± 492.94	1978.37 ± 526.04	0.7425
GSH (μM)									
Control group	0.37 ± 0.31	0.29 ± 0.16	0.37 ± 0.39	0.33 ± 0.27	0.9960	0.33 ± 0.31	0.34 ± 0.26	0.71 ± 0.43	0.2333
SCA patients	0.57 ± 0.37	0.73 ± 0.07	0.99 ± 0.53	0.87 ± 0.64	0.5681	0.38 ± 0.28[a]	0.71 ± 0.44	0.96 ± 0.24[b]	0.0360[#]

Wild [+] and null [−] genotype. I/I wild homozygous; I/V mutant homozygous. Data were expressed as mean ± standard deviation.
* Comparisons were made by Kruskal–Wallis test followed by post hoc Dunn test.
[#] Different letters indicate statistical difference.

better predict the relative risk generated by the co-inheritance of GST polymorphisms and Hb S mutation in the pathophysiology development.

Although oxidative stress and alterations in the activities of antioxidant enzymes have been extensively described in SCA, the results are sometimes contradictory, and their relationship with HU treatment remains unclear. We, therefore, examined the lipid peroxidation levels (TBARS) and the antioxidant capacity (TEAC levels, GST and CAT activities, and plasma GSH levels) in a control group (CG; $n=48$), SCA patients treated with HU [SCD (+HU); $n=13$], and SCA patients not previously treated with HU [SCA (−HU); $n=15$] (Fig. 1).

High levels of lipid peroxidation in SCA patients were reported [42–45], but we demonstrated in this study that the use of HU provides a 35.2% decrease in the lipid peroxidation levels on the SCA (+HU) group ($p<0.0001$), confirming its antioxidant property in vivo, as previously reported by Agil and Sadrzadeh [46] in an RBC model. Moreover, the SCA (+HU) group presented higher TEAC levels when compared to the control group ($p=0.002$), in accordance with a recent publication by our group [47]. The significant increase in TEAC levels is probably due to an indirect effect of the antioxidant property of HU use, counteracting the high levels of ROS generated by the disease. However, further studies are needed to understand this phenomenon.

The main role of GSTs in cells is related to the detoxification of xenobiotics and lipoperoxidation products, although some isoforms also contain peroxidase activity [12,48]. Because of this, the evaluation of GSTs has been used as an important biomarker of oxidative stress in many human diseases such as hypertension [41], Alzheimer's disease [49], non-alcoholic fatty liver disease [50], and cancer [51]. As in SCA, these diseases are also characterized by chronic inflammation closely associated with oxidative stress. However, there are no reports on the evaluation of GST activity in SCA patients. We did not find any significant difference in GST activity between the studied groups ($p=0.76$), suggesting that this enzyme was not affected by the disease. Although oxidative stress was present in SCA patients and GST accounts for antioxidant defenses, it may be hypothesized that other antioxidant enzymes present in RBCs such as SOD, CAT, GPx and peroxiredoxin [52–54] have a more prominent role on the oxidative stress responses. However, this remains unsolved and should be assessed by other studies.

The CAT activity was about 17% and 30% decreased in the SCA (+HU) and SCA (−HU) groups, respectively ($p<0.00001$), in agreement with the findings of Alsultan et al. [43] in SCA patients and with Dasgupta et al. [5] in transgenic sickle mouse models. Contrariwise, Manfredini et al. [42] and Cho et al. [7] did not observe differences in CAT activity between the evaluated control and SCA groups.

The statistical correlation analysis between CAT activity and TBARS levels (Fig. 2A) showed a negative correlation between these parameters ($r=-0.56$, $p<0.00001$), in which the increase in TBARS levels is associated with the decrease of CAT activity that may be due to its consumption by ROS generated by the chronic inflammatory process in these disorders.

The HU pharmacological effect (Hb F levels increase) seems to participate in the antioxidant defense, avoiding CAT consumption, since we found an association between the CAT activity and the Hb F levels in the SCA patients ($r=0.41$, $p=0.0293$) (Fig. 2B). These data agree with Dasgupta et al. (2010) [55] who showed that, in SCA transgenic mice, the Hb F protective effect is primarily mediated by decreased intravascular sickling, resulting in a decreased oxidative stress, and also in an increased nitric oxide bioavailability. Venkatesha et al. [56] submitted cultures of human non-malignant breast epithelial cells to high ROS levels and observed a significant increase in micronuclei frequency and in histone 2AX-phosphorylation. When these cell cultures were treated with CAT, it was shown that CAT blunted cell damages, validating its antioxidant action.

The tripeptide GSH is a very abundant intracellular free thiol that plays a critical role in regulating a variety of cellular functions, including xenobiotic detoxification, the synthesis of DNA and other endogenous

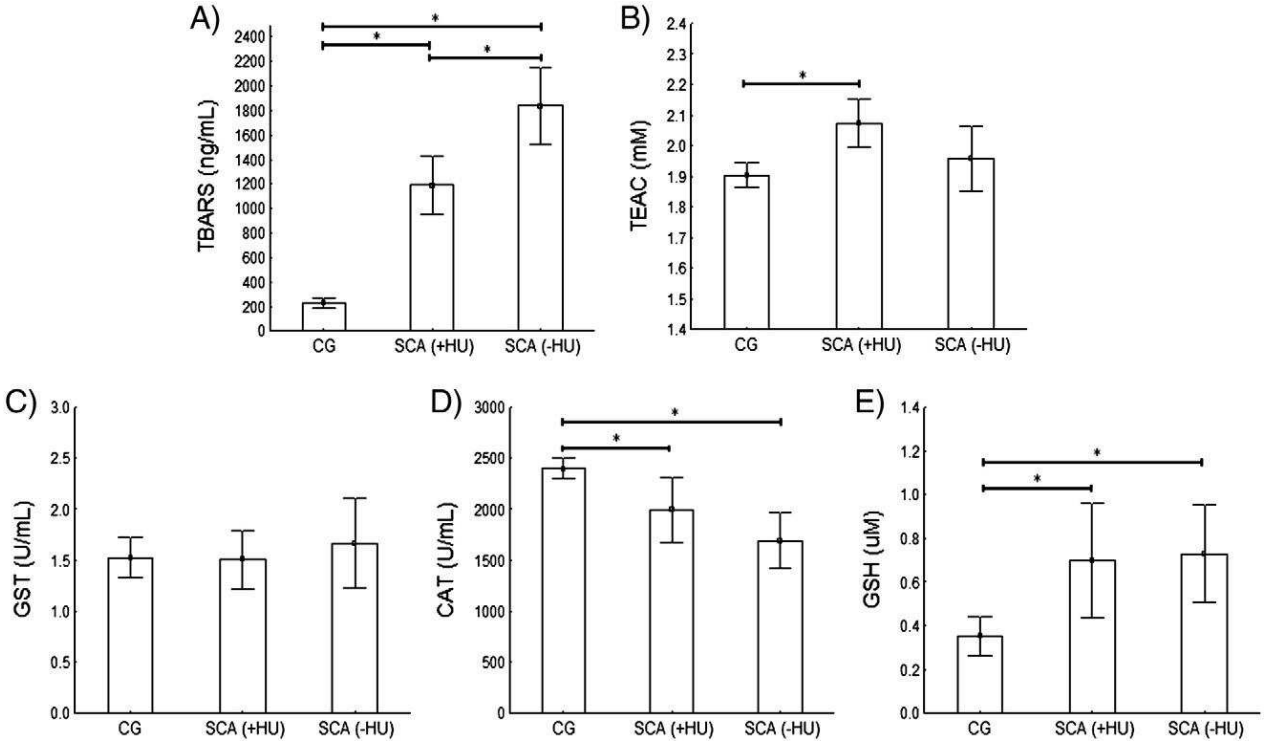

Fig. 1. The oxidative stress parameters in the control group and SCA patients. (A) TBARS levels were ~5 times and ~8 times higher in the SCA (+HU) and SCA (−HU), respectively, than in the control group (CG) ($p<0.0001$). (B) TEAC levels were higher in the SCA patients than in the control group ($p=0.002$). (C) GST activity showed no difference between the studied groups (−HU) ($p=0.76$). (D) CAT activity was lower in the SCA patients than in the control group ($p<0.00001$). (E) GSH levels were ~2 times higher in the SCA patients than in the control group ($p=0.0005$). *Indicates statistical difference according to the one-way ANOVA followed by post hoc Turkey test (TEAC and GST) or Kruskal–Wallis followed by post hoc Dunn test (TBARS, CAT and GSH).

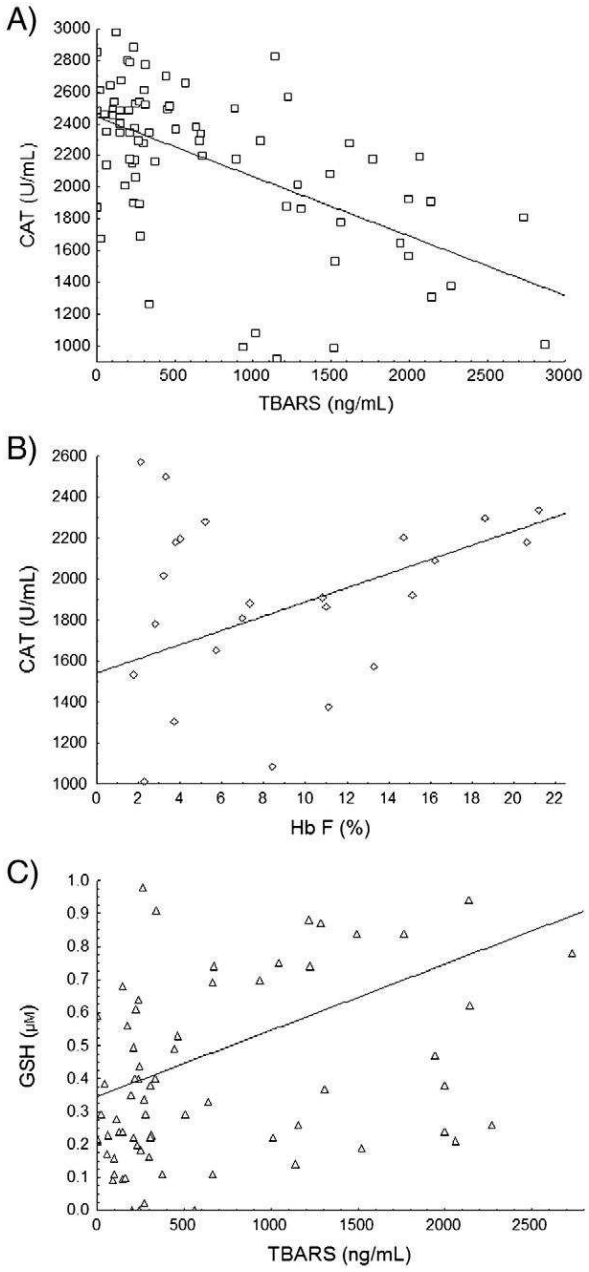

Fig. 2. Association between the biochemical parameters evaluated and Hb F levels. (A) Negative linear correlation between lipid peroxidation and CAT activity ($r = -0.56$, $p = 0.00001$). (B) Positive linear correlation between Hb F levels and CAT activity ($r = 0.41$, $p = 0.0293$). (C) Positive linear correlation between lipid peroxidation and GSH levels ($r = 0.37$, $p = 0.0008$).

compounds, modulation of gene expression, and the regulation of cell cycle. However, the most important and well-known function of GSH is as a non-enzymatic antioxidant [57]. There are several reports on the evaluation of erythrocyte or tissue GSH levels in SCA patients and transgenic mice [5,55,58–60], but to our knowledge, the present study was the first that has correlated plasma GSH levels with biochemical parameters in SCA. Human plasma contains very low GSH concentrations, in the range of 0.1 to 20 μM [61], which are consistent with our data. The plasma GSH levels were ~2 times higher in the SCA patients than the control group ($p = 0.0005$), and showed a positive correlation with the TBARS levels ($r = 0.37$, $p = 0.0008$) (Fig. 2C), which may be due to the hemolytic process of the SCA pathophysiology or a response to chronic stress, confirming its antioxidant function.

RBCs can synthesize GSH from cysteine, glycine and glutamic acid since they contain all the enzymes necessary for its biosynthesis, and a significant percentage of their GSH is produced de novo daily [62]. Furthermore, both glutathione disulfide (GSSG) and glutathione conjugates (GS-X) are actively exported from RBCs when their intracellular concentration is high. This de novo re-synthesis may balance the GSH loss due to GSSG and GS-X export, and is regulated by a feedback mechanism [63,64]. Giustarini et al. [65] demonstrated that RBCs significantly contribute to the plasma pool of GSH and suggested that abnormalities in RBCs can influence the plasma GSH levels, thus, SCA may interfere in the GSH de novo re-synthesis feedback mechanism. Dumaswala et al. [66] showed in vitro that supplementing the conventional additive with GSH precursor amino acids improved RBC GSH synthesis and maintenance, preventing RBC scavenging function which can be committed by ROS. However, further studies are needed to better understand this mechanism.

In conclusion, we observed that the co-inheritance of GST polymorphisms and Hb S showed low prognostic value for the biochemical parameters evaluated and significant differences in the biochemical parameters evaluated in SCA, except for GST activity. We suggest that these parameters may play important roles in other β-hemoglobinopathies or hemolytic anemia, which are associated with oxidative stress, but this remains to be further investigated. The use of HU improved antioxidant defenses of patients with SCA, also contributing to a higher CAT activity and TEAC levels, and a lower lipid peroxidation in SCA patients. With these results, we suggest that GSH can be used as an adjunct in reducing oxidative stress, improving the oxidative and inflammatory status of the SCA.

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References

[1] J.G. Taylor, D. Ackah, C. Cobb, et al., Mutations and polymorphisms in hemoglobin genes and the risk of pulmonary hypertension and death in sickle cell disease, Am. J. Hematol. 83 (2008) 6–14.
[2] S.H. Embury, N. Mohandas, C. Paszty, et al., In vivo blood flow abnormalities in the transgenic knockout sickle cell mouse, J. Clin. Invest. 103 (1999) 915–920.
[3] R.P. Hebbel, W.T. Morgan, J.W. Eaton, B.E. Hedlund, Accelerated autoxidation and heme loss due to instability of sickle hemoglobin, Proc. Natl Acad. Sci. U.S A. 85 (1988) 237–241.
[4] G.J. Kato, M.T. Gladwin, Mechanisms and clinical complications of hemolysis in sickle cell disease and thalassemia, in: M.H. Steinberg, B.G. Forget, D.R. Higgs, D.J. Weatherall (Eds.), Disorders of Hemoglobin, Cambridge University Press, Cambridge, 2009, pp. 201–224.
[5] T. Dasgupta, R.P. Hebbel, D.K. Kaul, Protective effect of arginine on oxidative stress in transgenic sickle mouse models, Free Radic. Biol. Med. 41 (2006) 1771–1780.
[6] N. Conran, C.F. Franco-Penteado, F.F. Costa, Newer aspects of the pathophysiology of sickle cell disease vaso-occlusion, Hemoglobin 33 (2009) 1–16.
[7] C.S. Cho, G.J. Kato, S.H. Yang, et al., Hydroxyurea-induced expression of glutathione peroxidase 1 in red blood cells of individuals with sickle cell anemia, Antioxid. Redox Signal. 13 (2010) 1–11.
[8] J.D. Hayes, R.C. Strange, Glutathione S-transferase polymorphisms and their biological consequences, Pharmacology 61 (2000) 154–166.
[9] J.D. Hayes, J.U. Flanagan, I.R. Jowsey, Glutathione transferases, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 51–88.
[10] J.D. Hayes, D.J. Pulford, The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 445–600.
[11] T.H. Rushmore, C.B. Pickett, Glutathione S-transferases, structure, regulation, and therapeutic implications, J. Biol. Chem. 268 (1993) 11475–11478.
[12] R. Rinaldi, E. Eliasson, S. Swedmark, R. Morgenstern, Reactive intermediates and the dynamics of glutathione transferases, Drug Metab. Dispos. 30 (2002) 1053–1058.

[13] V. Sharma, B. Kumar, R. Saxena, Glutathione S-transferase gene deletions and their effect on iron status in HbE/beta thalassemia patients, Ann. Hematol. 89 (2010) 411–414.
[14] R.E. Ware, B. Aygun, Advances in the use of hydroxyurea, Hematology Am. Soc. Hematol. Educ. Program. (2009) 62–69.
[15] Z.Y. Aliyu, A.R. Tumblin, G.J. Kato, Current therapy of sickle cell disease, Haematologica 91 (2006) 7–11.
[16] C. Lanaro, C.F. Franco-Penteado, D.M. Albuqueque, et al., Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy, J. Leukoc. Biol. 85 (2009) 235–242.
[17] C.R. Bonini-Domingos, Metodologias Laboratoriais Para o Diagnóstico de Hemoglobinopatias e Talassemias, NH, São José do Rio Preto, 2006.
[18] R.K. Saiki, S. Scharf, F. Faloona, et al., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia, Science 230 (1985) 1350–1354.
[19] S.Z. AbdelRahman, R.A. Elzein, W.A. Anwar, W.W. Au, A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies, Cancer Lett. 107 (1996) 229–233.
[20] N.L. Nock, C. Bock, C. Neslund-Dudas, et al., Polymorphisms in glutathione S-transferase genes increase risk of prostate cancer biochemical recurrence differentially by ethnicity and disease severity, Cancer Causes Control 20 (2009) 1915–1926.
[21] M. Uchiyama, M. Mihara, Determination of malonaldehyde precursor in tissues by thiobarbituric acid test, Anal. Biochem. 86 (1978) 271–278.
[22] R. Re, N. Pellegrini, A. Proteggente, et al., Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237.
[23] J.H. Keen, W.H. Habig, W.B. Jakoby, Mechanism for several activities of glutathione S-transferases, J. Biol. Chem. 251 (1976) 6183–6188.
[24] E. Beutler, Red cell metabolism. A manual of biochemical methods, Ann. Intern. Med. 83 (1975) 919.
[25] A. Rodriguez-Ariza, F. Toribio, J. Lopezbarea, Rapid-determination of glutathione status in fish liver using high-performance liquid-chromatography and electrochemical detection, J. Chromatogr. B Biomed. Appl. 656 (1994) 311–318.
[26] S.L. Thein, S. Menzel, Discovering the genetics underlying foetal haemoglobin production in adults, Br. J. Haematol. 145 (2009) 455–467.
[27] P. Van Delft, E. Lenters, M. Bakker-Verweij, et al., Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations, Int. J. Lab. Hematol. 31 (2009) 484–495.
[28] J.L. Kwiatkowski, E. Yim, S. Miller, R.J. Adams, Effect of transfusion therapy on transcranial doppler ultrasonography velocities in children with sickle cell disease, Pediatr. Blood Cancer (2010 Dec 23), -ediatr.
[29] S.L. Thein, Genetic modifiers of the â-haemoglobinopathies, Br. J. Haematol. 141 (2008) 357–366.
[30] P.G. Vekilov, Sickle-cell haemoglobin polymerization: is it the primary pathogenic event of sickle-cell anaemia? Br. J. Haematol. 139 (2007) 173–184.
[31] K.Y. Fertrin, F.F. Costa, Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment, Expert Rev. Hematol. 3 (2010) 443–458.
[32] A. Rossini, D.C. Rapozo, L.M. Amorim, et al., Frequencies of GSTM1, GSTT1, and GSTP1 polymorphisms in a Brazilian population, Genet. Mol. Res. 1 (2002) 233–240.
[33] Z. Mo, Y. Gao, Y. Cao, et al., An updating meta-analysis of the GSTM1, GSTT1, and GSTP1 polymorphisms and prostate cancer: a HuGE review, Prostate 69 (2009) 662–688.
[34] M.M. Lima Jr., M.N. Oliveira, F. Granja, et al., Lack of association of GSTT1, GSTM1, GSTO1, GSTP1 and CYP1A1 polymorphisms for susceptibility and outcome in Brazilian prostate cancer patients, Folia Biol. (Praha) 54 (2008) 102–108.
[35] M.A.S. Pinhel, M.A. Nakazone, J.C. Cacao, et al., Glutathione S-transferase variants increase susceptibility for late-onset Alzheimer's disease: association study and relationship with apolipoprotein E epsilon4 allele, Clin. Chem. Lab. Med. 46 (2008) 439–445.
[36] L.S. Ondei. Estresse oxidativo em pacientes beta talassêmicos e com deficiência de glicose-6-fosfato desidrogenase. 2009. Sao Jose do Rio Preto: Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista. Ref Type: Thesis/Dissertation.
[37] L.A.V. Magno, J. Talbot, T. Talbot, et al., Glutathione S-transferase variants in a Brazilian population, Pharmacology 83 (2009) 231–236.
[38] L. Tamer, M. Calikoglu, N.A. Ates, et al., Glutathione-S-transferase gene polymorphisms (GSTT1, GSTM1, GSTP1) as increased risk factors for asthma, Respirology 9 (2004) 493–498.
[39] S.L. Zhong, S.F. Zhou, X. Chen, et al., Relationship between genotype and enzyme activity of glutathione S-transferases M1 and P1 in Chinese, Eur. J. Pharm. Sci. 28 (2006) 77–85.
[40] L. Kilburn, M.F. Okcu, T. Wang, et al., Glutathione S-transferase polymorphisms are associated with survival in anaplastic glioma patients, Cancer 116 (2010) 2242–2249.
[41] S.S. Bessa, E.M.M. Ali, S.M. Hamdy, The role of glutathione S-transferase M1 and T1 gene polymorphisms and oxidative stress-related parameters in Egyptian patients with essential hypertension, Eur. J. Intern. Med. 20 (2009) 625–630.
[42] V. Manfredini, L.L. Lazzaretti, I.H. Griebeler, et al., Blood antioxidant parameters in sickle cell anemia patients in steady state, J. Natl Med. Assoc. 100 (2008) 897–902.
[43] A.I. Alsultan, M.A. Seif, T.T. Amin, et al., Relationship between oxidative stress, ferritin and insulin resistance in sickle cell disease, Eur. Rev. Med. Pharmacol. Sci. 14 (2010) 527–538.
[44] T. Repka, R.P. Hebbel, Hydroxyl radical formation by sickle erythrocyte-membranes—role of pathological iron deposits and cytoplasmic reducing agents, Blood 78 (1991) 2753–2758.
[45] E.D. Sess, M.A. Carbonneau, M. Meite, et al., Markers of lipid peroxidation, inflammatory proteins and plasma tocopherols in homozygotic and heterozygotic sickle cell anemia, Bull. Soc. Pathol. Exot. 91 (1998) 238–241.
[46] A. Agil, S.M.H. Sadrzadeh, Hydroxy-urea protects erythrocytes against oxidative damage, Redox Rep. 5 (2000) 29–34.
[47] E.L. Shimauti, D.G. Silva, E.A. de Almeida, et al., Serum melatonin level and oxidative stress in sickle cell anemia, Blood Cells Mol. Dis. 45 (2010) 297–301.
[48] P. Maher, Redox control of neural function: background, mechanisms, and significance, Antioxid. Redox Signal. 8 (2006) 1941–1970.
[49] D.T. Nam, M. Arseneault, V. Murthy, C. Ramassamy, Potential role of acrolein in neurodegeneration and in Alzheimer's disease, Curr. Mol. Pharmacol. 3 (2010) 66–78.
[50] R.N. Hardwick, C.D. Fisher, M.J. Canet et al. Diversity in antioxidant response enzymes in progressive stages of human non-alcoholic fatty liver disease. Drug Metab Dispos. In press. 30–8–2010.Ref Type: In Press.
[51] E.B. Burlakova, G.P. Zhizhina, S.M. Gurevich, et al., Biomarkers of oxidative stress and smoking in cancer patients, J. Cancer Res. Ther. 6 (2010) 47–53.
[52] R.M. Johnson, G. Goyette, Y. Ravindranath, Y.S. Ho, Hemoglobin autoxidation and regulation of endogenous H2O2 levels in erythrocytes, Free Radic. Biol. Med. 39 (2005) 1407–1417.
[53] S.G. Rhee, H.Z. Chae, K. Kim, Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling, Free Radic. Biol. Med. 38 (2005) 1543–1552.
[54] F.M. Low, M.B. Hampton, A.V. Peskin, C.C. Winterbourn, Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte, Blood 109 (2007) 2611–2617.
[55] T. Dasgupta, M.E. Fabry, D.K. Kaul, Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in transgenic-knockout sickle mice, Am. J. Physiol. Regul. Integr. Comp. Physiol. 298 (2010) R394–R402.
[56] V.A. Venkatesha, S. Venkataraman, E.H. Sarsour, et al., Catalase ameliorates polychlorinated biphenyl-induced cytotoxicity in nonmalignant human breast epithelial cells, Free Radic. Biol. Med. 45 (2008) 1094–1102.
[57] R.M. Liu, K.A.G. Pravia, Oxidative stress and glutathione in TGF-beta-mediated fibrogenesis, Free Radic. Biol. Med. 48 (2010) 1–15.
[58] V.L. Tatum, C.K. Chow, Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress, Free Radic. Res. 25 (1996) 133–139.
[59] M.A.F. Chaves, M.S.S. Leonart, A.J. do Nascimento, Oxidative process in erythrocytes of individuals with hemoglobin S, Hematology 13 (2008) 187–192.
[60] S.S. Somjee, R.P. Warrier, J.L. Thomson, et al., Advanced glycation end-products in sickle cell anaemia, Br. J. Haematol. 128 (2005) 112–118.
[61] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma, Anal. Biochem. 200 (1992) 218–229.
[62] P.D. Dass, E.W. Bermes, E.W. Holmes, Renal and hepatic output of glutathione in plasma and whole blood, Biochim. Biophys. Acta 1156 (1992) 99–102.
[63] R. Rossi, A. Milzani, I. le-Donne, et al., Different metabolizing ability of thiol reactants in human and rat blood—biochemical and pharmacological implications, J. Biol. Chem. 276 (2001) 7004–7010.
[64] S.R. Thom, M. Kang, D. Fisher, H. Ischiropoulos, Release of glutathione from erythrocytes and other markers of oxidative stress in carbon monoxide poisoning, J. Appl. Physiol. 82 (1997) 1424–1432.
[65] D. Giustarini, A. Milzani, I. le-Donne, R. Rossi, Red blood cells as a physiological source of glutathione for extracellular fluids, Blood Cells Mol. Dis. 40 (2008) 174–179.
[66] U.J. Dumaswala, L. Zhuo, S. Mahajan, et al., Glutathione protects chemokine-scavenging and antioxidative defense functions in human RBCs, Am. J. Physiol. Cell Physiol. 280 (2001) C867–C873.

The influence of hydroxyurea on oxidative stress in sickle cell anemia

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Objective: *The oxidative stress in 20 sickle cell anemia patients taking hydroxyurea and 13 sickle cell anemia patients who did not take hydroxyurea was compared with a control group of 96 individuals without any hemoglobinopathy.*

Methods: *Oxidative stress was assessed by thiobarbituric acid reactive species production, the Trolox-equivalent antioxidant capacity and plasma glutathione levels.*

Results: *Thiobarbituric acid reactive species values were higher in patients without specific medication, followed by patients taking hydroxyurea and the Control Group ($p < 0.0001$). The antioxidant capacity was higher in patients taking hydroxyurea and lower in the Control Group ($p = 0.0002$ for Trolox-equivalent antioxidant capacity and $p < 0.0292$ for plasma glutathione). Thiobarbituric acid reactive species levels were correlated with higher hemoglobin S levels ($r = 0.55$; $p = 0.0040$) and lower hemoglobin F concentrations ($r = -0.52$; $p = 0.0067$). On the other hand, plasma glutathione levels were negatively correlated with hemoglobin S levels ($r = -0.49$; $p = 0.0111$) and positively associated with hemoglobin F values ($r = 0.56$; $p = 0.0031$).*

Conclusion: *Sickle cell anemia patients have high oxidative stress and, conversely, increased antioxidant activity. The increase in hemoglobin F levels provided by hydroxyurea and its antioxidant action may explain the reduction in lipid peroxidation and increased antioxidant defenses in these individuals.*

Keywords: *Hemoglobinopathies; Oxidative stress; Anemia, sickle cell; Hydroxyurea*

Introduction

Sickle cell anemia (SCA) is one of the most common genetic disorders in the world. It is characterized by homozygous hemoglobin (Hb) S and represents the most severe form of sickle cell disease (SCD)[1]. Hb S is caused by a mutation in the β-globin gene in which the sixth amino acid is changed from glutamic acid to valine due to a substitution of adenine for thymine[2]. SCA presents a series of clinical manifestations which are influenced by genetic and environmental factors. These factors result in many phenotypes, mainly mediated by the polymerization of Hb S, hemolysis and cell adhesion to endothelium which leads to vascular occlusion[3,4]. Blood transfusions, folic acid supplementation and hydroxyurea (HU) are the most common treatments in SCA. HU is an oral drug whose main effect in SCA is the increased synthesis of fetal Hb (Hb F) which reduces the frequency of vaso-occlusive episodes, pain crises, hospitalizations and blood transfusions[5].

Oxidative stress is one of the factors that modulates the phenotypic expression of SCA. This stress influences the vaso-occlusive process by increasing the adhesive properties of erythrocytes, leukocytes and platelets to the endothelium[6]. Normal erythrocytes suffer oxidative stress resulting from the production of reactive oxygen species (ROS) due to the oxygen metabolism. This metabolic ROS production is increased in patients with hemoglobinopathies, causing oxidative damage such as lipid peroxidation. The release of Hb in plasma and ischemia-reperfusion cycles are characteristic of SCD, increasing oxidative stress and requiring a more effective antioxidant system[7,8].

The literature suggests that excess ROS has implications in the pathophysiology of SCA. Thus an evaluation of the oxidative stress in these patients may provide important information regarding the current use of medications, such as HU, and may lead to new therapeutic strategies. Therefore, considering the intense generation of ROS with the presence and hemolysis of Hb S in SCA, the influence of HU on oxidative stress was evaluated using cell damage markers and antioxidant capacity by comparing patients taking HU with those who were not taking the medication and a control group.

Methods

Subjects

Peripheral blood samples of 33 SCA patients (21 females and 12 males; mean age: 28 ± 15 years) from blood banks in São Paulo and Rio de Janeiro, southeastern Brazil were evaluated.

All patients were treated with folic acid supplementation however only 20 of the patients were taking HU. A Control Group was formed of 96 individuals without hemoglobinopathies and not taking HU (57 females and 39 males; mean age: 23 ± 6 years) from southeastern Brazil.

All SCA patients were screened using a questionnaire. Pregnant women, smokers and individuals who drank significant quantities of alcohol were excluded from the study as were patients who had had strokes, pain or hemolytic crisis or had received blood transfusions within the two months prior to the start of the study. Patients who had taken medications known to affect the analyzed parameters (such as acetylsalicylic acid, antibiotics or vitamins) within the 24 hours prior to sample collection were also excluded from the study. All subjects gave their informed consent and the study was approved by the Data Safety Monitoring Board (DSMB) according to Brazilian regulations.

Genotype investigation

All samples were submitted to classical hemoglobin diagnosis techniques including electrophoresis at alkaline and acid pH, to evaluate the Hb migration profile and high performance liquid chromatography (HPLC) to measure Hb fractions[9-11]. Genomic DNA was extracted employing the phenol-chloroform method for molecular analysis[12]. The amplification of the segment that encodes Hb S was performed using specific primers (sense: 5'-GGCAGAGCCATCTATTGCTTA-3'; antisense: 5'-ACCTTAGGGTTGCCCATAAC-3') and cleavage was achieved by the action of restriction endonuclease FastDigest DdeI (Fermentas, USA).[13]

Biochemical analysis

Oxidative stress analysis was evaluated based on the detection of lipid peroxidation and antioxidant capacity markers in plasma. The lipid peroxidation levels were calculated by the thiobarbituric acid reactive species (TBARS) technique. This method is based on the reaction of malondialdehyde and other aldehydes, which are by-products of membrane damage caused by ROS, with thiobarbituric acid (TBA) at low pH and high temperature forming a complex with maximum light absorption at 535 nm[14].

The antioxidant capacity was evaluated using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a potent antioxidant similar to vitamin E. The Trolox-equivalent antioxidant capacity (TEAC) method is based on the reaction between 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (SIGMA, A1888), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) and potassium persulfate ($K_2S_2O_8$). This reaction produces the radical cation $ABTS^{\bullet+}$, a green/blue chromophore. The addiction of one antioxidant to this radical cation reduces ABTS, resulting in a solution discoloration that is evaluated at 734 nm to determine the total antioxidant capacity[15].

Plasma glutathione (GSH) concentrations were determined in EDTA-treated plasma samples by the quantification in HPLC coupled to a coulometric electrochemical detector (Coulochem III ESA, Bedford, MA) set with a potential of 650 mV[16]. Under these conditions, GSH is clearly eluted in ~ 6 min. GSH was extracted from the plasma samples by adding perchloric acid to the plasma sample (10% final concentration). After vigorous stirring and 10 min on ice, the mixture was centrifuged at 825 g for 10 min at 4°C. The extract was then filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC. Calculations were based on a calibration curve previously constructed by injecting known GSH standards into the HPLC system.

Statistical analysis

Data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. Analysis of variance (ANOVA) was employed complemented by Tukey's test for data with normal distribution. The Kruskal-Wallis test was employed followed by Dunn's test for non-parametric data. The correlations between Hb concentrations and lipid peroxidation and plasma GSH levels were achieved by the Pearson linear correlation test. The level of significance was assumed as 0.05 and analysis was made using the Statistica 8.0 software.

Results

Hemoglobin fraction concentration in SCA patients

The Hb profiles of SCA individuals were compared depending on whether patients took HU or not. Results showed higher Hb F levels (p = 0.018) and lower Hb S concentrations (p = 0.015) among individuals taking HU compared to those who were not taking this medication (Table 1).

Table 1 - Differences between hemoglobin profiles of the SCA patients taking HU or not

	Sickle cell anemia patients		p-value
	(-HU)	(+HU)	
Hb S	86.63 ± 4.42	80.44 ± 7.47	0.015
Hb A_2	3.63 ± 1.28	4.11 ± 0.50	0.250
Hb F	6.47 ± 4.23	11.89 ± 6.92	0.018

-HU: patients not taking hydroxyurea; +HU: patients taking hydroxyurea.

Lipid peroxidation – thiobarbituric acid reactive species dosage

The generation of ROS was indirectly measured though the analysis of lipid peroxidation. The values were higher for SCA patients (1450 ± 549 ng/mL) compared to the Control Group (239 ± 159 ng/mL)(p < 0.0001). Patients receiving HU had lower lipid peroxidation than those without specific medication however they still had higher levels than the Control Group (p < 0.0001).

Correlation analysis was carried out comparing Hb S and Hb F levels and the TBARS dosage. Results showed that TBARS values in SCA patients were positively correlated with Hb S concentrations (r = 0.55; p = 0.0040) reflecting increased lipid peroxidation in the presence of Hb S (Figure 1A). Negative correlation was observed between TBARS and Hb F levels (r = -0.52; p = 0.0067), showing a protective effect of Hb F (Figure 1B).

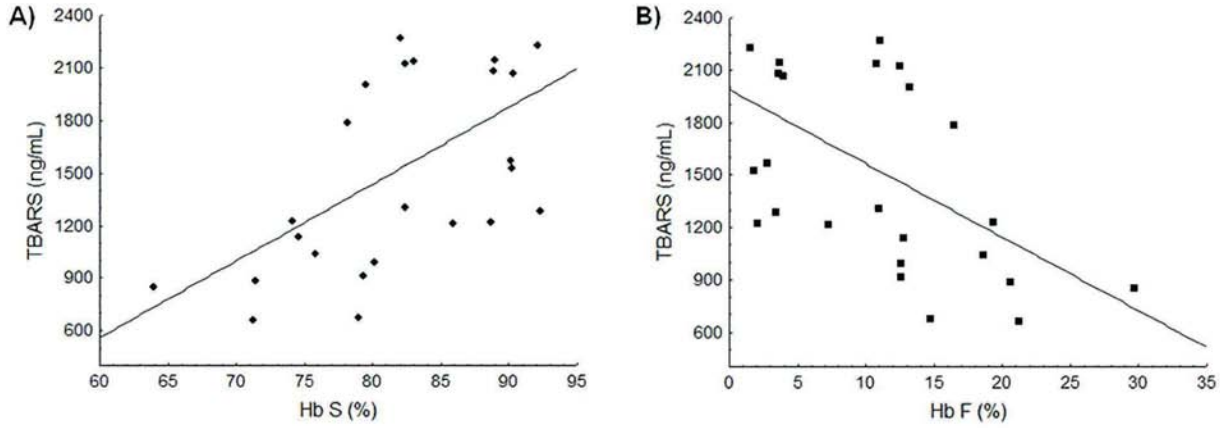

Figure 1 - Association between the Hb S and Hb F concentrations and lipid peroxidation levels. (A) Positive linear correlation between Hb S and TBARS levels (r = 0.55; p-value = 0.0040). (B) Negative linear correlation between Hb F and TBARS levels (r = -0.52; p-value = 0.0067).

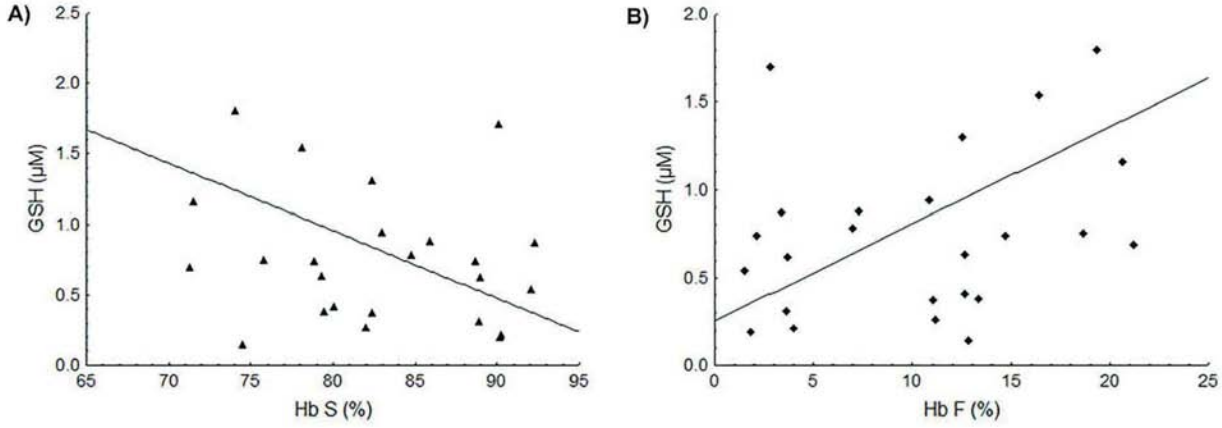

Figure 2 - Association between the Hb S and Hb F concentrations and the GSH levels. (A) Negative linear correlation between Hb S and GSH levels (r = -0.49; p-value = 0.0111). (B) Positive linear correlation between Hb F and GSH levels (r = 0.56; p-value = 0.0031).

Antioxidant Capacity – Trolox-equivalent antioxidant capacity assay and plasma glutathione levels

The overall antioxidant capacity analyzed by TEAC showed higher values for SCA patients (2.04 ± 0.15 mM) than for the Control Group (1.93 ± 0.15 mM; p = 0.0028). Patients taking HU had higher TEAC values when compared to the group of patients that did not take HU (p = 0.0002). No correlation was observed between Hb concentrations and TEAC levels.

The GSH concentration in the Control Group was 0.41 ± 0.38 µM with this value being about two times higher in SCA patients (0.88 ± 0.69 µM; p = 0.0292). Patients taking HU had higher GSH levels that were significantly different to the Control Group (p < 0.0001). Higher Hb S concentrations were correlated with decreased GSH levels (r = -0.49; p = 0.0111: Figure 2A) while Hb F concentrations were positively correlated with GSH values (r = 0.56; p = 0.0031: Figure 2B).

Discussion

The oxidative stress and antioxidant markers were evaluated in SCA patients who were either taking HU or not and the results were compared to the results of a control group. The correlation between Hb S levels and TBARS values, as well as lipid peroxidation in SCA patients suggest that oxidative stress may result from high levels of meta Hb S which is less stable than meta Hb A, leading to the formation of hemichromes and hemolysis with the release of heme iron. Oxidative stress may be even higher during vaso-occlusive crises and painful episodes[17].

Hb F levels were higher in SCA patients. It is known that in SCA patients, the concentration of Hb F ranges from 1% to 30% and it is inherited as a quantitative trait. A trend of increased Hb F levels in SCA patients taking HU was observed in this study which reflects the ability of HU to regulate Hb F expression[18].

SCA pathophysiology can be viewed as a cycle driven by hemolysis, oxidative stress, inflammation, cell adhesion to endothelium and vaso-occlusion. During hemolysis, iron is transformed from ferric to ferrous iron, thus producing meta Hb, with heme iron release. The hydrophobic character of free heme iron allows it to merge with cell membranes, increasing the susceptibility to oxidant-mediated destruction as well as the generation of ROS, characterizing lipid peroxidation. Thus, the level of TBARS is a good indicator of pro-oxidant stimuli, since it measures malondialdehyde and other aldehydes which are subproducts of cell membrane destruction[19].

Patients who are not taking HU but are taking folic acid, showed increased lipid peroxidation levels compared to patients taking HU. Folate acid is important in the formation of red blood cells because it participates in the purine and pyrimidine metabolism for DNA and RNA synthesis[20]. In turn, HU is a deoxyribonucleotide reductase which is able to increase Hb F synthesis due to its myelotoxicity. Studies have shown that this drug leads to increased Hb F levels in approximately 50% of patients with SCD. High Hb F concentrations may decrease the severity of illness because of inhibition in the polymerization of Hb S. Hb F alter contact sites between Hb molecules, impairing polymer formation with consequent reduction in the sickling process[21-23]. This is a potential reduction factor of hemolysis and the consequent cell damage caused by substances released during this process, for example, iron. Kaul et al. demonstrated that increased Hb F expression reduces oxidative stress in SCD transgenic mice[24]. In the current work, this antioxidant property of HU is observed by lower TBARS values obtained in patients in the group taking this drug compared to patients who were not.

The generation of free radicals is counteracted by enzymatic and non-enzymatic antioxidants, including antioxidants from the diet such as vitamins C and E and GSH[25]. Some studies have found decreased GSH concentrations in erythrocytes from SCD patients[26,27]. However, Reid et al. and Kiessling et al. showed that GSH synthesis is not impaired in SCA patients, thus suggesting that lower GSH levels may be explained by the high demand[28,29]. Reid et al. showed that GSH synthesis was increased in 57% of SCA patients compared to control subjects, indicating that the consumption of GSH may exceed its synthesis[28]. On the other hand, GSH levels may also be influenced by diet, for example, due to deficiencies in the amino acid precursors of GSH synthesis, especially cysteine[30].

Maintaining adequate levels of GSH is important for many critical cell functions with disruptions in these processes being observed in several human diseases. GSH deficiency is manifested primarily as an increased susceptibility to oxidative stress and cell damage, the results of which may be an important factor in the progression of the disease. On the other hand, high GSH levels may increase antioxidant capacity and resistance to oxidative stress[31].

Regarding treatment, HU was associated with increased antioxidant capacity. Liu et al. tested the activity of HU against free radicals such as DPPH (2,2-diphenyl-1-picril-hydrazyl) and hydroxyl radicals; they found that HU has antioxidant activity[32]. The higher values of GSH and TEAC in patients treated with HU corroborate this finding, since the antioxidant properties of HU may have curbed the consumption of the antioxidant systems evaluated.

Conclusion

The results of this study show that the influence of Hb S on the oxidative status is reflected by increased lipid peroxidation and antioxidant status in SCA patients. High Hb F concentrations are associated with less oxidative stress. Treatment using HU decreased lipid peroxidation and contributed to the body's antioxidant defenses.

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References

1. Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest. 2007;117(4): 850-8.
2. Higgs DR, Wood WG. Genetic complexity in sickle cell disease. Proc Natl Acad Sci USA. 2008;105(33):11595-6. Comment on: Proc Natl Acad Sci USA. 2008;105(33):11869-74.
3. Figueiredo MS. Fatores moduladores da gravidade da evolução clínica da anemia falciforme. Rev Bras Hematol Hemoter. 2007;29(3):215-7.
4. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. 2010;376(9757):2018-31.
5. Kovacic P. Hydroxyurea (therapeutics and mechanism): metabolism, carbamoyl nitroso, nitroxyl, radicals, cell signaling and clinical applications. Med Hypotheses. 2011;76(1):24-31.
6. Chaves MA, Leonart MS, do Nascimento AJ. Oxidative process in erythrocytes of individuals with hemoglobin S. Hematology. 2008;13(3):187-92.
7. Kuypers FA. Red cell membrane lipids in hemoglobinopathies. Curr Mol Med. 2008;8(7):633-8.
8. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT .Vasculopathy in sickle cell disease: biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol. 2009;84(9):618–25.
9. Marengo-Rowe AJ. Rapid electrophoresis and quantitation of haemoglobin on cellulose acetate. J Clin Pathol. 1965;18(6):790-2.
10. Vella F. Acid agar gel electrophoresis oh human hemoglobins. Am J Clin Pathol. 1968;49(3):440-2.
11. Bio-Rad Laboratories. Instruction Manual: Variant β-thalassemia short program. Hercules, California: Bio-Rad Laboratories; 1999.
12. Sambrook J, Fritcsh EF, Manatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
13. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985;230(4732):1350-4.
14. Percário S. Dosagem do dialdeído malônico. Newslab. 2004;6:46-50.
15. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9-10):1231-7.
16. Rodríguez-Ariza A, Toribio F, López-Barea J. Rapid determination of glutathione status in fish liver using high-performance liquid

chromatography and electrochemical detection. J Chromatogr B Biomed Appl. 1994;656(2):311-8.

17. Fibach E, Rachmilewitz E. The role of oxidative stress in hemolytic anemia. Curr Mol Med. 2008;8(7):609-19.
18. Green NS, Barral S. Genetic modifiers of HbF and response to hydroxyurea in sickle cell disease. Pediatric Blood Cancer. 2011;56(2):177-81.
19. Belcher JD, Beckman JD, Balla G, Balla J, Vercellotti G. Heme degradation and vascular injury. Antioxid Redox Signal. 2010;12(2):233-48.
20. Brasil. Ministério da Saúde. Agencia de Vigilância Sanitária – ANVISA. Manual de diagnóstico e tratamento de doenças falciformes [Internet]. Brasília: Ministério da Saúde; 2001.[cited 2011 Jan 12]. Available from: http://www.ebah.com.br/content/ABAAAA2R4AB/manual-diagnostico-tratamento-doencas-falciformes
21. Steinberg MH. Management of sickle cell disease. N Engl J Med. 1999;340(13):1021-30.
22. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. N Engl J Med. 1995;332(20):1317-22. Comment in: N Engl J Med. 1996;334(5):333-4; N Engl J Med. 1995;332(20):1372-4; N Engl J Med. 1995;333(15):1008-9; author reply 1009.
23. Silva LB, Gonçalves RP, Martins MF. Estudo da correlação entre os níveis de hemoglobina fetal e o prognóstico dos pacientes com anemia falciforme. Rev Bras Hematol Hemoter. 2009:31(6):417-20.
24. Kaul DK, Liu XD, Choong S, Belcher JD, Vercellotti GM, Hebbel RP. Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice. Am J Physiol Heart Circ Physiol. 2004;287(1):H293–301.
25. Hernanz A, Fernández-Vivancos E, Montiel C, Vazquez JJ, Arnalich F. Changes in the intracellular homocysteine and glutathione content associated with aging. Life Sci. 2000;67(11):1317-24.
26. Chan AC, Chow CK, Chiu D. Interaction of antioxidants and their implication in genetic anemia. Proc Soc Exp Biol Med. 1999;222(3):274-82.
27. Reid M, Jahoor F. Glutathione in disease. Curr Opin Clin Nutr Metab Care. 2001;4(1):65-71.
28. Reid M, Badaloo A, Forrester T, Jahoor F. In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. Am J Physiol Endocrinol Metab. 2006;291(1):E73-9.
29. Kiessling K, Roberts N, Gibson JS, Ellory JC. A comparison in normal individuals and sickle cell patients of reduced glutathione precursors and their transport between plasma and red cells. Hematol J. 2000;1(4):243-9.
30. Cresenzi CL, Lee JI, Stipanuk MH. Cysteine is the metabolic signal responsible for dietary regulation of hepatic cysteine dioxygenase and glutamate cysteine ligase in intact rats. J Nutr. 2003;133(9):2697-702.
31. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.
32. Liu YH, Wu WC, Lu YL, Lai YJ, Hou WC. Antioxidant and amine oxidase inhibitory activities of hydroxyrea. Biosci Biotechnol Biochem. 2010;74(6):1256-60.

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ORIGINAL ARTICLE

Oxidative stress and antioxidant capacity in sickle cell anaemia patients receiving different treatments and medications for different periods of time

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Abstract To evaluate, in a longitudinal study, the profile of lipid peroxidation and antioxidant capacity markers in sickle cell anaemia patients receiving different treatments and medication over different time periods. The three groups were: patients undergoing transfusion therapy and receiving iron chelator deferasirox (DFX group, n=20); patients receiving deferasirox and hydroxyurea (DFX + HU group, n=10), and patients receiving only folic acid (FA group, n=15). Thiobarbituric acid-reactive substance (TBARS) assays and trolox-equivalent antioxidant capacity (TEAC) assays were evaluated during two different periods of analysis, T0 and T1 (after ~388 days). Higher FA group TBARS values were observed compared with the DFX + HU group (p=0.016) at T0; and at T1, higher FA group TBARS values were also observed compared with both the DFX group (p=0.003) and the DFX + HU group (p=0.0002). No variation in TEAC values was seen between groups, at either T0 or T1. The mean values of TBARS and TEAC for both the DFX and DFX + HU groups decreased at T1. The antioxidant effects of HU and DFX were observed by through an increase in TEAC levels in DFX and DFX + HU groups when compared with those of normal subjects. Increased TEAC values were not recorded in the FA group, and lipid peroxidation was seen to decrease after DFX and HU use.

Keywords Sickle cell anaemia · Oxidative stress · Hydroxyurea · Deferasirox · Folic acid

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Introduction

Sickle cell anaemia (SCA) is one of the most common monogenic disorders in the world. It has been characterised as a multi-system disease, associated with episodes of acute illness and progressive organ damage [1]. Haemoglobin S (Hb S) is caused by a mutation in the β-globin gene in which the 17th nucleotide is changed from thymine to adenine and the sixth aminoacid in the β-globin chain becomes valine rather than glutamic acid [2].

In cases of hypoxia, Hb S polymerises, which defines the first indispensable event in the molecular pathogenesis of SCA [3]. The polymer grows, disrupting erythrocyte architecture and promoting cellular dehydration, with physical and oxidative cellular stress [4]. As a result of this process, intravascular haemolysis occurs, and free plasma Hb generates reactive oxygen species (ROS), such as superoxide and hydroxyl radicals [5], which are potent scavengers of nitric oxide (NO) [6], causing vascular endothelium dysfunction [7]. Moreover, the cycles of ischaemia and reperfusion cause oxidant stress. These

cycles also activate vascular oxidases [8] and lead to inflammatory stress. All of these changes increase the expression of endothelial cell-adhesion molecules and the synthesis of inflammatory cytokines, causing leucocytosis [9–11]. Thus, the consequence of oxidative stress may contribute to two major pathophysiological processes: vaso-occlusion with ischaemia/reperfusion injury and haemolytic anaemia [12, 13].

The most common treatments for SCA are folic acid (FA) supplementation, the use of hydroxyurea (HU), and blood transfusions. HU is often administered to induce increased synthesis of Hb F, and in many studies, it increased Hb F synthesis in approximately 60% of patients. In these studies, HU raised the rate of Hb and reduced the number of reticulocytes [14–16]. Other studies have shown that HU reduces the expression of adhesion molecules on the erythrocyte surface. It also reduces the number of granulocytes, monocytes and platelets, which, when increased, are risk factors for vaso-occlusion [17, 18]. HU also participates in NO production [19]. Transfusion therapy works by keeping Hb S concentration below 30%, increasing oxygenation, and to decreasing vascular complications [20]. Additionally, transfusions prevent primary and secondary strokes in children, and decrease the frequency of hospitalisation, vaso-occlusive events, acute chest syndrome and growth retardation [21]. However, in patients subject to iron overload, chelating agents are essential for survival. Among the available iron chelators, deferasirox (DFX) (Exjade™; Novartis Pharma AG, Basel, Switzerland) is a once-daily oral iron chelator which has been extensively evaluated in patients with a wide range of transfusion-dependent anaemia, including SCA, in both adult and paediatric patients [22]. Other drugs have also been used, but, as yet, there have been no large multi-centre studies proving their effectiveness [23].

In light of evidence suggesting that an excess of ROS has implications in SCA pathophysiology, the assessment of oxidative stress in these patients may provide significant information regarding the use of current medications and may lead to new therapeutic strategies. Therefore, the purpose of this longitudinal study was to evaluate the profile of lipid peroxidation and antioxidant capacity markers in SCA patients receiving different treatments and medication over different time periods.

Methods

Study design

This is a longitudinal study involving 45 SCA patients (20 males and 25 females with a mean age of 25.8 years old ranging between 11 and 45 years old) seen at the outpatient department of Santa Casa Medical School, Sao Paulo, Brazil.

The subjects were divided according to treatment and specific medication. All patients had been taking folic acid (5 mg/day) for at least 5 years, and the three groups were: patients undergoing transfusion therapy and receiving iron chelator deferasirox (DFX group, $n=20$); patients receiving iron chelator deferasirox and using hydroxyurea, (DFX + HU group, $n=10$) and patients receiving only folic acid (FA group, $n=15$). At the beginning of analysis, characterised as Time 0 (T0), the DFX group was exposed to iron chelation for 46.8±20.7 days, and the DFX + HU group was exposed to iron chelation for 39.1±29.9 days and to hydroxyurea for more than 3 years. After 388.6±58.7 days, the same patients' samples were again collected and evaluated. This period has been characterised as Time 1 (T1).

Patient eligibility

Eligible patients were 10 years or older at the beginning of the study, with SCA and well-defined treatment plans developed by the medical staff of Santa Casa Medical School.

Patients were excluded from the study if they were smokers, consumed alcoholic drinks, or if they had had a stroke, pain, or a haemolytic crisis in the 2 months prior to the start of the study, or if they had taken medications (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection. Patients with active hepatitis B and C, serum creatinine above normal levels, aspartate aminotransferase or alanine aminotransferase levels above 250 U/L, and patients who were pregnant were also excluded from this study. All subjects gave their informed consent, and the study was approved by the Data Safety Monitoring Board in line with Brazilian regulations. The demographic and clinical characteristics and β-globin haplotypes of all SCA patients are shown in Table 1.

Treatment plan

All patients with iron overload received DFX, except FA group patients. Iron overload was defined as the use of more than 20 units of red blood cell transfusions in the past and/or SF (serum ferritin) levels greater than 1,000 μg/L confirmed by at least two measurements during the 6 months leading up to enrolment for the formation this group. The patients began taking DFX at a dose of 20 mg/kg/day, and dose adjustments of 5–10 mg/kg/day (within a range of 5–30 mg/kg/day) were possible only after 12 weeks of treatment. Doses were adjusted, if necessary, every 3 months, with changes made by the haematologist responsible for patient care on a case-by-case basis. Decisions regarding changes in dosage were based on repeated measurements of SF and regularly

Table 1 Demographics, clinical data, and β-globin haplotypes data of SCA patients

Characteristics	Treatment/medication					
	DFX (n=20)		DFX + HU (n=10)		FA (n=15)	
Age (in years) mean±SD	25.2±12.2		30.9±11.1		23.7±8.9	
β-Globin haplotypes [n (%)]						
Bantu/Bantu	10 (50.0%)		05 (50.0%)		08 (53.3%)	
Bantu/Benin	04 (20.0%)		03 (30.0%)		01 (06.6%)	
Benin/Benin	02 (10.0%)		01 (10.0%)		04 (26.6%)	
Bantu/Atp	04 (20.0%)		01 (10.0%)		01 (06.6%)	
Number of RBC units transfused since birth [n (%)]						
<10	01 (05.0%)		–		04 (26.6%)	
10–20	04 (20.0%)		08 (80.0%)		07 (46.6%)	
>20	14 (70.0%)		02 (20.0%)		04 (26.6%)	
Number of days since last transfusion T0 [n (%)] and T1 [n (%)]						
<30 days	07 (35.0%)	09 (42.5%)	03 (30.0%)	03 (30.0%)	05 (33.3%)	07 (46.6%)
30–60 days	08 (40.0%)	05 (25.0%)	03 (30.0%)	02 (20.0%)	03 (20.0%)	05 (33.3%)
≥61 days	05 (25.0%)	06 (30.0%)	04 (40.0%)	05 (50.0%)	07 (46.6%)	08 (53.33%)
Number of sickle cell crises per person per year in the last 2 years [n (%)]						
0–2	11 (55.0%)		07 (70.0%)		02 (13.3%)	
3–5	07 (35.0%)		02 (20.0%)		04 (26.6%)	
≥6	02 (10.0%)		01 (10.0%)		09 (60.0%)	
Complications of sickle cell anaemia [n (%)]						
Cardiac complications	07 (35.0%)		04 (40.0%)		05 (33.3%)	
Muscular/skeletal/skin complications	06 (30.0%)		09 (90.0%)		06 (40.0%)	
Gastrointestinal/hepatobiliary complications	03 (15.0%)		04 (40.0%)		03 (20.0%)	
Splenic complications	02 (10.0%)		02 (20.0%)		04 (26.6%)	
Pulmonary complications	02 (10.0%)		03 (30.0%)		04 (26.6%)	
Neurologic complications	12 (60.0%)		02 (20.0%)		02 (13.3%)	

Definitions: Cardiac complications include cardiomegaly, cardiomyopathy, congestive heart failure, and hypertension; Muscular/skeletal/skin complications include avascular necrosis, dactilytis, leg ulcers, osteomyelitis, and osteoporosis; gastrointestinal/hepatobiliary complications include cholecycstitis, cholelithiasis/sludge, hepatic sequestration, and intrahepatic cholestasis. Splenic complications include acute splenic infarction, functional asplenia, hypersplenism, and acute splenic sequestration while pulmonary complications include acute chest syndrome and pulmonary hypertension. Neurologic complications—cerebrovascular accidents include aneurysm, haemorrhagic stroke, ischaemic or infarctive stroke, and silent cerebral infarction

DFX deferasirox, *HU* hydroxyurea, *FA* folic acid

measured laboratory safety parameters. This treatment was discontinued if SF levels reached 500 μg/dL or lower during two consecutive visits and was reinstated if levels subsequently rose above 1,000 μg/L.

The starting dose of HU (20 mg/kg/day) was increased by 5 mg/kg per day at 8-week intervals until a dose of 35 mg/kg/day was reached. The criteria used to decide whether to suspend the use of HU was the appearance of clinical evidence and/or laboratory evidence of intolerance or toxicity. Toxicity was defined as a decreased number of leukocytes (<4.000/mm^3), absolute neutrophil count (<2.000/mm^3), platelet count (<100.000/mm^3) or lowered Hb levels (less than 20% of base levels). Toxicity was also considered in cases of renal dysfunction, hepatic diseases or gastrointestinal disorders.

Biological samples

Three venous blood samples of 20 mL were collected from each patient in tubes with EDTA, tubes with heparin and tubes without any anticoagulants. EDTA samples were used for Hb profiles, haematological profiles and molecular analyses. The heparinised blood was incubated for 20 min at 37°C and centrifuged at 780×g for 20 min to separate the plasma and was then frozen at −80º C. These samples were used for thiobarbituric acid-reactive substance (TBARS) assays and trolox-equivalent antioxidant capacity (TEAC) assays. Samples without anticoagulants were used to measure serum iron (SI), serum ferritin (SF) and total iron-binding capacity (TIBC).

Haemoglobin phenotypes and haematologic profile

Hb was identified using electrophoresis on cellulose acetate at pH 8.4 and using agar electrophoresis at pH 6.2. Hb fraction quantification was obtained using high-performance liquid chromatography with the automated VARIANT™ equipment (Bio-Rad Laboratories, CA, USA) [24]. Haematologic profiles were obtained using the Cell-Dyn-3000™ automatic analyser (Abbott Laboratories, USA). Hb fraction values were obtained only at the beginning of the search for defined Hb phenotypes.

Haemoglobin genotypes and β^S-globin haplotypes

In all samples, the Hb genotype was developed through molecular analysis using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Segment amplification, which encodes Hb S, was accomplished through the use of specific primers, and amplicons were cleaved by the FastDigest™ DdeI restriction enzyme (Fermentas, CA, USA) [25]. Beta globin haplotypes were determined through the PCR-RFLP analysis of the following polymorphic restriction sites: γG (Hind III), γA (Hind III), ψβ (Hinc II), 3′ψβ (Hinc II) and 5′β (Hinf I), as previously described [26].

Biochemical analysis

Oxidative stress was evaluated by observing TBARS plasma levels, which themselves were calculated based on malondialdehyde and other aldehyde reactions with thiobarbituric acid at low pH levels and high temperatures. These formed a complex in which absorbance could be read at 535 nm. Normal values were considered to be up to 440 ng/mL [27, 28].

Antioxidant capacity of the samples was determined according to their equivalence to the antioxidant activity of trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) and the vitamin E analog [29, 30]. Normal values for TEAC were established under local laboratory analysis conditions using random volunteer samples with normal TBARS values and without haemoglobinopathies. The TEAC average was 2.12±0.10 mM, ranging from 1.85 to 2.31 mM [27].

Serum iron and TIBIC were measured using colorimetric and enzymatic assays with the automated Advia 1650™ system (Bayer Diagnostics, USA). Transferrin saturation was estimated as the ratio between SI and TIBC and is reported as a percentage. SF was determined using an immune assay with the Axsym System™ (Abbott Laboratories, USA).

Statistical analysis

Statistical analysis was performed using the Statistica 9.0 and Graphpad Prism 5.0 software. The data is expressed as mean±standard deviation and was tested for normal distribution using the Shapiro Wilk test. Data were also tested for homogeneity of variances using the Levene test. Means between the groups were compared by applying the *t* test or one-way ANOVA, followed by the post hoc Tukey test for parametric data, and either the Mann–Whitney test or Kruskal–Wallis test, followed by the post hoc Dunn test for non-parametric data. To assess the degree of association between the variables studied, the Pearson correlation for parametric data and the Spearman correlation for non-parametric data were used. Dependent *t* tests were utilised in the analysis of paired samples (T0 and T1) for parametric data, and the Wilcoxon matched-pairs test was used for non-parametric data. A p value of <0.05 was considered statistically significant.

Results

Neither gender nor age influenced haematological profiles, iron status or oxidative stress parameters in SCA patients (data not shown). Subjects were classified into three groups according to age (11–20, 21–30 and 31–45 years). The three groups were gender-paired and the parameters evaluated, but no significant p values were found when a two-way ANOVA test was performed.

The results obtained for all parameters evaluated are shown in Table 2, which highlights the differences in haematological profiles, haemoglobin fractions, iron statuses and TEAC and TBARS values between the groups studied. The differences have been outlined according to the time of treatment (T0 and T1). TBARS values were significant higher ($p<0.0001$) in all groups when compared with normal values. TBARS values in the FA group were 46.4% higher than those of the DFX + HU group ($p=0.016$) at T0. At T1, TBARS values in the FA group were 40.5% higher than those of the DFX group ($p=0.003$) and 69.4% higher than those of the DFX + HU group ($p=0.0002$). TEAC values did not differ between the groups, at either T0 or T1. However, when TEAC values were compared with normal subject values (see "Biochemical analysis" under "Methods"), the DFX group at T0 had TEAC values which were 2.75 times higher than normal values ($p=0.02$), the DFX + HU group at T0 had TEAC values which were 3.2 times higher than normal ($p=0.03$), and the FA group did not show such an increase.

With respect to haematimetric values, the FA group presented 1.50 and 1.75 times higher absolute values of

Table 2 Haematological and iron profiles and haemoglobin fractions and oxidative parameters in SCA patients, separated according to treatment/specific medication during the two analysis periods (T0 and T1)

	Initial analysis (T0)			Final analysis (T1)		
	DFX (n=20)	DFX + HU (n=10)	FA (n=15)	DFX (n=20)	DFX + HU (n=10)	FA (n=15)
Haematological profiles and haemoglobin fractions (mean±SD)						
Haemoglobin (g/dL)	8.42±0.98	7.84±1.23	8.52±1.55	8.82±1.28	8.83±1.17	8.32±1.93
MCV (fL)	93.76±5.72	99.98±9.70	88.48±14.40	99.31±6.31	95.78±6.36	89.62±11.40
MCH (pg)	31.63±1.43	31.71±2.35	29.20±4.64	30.83±3.05	32.32±3.01	30.11±4.35
Leucocytes (/mm^3)	10.74±2.18	8.90±2.75	9.63±1.24	11.60±3.20	10.15±2.52	12.03±2.05
Lymphocytes (/mm^3)	3.17±0.85	2.61±0.84	2.97±0.52	3.73±1.47	3.71±1.51	4.33±1.44
Neutrophils (/mm^3)	5.96±1.87	4.97±1.72	4.85±1.15	6.40±2.35	5.21±1.65	5.74±1.54
Monocytes (/mm^3)	0.81±0.27[a]	0.72±0.35[a]	1.26±0.25[b]	0.77±0.47[a]	0.55±0.28[a]	1.18±0.41[b]
Eosinophils (/mm^3)	0.51±0.38	0.46±0.45	0.42±0.25	0.52±0.23	0.50±0.32	0.42±0.34
Hb F (%)	4.95±4.12[e]	10.87±9.46[f]	5.70±4.64[e, f]	NA	NA	NA
Hb A_2 (%)	2.47±0.99	2.68±0.42	3.47±1.88	NA	NA	NA
Hb S (%)	57.23±19.17	67.42±21.61	70.13±19.68	NA	NA	NA
Iron profile (mean±SD)						
Serum iron (μg/dL)	178.35±72.91	160.90±50.24	141.13±46.12	137.55±49.10[a, b]	96.22±30.75[a]	159.53±47.28[b]
Serum ferritin (μg/dL)	2,503.5±1,514.3	3,192.9±1,796.7	1,712.8±1,060.5	2,002.0±808.3	2,376.2±1,636.7	1,866.1±1,791.5
TIBC (μg/dL)	310.95±108.83	284.56±55.23	273.87±56.74	277.43±98.57	246.44±56.65	270.47±41.49
Transferrin saturation (%)	58.86±16.90	57.27±17.45	52.62±16.07	51.65±17.20[e, f]	39.35±9.29[e]	61.10±24.37[f]
Oxidative stress parameters (mean±SD)						
TBARS (ng/mL)	749.1±254.4[c, d]	559.4±128.1[c]	818.9±164.5[d]	650.8±188.5[a]	539.9±122.2[a]	914.8±192.4[b]
TEAC (mM)	2.18±0.14	2.19±0.05	2.15±0.10	2.08±0.13	2.10±0.09	2.16±0.09

NA not available, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *TIBC* total iron-binding capacity, *TBARS* thiobarbituric acid-reactive substances, *TEAC* trolox equivalent antioxidant capacity

Normal values: TBARS (up to 440 ng/mL) and TEAC (2.12±0.10 mM)

[a, b] Different letters indicate statistical difference (one-way ANOVA, post hoc Tukey test $p<0.001$)

[c, d] Different letters indicate statistical difference (one-way ANOVA, post hoc Tukey test $p<0.05$)

[e, f] Different letters indicate statistical difference (Kruskal Wallis, post hoc Dunn test $p<0.05$)

monocytes compared with the DFX and DFX + HU groups ($p<0.001$) at T0, and 1.53 and 2.14 times higher quantities of monocytes compared with the DFX and DFX + HU groups at T1 ($p<0.001$).

The serum ferritin values and transferrin saturation in all groups were above normal values. At T1, the DFX + HU group presented values of serum iron ($p<0.001$) and transferrin saturation ($p=0.03$) which were lower than those observed in the FA group.

The application of paired analysis between all groups revealed that, in the DFX group, Hb values ($p=0.034$) and MCV values ($p=0.033$) were higher at T1. Figure 1 shows the decrease in the mean values of TBARS and TEAC for the DFX and DFX + HU groups at T1, in contrast to the greater lipid peroxidation of the FA group. Figure 2 shows that the DFX and DFX + HU groups had a significant decrease in all iron parameters along time but the FA group did not.

The degree of relation between TBARS and TEAC was significant at T0 ($p=0.023$, $r=0.34$) and T1 ($p=0.01$, $r=0.46$). When grouped, individuals at T0 and T1 showed a significant positive correlation ($p<0.001$ and $r=0.59$), as shown in Fig. 3a. No correlation was observed between Hb fractions (Hb S and Hb F) and TEAC values, but a significant negative correlation was observed between Hb F and TBARS values ($p=0.014$, $r=-0.36$) (Fig. 3b). The iron parameters related to TBARS and TEAC during the two periods showed a significant positive correlation between variables: TBARS and serum iron [T0 ($p=0.02$, $r=0.33$), T1 ($p=0.009$/$r=0.38$)], and TBARS and TIBIC [T0 ($p=0.01$, $r=0.35$), T1 ($p=0.01$/$r=0.36$)], both at T0 and T1 (Fig. 4). Correlation analyses were also performed between leukocyte, neutrophil, lymphocyte, eosinophil and monocyte values (Table 2) and TBARS and TEAC levels, but only the FA group showed a significant positive correlation for absolute monocytes and TBARS, at both T0 ($p<0.01$) and T1 ($p<0.01$). When grouped, all

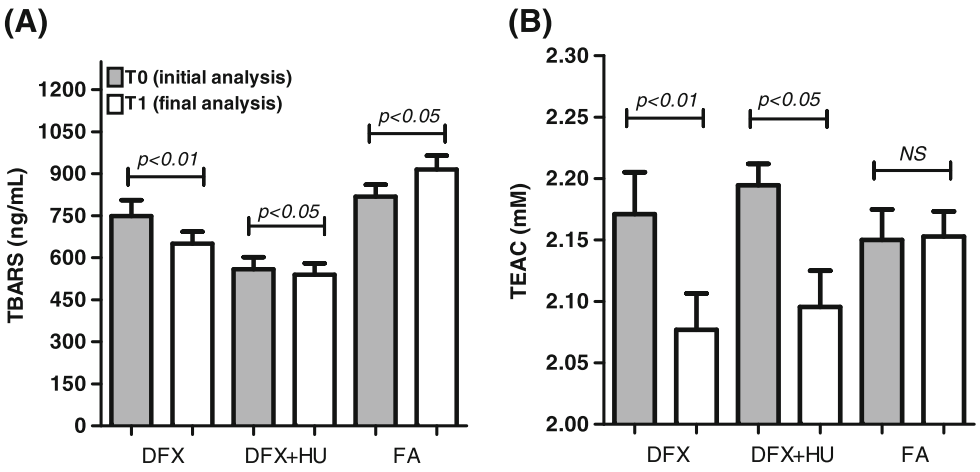

Fig. 1 Paired analyses of lipid peroxidation (TBARS) and antioxidant capacity (TEAC) in SCA patients under different treatments/medications. (**A**) DFX group: TBARS values decreased ~13% from T0 to T1; DFX + HU: TBARS values decreased ~3.5% from T0 to T1; FA group: TBARS values increased ~11.7% from T0 to T1. (**B**) DFX group: TEAC values decreased ~4.6% from T0 to T1; DFX + HU: TEAC values decreased ~4.1% from T0 to T1; FA group: not significant

individuals in the FA group showed a significant correlation ($p<0.001$, $r=0.58$) as shown in Fig. 5.

Discussion

In this study, we have evaluated oxidative stress markers in SCA patients receiving different treatments and medications over different time periods. A decrease in lipid peroxidation values was found in the group which received DFX associated with HU. After a longer period of DFX use, there was a reduction in lipid peroxidation and iron values, and in the FA group, the lack of treatment or specific medication may explain the high TBARS values.

Several studies have demonstrated significant increases in stress markers and differing behaviour in antioxidant

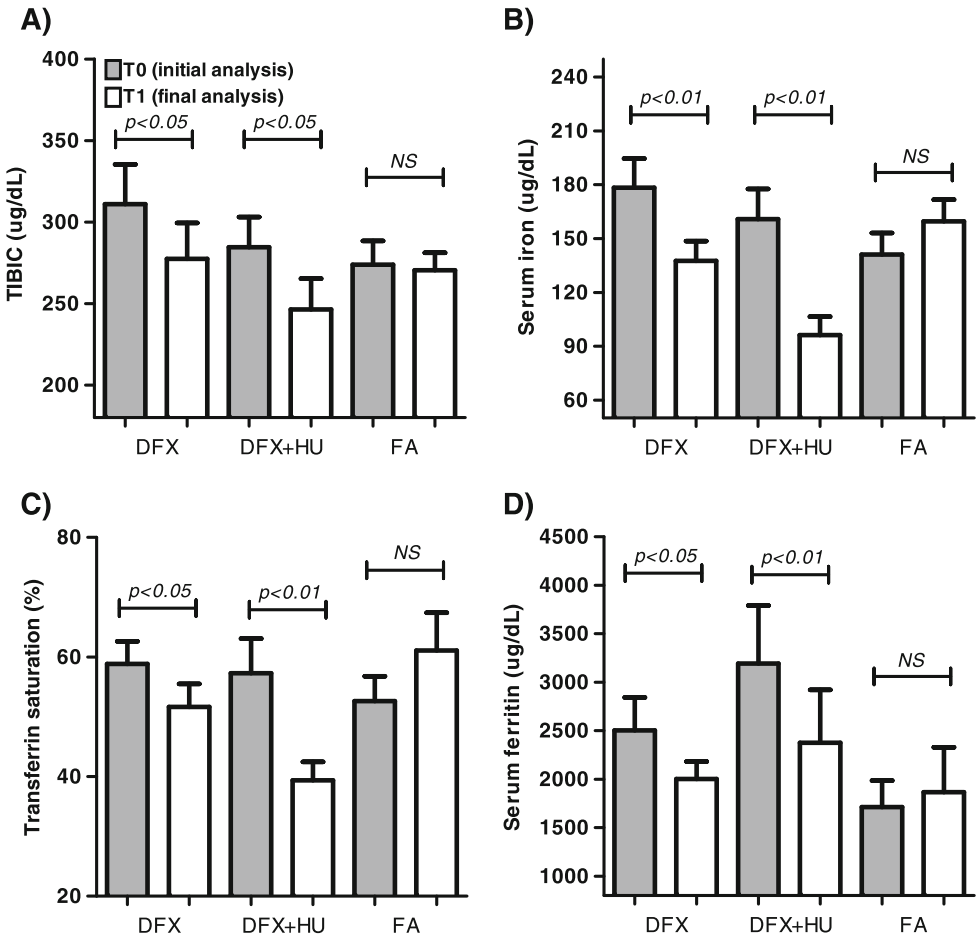

Fig. 2 Paired analyses of iron status in SCA patients under different treatments/medications. TIBIC (total iron-binding capacity); SI (serum iron); TS (transferrin saturation); SF (serum ferritin). (**A**) DFX group: TIBIC values decreased ~10.8% from T0 to T1; DFX + HU: TIBIC values decreased ~13.4% from T0 to T1; FA group: not significant. (**B**) DFX group: SI values decreased ~22.8% from T0 to T1; DFX + HU: SI values decreased ~40.2% from T0 to T1; FA group: not significant. (**C**) DFX group: TS decreased ~12.2% from T0 to T1; DFX + HU: TS decreased ~31.31% from T0 to T1; FA group: not significant. (**D**) DFX group: SF decreased ~20.1% from T0 to T1; DFX + HU: SF decreased ~25.6% from T0 to T1; FA group: not significant

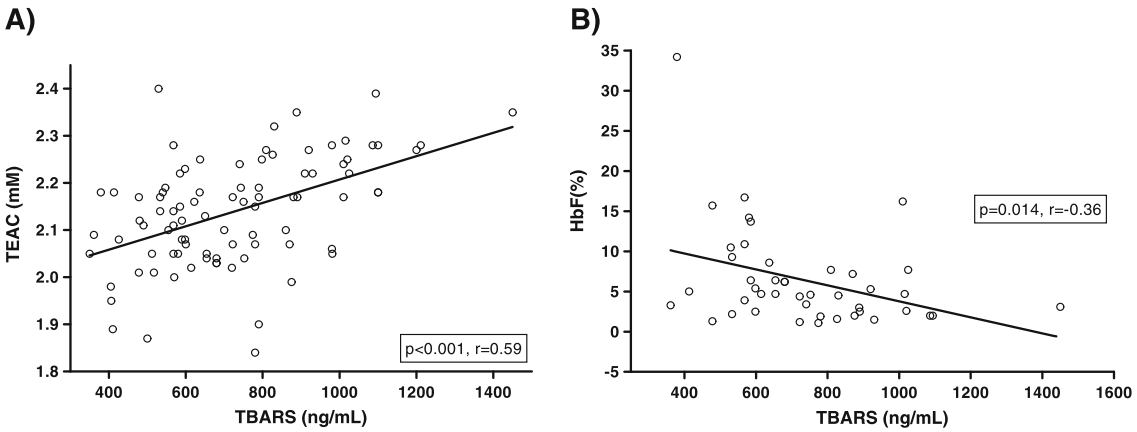

Fig. 3 Correlation analyses in all SCA patients independent of medication type. (**A**) The degree of relation between TBARS and TEAC was significantly positive ($p<0.001$, $r=0.59$); when TBARS values increased, values of TEAC tended to increase. (**B**) The degree of relation between TBARS and Hb F was significantly negative ($p=0.014$, $r=-0.36$); when Hb F values increased, values of TBARS tended to decrease

defense systems in SCA patients when compared with normal subjects [31–34], but no longitudinal study has related oxidative parameters to the use of different medications, as has been presented here.

Under normal physiological conditions, antioxidant defences ensure that basal fluxes of ROS do not negatively affect the body [35]. Nevertheless, in cases of SCA, this process is altered due to the increased production of ROS, which itself is caused by the intrinsic mechanisms of the disease, such as increased activity of several oxidases (NADPH oxidase and endothelial xanthine oxidase) [8, 36], auto-oxidation of Hb S [37], the release of haem iron, increased asymmetric dimethylarginine [38, 39], uncoupling of nitric oxide synthase activity and decreased levels of NO [40]. As the antioxidant defense systems in SCA are affected and/or are not strong enough to neutralise the excessive production of ROS [41, 42], chronic oxidative stress is a critical factor in endothelial dysfunction, inflammation and damage to multiple organs [43].

Among the existing biomarkers, the evaluation of TBARS is an indirect quantification of malondialdehyde (MDA) and other aldehydes resulting from the lipid peroxidation process, which makes it a good indicator of pro-oxidant stimuli [44–46]. The antioxidant capacity of the individual can be assessed using different techniques. Unlike other studies in which specific antioxidants are measured, this study has assessed the overall soluble antioxidants present in plasma (TEAC), revealing its utility as a biomarker of the body's defense response to oxidative stimuli due to either pathological processes or to pharmacological treatment [47–49]. The results of this study have revealed the direct relationship between the two markers: When TBARS values increase, TEAC values tend to increase as well, that is, the high concentration of oxidising

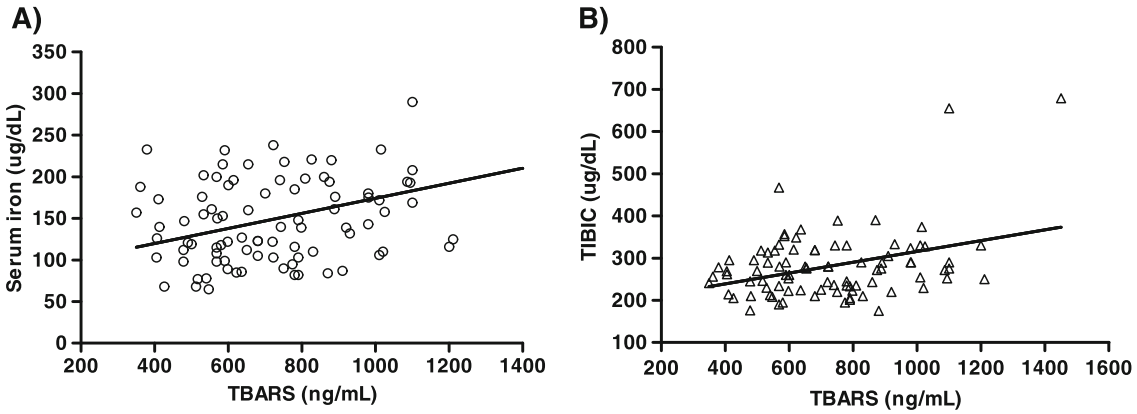

Fig. 4 Correlation analyses between lipid peroxidation and serum iron and between lipid peroxidation and total iron-binding capacity in SCA patients. (**A**) The degree of relation between TBARS and serum iron was significantly positive ($p=0.0006/r=0.35$) in all SCA patients ($n=90$). (**B**) The degree of relation between TBARS and TIBIC ($p=0.0007/r=0.35$) in all SCA patients ($n=90$)

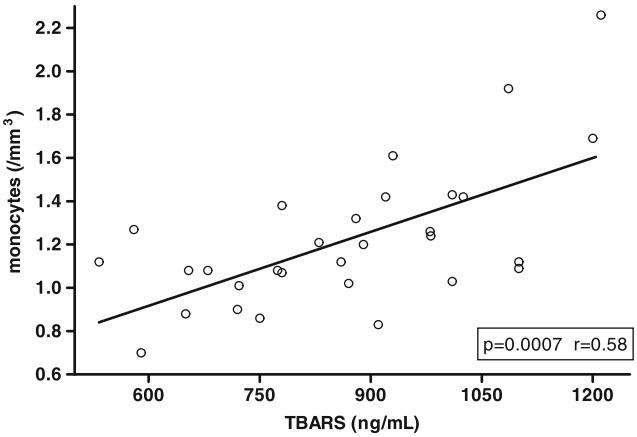

Fig. 5 Correlation analysis between lipid peroxidation and absolute monocytes values in SCA patients receiving only folic acid. The degree of relation between the two variables was significant (p= 0.0007 and r=0.58); when TBARS values increased, values of monocytes tended to increase

agents seems to increase antioxidant capacity so that it may counteract the action of reactive species. These findings have corroborated the results of two other recent studies in which the same behaviour was recorded for these variables [50, 51].

As the increase in Hb F concentration either inhibits or retards Hb S polymerisation [52], our study has shown that increased Hb F decreases levels of lipid peroxidation and therefore plays a protective effect on sickling and haemolysis, lowering the release of oxidants. These results corroborate a previous in vivo study on knockout mice. This study reported a decrease in the occurrence of the intravascular sickling process, increased NO bioavailability and a decrease in the occurrence of lipid peroxidation in animals that had increased Hb F [53].

As was expected, TBARS values were found to be higher than in normal subjects in the patients evaluated. At T0, the DFX + HU group presented lower lipid peroxidation when compared with the FA and DFX groups. As the time of DFX use in the study groups was short (46.8 and 39.1 days) and the dose adjustments were possible only after 12 weeks of the study treatment because of adherence the patient to treatment (~85 days), we believe that HU had an antioxidant activity independent of chelating iron, mainly because of patients using HU for more 3 years and the optimal dosage adjusted. HU is a nitric oxide donor drug which prevents vasoconstriction, decreases the expression of adhesion molecules, reduces the number of leukocytes [14, 54] and, consequently, decreases the processes of vaso-occlusion and ischaemia or reperfusion in microcirculation [35, 37, 39]. All of these events can cause significant increases in ROS production, but if these processes are reduced by HU, a lower production of pro-oxidant agents can be expected. Thus, our results have reinforced the antioxidant action of HU. A similar result has also been found in some in vivo experiments in which HU protected the red cell membrane when exposed to oxidising agents, thus reducing lipid peroxidation [55].

The antioxidant and iron-chelating effects of DFX could only be seen at T1, even though the difference between "adjustment dose of DFX" and "DFX dose in T1" were small, time limitations of DFX in its protection against oxidative stress. Studies on the action of DFX in both sickle cell disease (SCD) and beta thalassaemia major patients have demonstrated a progressive reduction of non-transferrin-bound iron (NTBI) after multiple doses [39]. In other studies, both a high iron-chelating effectiveness and a rapid accessibility of DFX to intracellular labile iron compartments in cardiomyocytes have been reported [56]. The cardioprotective effect was demonstrated both in patients with beta thalassaemia major and in SCD patients who had transfusional iron overload [57].

Though the mechanisms of antioxidant defences in SCA are affected by lower levels of both non-enzymatic and enzymatic systems [41, 42, 51], the groups receiving DFX and DFX + HU presented with higher TEAC values when compared with those of normal subjects, which suggests an increase in antioxidant protection caused by the treatment. However, results were not the same for the FA group, which had no increase in TEAC values but which did have high TBARS levels. These results indicate an additional aggravating factor for this group, as the excess of ROS is not being balanced out. Nevertheless, other antioxidants not examined in this study may be contributing to the body's protection against ROS in FA patients. This possibility must be further studied.

In the FA group, the increase in reactive radicals may be reflected in the leukocyte profiles. The significant positive correlation between TBARS and absolute values of monocytes suggests that, as lipid peroxidation increases, there is also an increase in the absolute values of monocytes. Other studies have shown that oxidative stress increases the adhesive properties of red cells, platelets and leukocytes [58]. When human umbilical vein endothelial cells were cultivated in contact with sickle cell erythrocytes stimulated by oxidizing agents, the expression of cell adhesion molecules increased (ICAM-1, E-selectin and VCAM-1), as did the transendothelial migration of monocytes and the phosphorylation of PECAM-1 [59]. These results suggest that the adherence of sickle cell erythrocytes to endothelial cells generates oxidative stress, which leads to increased adhesion in monocytes and sickle reticulocytes, which, in turn, may contribute to the development of vaso-occlusive processes.

Iron overload in SCA is usually associated with recurrent haemolysis, frequent blood transfusions and an iron-rich

diet. SCA patients require regular blood transfusions to manage acute and chronic complications alike [3, 20, 60]. As humans have no physiological mechanism to actively eliminate the iron overload from the breakdown of transfused red blood cells, the excess iron is deposited as haemossiderin and ferritin in the liver, spleen, endocrine organs and myocardium. The accumulation of toxic amounts of iron causes tissue damage and leads to complications such as liver failure, hypothyroidism, diabetes mellitus and cardiac complications later in life [61, 62].

Iron is an important catalyst of oxidation reactions of biomolecules, as observed in the Fenton reaction and the Haber–Weiss reaction, which generate different types of ROS, such as a superoxide radical ($O_2 \cdot -$), a hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2). When these products are produced in excess, they cause oxidative stress [63]. The positive correlations found between quantities of iron and rates of lipid peroxidation indicate that the rate of lipid peroxidation tends to increase as quantities of serum iron and TIBIC increase, at both T0 and T1. Thus, though the iron parameters used in this study are not direct ways to measure free iron or toxic irons, such as labile plasma (LPI) and NTBI [64, 65], we still observed iron overload in the patients involved. A decrease in the parameters resulted in a decrease in lipid peroxidation, particularly in groups which made use of the chelator. This correlation supports findings involving SCA patients receiving transfusion therapy with iron overload, in which an association between high liver iron concentration and increased levels of MDA was reported [34].

Conclusions

All SCA patients in this study presented high levels of lipid peroxidation, but the DFX + HU group had lower TBARS values, results which demonstrate the antioxidant action of HU, because an initial analysis of TBARS values in the DFX and FA groups showed the same values. After a long period of DFX use, patients exhibited an antioxidant response with concomitant reduction of TBARS values, as well as a reduction in iron parameters. The antioxidant effects of HU and DFX were observed in two ways: an increase in TEAC levels in the DFX and DFX + HU groups when compared with values of normal subjects, and an increase in the TEAC values which did not occur in the group that did not receive specific drugs (the FA group) even with the high levels of TBARS. Patients in the FA group had higher levels of lipid peroxidation and reduced antioxidant capacity, resulting in increased absolute values of monocytes.

It is important to note that further studies are needed to better characterise the antioxidant action of HU and DFX with the inclusion of other markers of antioxidant capacity and also of oxidative stress.

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References

1. Weatherall D, Hofman K, Rodgers G, Ruffin J, Hrynkow S (2005) A case for developing north–south partnerships for research in sickle cell disease. Blood 105:921–923
2. Bunn HF (1997) Mechanisms of disease—pathogenesis and treatment of sickle cell disease. N Engl J Med 337:762–769
3. Steinberg MH (1999) Management of sickle cell disease. N Engl J Med 340:1021–1030
4. Vekilov PG (2007) Sickle-cell haemoglobin polymerization: is it the primary pathogenic event of sickle-cell anaemia? Br J Haematol 139:173–184
5. Repka T, Hebbel RP (1991) Hydroxyl radical formation by sickle erythrocyte-membranes—role of pathological iron deposits and cytoplasmic reducing agents. Blood 78:2753–2758
6. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO III, Schechter AN et al (2002) Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med 8:1383–1389
7. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT (2009) Vasculopathy in sickle cell disease: biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol 84:618–625
8. Wood KC, Hebbel RP, Granger DN (2005) Endothelial cell NADPH oxidase mediates the cerebral microvascular dysfunction in sickle cell transgenic mice. FASEB J 19:989–991
9. Belcher JD, Mahaseth H, Welch TE, Vilback AE, Sonbol KM, Kalambur VS et al (2005) Critical role of endothelial cell activation in hypoxia-induced vasoocclusion in transgenic sickle mice. Am J Physiol Heart Circ Physiol 288:H2715–H2725
10. Belcher JD, Bryant CJ, Nguyen J, Bowlin PR, Kielbik MC, Bischof JC et al (2003) Transgenic sickle mice have vascular inflammation. Blood 101:3953–3959
11. Frenette PS (2002) Sickle cell vaso-occlusion: multistep and multicellular paradigm. Curr Opin Hematol 9:101–106
12. Conran N, Franco-Penteado CF, Costa FF (2009) Newer aspects of the pathophysiology of sickle cell disease vaso-occlusion. Hemoglobin 33:1–16
13. Rees DC, Williams TN, Gladwin MT (2010) Sickle-cell disease. Lancet 376:2018–2031
14. Covas DT, de Lucena Angulo I, Vianna Bonini PP, Zago MA (2004) Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia. Haematologica 89:273–280
15. Kinney TR, Helms RW, O'Branski EE, Ohene-Frempong K, Wang W, Daeschner C et al (1999) Safety of hydroxyurea in children with sickle cell anemia: results of the HUG-KIDS study, a phase I/II trial. Pediatric Hydroxyurea Group. Blood 94:1550–1554
16. Schnog JB, Duits AJ, Muskiet FA, ten Cate H, Rojer RA, Brandjes DP (2004) Sickle cell disease; a general overview. Neth J Med 62:364–374
17. Halsey C, Roberts IAG (2003) The role of hydroxyurea in sickle cell disease. Br J Haematol 120:177–186

18. Hillery CA, Du MC, Wang WC, Scott JP (2000) Hydroxyurea therapy decreases the in vitro adhesion of sickle erythrocytes to thrombospondin and laminin. Br J Haematol 109:322–327
19. Lou TF, Singh M, Mackie A, Li W, Pace BS (2009) Hydroxyurea generates nitric oxide in human erythroid cells: mechanisms for gamma-globin gene activation. Exp Biol Med 234:1374–1382
20. Adams RJ, Mckie VC, Hsu L, Files B, Vichinsky E, Pegelow C et al (1998) Prevention of a first stroke by transfusions in children with sickle, cell anemia and abnormal results on transcranial Doppler ultrasonography. N Engl J Med 339:5–11
21. Vichinsky E, Butensky E, Fung E, Hudes M, Theil E, Ferrell L et al (2005) Comparison of organ dysfunction in transfused patients with SCD or beta thalassemia. Am J Hematol 80:70–74
22. Cappellini MD, Porter J, El-Beshlawy A, Li CK, Seymour JF, Elalfy M et al (2010) Tailoring iron chelation by iron intake and serum ferritin: the prospective EPIC study of deferasirox in 1744 patients with transfusion-dependent anemias. Haematologica 95:557–566
23. Steinberg MH (2008) Sickle cell anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches. Scientific World Journal 8:1295–1324
24. Bonini-Domingos CR (2006) Metodologias laboratoriais para o diagnóstico de hemoglobinopatias e talassemias. NH, São José do Rio Preto
25. Belini E, Cancado RD, Domingos CRB (2010) The Xmnl polymorphic site 5′ to the gene G gamma in a Brazilian patient with sickle cell anaemia—fetal haemoglobin concentration, haematology and clinical features. Arch Med Sci 6:822–825
26. Sutton M, Bouhassira EE, Nagel RL (1989) Polymerase chain-reaction amplification applied to the determination of beta-like globin gene-cluster haplotypes. Am J Hematol 32:66–69
27. Ondei LS, Silveira LM, Leite AA, Souza DR, Pinhel MA, Percario S et al (2009) Lipid peroxidation and antioxidant capacity of G6PD-deficient patients with A-(202G>A) mutation. Genet Mol Res 8:1345–1351
28. Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–278
29. Miller NJ, Riceevans C, Davies MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 84:407–412
30. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237
31. Manfredini V, Lazzaretti LL, Griebeler IH, Santin AP, Brandao VDM, Wagner S et al (2008) Blood antioxidant parameters in sickle cell anemia patients in steady state. J Natl Med Assoc 100:897–902
32. Jain SK, Ross JD, Levy GJ, Duett J (1990) The effect of malonyldialdehyde on viscosity of normal and sickle red blood cells. Biochem Med Metab Biol 44:37–41
33. Sertac A, Bingol F, Aydin S, Uslu A (1997) Peroxidative damage in sickle-cell erythrocyte ghosts: protective effect of allopurinol. Gen Pharmacol 28:427–428
34. Walter PB, Fung EB, Killilea DW, Jiang Q, Hudes M, Madden J et al (2006) Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. Br J Haematol 135:254–263
35. Wood KC, Granger DN (2007) Sickle cell disease: role of reactive oxygen and nitrogen metabolites. Clin Exp Pharmacol Physiol 34:926–932
36. Aslan M, Ryan TM, Adler B, Townes TM, Parks DA, Thompson JA et al (2001) Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. Proc Natl Acad Sci USA 98:15215–15220
37. Hebbel RP, Eaton JW, Balasingam M, Steinberg MH (1982) Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 70:1253–1259
38. Landburg PP, Teerlink T, Biemond BJ, Brandjes DP, Muskiet FA, Duits AJ et al (2010) Plasma asymmetric dimethylarginine concentrations in sickle cell disease are related to the hemolytic phenotype. Blood Cells Mol Dis 44:229–232
39. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL (1996) Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci USA 93:6770–6774
40. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V et al (2005) Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. JAMA 294:81–90
41. Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, Fibach E (2006) Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. Br J Haematol 132:108–113
42. Schacter L, Warth JA, Gordon EM, Prasad A, Klein BL (1988) Altered amount and activity of superoxide dismutase in sickle cell anemia. FASEB J 2:237–243
43. Hebbel RP, Osarogiagbon R, Kaul D (2004) The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. Microcirculation 11:129–151
44. Cighetti G, Duca L, Bortone L, Sala S, Nava I, Fiorelli G et al (2002) Oxidative status and malondialdehyde in beta-thalassaemia patients. Eur J Clin Invest 32:55–60
45. Hebbel RP, Morgan WT, Eaton JW, Hedlund BE (1988) Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. Proc Natl Acad Sci USA 85:237–241
46. Scott MD (2006) H2O2 injury in beta thalassemic erythrocytes: protective role of catalase and the prooxidant effects of GSH. Free Radic Biol Med 40:1264–1272
47. Cao GH, Prior RL (1998) Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 44:1309–1315
48. Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 37:277–285
49. Fasola F, Adedapo K, Anetor J, Kuti M (2007) Total antioxidants status and some hematological values in sickle cell disease patients in steady state. J Natl Med Assoc 99:891–894
50. Shimauti EL, Silva DG, de Almeida EA, Zamaro PJ, Belini Junior E, Bonini-Domingos CR (2010) Serum melatonin level and oxidative stress in sickle cell anemia. Blood Cells Mol Dis 45:297–301
51. Silva DG, Belini Junior E, Torres LS, Ricci Junior O, Lobo CC, Bonini-Domingos CR et al (2011) Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia. Blood Cells Mol Dis 47:23–28
52. Poillon WN, Kim BC, Rodgers GP, Noguchi CT, Schechter AN (1993) Sparing effect of hemoglobin-F and hemoglobin-A2 on the polymerization of hemoglobin-S at physiological ligand saturations. Proc Natl Acad Sci U S A 90:5039–5043
53. Dasgupta T, Fabry ME, Kaul DK (2010) Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in transgenic-knockout sickle mice. Am J Physiol Regul Integr Comp Physiol 298:R394–R402
54. Steinberg MH (2009) Genetic etiologies for phenotypic diversity in sickle cell anemia. Scientific World Journal 9:46–67
55. Agil A, Sadrzadeh SMH (2000) Hydroxy-urea protects erythrocytes against oxidative damage. Redox Rep 5:29–34

56. Glickstein H, Ben El R, Link G, Breuer W, Konijn AM, Hershko C et al (2006) Action of chelators in iron-loaded cardiac cells: accessibility to intracellular labile iron and functional consequences. Blood 108:3195–3203
57. Wood JC, Tyszka JM, Carson S, Nelson MD, Coates TD (2004) Myocardial iron loading in transfusion-dependent thalassemia and sickle cell disease. Blood 103:1934–1936
58. Kaul DK, Liu XD, Choong S, Belcher JD, Vercellotti GM, Hebbel RP (2004) Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice. Am J Physiol Heart Circ Physiol 287: H293–H301
59. Sultana C, Shen YM, Rattan V, Johnson C, Kalra VK (1998) Interaction of sickle erythrocytes with endothelial cells in the presence of endothelial cell conditioned medium induces oxidant stress leading to transendothelial migration of monocytes. Blood 92:3924–3935
60. Vichinsky EP, Ohene-Frempong K (2011) Approaches to transfusion therapy and iron overload in patients with sickle cell disease: results of an international survey. Pediatr Hematol Oncol 28:37–42
61. Olivieri NF (1999) The beta-thalassemias. N Engl J Med 341:99–109
62. Porter JB (2001) Practical management of iron overload. Br J Haematol 115:239–252
63. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47–95
64. Kolb AM, Smit NPM, Lentz-Ljuboje R, Osanto S, van Pelt J (2009) Non-transferrin bound iron measurement is influenced by chelator concentration. Anal Biochem 385:13–19
65. Koren A, Fink D, Admoni O, Tennenbaum-Rakover Y, Levin C (2010) Non-transferrin bound labile plasma iron and iron overload in sickle cell disease: a comparative study between sickle cell disease and beta thalassemic patients. Eur J Haematol 84:72–78

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Review Article

Oxidative stress in sickle cell disease: An overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies

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ABSTRACT

Erythrocytes have an environment of continuous pro-oxidant generation due to the presence of hemoglobin (Hb), which represents an additional and quantitatively significant source of superoxide ($O_2^{\bullet -}$) generation in biological systems. To counteract oxidative stress, erythrocytes have a self-sustaining antioxidant defense system. Thus, red blood cells uniquely function to protect Hb via a selective barrier allowing gaseous and other ligand transport as well as providing antioxidant protection not only to themselves but also to other tissues and organs in the body. Sickle hemoglobin molecules suffer repeated polymerization/depolymerization generating greater amounts of reactive oxygen species, which can lead to a cyclic cascade characterized by blood cell adhesion, hemolysis, vaso-occlusion, and ischemia–reperfusion injury. In other words, sickle cell disease is intimately linked to a pathophysiologic condition of multiple sources of pro-oxidant processes with consequent chronic and systemic oxidative stress. For this reason, newer therapeutic agents that can target oxidative stress may constitute a valuable means for preventing or delaying the development of organ complications.

Contents

Introduction

Sickle cell disease (SCD) encompasses inherited hemolytic anemia due to a single point mutation in the β-globin gene ($HBB^{glu6val}$)

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that results in the formation of sickle hemoglobin (HbS) [1]. The phenotypic expression of SCD patients is complex and characterized by intermittent vaso-occlusion events, increased susceptibility to infections, chronic inflammatory disease, and microvascular damage in all organs [2–5].

The molecular basis for HbS formation is known, but the mutation by itself is not sufficient to explain the heterogeneous phenotype found in SCD patients, such as pain episodes, acute chest syndrome, neurological complications, leg ulcers, and other symptoms. However, since the first description of sickle-shaped erythrocytes in 1910 by Herrick [6], the understanding of SCD pathophysiology has gradually increased.

Among the new evidence, oxidative stress processes have been increasingly related to SCD pathophysiology [7]. The increased production of pro-oxidant elements is caused by intrinsic disease mechanisms, such as increased activity of several oxidases (NADPH oxidase and endothelial xanthine oxidase) [8,9], HbS autoxidation [10], heme iron release, increased asymmetric dimethylarginine [11,12], uncoupling of nitric oxide synthase (NOS) activity, and decreased nitric oxide (NO) levels [13]. As the antioxidant defense systems in SCD are affected and/or are not efficient enough to neutralize excessive production of oxidant species [14,15], chronic oxidative stress is established, being a critical factor in endothelial dysfunction, inflammation, and damage to multiple organs [16].

Even with better understanding of several mechanisms involved in SCD, e.g., the consequences of HbS polymerization, clinical management of the disease is still basic and, although much evidence gives support to the use of blood transfusion and hydroxycarbamide in some circumstances, no drugs have been specifically developed to target the pathophysiology of this disease [17]. Many studies have been carried out to elucidate oxidative stress in SCD or even in red blood cells (RBCs), and some antioxidant therapeutic possibilities, but to our knowledge this is the first work focusing specifically on normal and sickle erythrocyte redox metabolism, and its implications for body antioxidant systems and SCD pathophysiology, and on current antioxidant strategies to attenuate oxidative stress, thus improving SCD patients' condition. In this review, we briefly overview the implications of the HbS presence in erythrocyte redox metabolism and the use of antioxidant therapeutic strategies in SCD.

Erythrocyte role in the maintenance of body antioxidant systems

Reactive oxygen species (ROS) production is a steady-state event in respiring cells. In erythrocytes, Hb can represent an additional and quantitatively significant source of superoxide ($O_2^{\bullet -}$) generation in biological systems. There is an electron transfer in the bonding interaction between the heme and the O_2 in oxygenated Hb [18]. When Hb deoxygenates, the heme iron normally remains in the Fe(II) ferrous state. In this exchange, alterations wherein Hb autoxidizes result in methemoglobin and $O_2^{\bullet -}$ formation [19]. There is a normal "physiologic" rate of red cell methemoglobin formation that provides a continual source of $O_2^{\bullet -}$ production, which in turn generates hydrogen peroxide (H_2O_2) and O_2 as enzymatic dismutation by-products [20]. Thus, perturbations in erythrocyte function and structure can lead to an enhanced flow of pro-oxidant generation that can lead to oxidative stress.

To counteract oxidative stress, erythrocytes have a self-sustaining activity of antioxidant defense enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), in addition to low-molecular-weight antioxidants, such as glutathione (GSH) and vitamins E and C [21]. Furthermore, RBCs have a plasma membrane redox system that transfers electrons from intracellular substrates to extracellular electron acceptors, which may be NAD^+ or vitamin C [22]. Therefore, RBCs uniquely function to protect Hb via a selective barrier allowing gaseous and other ligand transport as well as providing enzymatic mechanisms to maintain Hb in a functional nontoxic state [23], providing antioxidant protection not only to themselves but also to other tissues and organs in the body [24].

In healthy individuals, the erythrocyte number exceeds 4×10^{12}/L in circulating blood, which comprises approximately one-quarter of the total body cell number [25–27]. Thus, erythrocytes are among the most abundant cell types in a human body [28–30]. The usual erythrocyte life span approaches 100–120 days [25–27]. Thus, in an individual with 5 L of blood, more than 10^{11} erythrocytes are newly formed and removed each day [25–27], meaning the erythrocyte generation rate is closely coordinated with their removal by the reticuloendothelial system [31]. Erythrocytes lack nuclei and mitochondria, critical elements in the apoptosis machinery. Thus, dying erythrocytes were considered to be eliminated by mechanisms other than apoptosis [32–34]. However, aged erythrocyte clearance shares apoptosis hallmarks, such as cell shrinkage, cell membrane blebbing, and cell membrane scrambling leading to phosphatidylserine exposure at the cell surface [35]. Owing to similarities to apoptosis, the term eryptosis has been coined to describe the suicidal erythrocyte death [36].

Eryptosis may be elicited by several cell stressors, including osmotic shock [37], energy depletion [38], and oxidative stress [38–40]. Before senescence, erythrocytes may experience injury, which may compromise their integrity and survival [35]. On average RBCs pass once a minute through the lungs, where they are exposed to oxidative stress. More than once an hour they travel through the kidney medulla, where they face osmotic shock. Erythrocytes have to squeeze through capillaries that are smaller than the cells [36]. Thus, the erythrocyte's integrity is constantly challenged [41]. Human erythrocytes are also exposed to pro-oxidative actions of ROS produced in the circulation [21] as well as to a wide range of environments for each vascular circuit and are exposed to a variety of xenobiotics along their lifetime [41]. Under these conditions affected erythrocytes may undergo eryptosis, leading to removal of injured erythrocytes before hemolysis. Enhanced eryptosis may also result from genetic defects of erythrocyte enzymes, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, or of Hb synthesis, such as thalassemia or SCD [26].

Sickle erythrocytes: a pro-oxidant machine

Sickle erythrocytes are characterized by the presence of HbS, instead of the normal HbA. Upon deoxygenation within the microcirculation, HbS molecules alter their configuration, exposing some hydrophobic residues that rapidly associate with hydrophobic regions of adjacent molecules in the aqueous cell compartment [42]. This process of self-assembly or polymerization/sickling generates rigid HbS fibers that distort and damage the RBC membrane and cytoskeleton and alter RBC biochemical properties. HbS polymerization is reversible; fibers "melt" as oxygen is taken up by the HbS and the normal discoid shape returns [42].

One of the important pro-oxidant sources in SCD are sickle erythrocytes, in which the unstable autoxidative HbS and increased metabolic turnover due to recurrent HbS polymerizations and depolymerizations cause increased ROS generation [43,44]. The higher energy expenditure due to the increased metabolic turnover upon polymerization and depolymerization

results in higher ROS production in sickle erythrocytes [43,45]. Hence, sickle erythrocytes have been reported to generate twofold greater amounts of $O_2^{\bullet -}$, H_2O_2, hydroxyl radical ($HO^{\bullet}$), and lipid oxidation products compared with HbA-containing erythrocytes [18,44,46–49]. The increased and unremitting pro-oxidant generation in SCD results in excessive antioxidant consumption and thus antioxidant deficiency [43,50,51]. RBC SOD activity has been shown to increase in some SCD studies [52–54], which concluded that this may be a defense mechanism in response to increased oxidative stress and could possibly cause a H_2O_2 buildup, whereas other works showed decreased SOD activity [15,55,56]. According to Schacter et al. [15], decreased RBC SOD is related to disease severity in SCD patients compared with healthy volunteers.

H_2O_2 is produced either via a two-electron transfer or as a result of sickling and it is removed by two main antioxidant enzymes: GPx and CAT [18,52,57]. CAT is usually more important than GPx because of its ability to degrade H_2O_2 without consuming cellular reducing equivalents (GSH or NADPH), which is an energy-efficient way of removing H_2O_2 [41]. There is a discrepancy between studies on SCD CAT levels; some studies have shown decreased CAT activity in transgenic sickle mouse models [56] and likewise in SCD patients [55,58]. In contrast, Manfredini et al. [54] observed increased CAT levels in SCD patients, in agreement with another study done by Das and Nair [52]. The increase in CAT might be a protective effect to scavenge H_2O_2, whereas the decreased levels might be due to the overwhelming and chronic oxidative stress [59]. An additional hypothesis is that elevated antioxidant enzyme levels in sickle erythrocytes might also be a consequence of higher reticulocyte content in SCD patients' blood.

Another important enzyme related to the antioxidant defense in erythrocytes is G6PD, the regulatory enzyme of the pentose–phosphate (PP) pathway. Because RBCs do not contain mitochondria, the PP pathway is the only source of NADPH in erythrocytes [60], playing an important role in the NADPH-dependent antioxidant defense [61,62]. G6PD deficiency is the most common human enzyme defect, affecting over 400 million people worldwide [60], and it has been associated with increased oxidative stress [63]. The interaction of G6PD deficiency and SCD has been of interest; various studies have suggested that G6PD deficiency has a beneficial [64,65], deleterious [66,67], or no influence upon the course of SCD [68,69]. However, the influence of the interaction of these disorders on oxidative stress markers is still unclear.

Glutathione, a cofactor for GPx to reduce H_2O_2, is easily oxidized to glutathione disulfide (GSSG) by oxidant compounds. GSH concentration is significantly reduced in SCD patients [51,57], with some studies noting a 50% decrease in sickle erythrocytes compared with normal erythrocytes [70]. GPx concentrations are also reduced [55,71], with a direct relationship to the disease severity [56]. A contrasting study by Gizi et al. [53] reported higher GPx activity compared to controls, whereas no significant difference was found regarding the activity of GR, a key enzyme in the regeneration of GSH from GSSG by using electrons from NADPH. As commented, sickle erythrocytes are characterized by decreased GSH concentrations [43,72], even though its de novo synthesis and recycling rate have been shown to be higher compared to healthy erythrocytes [51]. These studies suggest that the pro-oxidant overabundance may lead to the consumption or inactivation of these protective elements.

Under normal conditions 95% of the intracellular glutathione is present in its reduced form (GSH) [73]. The disulfide form (GSSG) can be either recycled to GSH or removed from the intracellular environment through specialized transporters, such as multidrug resistance-associated protein-1 (encoded by the ABCC1 gene), located in the erythrocyte cell membrane [74,75]. The mechanism for sickle erythrocyte GSH deficiency has not been determined yet. Nur et al. [76] demonstrated an increased GSSG efflux in sickle erythrocytes that can be a protective action, because GSSG is an oxidant itself and its enhanced excretion under oxidative conditions prevents the potentially toxic effects of its intracellular accumulation [77]. But increased GSSG efflux could play an important role in GSH depletion in these cells. Erythrocytes are not only a main ROS source in SCD but also endure the brunt of the intracellular oxidative stress [44,78].

Other low-molecular-weight antioxidants suffer alterations due to excessive ROS generation. SCD patients have approximately a 40% reduction in plasma carotene level and a 30% reduction in vitamin E levels [70,79] and reduced ascorbic acid levels [80]. More recently, Gizi et al. [53] also showed lower levels of vitamins A, C, and E and antioxidant compounds (an overall antioxidant capacity measure) in SCD patients compared with normal counterparts. In contrast, Shimauti et al. [81] reported higher antioxidant compounds, but ones that correlated positively with lipid peroxidation in SCD patients, suggesting that even higher antioxidant levels were not enough to counteract oxidative stress in SCD patients.

High lipid peroxidation levels have been extensively described in SCD [47,54–56,58,81–87], because sickle erythrocytes showed both an increased endogenous extent of oxidized lipid and an increased susceptibility to further lipid peroxidation, compared with HbA-containing erythrocytes [48,52,88,89], and also a rise in intracellular Ca^{2+} [90]. In addition to lipid peroxidation, increased intra- and extraerythrocytic oxidative stress induces membrane instability with high phosphatidylserine (PS) exposure percentage, contributing to accelerated intravascular hemolysis [5,91–93] that drives increased endothelial ROS generation. Furthermore, externalization of PS at the outer side of erythrocytes may act as a catalytic surface on which various coagulation factors can interact, resulting in a condition of hypercoagulability [94].

The repeated polymerization/depolymerization process can lead to a vicious cycle inciting blood cell adhesion, hemolysis, vaso-occlusion, and ischemia–reperfusion injury [59], in other words, multiple sources of pro-oxidant processes with consequent chronic and systemic oxidative stress. For this reason, newer therapeutic agents that can target oxidative stress may constitute a valuable means of preventing or delaying the development of organ complications [5,95].

Antioxidant therapeutic strategies in SCD

Several studies have demonstrated a significant increase in stress markers in SCD, and protective mechanisms such as antioxidants are often decreased [7,54,92,96,97]. In SCD the benefit of life-long treatment with antioxidants may be more promising, because the onslaught of oxidizing agents is widespread in the body and probably commences in the first year of life and continues from then on at a high intensity [95]. Thus, the demonstration of a reduction in oxidative stress parameters and of the prevention of pathophysiologic events due to the use of drugs with antioxidant properties in SCD patients indicates that the use of therapeutic antioxidants may represent a fundamental issue in the attenuation of several oxidative stress consequences of the disease. However, owing to the complexity of the SCD pathophysiology, there are few available clinical trials, mainly phase III ones, which limits the evaluation of the impact of antioxidant therapies on disease symptoms. However, in vitro studies, observations in animal models, and studies involving SCD patients have shown interesting and optimistic results (Table 1).

Hydroxycarbamide

Currently, hydroxycarbamide, or hydroxyurea (HU), is the only disease-modifying therapy approved for SCD; the increased synthesis of fetal Hb (HbF) is the main effect of this drug [98].

Table 1
Summary of main antioxidant effects of various therapeutic strategies on the oxidative stress attenuation in SCD.

Drug/treatment	Main antioxidant effect on oxidative stress in SCD
Hydroxyurea	Decreases lipid peroxidation and enhances NO metabolites
Iron chelators	Decreases iron toxicity and lipid peroxidation
Glutamine	Increases the NAD redox potential and NO generation
α-Lipoic acid	Decreases plasma protein carbonyls, increases NF-κB and glutathione synthesis
Acetyl-L-carnitine	Decreases lipid peroxidation
Statins	Increases NO metabolites
Zinc	Increases antioxidant power and plasma nitrite and nitrate
L-Arginine	Increases plasma NO
Inhaled NO	Increases NO bioavailability
Tetrahydrobiopterin	Increases eNOS activity and NO production
N-acetylcysteine	Increases glutathione synthesis
Vitamin E	Decreases lipid peroxidation and ROS production
Vitamin C	Decreases ROS production and increases glutathione concentration

NO, nitric oxide; eNOS, endothelial nitric oxide synthase, NAD, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

The augmented HbF expression reduces several oxidative stress biomarkers and NO scavenging in both sickle cell mice and SCD patients [58,86,99,100]. Other results also showed that increasing concentrations of HbF, a protective effect of HU, is directly proportional to the increase in CAT activity and GSH levels and decreased lipid peroxidation [58,99]. Thus, HbF reduces the number of painful vaso-occlusive crises and seems to reduce hemolysis [101,102].

Another pathway through which HU enhances antioxidant capacity of sickle erythrocytes is by inducing GPx1 expression, whose activity plays a role in reducing membrane lipid peroxidation, promoting membrane stability, and thereby probably reducing hemolysis [103]. In addition to this observation, HU can directly protect the RBC membrane when exposed to oxidizing agents, reducing lipid peroxidation [104]. Ghatpande et al. [105] demonstrated, through analysis of the sickle RBC membrane, that in vitro HU treatment increased antioxidant enzymes, such as catalase, thioredoxin peroxidase, and flavin reductase, suggesting an RBC-adaptive response to oxidative damage.

Furthermore, HU showed a capacity as a NO donor, based on reports of enhanced NO metabolite levels as well as cGMP in SCD patients within 1–2 h after its administration [106–108]. By reducing vaso-occlusion and hemolysis alone, HU administration could have a major impact on total oxidative stress in SCD. It is important to note that in addition to the effect of increasing HbF levels, HU also can reduce hemolysis markers, increase total Hb amount, and decrease reticulocyte number and adhesion molecule expression, among others [109–111].

Iron chelators

Iron overload in SCD is usually associated with recurrent hemolysis, frequent blood transfusions, and an iron-rich diet. SCD patients require regular blood transfusions to manage acute and chronic complications [112–114]. As humans have no physiological mechanism to actively eliminate iron overload, the excessive labile iron in the circulation promotes ROS generation through Fenton and Haber–Weiss reactions [115]. Thus, to avoid excessive oxidation of biomolecules by ROS due to iron overload, SCD patients are often treated with iron chelators.

Among the available iron chelators, deferiprone has been shown in vitro and in vivo to decrease iron associated with sickle erythrocyte membranes and H_2O_2-mediated lipid peroxidation end-products, suggesting that enhanced sickle erythrocyte oxidant production and cell damage are partially dependent on catalytically active iron [116]. Desferoxamine, a cell-impermeative chelator, has been shown to attenuate blood cell adhesion in cerebral venules of SCD mice [9]. Deferasirox has shown efficacy in iron chelating, reducing oxidative stress parameters in β-thalassemia and myelodysplastic syndrome patients that were transfusion-dependent [117–120]. In SCD patients, Belini Junior et al. [121] demonstrated, in a longitudinal study, the antioxidant and iron-chelating effects of deferasirox. SCD patients showed decreased lipid peroxidation levels and iron parameters, in addition to increased overall antioxidant capacity levels.

Glutamine

Glutamine plays an additional antioxidant role through preservation of intracellular NADPH levels, required for GSH recycling. In vitro studies with glutamine have shown an antisickling property in RBCs [122,123]. Glutamine oral supplementation in SCD increases the NAD redox potential and may decrease sickle erythrocyte adhesiveness [124]. RBC glutamine depletion has been associated with elevated tricuspid regurgitant jet velocity, hemolysis, and oxidative stress markers in SCD patients [13]. These observations have led to a phase II trial of oral glutamine therapy for hemolysis-associated pulmonary hypertension in SCD and thalassemia (NCT01048905). Another phase III clinical trial is currently under way to determine whether a 48-week course of glutamine supplementation reduces vaso-occlusive events in adults with SCD (NCT01179217).

α-Lipoic acid and acetyl-L-carnitine

In vitro supplementation of α-lipoic acid is known to have potent antioxidant properties. It can inhibit sickling by 50% [125]; decrease plasma protein carbonyls, urinary isoprostanes, and low-density lipoprotein oxidation [126]; protect peroxyl radical-induced hemolysis; and increase GSH synthesis [127]. The cellular stress response is related to increasing antioxidant gene expression, NF-κB, and GSH synthesis [128].

Acetyl-L-carnitine is an essential nutrient that facilitates the entry of long-chain fatty acids into the mitochondria and decreases lipid peroxidation in tissue. Based on in vitro observations, Ronca et al. [129] suggest that this nutrient may be beneficial in maintaining the normal shape of sickle erythrocytes at low oxygen tension and in decreasing the peroxidative damages that accumulate during RBC life. A recent study in SCD also suggests a benefit in cardiac function [130].

The association of α-lipoic acid and acetyl-L-carnitine has a synergistic antioxidant effect in reversing oxidative stress arising from iron overload [131], and a phase II trial is now evaluating their efficacy in decreasing vaso-occlusive events (NCT01054768).

Statins

Statins are believed to mediate their effects through prevention of endothelial injury via direct action on NO production [132,133]. The clinical benefit derived from statins is mediated via improved endothelial function, as shown in diseases such as diabetes, stroke, metabolic syndrome, and cardiovascular disease, but their effects in SCD are not known [134]. In SCD, results from transgenic sickle cell mice showed that lovastatin (a type of fermentation-derived statin) inhibits endothelial expression of tissue factors induced by hypoxia and reperfusion injury [135]. Hoppe et al. [136], in a pilot study, observed increased plasma NO metabolites and decreased C-reactive protein in SCD patients using simvastatin (a type of

fermentation-derived statin), suggesting a potential therapeutic role for SCD vasculopathies.

Other clinical trials involving SCD are in progress using simvastatin (NCT00508027) and atorvastatin (NCT00072826); thus statins may prove to be a viable option for stopping the vasculopathy progression.

Zinc supplementation

Zinc deficiency is common in adult SCD patients [137–139]. Zinc supplementation of SCD patients has been shown to decrease oxidative stress, incidence of infection, and inflammatory cytokine generation [140]. The mechanism of zinc functioning as a site-specific antioxidant has been widely investigated. At least two mechanisms have been proposed: (i) the competition of zinc with iron and copper for binding to cell membranes and some proteins, which displaces these redox-active metals, and (ii) zinc can bind the SH groups in proteins, which protects them from oxidation [141]. Furthermore, zinc has been recognized as an inhibitor of NADPH oxidases to reduce generation of ROS and it is also a cofactor of SOD. Another study found that zinc reduces the irreversibility of sickled cells, but this antioxidant did not accomplish a clinically measurable ameliorating effect, such as reducing the degree of hemolysis [142].

L-Arginine, tetrahydrobiopterin (R-BH_4), and inhaled NO

In SCD patients, NO bioavailability can be reduced in several ways, e.g., via an increase in free $O_2^{\bullet -}$, through the products of hemolysis (free heme and arginase), and through the "uncoupling" of endothelial NOS (eNOS) [59]. In addition to its role in vascular tone, blood flow, and adhesion, NO is also known to possess antioxidant properties, two of the most noteworthy being $O_2^{\bullet -}$ scavenging and heme oxygenase induction [143]. Because of the importance in maintaining proper NO levels, exogenous NO treatment is often beneficial.

In addition to the evidence for HU increasing NO metabolite levels, arginine therapy in transgenic knockout sickle mice has been demonstrated to improve NO bioavailability and microvascular function and reduce hemolysis and oxidative stress [56,144]. In SCD patients with pulmonary hypertension, arginine supplementation increases plasma NO and rapidly decreases pulmonary artery pressure [145]. When given in combination with HU, arginine therapy of SCD patients seems to increase NO bioavailability [146,147]. Otherwise, a phase II clinical trial found that NO amounts did not increase in patients using arginine compared to a placebo group (NCT00513617).

Normally, through the catalytic action of eNOS, R-BH_4 transfers an electron in the oxygenase domain, converting L-arginine into NO and L-citrulline. A deficiency of R-BH_4 can lead to the production of $O_2^{\bullet -}$ via the uncoupling of eNOS [148]. In cultured endothelial cells, a deficiency of R-BH_4 was shown to uncouple eNOS, whereas its supplementation completely restored eNOS activity and increased NO production [149]. The combined effects of these studies demonstrate the vital role of R-BH_4 in assisting in eNOS function and thus its potential as a treatment for SCD [150].

Measurements of decreased exhaled NO in SCD patients with acute chest syndrome (ACS) suggest that these patients may benefit from NO therapy [143,151]. A case report showed a variety of beneficial effects, including increased plasma NOx, in inhaled NO treatment used to treat SCD patients with ACS and nonhemorrhagic stroke [152]. Indeed, it has been reported that inhaled NO improves tissue oxygenation and reduces pain in SCD patients [153,154], although it remains unclear whether this effect reflects an improved oxygen affinity of HbS [155].

Other approaches to increasing NO bioavailability include phase I and II clinical trials evaluating sodium nitrite, niacin, and R-BH_4 (NCT01033227, NCT00508989).

N-acetylcysteine

N-acetylcysteine (NAC) is an important antioxidant with pleiotropic effects on inflammation and vasomotor function. NAC readily enters cells and within the cytoplasm it is converted to L-cysteine, which is a precursor of GSH [156,157]. Gibson et al. [158] demonstrated, in vitro, that NAC could effectively block the formation of dense cells and irreversible sickle cells. SCD patients treated with NAC at a dose of 2400 mg increased intracellular GSH levels and reduced dense cell formation [159]. In another study it was observed that NAC at both 1200- and 2400-mg doses seemed to decrease hemolysis (detected by reductions in cell-free hemoglobin concentrations) and oxidative stress (evidenced by reductions in sickle erythrocyte phosphatidylserine expression and plasma levels of advanced glycation end-products) [160].

Vitamin E and vitamin C

Supplementation of vitamins C and E in SCD patients has conflicting reports in the literature. In vitro, preincubation of sickle erythrocytes with vitamin E decreased the susceptibility to peroxidation. This finding was also observed in vivo, whereby those individuals with vitamin E deficiency showed increased levels of lipid peroxidation compared to control subjects [161,162]. Ohnishi et al. [125], both in an ex vivo study and in a pilot clinical trial, demonstrated that a cocktail consisting of daily doses of vitamin E, vitamin C, and garlic extract may be beneficial to SCD patients. Another study found that after only 10 weeks of vitamin E supplementation, the irreversibly sickled red cells decreased from 25 to 11% [163]. Amer et al. [14] also observed a decrease in sickled RBC and ROS markers and an increase in GSH concentration. In a study with SCD children supplemented with α-tocopherol, the percentage of irreversibly sickled cells was significantly reduced and the resistance of the cells to lysis was increased [164]. On the other hand, in other studies the vitamin E supplementation did not improve anemia or markers of hemolysis, oxidative stress, and inflammation in SCD patients [142,165].

Vitamin C supplementation efficiently decreased ROS production while it increased GSH concentration. It may be through these effects that ascorbic acid supplements were shown to also prevent H_2O_2-induced hemolysis [14]. Ascorbic acid and dehydroascorbic acid supplements have also shown to inhibit dense RBC formation and to decrease lipid peroxidation levels in SCD patients [126], despite Arruda et al. [165] and Muskiet et al. [142] having found no significant differences in SCD patients.

Conclusions

The presence of HbS in erythrocytes not only causes erythrocyte deformation and biochemical alterations that enhance hemolysis and eryptosis, but is also responsible for increases in ROS production and oxidative stress. Nevertheless the classical therapeutic strategies for SCD treatment (i.e., blood transfusion and hydroxycarbamide) have mainly focused on increasing the non-sickled cell amounts or HbF levels. Several studies have pointed toward the great efficacy of therapeutic drugs that decrease ROS consequences in SCD, i.e., decrease lipid peroxidation levels in plasma, including hydroxycarbamide. These studies often indicate that oxidative stress mitigation by various antioxidant therapeutic approaches generally results in the amelioration of the health conditions of SCD patients, but for the most part they refer only to

blood oxidative stress parameters. There are few studies regarding oxidative stress in SCD that have focused on the role of the erythrocytes in ROS generation and the molecular strategies triggered by these cells that could be important for the attenuation of general blood oxidative stress. Therefore more studies on oxidative stress biomarkers and antioxidant therapeutic strategies that reduce oxidative stress generation in erythrocytes, as discussed in this review, remain a worthy and promising goal, which might lead to more specific and effective prognostic information for SCD patients and to the development of more efficacious therapeutic drugs for mitigating oxidative stress in sickle erythrocytes, thus alleviating the disease outcome.

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References

[1] Steinberg, M.; Sebastiani, P. Genetic modifiers of sickle cell disease. *Am. J. Hematol.* **87:**795–803; 2012.
[2] Barabino, G.; Platt, M.; Kaul, D. Sickle cell biomechanics. *Annu. Rev. Biomed. Eng.* **12:**345–367; 2010.
[3] Steinberg, M. Predicting clinical severity in sickle cell anaemia. *Br. J. Haematol.* **129:**465–481; 2005.
[4] van Beers, E.; van Tuijn, C.; Mac Gillavry, M.; van der Giessen, A.; Schnog, J. J.; Biemond, B., CURAMA Study Group. Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. *Haematologica* **93:**757–760; 2008.
[5] Wood, K. C.; Granger, D. N. Sickle cell disease: role of reactive oxygen and nitrogen metabolites. *Clin. Exp. Pharmacol. Physiol.* **34:**926–932; 2007.
[6] Herrick, J. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. 1910. *Yale J. Biol. Med.* **74**:179-184; 2001.
[7] Rees, D.; Gibson, J. Biomarkers in sickle cell disease. *Br. J. Haematol.* **156:**433–445; 2012.
[8] Aslan, M.; Ryan, T.; Adler, B.; Townes, T.; Parks, D.; Thompson, J.; Tousson, A.; Gladwin, M.; Patel, R.; Tarpey, M.; Batinic-Haberle, I.; White, C.; Freeman, B. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proc. Natl. Acad. Sci. USA* **98:**15215–15220; 2001.
[9] Wood, K.; Hebbel, R.; Granger, D. Endothelial cell NADPH oxidase mediates the cerebral microvascular dysfunction in sickle cell transgenic mice. *FASEB J* **19:**989–991; 2005.
[10] Hebbel, R. P.; Eaton, J. W.; Balasingam, M.; Steinberg, M. H. Spontaneous oxygen radical generation by sickle erythrocytes. *J. Clin. Invest.* **70:**1253–-1259; 1982.
[11] Landburg, P. P.; Teerlink, T.; Biemond, B. J.; Brandjes, D. P.; Muskiet, F. A.; Duits, A. J.; Schnog, J. B. Plasma asymmetric dimethylarginine concentrations in sickle cell disease are related to the hemolytic phenotype. *Blood Cells Mol. Dis.* **44:**229–232; 2010.
[12] Xia, Y.; Dawson, V.; Dawson, T.; Snyder, S.; Zweier, J. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. USA* **93:**6770–-6774; 1996.
[13] Morris, C. R.; Kato, G. J.; Poljakovic, M.; Wang, X.; Blackwelder, W. C.; Sachdev, V.; Hazen, S. L.; Vichinsky, E. P.; Morris Jr S. M.; Gladwin, M. T. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA* **294:**81–90; 2005.
[14] Amer, J.; Ghoti, H.; Rachmilewitz, E.; Koren, A.; Levin, C.; Fibach, E. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. *Br. J. Haematol.* **132:**108–113; 2006.
[15] Schacter, L.; Warth, J. A.; Gordon, E. M.; Prasad, A.; Klein, B. L. Altered amount and activity of superoxide dismutase in sickle cell anemia. *J. Fed. Am. Soc. Exp. Biol* **2:**237–243; 1988.
[16] Hebbel, R. P.; Osarogiagbon, R.; Kaul, D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. *Microcirculation* **11:**129–-151; 2004.
[17] Rees, D. C.; Williams, T. N.; Gladwin, M. T. Sickle-cell disease. *Lancet* **376:**2018–2031; 2010.
[18] Aslan, M.; Thornley-Brown, D.; Freeman, B. A. Reactive species in sickle cell disease. *Ann. N. Y. Acad. Sci.* **899:**375–391; 2000.
[19] Carrell, R. W.; Winterbourn, C. C.; Rachmilewitz, E. A. Activated oxygen and haemolysis. *Br. J. Haematol.* **30:**259–264; 1975.
[20] Freeman, B. A.; Crapo, J. D. Biology of disease: free radicals and tissue injury. *Lab. Invest.* **47:**412–426; 1982.
[21] Perrone, S.; Tataranno, M. L.; Stazzoni, G.; Del, V. A.; Buonocore, G. Oxidative injury in neonatal erythrocytes. *J. Matern.–Fetal Neonat. Med* **25:**104–108; 2012.
[22] Rizvi, S. I.; Jha, R.; Maurya, P. K. Erythrocyte plasma membrane redox system in human aging. *Rejuvenation Res* **9:**470–474; 2006.
[23] Cappadoro, M.; Giribaldi, G.; O'Brien, E.; Turrini, F.; Mannu, F.; Ulliers, D.; Simula, G.; Luzzatto, L.; Arese, P. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by Plasmodium falciparum may explain malaria protection in G6PD deficiency. *Blood* **92:**2527–2534; 1998.
[24] Siems, W. G.; Sommerburg, O.; Grune, T. Erythrocyte free radical and energy metabolism. *Clin. Nephrol.* **53:**S9–17; 2000.
[25] Foller, M.; Huber, S. M.; Lang, F. Erythrocyte programmed cell death. *IUBMB Life* **60:**661–668; 2008.
[26] Lang, E.; Qadri, S. M.; Lang, F. Killing me softly—suicidal erythrocyte death. *Int. J. Biochem. Cell Biol.* **44:**1236–1243; 2012.
[27] Lang, F.; Gulbins, E.; Lerche, H.; Huber, S. M.; Kempe, D. S.; Foller, M. Eryptosis, a window to systemic disease. *Cell. Physiol. Biochem.* **22:**373–380; 2008.
[28] Arese, P.; Turrini, F.; Schwarzer, E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell. Physiol. Biochem.* **16:**133–146; 2005.
[29] Bosman, G. J.; Willekens, F. L.; Werre, J. M. Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell. Physiol. Biochem.* **16:**1–8; 2005.
[30] Kiefer, C. R.; Snyder, L. M. Oxidation and erythrocyte senescence. *Curr. Opin. Hematol.* **7:**113–116; 2000.
[31] (de) Oliveira, S.; Saldanha, C. An overview about erythrocyte membrane. *Clin. Hemorheol. Microcirc.* **44:**63–74; 2010.
[32] Berg, C. P.; Engels, I. H.; Rothbart, A.; Lauber, K.; Renz, A.; Schlosser, S. F.; Schulze-Osthoff, K.; Wesselborg, S. Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ.* **8:**1197–1206; 2001.
[33] Bratosin, D.; Estaquier, J.; Petit, F.; Arnoult, D.; Quatannens, B.; Tissier, J. P.; Slomianny, C.; Sartiaux, C.; Alonso, C.; Huart, J. J.; Montreuil, J.; Ameisen, J. C. Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ.* **8:**1143–1156; 2001.
[34] Daugas, E.; Cande, C.; Kroemer, G. Erythrocytes: death of a mummy. *Cell Death Differ.* **8:**1131–1133; 2001.
[35] Lang, E.; Qadri, S. M.; Zelenak, C.; Gu, S.; Rotte, A.; Draeger, A.; Lang, F. Inhibition of suicidal erythrocyte death by blebbistatin. *Am. J. Physiol. Cell Physiol* **301:**C490–C498; 2011.
[36] Lang, K. S.; Lang, P. A.; Bauer, C.; Duranton, C.; Wieder, T.; Huber, S. M.; Lang, F. Mechanisms of suicidal erythrocyte death. *Cell. Physiol. Biochem.* **15:**195–202; 2005.
[37] Lang, K. S.; Duranton, C.; Poehlmann, H.; Myssina, S.; Bauer, C.; Lang, F.; Wieder, T.; Huber, S. M. Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ.* **10:**249–256; 2003.
[38] Lang, K. S.; Roll, B.; Myssina, S.; Schittenhelm, M.; Scheel-Walter, H. G.; Kanz, L.; Fritz, J.; Lang, F.; Huber, S. M.; Wieder, T. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell. Physiol. Biochem.* **12:**365–372; 2002.
[39] Barvitenko, N. N.; Adragna, N. C.; Weber, R. E. Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell. Physiol. Biochem.* **15:**1–18; 2005.
[40] Bracci, R.; Perrone, S.; Buonocore, G. Oxidant injury in neonatal erythrocytes during the perinatal period. *Acta Paediatr.* **91:**130–134; 2002.
[41] Pandey, K. B.; Rizvi, S. I. Biomarkers of oxidative stress in red blood cells. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub* **155:**131–136; 2011.
[42] Stuart, M. J.; Nagel, R. L. Sickle-cell disease. *Lancet* **364:**1343–1360; 2004.
[43] Banerjee, T.; Kuypers, F. A. Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells. *Br. J. Haematol.* **124:**391–402; 2004.
[44] Hebbel, R. P.; Morgan, W. T.; Eaton, J. W.; Hedlund, B. E. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. *Proc. Natl. Acad. Sci. USA* **85:**237–241; 1988.
[45] Akohoue, S. A.; Shankar, S.; Milne, G. L.; Morrow, J.; Chen, K. Y.; Ajayi, W. U.; Buchowski, M. S. Energy expenditure, inflammation, and oxidative stress in steady-state adolescents with sickle cell anemia. *Pediatr. Res.* **61:**233–238; 2007.
[46] Hebbel, R. P. Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology. *Blood* **77:**214–237; 1991.
[47] Klings, E. S.; Farber, H. W. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. *Respir. Res* **2:**280–285; 2001.
[48] Rice-Evans, C.; Omorphos, S. C.; Baysal, E. Sickle cell membranes and oxidative damage. *Biochem. J.* **237:**265–269; 1986.
[49] Sheng, K.; Shariff, M.; Hebbel, R. P. Comparative oxidation of hemoglobins A and S. *Blood* **91:**3467–3470; 1998.
[50] Chaves, M. A.; Leonart, M. S.; do Nascimento, A. J. Oxidative process in erythrocytes of individuals with hemoglobin S. *Hematology* **13:**187–192; 2008.
[51] Reid, M.; Badaloo, A.; Forrester, T.; Jahoor, F. In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. *Am. J. Physiol. Endocrinol. Metab.* **291:**E73–E79; 2006.
[52] Das, S. K.; Nair, R. C. Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation of normal and sickled erythrocytes. *Br. J. Haematol.* **44:**87–92; 1980.

[53] Gizi, A.; Papassotiriou, I.; Apostolakou, F.; Lazaropoulou, C.; Papastamataki, M.; Kanavaki, I.; Kalotychou, V.; Goussetis, E.; Kattamis, A.; Rombos, I.; Kanavakis, E. Assessment of oxidative stress in patients with sickle cell disease: the glutathione system and the oxidant–antioxidant status. *Blood Cells Mol. Dis.* **46:**220–225; 2011.

[54] Manfredini, V.; Lazzaretti, L. L.; Griebeler, I. H.; Santin, A. P.; Brandao, V. D.; Wagner, S.; Castro, S. M.; Peralba, M. C.; Benfato, M. S. Blood antioxidant parameters in sickle cell anemia patients in steady state. *JAMA* **100:**897–902; 2008.

[55] Alsultan, A. I.; Seif, M. A.; Amin, T. T.; Naboli, M.; Alsuliman, A. M. Relationship between oxidative stress, ferritin and insulin resistance in sickle cell disease. *Eur. Rev. Med. Pharmacol. Sci.* **14:**527–538; 2010.

[56] Dasgupta, T.; Hebbel, R. P.; Kaul, D. K. Protective effect of arginine on oxidative stress in transgenic sickle mouse models. *Free Radic. Biol. Med.* **41:**1771–1780; 2006.

[57] Morris, C. R.; Suh, J. H.; Hagar, W.; Larkin, S.; Bland, D. A.; Steinberg, M. H.; Vichinsky, E. P.; Shigenaga, M.; Ames, B.; Kuypers, F. A.; Klings, E. S. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. *Blood* **111:**402–410; 2008.

[58] Silva, D. G.; Belini, J. E.; Torres, L. S.; Ricci, J. O.; Lobo, C. C.; Bonini-Domingos, C. R. de Almeida, E. A. Relationship between oxidative stress, glutathione S-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia. *Blood Cells Mol. Dis.* **47:**23–28; 2011.

[59] Chirico, E. N.; Pialoux, V. Role of oxidative stress in the pathogenesis of sickle cell disease. *IUBMB Life* **64:**72–80; 2012.

[60] Cappellini, M. D.; Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. *Lancet* **371:**64–74; 2008.

[61] Faut, M.; Paiz, A.; San Martin de Viale, L. C.; Mazzetti, M. B. Alterations of the redox state, pentose pathway and glutathione metabolism in an acute porphyria model: their impact on heme pathway. *Exp. Biol. Med. (Maywood)* **238:**133–143; 2013.

[62] Pandolfi, P. P.; Sonati, F.; Rivi, R.; Mason, P.; Grosveld, F.; Luzzatto, L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J* **14:**5209–5215; 1995.

[63] Hecker, P. A.; Lionetti, V.; Ribeiro Jr R. F.; Rastogi, S.; Brown, B. H.; O'Connell, K. A.; Cox, J. W.; Shekar, K. C.; Gamble, D. M.; Sabbah, H. N.; Leopold, J. A.; Gupte, S. A.; Recchia, F. A.; Stanley, W. C. Glucose 6-phosphate dehydrogenase deficiency increases redox stress and moderately accelerates the development of heart failure. *Circ. Heart Fail* **6:**118–126; 2013.

[64] Lewis, R. A.; Kay, R. W.; Hathorn, M. Sickle cell disease and glucose-6-phosphate dehydrogenase. *Acta Haematol.* **36:**399–411; 1966.

[65] Piomelli, S.; Reindorf, C. A.; Arzanian, M. T.; Corash, L. M. Clinical and biochemical interactions of glucose-6-phosphate dehydrogenase deficiency and sickle-cell anemia. *N. Engl. J. Med.* **287:**213–217; 1972.

[66] Konotey-Ahulu, F. I. Glucose-6-phosphate dehydrogenase deficiency and sickle-cell anemia. *N. Engl. J. Med.* **287:**887–888; 1972.

[67] Smits, H. L.; Oski, F. A.; Brody, J. I. The hemolytic crisis of sickle cell disease: the role of glucose-6-phosphate dehydrogenase deficiency. *J. Pediatr.* **74:**544–551; 1969.

[68] Bienzle, U.; Sodeinde, O.; Effiong, C. E.; Luzzatto, L. Glucose 6-phosphate dehydrogenase deficiency and sickle cell anemia: frequency and features of the association in an African community. *Blood* **46:**591–597; 1975.

[69] Gibbs, W. N.; Wardle, J.; Serjeant, G. R. Glucose-6-phosphate dehydrogenase deficiency and homozygous sickle cell disease in Jamaica. *Br. J. Haematol.* **45:**73–80; 1980.

[70] Tatum, V. L.; Chow, C. K. Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress. *Free Radic. Biol. Med.* **25:**133–-139; 1996.

[71] Natta, C. L.; Chen, L. C.; Chow, C. K. Selenium and glutathione peroxidase levels in sickle cell anemia. *Acta Haematol.* **83:**130–132; 1990.

[72] Somjee, S. S.; Warrier, R. P.; Thomson, J. L.; Ory-Ascani, J.; Hempe, J. M. Advanced glycation end-products in sickle cell anaemia. *Br. J. Haematol.* **128:**112–118; 2005.

[73] Reid, M.; Jahoor, F. Glutathione in disease. *Curr. Opin. Clin. Nutr. Metab. Care* **4:**65–71; 2001.

[74] Cole, S. P.; Deeley, R. G. Transport of glutathione and glutathione conjugates by MRP1. *Trends Pharmacol. Sci.* **27:**438–446; 2006.

[75] Pulaski, L.; Jedlitschky, G.; Leier, I.; Buchholz, U.; Keppler, D. Identification of the multidrug-resistance protein (MRP) as the glutathione-S-conjugate export pump of erythrocytes. *Eur. J. Biochem.* **241:**644–648; 1996.

[76] Nur, E.; Verwijs, M.; de Waart, D. R.; Schnog, J. J.; Otten, H. M.; Brandjes, D. P.; Biemond, B. J.; Elferink, R. P. Increased efflux of oxidized glutathione (GSSG) causes glutathione depletion and potentially diminishes antioxidant defense in sickle erythrocytes. *Biochim. Biophys. Acta* **1812:**1412–1417; 2011.

[77] Prchal, J.; Srivastava, S. K.; Beutler, E. Active transport of GSSG from reconstituted erythrocyte ghosts. *Blood* **46:**111–117; 1975.

[78] Wood, B. L.; Gibson, D. F.; Tait, J. F. Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* **88:**1873–1880; 1996.

[79] Chiu, D.; Lubin, B. Abnormal vitamin E and glutathione peroxidase levels in sickle cell anemia: evidence for increased susceptibility to lipid peroxidation in vivo. *J. Lab. Clin. Med* **94:**542–548; 1979.

[80] Jain, S. K.; Williams, D. M. Reduced levels of plasma ascorbic acid (vitamin C) in sickle cell disease patients: its possible role in the oxidant damage to sickle cells in vivo. *Clin. Chim. Acta* **149:**257–261; 1985.

[81] Shimauti, E. L.; Silva, D. G.; de Almeida, E. A.; Zamaro, P. J.; Belini, J. E.; Bonini-Domingos, C. R. Serum melatonin level and oxidative stress in sickle cell anemia. *Blood Cells Mol. Dis.* **45:**297–301; 2010.

[82] Oztas, Y. E.; Sabuncuoglu, S.; Unal, S.; Ozgunes, H.; Ozgunes, N. Hypocholesterolemia is associated negatively with hemolysate lipid peroxidation in sickle cell anemia patients. *Clin. Exp. Med.* **11:**195–198; 2011.

[83] Rusanova, I.; Escames, G.; Cossio, G.; de Borace, R. G.; Moreno, B.; Chahboune, M.; Lopez, L. C.; Diez, T.; cuna-Castroviejo, D. Oxidative stress status, clinical outcome, and beta-globin gene cluster haplotypes in pediatric patients with sickle cell disease. *Eur. J. Haematol.* **85:**529–537; 2010.

[84] Sess, E. D.; Carbonneau, M. A.; Meite, M.; Peuchant, E.; Dumont, M. F.; Receveur, M. C.; Thomas, M. J.; Perromat, A.; Sangare, A.; Le, B. M.; Clerc, M. [Markers of lipid peroxidation, inflammatory proteins and plasma tocopherols in homozygotic and heterozygotic sickle cell anemia]. *Bull. Soc. Pathol. Exot.* **91:**238–241; 1998.

[85] Titus, J.; Chari, S.; Gupta, M.; Parekh, N. Pro-oxidant and anti-oxidant status in patients of sickle cell anaemia. *Indian J. Clin. Biochem.* **19:**168–172; 2004.

[86] Torres, L. S.; da Silva, D. G.; Belini, J. E.; de Almeida, E. A.; Lobo, C. L.; Cancado, R. D.; Ruiz, M. A.; Bonini-Domingos, C. R. The influence of hydroxyurea on oxidative stress in sickle cell anemia. *Rev. Bras. Hematol. Hemoter* **34:**421–425; 2012.

[87] Viana-Baracioli, L. M.; Tukamoto Junior, N. C.; Ricci Junior, O.; Mattos, L. C.; Angulo, I. L.; Bonini-Domingos, C. R. Comparison of oxidative stress and the frequency of polymorphisms in the HFE gene between hemoglobin S trait blood donors and sickle cell disease patients. *Genet. Mol. Res* **10:**3446–3454; 2011.

[88] Essien, E. U. Increased susceptibility of erythrocyte membrane lipids to peroxidation in sickle cell disease. *Cent. Afr. J. Med.* **40:**217–220; 1994.

[89] Jain, S. K.; Shohet, S. B. A novel phospholipid in irreversibly sickled cells: evidence for in vivo peroxidative membrane damage in sickle cell disease. *Blood* **63:**362–367; 1984.

[90] Jain, S. K.; Shohet, S. B. Calcium potentiates the peroxidation of erythrocyte membrane lipids. *Biochim. Biophys. Acta* **642:**46–54; 1981.

[91] Repka, T.; Hebbel, R. P. Hydroxyl radical formation by sickle erythrocyte membranes: role of pathologic iron deposits and cytoplasmic reducing agents. *Blood* **78:**2753–2758; 1991.

[92] Walter, P. B.; Fung, E. B.; Killilea, D. W.; Jiang, Q.; Hudes, M.; Madden, J.; Porter, J.; Evans, P.; Vichinsky, E.; Harmatz, P. Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. *Br. J. Haematol.* **135:**254–263; 2006.

[93] Jain, S. K. The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. *J. Biol. Chem* **259:**3391–3394; 1984.

[94] Jain, S. K. In vivo externalization of phosphatidylserine and phosphatidylethanolamine in the membrane bilayer and hypercoagulability by the lipid peroxidation of erythrocytes in rats. *J. Clin. Invest* **76:**281–286; 1985.

[95] Nur, E.; Biemond, B. J.; Otten, H. M.; Brandjes, D. P.; Schnog, J. J. Oxidative stress in sickle cell disease; pathophysiology and potential implications for disease management. *Am. J. Hematol.* **86:**484–489; 2011.

[96] Jain, S.; Ross, J.; Levy, G.; Duett, J. The effect of malonyldialdehyde on viscosity of normal and sickle red blood cells. *Biochem. Med. Metab. Biol.* **44:**37–41; 1990.

[97] Sertac, A.; Bingol, F.; Aydin, S.; Uslu, A. Peroxidative damage in sickle-cell erythrocyte ghosts: protective effect of allopurinol. *Gen. Pharmacol.* **28:** 427–428; 1997.

[98] Zimmerman, S. A.; Schultz, W. H.; Davis, J. S.; Pickens, C. V.; Mortier, N. A.; Howard, T. A.; Ware, R. E. Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. *Blood* **103:**2039–2045; 2004.

[99] Dasgupta, T.; Fabry, M.; Kaul, D. Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in transgenic-knockout sickle mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **298:**402; 2010.

[100] Kaul, D. K.; Liu, X. D.; Chang, H. Y.; Nagel, R. L.; Fabry, M. E. Effect of fetal hemoglobin on microvascular regulation in sickle transgenic-knockout mice. *J. Clin. Invest.* **114:**1136–1145; 2004.

[101] Charache, S.; Terrin, M.; Moore, R.; Dover, G.; Barton, F.; Eckert, S.; McMahon, R.; Bonds, D. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N. Engl. J. Med.* **332:**1317–1322; 1995.

[102] Rodgers, G.; Dover, G.; Noguchi, C.; Schechter, A.; Nienhuis, A. Hematologic responses of patients with sickle cell disease to treatment with hydroxyurea. *N. Engl. J. Med.* **322:**1037–1045; 1990.

[103] Cho, C. S.; Kato, G.; Yang, S.; Bae, S.; Lee, J.; Gladwin, M.; Rhee, S. Hydroxyurea-induced expression of glutathione peroxidase 1 in red blood cells of individuals with sickle cell anemia. *Antioxid. Redox Signaling* **13:**1–11; 2010.

[104] Agil, A.; Sadrzadeh, S. M. Hydroxy-urea protects erythrocytes against oxidative damage. *Redox Rep.* **5:**29–34; 2000.

[105] Ghatpande, S. S.; Choudhary, P. K.; Quinn, C. T.; Goodman, S. R. Pharmacoproteomic study of hydroxyurea-induced modifications in the sickle red blood cell membrane proteome. *Exp. Biol. Med. (Maywood)* **233:**1510–-1517; 2008.

[106] Gladwin, M.; Shelhamer, J.; Ognibene, F.; Pease-Fye, M.; Nichols, J.; Link, B.; Patel, D.; Jankowski, M.; Pannell, L.; Schechter, A.; Rodgers, G. Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. *Br. J. Haematol.* **116:**436–444; 2002.

[107] Huang, J.; Kim-Shapiro, D.; King, S. Catalase-mediated nitric oxide formation from hydroxyurea. *J. Med. Chem.* **47:**3495–3501; 2004.

[108] King, S. Nitric oxide production from hydroxyurea. *Free Radic. Biol. Med.* **37:**737–744; 2004.

[109] Covas, D. T.; de Lucena Angulo, I.; Vianna Bonini Palma, P.; Zago, M. A. Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia. *Haematologica* **89:**273–280; 2004.

[110] Hillery, C. A.; Du, M. C.; Wang, W. C.; Scott, J. P. Hydroxyurea therapy decreases the in vitro adhesion of sickle erythrocytes to thrombospondin and laminin. *Br. J. Haematol.* **109:**322–327; 2000.

[111] Schnog, J. B.; Duits, A. J.; Muskiet, F. A.; ten Cate, H.; Rojer, R. A.; Brandjes, D. P. Sickle cell disease: a general overview. *Neth. J. Med.* **62:**364–374; 2004.

[112] Adams, R.; McKie, V.; Hsu, L.; Files, B.; Vichinsky, E.; Pegelow, C.; Abboud, M.; Gallagher, D.; Kutlar, A.; Nichols, F.; Bonds, D.; Brambilla, D. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *N. Engl. J. Med.* **339:**5–11; 1998.

[113] Steinberg, M. H. Management of sickle cell disease. *N. Engl. J. Med.* **340:**1021-–1030; 1999.

[114] Vichinsky, E.; Ohene-Frempong, K., Transfusion Committee. Approaches to transfusion therapy and iron overload in patients with sickle cell disease: results of an international survey. *Pediatr. Hematol. Oncol* **28:**37–42; 2011.

[115] Dröge, W. Free radicals in the physiological control of cell function. *Physiol. Rev.* **82:**47–95; 2002.

[116] Shalev, O.; Repka, T.; Goldfarb, A.; Grinberg, L.; Abrahamov, A.; Olivieri, N.; Rachmilewitz, E.; Hebbel, R. Deferiprone (L1) chelates pathologic iron deposits from membranes of intact thalassemic and sickle red blood cells both in vitro and in vivo. *Blood* **86:**2008–2013; 1995.

[117] Breccia, M.; Alimena, G. Efficacy and safety of deferasirox in myelodysplastic syndromes. Ann. Hematol. 2013.

[118] Ghoti, H.; Fibach, E.; Merkel, D.; Perez-Avraham, G.; Grisariu, S.; Rachmilewitz, E. A. Changes in parameters of oxidative stress and free iron biomarkers during treatment with deferasirox in iron-overloaded patients with myelodysplastic syndromes. *Haematologica* **95:**1433–1434; 2010.

[119] Saigo, K.; Kono, M.; Takagi, Y.; Takenokuchi, M.; Hiramatsu, Y.; Tada, H.; Hishita, T.; Misawa, M.; Imoto, S.; Imashuku, S. Deferasirox reduces oxidative stress in patients with transfusion dependency. *J. Clin. Med. Res* **5:**57–60; 2013.

[120] Walter, P. B.; Macklin, E. A.; Porter, J.; Evans, P.; Kwiatkowski, J. L.; Neufeld, E. J.; Coates, T.; Giardina, P. J.; Vichinsky, E.; Olivieri, N.; Alberti, D.; Holland, J.; Harmatz, P. Inflammation and oxidant-stress in beta-thalassemia patients treated with iron chelators deferasirox (ICL670) or deferoxamine: an ancillary study of the Novartis CICL670A0107 trial. *Haematologica* **93:**817–825; 2008.

[121] Belini Junior, E.; da Silva, D. G.; de Souza, T. L.; de Almeida, E. A.; Cancado, R. D.; Chiattone, C.; Bonini-Domingos, C. R. Oxidative stress and antioxidant capacity in sickle cell anaemia patients receiving different treatments and medications for different periods of time. *Ann. Hematol.* **91:**479–489; 2012.

[122] Mackenzie, R.; Gleason, E.; Schatzman, G.; Cawein, M. An in vitro method for screening compounds for the effect on the rate of sickling of erythrocytes. *J. Int. Med. Res.* **4:**375–381; 1976.

[123] Rumen, N. Inhibition of sickling in erythrocytes by amino acids. *Blood* **45:**45–48; 1975.

[124] Niihara, Y.; Matsui, N. M.; Shen, Y. M.; Akiyama, D. A.; Johnson, C. S.; Sunga, M. A.; Magpayo, J.; Embury, S. H.; Kalra, V. K.; Cho, S. H.; Tanaka, K. R. L-Glutamine therapy reduces endothelial adhesion of sickle red blood cells to human umbilical vein endothelial cells. *BMC Blood Disord.* **5:**4; 2005.

[125] Ohnishi, S.; Ohnishi, T.; Ogunmola, G. Sickle cell anemia: a potential nutritional approach for a molecular disease. *Nutrition (Burbank, CA)* **16:**330–338; 2000.

[126] Marangon, K.; Devaraj, S.; Tirosh, O.; Packer, L.; Jialal, I. Comparison of the effect of α-lipoic acid and α-tocopherol supplementation on measures of oxidative stress. *Free Radic. Biol. Med.* **27:**1114–1121; 1999.

[127] Lexis, L.; Fassett, R.; Coombes, J. Alpha-tocopherol and alpha-lipoic acid enhance the erythrocyte antioxidant defence in cyclosporine A-treated rats. *Basic Clin. Pharmacol. Toxicol* **98:**68–73; 2006.

[128] Vichinsky, E. Emerging 'A' therapies in hemoglobinopathies: agonists, antagonists, antioxidants, and arginine. *Hematology Am. Soc. Hematol. Educ. Program* **2012:**271–275; 2012.

[129] Ronca, F.; Palmieri, L.; Malengo, S.; Bertelli, A. Effect of L-propionyl carnitine on in-vitro membrane alteration of sickle-cell anaemia erythrocytes. *Int. J. Tissue React.* **16:**187–194; 1994.

[130] El-Beshlawy, A.; bd El Raouf, E.; Mostafa, F.; Talaat, M.; Isma'eel, H.; Aoun, E.; Hoffbrand, A.; Taher, A. Diastolic dysfunction and pulmonary hypertension in sickle cell anemia: is there a role for L-carnitine treatment? *Acta Haematol.* **115:**91–96; 2006.

[131] Lal, A.; Atamna, W.; Killilea, D.; Suh, J.; Ames, B. Lipoic acid and acetyl-carnitine reverse iron-induced oxidative stress in human fibroblasts. *Redox Rep.* **13:**2–10; 2008.

[132] Amin-Hanjani, S.; Stagliano, N. E.; Yamada, M.; Huang, P. L.; Liao, J. K.; Moskowitz, M. A. Mevastatin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nitric oxide synthase in mice. *Stroke* **32:**980–986; 2001.

[133] Asahi, M.; Huang, Z.; Thomas, S.; Yoshimura, S.; Sumii, T.; Mori, T.; Qiu, J.; min-Hanjani, S.; Huang, P. L.; Liao, J. K.; Lo, E. H.; Moskowitz, M. A. Protective effects of statins involving both eNOS and tPA in focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **25:**722–729; 2005.

[134] Adam, S. S.; Hoppe, C. Potential role for statins in sickle cell disease. *Pediatr. Blood Cancer* **60:**550–557; 2013.

[135] Solovey, A.; Kollander, R.; Shet, A.; Milbauer, L. C.; Choong, S.; Panoskaltsis-Mortari, A.; Blazar, B. R.; Kelm Jr R. J.; Hebbel, R. P. Endothelial cell expression of tissue factor in sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin. *Blood* **104:**840–846; 2004.

[136] Hoppe, C.; Kuypers, F.; Larkin, S.; Hagar, W.; Vichinsky, E.; Styles, L. A pilot study of the short-term use of simvastatin in sickle cell disease: effects on markers of vascular dysfunction. *Br. J. Haematol.* **153:**655–663; 2011.

[137] Niell, H. B.; Leach, B. E.; Kraus, A. P. Zinc metabolism in sickle cell anemia. *JAMA* **242:**2686–2687; 1979.

[138] Prasad, A. S.; Schoomaker, E. B.; Ortega, J.; Brewer, G. J.; Oberleas, D.; Oelshlegel Jr. F. J. Zinc deficiency in sickle cell disease. *Clin. Chem.* **21:** 582–587; 1975.

[139] Prasad, A. S.; Abbasi, A. A.; Rabbani, P.; DuMouchelle, E. Effect of zinc supplementation on serum testosterone level in adult male sickle cell anemia subjects. *Am. J. Hematol.* **10:**119–127; 1981.

[140] Bao, B.; Prasad, A. S.; Beck, F. W.; Snell, D.; Suneja, A.; Sarkar, F. H.; Doshi, N.; Fitzgerald, J. T.; Swerdlow, P. Zinc supplementation decreases oxidative stress, incidence of infection, and generation of inflammatory cytokines in sickle cell disease patients. *Transl. Res.* **152:**67–80; 2008.

[141] Bettger, W. J. Zinc and selenium, site-specific versus general antioxidation. *Can. J. Physiol. Pharmacol.* **71:**721–724; 1993.

[142] Muskiet, F. A.; Muskiet, F. D.; Meiborg, G.; Schermer, J. G. Supplementation of patients with homozygous sickle cell disease with zinc, alpha-tocopherol, vitamin C, soybean oil, and fish oil. *Am. J. Clin. Nutr.* **54:**736–744; 1991.

[143] Wood, K. C.; Hsu, L. L.; Gladwin, M. T. Sickle cell disease vasculopathy: a state of nitric oxide resistance. *Free Radic. Biol. Med.* **44:**1506–1528; 2008.

[144] Kaul, D. K.; Zhang, X.; Dasgupta, T.; Fabry, M. E. Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. *Am. J. Physiol. Heart Circ. Physiol* **295:**H39–H47; 2008.

[145] Morris, C. R.; Morris Jr S. M.; Hagar, W.; van, W. J.; Claster, S.; Kepka-Lenhart, D.; Machado, L.; Kuypers, F. A.; Vichinsky, E. P. Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? *Am. J. Respir. Crit. Care Med.* **168:**63–69; 2003.

[146] Elias, D. B.; Barbosa, M. C.; Rocha, L. B.; Dutra, L. L.; Silva, H. F.; Martins, A. M.; Goncalves, R. P. L-Arginine as an adjuvant drug in the treatment of sickle cell anaemia. *Br. J. Haematol.* **160:**410–412; 2013.

[147] Morris, C. R.; Vichinsky, E. P.; van, W. J.; Machado, L.; Kepka-Lenhart, D.; Morris Jr S. M.; Kuypers, F. A. Hydroxyurea and arginine therapy: impact on nitric oxide production in sickle cell disease. *J. Pediatr. Hematol. Oncol.* **25:**629–634; 2003.

[148] Wood, K. C.; Hebbel, R. P.; Lefer, D. J.; Granger, D. N. Critical role of endothelial cell-derived nitric oxide synthase in sickle cell disease-induced microvascular dysfunction. *Free Radic. Biol. Med.* **40:**1443–1453; 2006.

[149] Vasquez-Vivar, J. Tetrahydrobiopterin, superoxide, and vascular dysfunction. *Free Radic. Biol. Med.* **47:**1108–1119; 2009.

[150] Hsu, L.; Ataga, K. I.; Gordeuk, V. R. *Tetrahydrobiopterin (6R-BH4): novel therapy for endothelial dysfunction in sickle cell disease*. . Blood (ASH Annual Meeting Abstracts) 122(11); 2008. ([Abstract]).

[151] Girgis, R. E.; Qureshi, M. A.; Abrams, J.; Swerdlow, P. Decreased exhaled nitric oxide in sickle cell disease: relationship with chronic lung involvement. *Am. J. Hematol.* **72:**177–184; 2003.

[152] Montero-Huerta, P.; Hess, D. R.; Head, C. A. Inhaled nitric oxide for treatment of sickle cell stroke. *Anesthesiology* **105:**619–621; 2006.

[153] Weiner, D. L.; Hibberd, P. L.; Betit, P.; Cooper, A. B.; Botelho, C. A.; Brugnara, C. Preliminary assessment of inhaled nitric oxide for acute vaso-occlusive crisis in pediatric patients with sickle cell disease. *JAMA* **289:** 1136–1142; 2003.

[154] Zuzak, K. J.; Gladwin, M. T.; Cannon, R. O.; Levin, I. I. I. I. W. Imaging hemoglobin oxygen saturation in sickle cell disease patients using noninvasive visible reflectance hyperspectral techniques: effects of nitric oxide. *Am. J. Physiol. Heart Circ. Physiol* **285:**H1183–H1189; 2003.

[155] Gladwin, M. T.; Schechter, A. N.; Shelhamer, J. H.; Pannell, L. K.; Conway, D. A.; Hrinczenko, B. W.; Nichols, J. S.; Pease-Fye, M. E.; Noguchi, C. T.; Rodgers, G. P.; Ognibene, F. P. Inhaled nitric oxide augments nitric oxide transport on sickle cell hemoglobin without affecting oxygen affinity. *J. Clin. Invest.* **104:**937–945; 1999.

[156] Faintuch, J.; Aguilar, P. B.; Nadalin, W. Relevance of N-acetylcysteine in clinical practice: fact, myth or consequence? *Nutrition* **15:**177–179; 1999.

[157] Zafarullah, M.; Li, W. Q.; Sylvester, J.; Ahmad, M. Molecular mechanisms of N-acetylcysteine actions. *Cell Mol. Life Sci.* **60:**6–20; 2003.

[158] Gibson, X. A.; Shartava, A.; McIntyre, J.; Monteiro, C. A.; Zhang, Y.; Shah, A.; Campbell, N. F.; Goodman, S. R. The efficacy of reducing agents or antioxidants in blocking the formation of dense cells and irreversibly sickled cells in vitro. *Blood* **91:**4373–4378; 1998.

[159] Pace, B. S.; Shartava, A.; Pack-Mabien, A.; Mulekar, M.; Ardia, A.; Goodman, S. R. Effects of N-acetylcysteine on dense cell formation in sickle cell disease. *Am. J. Hematol.* **73:**26–32; 2003.

[160] Nur, E.; Brandjes, D.; Teerlink, T.; Otten, H. M.; Oude Elferink, R.; Muskiet, F.; Evers, L.; ten Cate, H.; Biemond, B.; Duits, A.; Schnog, J. J., CURAMA Study Group. N-acetylcysteine reduces oxidative stress in sickle cell patients. *Ann. Hematol.* **91:**1097–1105; 2012.

[161] Chiu, D.; Vichinsky, E.; Yee, M.; Kleman, K.; Lubin, B. Peroxidation, vitamin E, and sickle-cell anemia. *Ann. N. Y. Acad. Sci.* **393:**323–335; 1982.
[162] Gbenebitse, S.; Jaja, S. I.; Kehinde, M. O. Effect of changes in plasma vitamin E level of vascular responses and lipid peroxidation in sickle cell anaemia subjects. *Niger. Postgrad. Med. J.* **12:**81–84; 2005.
[163] Natta, C. L.; Machlin, L. J.; Brin, M. A decrease in irreversibly sickled erythrocytes in sickle cell anemia patients given vitamin E. *Am. J. Clin. Nutr.* **33:**968–971; 1980.
[164] Jaja, S. I.; Aigbe, P. E.; Gbenebitse, S.; Temiye, E. O. Changes in erythrocytes following supplementation with alpha-tocopherol in children suffering from sickle cell anaemia. *Niger. Postgrad. Med. J.* **12:**110–114; 2005.
[165] Arruda, M. M.; Mecabo, G.; Rodrigues, C. A.; Matsuda, S. S.; Rabelo, I. B.; Figueiredo, M. S. Antioxidant vitamins C and E supplementation increases markers of haemolysis in sickle cell anaemia patients: a randomized, double-blind, placebo-controlled trial. *Br. J. Haematol.* **160:**688–700; 2013.

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Influence of β^S allele in the lipid peroxidation and antioxidant capacity parameters

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Sickle cell anemia, sickle cell trait, oxidative stress, TBARS, TEAC

SUMMARY

Introduction: The oxidative process plays a fundamental role in the pathophysiology of sickle cell anemia (SCA), and population and environmental characteristics may influence redox balance. The aim of this study was to evaluate lipid peroxidation and antioxidant capacity in Brazilian Hb S carriers undergoing different therapies.
Methods: Blood samples from 270 individuals were analyzed (Hb SS, $n = 68$; Hb AS, $n = 53$, and Hb AA, $n = 149$). Hemoglobin genotypes were assessed through cytological, electrophoretic, chromatographic, and molecular methods. Plasma lipid peroxidation and antioxidant capacity were measured by spectrophotometric methods.
Results: Patients with SCA who used iron-chelating drugs combined with hydroxyurea, associated with regular transfusions, showed lower levels of TBARS ($P \leq 0.05$), higher levels of TEAC ($P \leq 0.01$), and lower TBARS/TEAC ratio (R = 255.8). The redox profile of Hb AS subjects was not statistically different ($P > 0.05$) from that of Hb AA subjects.
Conclusion: The data suggest that oxidative stress is lower in the patients with SCA who received regular blood transfusions associated with the combined use of HU and iron chelators than the group received only HU. The redox system of the Hb AS carriers is compatible with the control group.

INTRODUCTION

The hemoglobin S (Hb S) molecule, originated by the GAG to GTG transversion in the sixth codon of the β globin gene on chromosome 11, responsible for replacing glutamic acid with valine, leads to physicochemical changes in the molecule [1]. It is estimated that nearly 4% of the Brazilian population and 6% to 10% of African descendents are asymptomatic carriers (Hb AS), and 25 to 30 thousand Brazilians have sickle cell anemia (SCA) [2, 3]. Due to its high prevalence and high morbidity and mortality rates, sickle cell disease is considered a public health problem in Brazil [4]. As a result of the interaction of multiple factors, one of the consequences of the disease is stiff and deformed erythrocytes, causing microvascular occlusion associated with endothelial and oxidative damage and chronic hemolytic anemia [5]. The factors that predispose to hemolyisis result from Hb S deoxygenation and methemoglobinization, followed by the formation of hemichromes that bind excessively to the sickle cell erythrocyte membrane, facilitating the generation of reactive oxygen species (ROS) [6, 7]. The externalization of phosphatidylserine on the sickle cell erythrocyte membrane due to ionic imbalance and lipid peroxidation (LPO) [8, 9], as well as the consumption of nitric oxide by plasma heme released by hemolysis, can lead to vaso-occlusion [5, 10]. Oxidative status is determined by the balance between pro-oxidants and antioxidants. The chronic imbalance in the redox system of sickle cells may contribute to the worsening of SCA symptoms and shortening of erythrocyte survival [9, 11, 12]. The response to the more harmful effect of free radicals is the LPO of the erythrocyte membrane, induced by ROS such as superoxide ion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), and peroxyl radicals ($ROO^{\cdot}$) [13]. Iron resulting from autoxidation and Hb S degradation plays a determinant role in the initiation of this process, because it increases the generation of the $^{\cdot}OH$ radical, which is considered the most reactive and harmful form of ROS [5, 14, 15].

The organism defends against free radical-mediated aggressions by means of enzymatic and non-enzymaticantioxidant defense systems and of the metal-chelating system as well [5]. It has been reported that the activities of enzymatic antioxidants are increased in the presence of cofactors such as selenium and transition metals, and also by inflammatory cytokines, bacterial endotoxins, and vitamin E [16, 17]. This way, appropriate and fairly well-described methods may be used to assess redox state, for example Trolox equivalent antioxidant capacity (TEAC), as a overall measure of antioxidant capacity, and thiobarbituric acid reactive substances (TBARS), a LPO marker [18].

Population characteristics, environmental influences, and type of treatment may also influence the capacity of antioxidant defense in Hb S. Thus, evaluating Hb S individuals from different regions of the country, who undergo different environmental and therapeutic conditions, can provide grounds to examine situations associated with a better redox balance in SCA. Sickle cell traits, contrary to SCA patients, are considered as carriers of a benign condition; however, there have been reports of the occurrence of clinical manifestations, ranging from mild to severe, when traits are exposed to adverse situations [12, 19, 20]. Thus, the aim of this study was to evaluate lipid peroxidation and antioxidant capacity in Hb S carriers in Southern and Southeastern Brazil undergoing different therapies, compared with individuals without hemoglobinopathies as a control group.

METHODS

Blood samples from 270 individuals randomized (age ranged from 9 to 66 years) were analyzed, 68 patients with SCA in steady state seen at the outpatient department of Hematology Centers, 53 Hb AS carriers, and 149 individuals without hemoglobinopathies (Hb AA). Inclusion criteria were being non-smoker, not pregnant, non-alcoholic, no exposure to chemicals (e.g. solvents, glue, paints, pesticides, herbicides, fertilizers, motor oil, gasoline, and radioactive material), not be taking vitamin E supplementation at a dose higher than 800 UI/day [18], and whether patients had a stroke, pain, or a hemolytic crisis in the 2 months prior to the start of the study were excluded.

Patients with SCA and Hb AS carriers came from the northwest region of Paraná state (NWPR) (Hematology center, referral public hospital and other health units), the northwest region of São Paulo state (NWSP - Hematology center), and the east region of São Paulo (ESP - Hematology center). The NWPR and NWSP sites are regions with high agricultural activity,

with approximately 500 000 inhabitants each, and ESP is a region with high air pollution and with approximately 12 million inhabitants. All patients attended at the Hematology center were under follow-up according to Clinical Protocols and Therapeutic Guidelines established by the Ministry of Health of Brazil for sickle cell disease [21].

The patients with SCA from ESP were under regular transfusion therapy (received of more than 20 units of red blood cell in the past) and received iron chelator deferasirox for over 2 years, and with the adjusted dose of deferasirox (range 5–30 mg/kg/day). All patients from ESP on the use of hydroxyurea (HU) were in the maximum tolerated dose of 35 mg/kg/day for more than 2 year of use. The patients with SCA from NWPR and NWSP received sporadic blood transfusions and did not receive iron chelator and hyper transfusions. The use of HU time was shorter than 1 year.

The Hb AA group (control) comprised students from a public higher education institution and other voluntary blood donors.

After informed consent, 4 mL of venous blood samples was collected in ethylenediamine tetraacetic acid (EDTA) tubes for hematological tests, screening for hemoglobinopathies, and molecular biology. Furthermore, 7 mL was collected in heparin tubes for the evaluation of oxidative stress biomarkers.

The tests for hemoglobinopathy screening consisted of hemoglobin electrophoresis at pH 8.6 and pH 6.2 [22, 23]. Quantitative analysis of hemoglobin fractions was performed by high-performance liquid chromatography (HPLC) using a Variant equipment (Bio-Rad) [24]. The Hb S genotypes were confirmed by molecular analysis (PCR-RFLP). The amplification of the segment that encodes the β^S gene was performed by specific primers, followed by restriction analysis using *DdeI* enzyme (New England Biolabs, MA, USA) [25].

Lipid peroxidation was assessed by estimating TBARS levels according to the method described by Mihara and Uchiyama [26] with a reference value from 0 to 440 ng/mL. The overall antioxidant capacity was determined by TEAC, which was performed as described by Miller *et al.* [27] and Re *et al.* [28] to evaluate plasma non-enzymatic antioxidants. Reference values established for TEAC, considering regional ethnic variation and laboratory conditions, were 1.83–2.07 mM/L.

Data were expressed as medians, and Statistica software, version 8.0, was used for statistical analyses. Results were assessed as for normality and homogeneity of variance. As data did not meet these assumptions, the Mann–Whitney and Kruskal–Wallis nonparametric tests were used, complemented by the *U* test and the Dunn's multiple comparison test. The Spearman's correlation test was used for correlation analyses. The significance level for the tests was set at 5% ($\alpha = 0.05$).

RESULTS

Of the total of 68 Hb SS subjects, 25% ($n = 17$) came from NWPR; 23.5% ($n = 16$) from NWP; and 51.5% ($n = 35$) from ESP. Among sickle cell traits (Hb AS), 56.6% ($n = 30$) were voluntary blood donors from NWPR and 43.4% ($n = 23$) were blood donors from the hematology center of NWSP. Age ranged from 9 to 50 years in the Hb SS group, from 14 to 66 year in the Hb AS group and from 11 to 55 years in the group without hemoglobinopathy (Hb AA) (Table 1).

The last Hb S, Hb F, and Hb A concentrations resulting from the transfusion process were compared in Hb S individuals from the three localities and showed statistically significant differences ($P = 0.01$, $P = 0.01$, and $P < 0.001$, respectively). The higher concentrations of Hb S and Hb F were found in subjects from NWPR, and the lower level of Hb S was observed in subjects from ESP. As for Hb A, the higher concentration was observed in samples from ESP and is a vestige of transfusion processes (Table 1). However, when comparing patients with and without use of HU between the different groups, we found no significant difference in the level of Hb F ($P > 0.05$). All patients were using HU showed higher Hb F when compared with patients without the use of HU ($P < 0.001$).

When it comes to hematological parameters, Hb concentration in the SS group from ESP was significantly higher compared with that of SS groups from NWPR and NWSP ($P < 0.001$), and the degree of reticulocytosis and total leukocyte showed significantly reduced levels in the SS group from ESP compared with those of the other localities ($P < 0.001$).

As expected, the levels of TBARS in Hb SS groups from NWPR, NWSP, and ESP were significantly higher compared with those of the control group (Hb AA)

Table 1. Demographic characteristics and hematological/clinical profile of study groups, according to region of origin

	NWPR	ESP	NWSP
N	77	35	158
Gender F/M (%)	46.8/53.2	77.1/22.9	37.3/62.7
Age (years)	28 (10 to 55)	24 (9 to 50)	30 (18 to 66)
Genotype (*n*)			
AA	30	–	119
AS	30	–	23
SS	17	35	16
Hematological data (HbSS)			
Hb (g/dL)	7.6^{a} (5.9–9.7)	8.1^{b} (5.6–10.8)	7.8^{a} (4.7–12.8)
Reticulocytes (%)	11.2^{a} (6.3–36.9)	6.8^{b} (0.7–14.8)	–*
Leukocytes (x10^9/L)	12.6^{a} (8.6–18.8)	10.2^{b} (5.35–15.6)	$13.3^{a,c}$ (7.1–23.7)
Hb F (%)	10.2^{a} (1.2–24.2)	4.5^{b} (1.1–34.2)	4.25^{b} (0.6–13.3)
Hb S (%)	83.5^{a} (55.3–93.9)	68.6^{b} (30.4–94.1)	$76.4^{a,b}$ (25.6–93.3)
Hb A (%)	1.9^{a} (0–33)	20^{b} (1.1–61.7)	5.5^{b} (1.5–60.6)
Treatment (Hb SS) (%)			
Iron chelation therapy	5.9	74.3	18.7
Hydroxyurea	53	31.4	25
Folic acid	88.2	100	100
Blood transfusion†	5.9	68.6	43.8
Clinical complications (Hb SS) (%)			
Osteonecrosis	23.5	20	0
Ulcer of the leg	5.8	22.9	50
Acute chest syndrome	5.9	2.85	6.3
Stroke	0	34.3	12.5
Priapism (% M)	0	25	33.3
Cholelithiasis/cholecystectomy	35.2	30	25
Retinopathy	0	5.7	0
Pneumonia	23.5	–	–
Joint and muscular pain	29.4	–	–
Cardiac abnormalities‡	41.2	–	–
Number of painful crisis in the last three years (%)			
0–2	70.6	60	43.8
3–5	23.5	22.9	12.5
≥6	5.9	17.1	43.8

F, female; M, male; ESP, east region of São Paulo state; NWPR, northwest region of Paraná state; NWSP, northwest region of São Paulo state.
Different superscript letters indicate statistical differences, $P < 0.05$ (Kruskal–Wallis followed by Dunn's multiple comparison test or Mann–Whitney).
*Data not shown due to missing information on medical records.
†Up to 60 days prior to data collection.
‡Mitral insufficiency, cardiomegaly, heart murmur.

($P < 0.01$). TEAC values for Hb SS groups from these localities were also higher compared with those of the Hb AA group ($P < 0.01$). Conversely, when these values were compared between the three localities, a significant increase in TBARS was found in the groups from NWPR ($P = 0.01$) and NWSP ($P = 0.04$) in relation to the group from ESP (Figure 1).

TEAC values were significantly different between the three localities ($P < 0.001$), with highest levels in ESP group and lowest levels in NWPR group (Figure 2).

Table 2 shows the redox profile of Hb SS subjects from the three regions studied who were treated with folic acid alone. Data indicated that there was no sta-

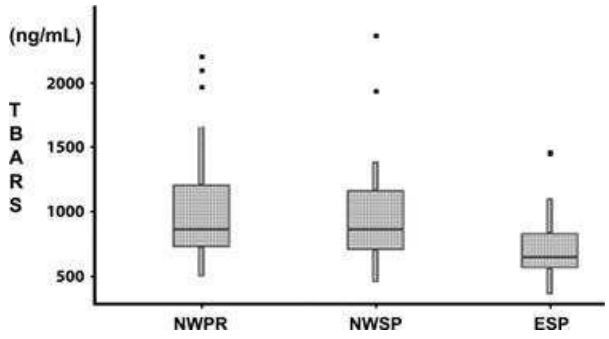

Figure 1. Comparative analysis of TBARS levels in Hb SS subject from NWPR, NWSP, and ESP, using the Kruskal–Wallis test complemented by the Dunn's with a 0.05 significance level. Between NWPR and NWSP ($P = 1$); NWPR and ESP ($P = 0.01$); NWSP and ESP ($P = 0.04$). ESP-east region of São Paulo state; NWPR-northwest region of Paraná state; NWSP-northwest region of São Paulo state.

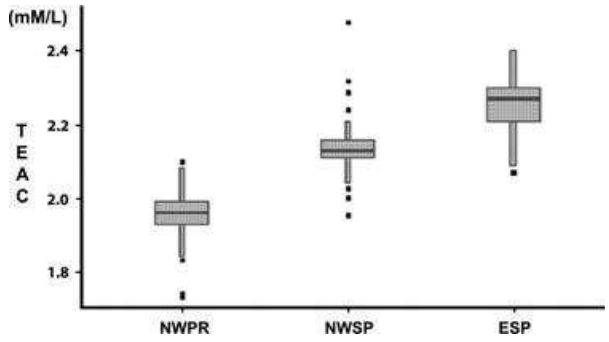

Figure 2. Comparative analysis of TEAC levels in Hb SS subject from NWPR, NWSP, and ESP, using the Kruskal–Wallis test complemented by the Dunn's with a 0.05 significance level. Between NWPR and NWSP ($P = 0.005$); NWPR and ESP ($P < 0.001$); NWSP and ESP ($P = 0.03$). ESP-east region of São Paulo state; NWPR-northwest region of Paraná state; NWSP-northwest region of São Paulo state.

tistically significant difference ($P > 0.05$) in the level of TBARS between the localities, although there was a clear difference in transfusion frequency. However, TEAC had a statistically significant reduction ($P < 0.01$) in the group from NWPR, which showed lower transfusion frequency compared with the other two groups. TBARS/TEAC ratio, expressed by the R index, exhibited higher levels in the group from NWPR.

Table 2. Distribution of medians for TBARS and TEAC, TBARS/TEAC ratio (R index) and transfusion frequency among Hb SS subjects from three different localities who were given folic acid alone. Oxidative status was inferred using the R index for the AA group

	ESP ($n = 7$)	NWSP ($n = 10$)	NWPR ($n = 7$)	P
TBARS (ng/mL)	780[a]	892.5[a]	910.9[a]	0.251
TEAC (mM/L)	2.3[b]	2.15[b]	1.98[c]	<0.001
R-SS (R- AA)	339.1 (261.3)	415.1 (261.3)	460 (261.3)	–
B.T.* (%)	85.7	40	14.2	–

Different superscript letters indicate statistical differences; (R-AA)-R index for the control group; Kruskal–Wallis test complemented by the Dunn's test with a 5% significance level.
ESP, east region of São Paulo state; NWPR, northwest region of Paraná state; NWSP, northwest region of São Paulo state; B.T. *, Blood transfusion in the last 60 days prior to data collection.

Table 3 shows that the group that was given combined antioxidant therapy, hydroxyurea, and iron chelation (HU+IC) and that had a higher frequency of blood transfusions presented a significantly lower level of TBARS ($P < 0.01$) and a higher level of TEAC ($P < 0.01$) when compared with the group with lower transfusion frequency and isolate use of antioxidant (HU). The R index was lower in the group given HU+IC.

Comparing the data from Hb AS subjects from NWPR and NWSP with those from the control group (Hb AA), it was found that the difference between the levels of TBARS and TEAC did not show statistical significance ($P > 0.05$), except for TEAC in the group from NWSP, which had a significant increase ($P < 0.01$). A comparison of the data from NWPR and NWSP localities revealed that the level of TBARS was significantly higher ($P < 0.001$), TEAC was significantly lower ($P < 0.001$), and a significantly higher total leukocyte count (7.6×10^9/L for NWPR; 6.3×10^9/L for NWSP) ($P < 0.01$) in the group from NWPR.

The Spearman's correlation test showed that Hb AS carriers presented a positive relationship between TBARS and TEAC ($r = 0.388$, $P = 0.033$), which indicates the efficacy of the response of the total antioxidant defense system against ROS. However, no correlation

Table 3. Distribution of medians for TBARS and TEAC, –TBARS/TEAC ratio (R index) and transfusion frequency among Hb SS subjects who underwent combined and isolated antioxidant therapy. Oxidative status was inferred using the R index for the AA group

	TBARS (ng/mL)	TEAC (mM/L)	R-SS (R- AA)	B.T.*- (%)
HU+IC (n = 9)	568	2.22	255.8 (261.3)	77.7
HU (n = 8)	907.1	1.99	455.8 (261.3)	25
P	0.007	<0.001	–	–

HU, hydroxyurea; IC, iron chelation; R-AA, R index for the control group; B.T.*, blood transfusion in the last 60 days prior to data collection; IC, iron chelation therapy; Mann–Whitney's test complemented by the U test with a 5% significance level.

was found between TBARS and TEAC or any of the parameters analyzed in the Hb SS group, except between TBARS and leukocytes (r = 0.31; P = 0.008), and between TEAC and Hb A (r = 0.35; P = 0.003), which showed a significant positive correlation.

DISCUSSION

Sickle cell erythrocyte membranes are susceptible to continuous oxidative stress, due to the production of lipid alkoxy ($RO^{\cdot}$) and $ROO^{\cdot}$ radicals, both of them catalyzed by iron, thus contributing to cell damage [14]. The extent of oxidative stress, evaluated by TBARS, reflects the LPO of cell membranes, whose increase is indicative of imbalance between ROS and antioxidant defense [29]. This study, consistent with SCA biology showed a significant increase in both TBARS and TEAC levels in Hb SS samples from all localities, in comparison with the Hb AA group, as described previously in patients with SCA [30]. To protect cells against oxidative stress, the antioxidant defense system seems to respond adaptively by increasing its activity. However, this adaptation is not sufficient to protect against organic damage when the magnitude of ROS is excessive [31]. In a study conducted in an animal model, the increase in LPO levels associated with a marked reduction in the antioxidant defense system is reflective of the disease severity, which is proportional to Hb S concentration [5].

This study revealed that the lower degree of oxidative stress was found in the Hb SS group that received regular blood transfusions, associated with the combined use of pharmacological agents with antioxidant properties (HU+IC). Data suggest that Hb A resulting from regular transfusion processes, even at high concentrations, does not seem to optimize the protective effect against oxidative damage when it is not associated with the combined use of pharmacological agents with synergistic effects on antioxidation. Hence, individuals who received regular transfusions and were given folic acid alone showed higher R index compared with those treated with combined antioxidants.

Similar data were observed by Rice-Evans, Omorphos, and Baysal [14] who showed that the combined use of antioxidants, such as metal-chelating agents and ascorbate, leads to a higher reduction in LPO than the isolated use of these antioxidants. Drug action with antioxidant properties associated with a low Hb S concentration and a considerable presence of Hb A, resulting from the transfusion process, may have contributed to the optimization of the protective effect against LPO. In the group with higher R index (Hb SS from NWPR), in which the use of iron chelation therapy and blood transfusions was not usual, there was an association between higher Hb S concentrations and lower levels of Hb A. Iron-chelating agents suppress LPO in the erythrocyte membrane because they do not provide the metals to catalyze the conversion of lipid hydroperoxide to highly reactive radicals, such as $RO^{\cdot}$ and $ROO^{\cdot}$ [14]. HU, besides having several beneficial effects on the erythrocyte lineage, such as the reduction in the expression of adhesion molecules located on the surface of red blood cells and platelets and the increase in Hb F and total Hb [32], has been recently pointed out as a dose-dependent scavenger of the $^{\cdot}OH$ radical [33]. Thus, there is evidence that both iron-chelating agents and HU have a powerful antioxidant activity in SCA. Another study that confirms our results, the authors demonstrated in a longitudinal study, in which decreased TBARS values and increased TEAC values in patients receiving deferasirox (iron chelator) and hydroxyurea for approximately 390 days [34].

In our study, the isolated effect of HU showed no decrease in oxidative stress parameters. When separate patients were using only HU, they belong to the NWPR group that despite the high levels of Hb F, the ratio R is greater than the ESP group. We believe the

time of use of hydroxyurea may have affected in this result, because the patients of NWPR group used HU <1 year, unlike the ESP group who used the drug for more than two years. However, in other studies, the authors showed that HU was responsible for improving the parameters of oxidative stress in patients who used the drug for more than three years. This effect can probably be at increased Hb F and other actions of HU [34]. The Hb F concentration increase inhibits the polymerization and sickling of Hb S, which attenuates the oxidants production, thus playing the protecting effect against LPO and hemolysis [34, 35].

Some authors have suggested that a concentration higher than 25% of Hb F is necessary to eliminate Hb S polymerization completely [36, 37], but the rate observed in our study was behind that rate. The extent of deoxygenated Hb S polymerization depends on the intracellular concentration of Hb S and Hb non-S [36].

The increase in Hb F and Hb A levels makes it difficult for Hb S molecules to undergo polymerization and sickling, thus reducing the severity and the intensity of clinical manifestations. Therefore, in patients who had Hb A, although at lower levels compared with Hb S, it was sufficient to inhibit the formation of polymers [38]. Our results corroborate these observations, as the positive correlation between Hb A and TEAC among Hb SS groups suggests a protective effect against LPO in SCA. It is interesting to note that high Hb F levels may have been influenced by non-target factors in this study, such as the three major loci – Xmn1-HBG2 single-nucleotide polymorphism, HBS1L-MYB intergenic region on chromosome 6q, and BCL11A – that contribute 20–50% of the trait Hb F variance in patients with SCA [39].

Painful crises have been considered an important parameter to estimate the severity of sickle cell disease (SCD). However, this study found cholelithiasis and osteonecrosis, associated with high levels of TBARS, even in subjects with no painful crises or with a low rate of painful crises in the last three years. These observations suggest that the rate of painful crises does not depend on TBARS levels and clinical complications. The retrospective nature of this information may have incurred systematic errors, but our data corroborate those of Van Beers *et al.* [40], who found that clinical complications and organic damage in sickle cell patients are not related to painful crises and occur even in patients who seem clinically stable.

Carriers of sickle cell traits have redox balance compatible with the control group. These results corroborate data from literature that demonstrate greater preservation of antioxidants in heterozygous individuals, providing thus higher efficacy in antioxidant defense and improved protection against LPO [41].

Oxidative stress plays a major role in the pathophysiology of SCA, as there was evidence of oxidative damage even in steady state of the disease, despite the increase in the antioxidant defense system. The combined use of antioxidant agents associated with a regular transfusion process mitigated the level of oxidative stress in the SCA group. It has been reported that treatments with antioxidant agents, such as α-Tocopherol, vitamin C, N-acetyl L-cysteine, have been reducing LPO and providing improvements in abnormalities induced by oxidative stress in SCA [42]. Further studies involving the appropriate dosing for the combination of pharmacological agents that have a synergistic effect on antioxidant defense would be necessary, with the purpose of minimizing LPO and mitigating the clinical severity of SCA and thus contribute to improvement in the quality of life of the patients.

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REFERENCES

1. Galiza Neto GCD, Pitombeira MDS. [Molecular aspects for sickle cell anemia]. J Bras Patol Med Lab 2003;39:51–6. [Article in Portuguese].
2. Cançado RD, Jesus JA. [Sickle cell disease in Brazil]. Rev Bras Hematol Hemoter 2007;29:204–6. [Article in Portuguese].
3. Simões BP, Pieroni F, Barros GMN, Machado CL, Cançado RD, Salvino MA,

Angulo I, Voltarelli JC. [Brazilian consensus meeting on stem cell transplantation: hemoglobinopathies committee]. Rev Bras Hematol Hemoter 2010;32:46–53. [Article in Portuguese]
4. Brasil, National Health Surveillance Agency. Manual of diagnosis and treatment of sickle cell disease. Brasília: Ministério da Saúde; 2002.
5. Dasgupta T, Hebbel RP, Kaul DK. Protective effect of arginine on oxidative stress in transgenic sickle mouse models. Free Radic Biol Med 2006;41:1771–80.
6. Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest 2007;117:850–8.
7. Booth C, Inusa B, Obaro SK. Infection in sickle cell disease: a review. Int J Infect Dis 2010;14:e2–12.
8. Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrossek V, Ishii S, Shimizu T, Waidamann M, Hessler G, Huber SM, Lang F, Wieder T. Stimulation of erythrocyte ceramide formation by platelet-activating factor. J Cell Sci 2005;118(Pt 6):1233–43.
9. de Jong K, Larkin SK, Styles LA, Bookchin RM, Kuypers FA. Characterization of the phosphatidylserine-exposing subpopulation of sickle cells. Blood 2001;98:860–7.
10. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT. Vasculopathy in sickle cell disease: biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol 2009;84: 618–25.
11. Fibach E, Rachmilewitz E. The role of oxidative stress in hemolytic anemia. Curr Mol Med 2008;8:609–19.
12. Silva Filho IL, Gonçalves MS, Adôrno EV, Campos DP, Fleury MK. [Screening of abnormal hemoglobin and the evaluation of oxidative degeneration of hemoglobin among workers with the sickle cell trait (HbAS), exposed to occupational hazards]. Rev Bras Hematol Hemoter 2005;27:183–7. [Article in Portuguese].
13. Ferreira ALA, Matsubara LS. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. Rev Assoc Med Bras 1997;43:61–8.
14. Rice-Evans C, Omorphos SC, Baysal E. Sickle cell membranes and oxidative damage. Biochem J 1986;237:265–9.
15. Jarolim P, Lahav M, Liu SC, Palek J. Effect of hemoglobin oxidation products on the stability of red cell membrane skeletons and the associations of skeletal proteins: correlation with a release of hemin. Blood 1990;76:2125–31.
16. Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev 1994;74:139–62.
17. Harris ED. Regulation of antioxidant enzymes. FASEB J 1992;6:2675–83.
18. Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, Packer L. Factors associated with oxidative stress in human populations. Am J Epidemiol 2002;156:274–85.
19. Murao M, Ferraz MHC. [Sickle cell trait - heterozygous for the hemoglobin S]. Rev Bras Hematol Hemoter 2007;29:223–5. [Article in Portuguese]
20. Tsaras G, Owusu-Ansah A, Boateng FO, Amoateng-Adjepong Y. Complications associated with sickle cell trait: a brief narrative review. Am J Med 2009;122:507–12.
21. BRAZIL, Ministry of Health Department of Health Care, Department of Health Care. Clinical Protocols and Therapeutic Guidelines. Brasilia; 2011; Ordinance 853.
22. Marengo-Rowe AJ. Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. J Clin Pathol 1965;18: 790–2.
23. Vella F. Acid-agar gel electrophoresis of human hemoglobins. Am J Clin Pathol 1968;49:440–2.
24. Bio-Rad. Variant b-Thalassemia short program instruction manual. Hercules, CA: Bio-rad laboratories; 2006.
25. Belini Júnior E, Cançado RD, Bonini-Domingos CR. The Xmnl polymorphic site 5 ′ to the gene G(y) in a Brazilian patient with sickle cell anaemia - fetal haemoglobin concentration, haematology and clinical features. Arch Med Sci 2010;6:822–5.
26. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271–8.
27. Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci (Lond) 1993;84:407–12.
28. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231–7.
29. Brigelius-Flohe R. Commentary: oxidative stress reconsidered. Genes Nutr 2009;4: 161–3.
30. Shimauti EL, Silva DG, de Almeida EA, Zamaro PJ, Belini Junior E, Bonini-Domingos CR. Serum melatonin level and oxidative stress in sickle cell anemia. Blood Cells Mol Dis 2010;45:297–301.
31. Manfredini V, Lazzaretti LL, Griebeler IH, Santin AP, Brandao VD, Wagner S, Castro SM, Peralba Mdo C, Benfato MS. Blood antioxidant parameters in sickle cell anemia patients in steady state. J Natl Med Assoc 2008;100:897–902.
32. Covas DT, de Lucena Angulo I, Vianna Bonini Palma P, Zago MA. Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia. Haematologica 2004;89:273–80.
33. Liu YH, Wu WC, Lu YL, Lai YJ, Hou WC. Antioxidant and amine oxidase inhibitory activities of hydroxyurea. Biosci Biotechnol Biochem 2010;74:1256–60.
34. Belini Junior E, da Silva DG, Torres Lde S, de Almeida EA, Cancado RD, Chiattone C, Bonini-Domingos CR. Oxidative stress and antioxidant capacity in sickle cell anaemia patients receiving different treatments and medications for different periods of time. Ann Hematol 2012;91:479–89.
35. Torres LS, Silva DG, Belini Junior E, Almeida EA, Lobo CL, Cançado RD, Ruiz MA, Bonini-Domingos CR. The influence of hydroxyurea on oxidative stress in sickle cell anemia. Rev Bras Hematol Hemoter 2012;34:421–5.
36. Noguchi CT, Torchia DA, Schechter AN. Intracellular polymerization of sickle hemoglobin. Effects of cell heterogeneity. J Clin Invest 1983;72:846–52.
37. Atweh GF, Loukopoulos D. Pharmacological induction of fetal hemoglobin in sickle cell disease and beta-thalassemia. Semin Hematol 2001;38:367–73.
38. Zago MA, Pinto ACS. [The pathophysiology of sickle cell disease: from the genetic mutation to multiorgan disfunction]. Rev Bras Hematol Hemoter 2007;29:207–14. [Article in Portuguese].
39. Thein SL, Menzel S. Discovering the genetics underlying foetal haemoglobin production in adults. Br J Haematol 2009;145:455–67.
40. Van Beers EJ, Van Tuijn CFG, Mac Gillavry MR, Van der Giessen A, Schnog JJB, Biemond BJ. Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. Haematologica 2008;93:757–60.
41. Sess ED, Carbonneau MA, Meïté M, Peuchant E, Dumont MF, Receveur MC, Thomas MJ, Perromat A, Sangaré A, Le BrasM, Clerc M. [Markers of lipid peroxidation, inflammatory proteins and plasma tocopherols in homozygotic and heterozygotic sickle cell anemia]. Bull Soc Pathol Exot 1998;91:238–41. [Article in French].
42. Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, Fibach E. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. Br J Haematol 2006;132: 108–13.

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Genetic and biochemical markers of hydroxyurea therapeutic response in sickle cell anemia

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Abstract

Background: Sickle cell anemia (SCA) presents a complex pathophysiology which can be affected by a number of modifying factors, including genetic and biochemical ones. In Brazil, there have been no studies verifying β^S-haplotypes effect on oxidative stress parameters. This study evaluated β^S-haplotypes and Hb F levels effects on oxidative stress markers and their relationship with hydroxyurea (HU) treatment in SCA patients.

Methods: The studied group was composed by 28 SCA patients. Thirteen of these patients were treated with HU and 15 of them were not. We used molecular methodology (PCR-RFLP) for hemoglobin S genotype confirmation and haplotypes identification. Biochemical parameters were measured using spectrophotometric methods (Thiobarbituric-acid-reactive substances and Trolox equivalent antioxidant capacity levels, catalase and GST activities) and plasma glutathione levels by High-performance liquid chromatography coupled to electrochemical detection.

Results: We found the highest frequency of Bantu haplotype (48.2%) which was followed by Benin (32.1%). We observed also the presence of Cameroon haplotype, rare in Brazilian population and 19.7% of atypical haplotypes. The protective Hb F effect was confirmed in SCA patients because these patients showed an increase in Hb F levels that resulted in a 41.3% decrease on the lipid peroxidation levels ($r = -0.74$, $p=0.01$). Other biochemical parameters have not shown differential expression according to patient's haplotypes. Bantu haplotype presence was related to the highest lipid peroxidation levels in patients ($p < 0,01$), but it also conferred a differential response to HU treatment, raising Hb F levels in 52.6% ($p = 0.03$) when compared with the group with the same molecular profile without HU usage.

Conclusions: SCA patients with Bantu haplotype showed the worst oxidative status. However these patients also demonstrated a better response to the treatment with HU. Such treatment seems to have presented a "haplotype-dependent" pharmacological effect.

Keywords: Hemoglobin S, Beta-S-gene cluster haplotypes, Oxidative stress, Antioxidant capacity

Background

Sickle cell anemia (SCA) is a chronic and progressively debilitating medical condition featuring ongoing hemolytic anemia and recurrent acute vaso-occlusive events [1]. It is characterized by a clinical course highly variable, ranging from death in early childhood [2] to a normal life span with few complications [3]. This feature reflects the complex pathophysiology of SCA which can be affected by a number of modifying factors including haplotype of β-globin gene cluster [4], coinheritance of polymorphisms associated with clinical aspects [5,6] and treatment response [7], hemoglobin fetal (Hb F) levels [8], chronic inflammation and oxidative states [9,10] as well as gender [4].

There are five distinct haplotypes linked to the β^S-mutation and they are known as Benin (Ben), Bantu or Central African Republic, Senegal (Sen), Cameroon (Camer) and Indian-Arab haplotypes. These ones are classified according to the geographical region in which they were originally identified [11,12]. Analysis of β^S polymorphisms is of genetic and anthropologic interest, but it may also be related to

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disease severity as well as variations in drug response [13,14]. Bantu haplotype has been associated with more severe disease outcome and a high organ damage incidence. Benin haplotype has been associated with intermediate disease severity. On the other hand, Senegal and Indian-Arab haplotypes have been associated with milder disease severity [13,15] due to their higher Hb F levels related to the C → T mutation at position -158 *Xmn*I in the $^{G}\gamma$-globin gene promoter region [15].

Hydroxyurea (HU) administration seems to be the best available treatment option for SCA patients [1,16,17]. HU is an antineoplastic drug which its main pharmacological action is to increase Hb F levels. It has other potentially beneficial effects including improved nitric oxide (NO) metabolism, reduced red cell–endothelial interaction and decreased erythrocyte density [1]. Although highly effective for most SCA patients, there is a considerable inter-patient variability creating a broad spectrum of Hb F induction [1,18]. HU mechanisms of action for Hb F induction remain incompletely understood. Hb F induction by HU has been correlated to cell cycle inhibition leading to activation of stress erythropoiesis [1,19-21]. Other studies have suggested that Hb F induction by HU is mediated more specifically via nitric oxide-dependent transcriptional mechanisms [22,23] and cyclic nucleotides [24,25] and initial evidence for some epigenetic regulation [26].

Many studies have been carried out trying to establish a relation between β^S-haplotypes and SCA phenotype. These haplotype-phenotype associations are not definitely established and no clear correlation has emerged [6,27-29] to date, though. In Brazil, there have been no studies verifying β^S-haplotypes effect on oxidative stress parameters. Therefore this work aimed at studying β^S-haplotype effects and Hb F levels on oxidative stress markers and their relationship with HU treatment.

Methods

Patients

Eligible patients were 10 years or older at the beginning of the study and they were diagnosed with SCA. They all had access to the same medication protocol. The studied group was composed by 28 SCA patients (11 males and 17 females; mean age: 27.7 years old; range: 10-65 years old) in clinical follow-up in Sao Jose do Rio Preto (SP) and Rio de Janeiro (RJ). All the patients are from the southeast region of Brazil.

All SCA patients were screened using a questionnaire. Pregnant, smokers or drinkers were excluded from the study, as well as anyone who had had a stroke, pain and/or hemolytic crisis or had received blood transfusion within two months prior to the start of the study. The medications used by SCA patients were previously checked and the ones taking any other medication known to affect the parameters analyzed (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection were also excluded. All subjects gave their informed consent and the study was reviewed and ethically approved by the Data Safety Monitoring Board (DSMB) according to Brazilian Regulations and Ethical Committee of Sao Paulo State University (0015.0.229.000-09).

Biological samples

Blood samples (11 mL) were collected through venipuncture in heparinized and ethylenediamine tetraacetic acid (EDTA) tubes. The heparinized blood (7 mL) was incubated for 20 min at 37°C and then centrifuged at 206 g for 20 min to separate plasma for Thiobarbituric-acid-reactive substances (TBARS) and Trolox equivalent antioxidant capacity (TEAC) analysis. The EDTA sample fraction (4 mL) was aliquoted: 2 mL used for the hemoglobinopathies tests, genotypic determination and catalase (CAT) and glutathione *S*-transferase (GST) enzymatic activities analysis and the other 2 mL were submitted to centrifugation at 825 g for 10 min to obtain plasma and then were frozen at–80°C for glutathione (GSH) levels determination.

Hemoglobin phenotypes, genotypes and β^S-globin haplotypes

Hb identification was performed using electrophoresis on cellulose acetate pH 8.4 and agar electrophoresis at pH 6.2. Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANT™ equipment (Bio-Rad Laboratories, CA, USA) [30]. Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald-Giemsa. In all patient samples, Hb S genotype was developed by molecular analysis using PCR-RFLP. The segment amplification that encodes β^S gene was accomplished by specific primers and amplicon was cleaved by the *Dde*I restriction endonuclease (New England BioLabs, MA, USA) [31]. Beta globin haplotypes were determined through the PCR-RFLP analysis of the following polymorphic restriction sites: γG (Hind III), γA (Hind III), ψβ (Hinc II), 3′ψβ (Hinc II) and 5′β (Hinf I), as previously described [32].

Biochemical analysis

Lipid peroxidation levels were assessed in heparinized plasma using TBARS assay [33]. Antioxidant capacity was also determined in heparinized plasma samples according to their equivalence to Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) [34]. For total GST activity, blood samples were diluted in a 3.5 μM 2-mercaptoethanol 10 μM NADP 2.7 mM EDTA hemolyzing solution (1:20, v/v) and then assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm. The assay was carried out in 0.2 M K-phosphate buffer pH 6.5, 1 mM CDNB, 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}$

cm^{-1}) [35]. For CAT activity analysis, blood samples were diluted in ultrapure water (1:50, v/v) and then 10 μL were used to measure CAT activity, by the decrease in absorbance at 240 nm (ε = 0.04 mM^{-1} cm^{-1}) due to consumption of H_2O_2 (10 mM H_2O_2 in 1 M Tris–HCl buffer pH 8.0 containing 5 mM EDTA) [36].

GSH concentration was determined in EDTA plasma samples using HPLC coupled to a coulometric electrochemical detector (Coulochem III ESA, Bedford, MA) [37]. Under these conditions, GSH clearly eluted in ~ 6 min. GSH was extracted from the plasma samples by adding perchloric acid to the plasma sample (10% final concentration). After vigorous stirring and remaining 10 min on ice, the mixture was centrifuged at 825 g for 10 min at 4°C. The extract was then filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC system. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into HPLC system.

Statistical analysis

Statistical analysis was performed in groups with at least three individuals using the Statistica 9.0 software (Statsoft Inc.). Data were tested regarding normality and homogeneity of variances assumptions according to Shapiro-Wilk test and Levene's test, respectively. Groups that met the assumptions (parametric data) were compared by applying t test or one-way ANOVA followed by Fisher's post hoc. Those groups that did not meet the assumptions (non-parametric data) were compared by Mann–Whitney test or Kruskal-Wallis followed by Dunn's post hoc test. In order to assess association degree between the studied variables, we used Pearson's correlation for parametric data and Spearman's rank correlation for non-parametric data. In order to assess age and gender influence on the values of oxidative stress markers, we classified SCA patients into two age groups (≤ 20 and > 20 years) and we applied factorial ANOVA.

Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

Table 1 Characterization of atypical β^S-haplotypes alleles

	Restriction sites					
β^S-Haplotypes	*Xmn* I	*Hind* III		*Hinc* II		*Hinf* I
	5′γ ^{G}	γ ^{G}	γ ^{A}	Ψβ	3′ψβ	5′ β
Atypical 1	-	-	-	-	-	-
Atypical 2	-	+	-	-	+	-
Atypical 3	-	-	+	-	+	-

Results

Through β^S-haplotypes molecular analysis we found nine different combinations of restriction sites, resulting in the following specific combinations: Bantu, Benin, Cameroon and three atypical. The atypical patterns were classified by the numbers 1, 2 and 3, they do not fall into any of the classifications previously reported in the literature (Table 1).

We identified eight (28.5%) patients with haplotype Bantu/Bantu, 10 (35.7%) Bantu/Benin, two (7.1%) Benin/Benin, one (3.6%) Benin/Cameroon, one (3.6%) Bantu/Atypical 1, one (3.6%) Benin/Atypical 1, one (3.6%) Benin/Atypical 2, one (3.6%) Benin/Atypical 3 and three (10.8%) Atypical 2/Atypical 2. From 56 chromosomes analyzed, the allelic frequency observed was: 27 (48.2%) alleles Bantu, 18 (32.1%) Benin, one (1.8%) Cameroon and 10 (19.7%) Atypical, from the atypical ones, two (3.6%) Atypical 1, seven (12.5%) Atypical 2 and one (1.8%) Atypical 3.

For biochemical parameters assessment, firstly we checked whether age and gender could influence the values of studied markers (TBARS and TEAC levels, GST and CAT enzyme activities and plasma GSH levels) to avoid biases. We found no statistically significant difference for any of the evaluated parameters, as shown in Table 2.

The influence of haplotypes and HU treatment over Hb F concentration and on biochemical markers was determined by subgroups formation - haplotype and HU use (+ HU) and haplotype without HU use (–HU). The values and/or mean of ana\lyzed parameters according to subgroup are presented in Table 3.

Between the subgroups submitted to statistical comparisons, we assessed haplotypes effect on SCA phenotypic

Table 2 Analysis of the age and gender interference on the biochemical markers values in SCA patients

	Age[#]		P values*	Gender[#]		P values*
	≤ 20 years n = 09	> 20 years n = 19		Male n = 11	Female n = 17	
TBARS (ng/mL)	1452.94 ± 699.00	1577.07 ± 539.85	0.4950	1345.94 ± 413.13	1660.90 ± 655.72	0.0719
TEAC (mM)	1.97 ± 0.21	2.03 ± 0.15	0.6737	2.01 ± 0.23	2.01 ± 0.12	0.7292
GST (U/mL)	1.77 ± 0.94	1.51 ± 0.49	0.2394	1.50 ± 0.56	1.64 ± 0.72	0.3757
CAT (U/mL)	1660.80 ± 525.41	1912.16 ± 517.83	0.0957	1831.30 ± 634.04	1831.40 ± 461.03	0.7524
GSH (μM)	0.74 ± 0.49	0.70 ± 0.39	0.3644	0.59 ± 0.52	0.79 ± 0.31	0.2791

*Comparisons were made by factorial ANOVA.
[#]There were no significant interactions between independent variables: age and gender ($p > 0.05$).

Table 3 Descriptive analysis of the β^S-haplotypes interference in the phenotypic expression of SCA patients

		Parameters					
		Hb F (%)	TBARS (ng/mL)	TEAC (mM)	GST (U/mL)	CAT (U/mL)	GSH (μM)
Haplotypes (+HU)	**n**						
Bantu/Bantu	2	1.95	1201.18	2.17	1.26	1742.95	1.30
Bantu/Benin	6*	17.42	1066.26	2.03	1.50	2294.01	0.62
Benin/Benin	1	5.2	1616.62	2.17	1.44	2278.17	1.10
Benin/Camer	0	———	———	———	———	———	———
Bantu/Atp1	0	———	———	———	———	———	———
Benin/Atp1	1	1.8	1524.30	2.20	1.20	1531.69	0.19
Benin/Atp2	1	8.4	1012.00	2.01	2.00	1084.51	0.22
Benin/Atp3	1	7.3	1216.92	2.10	1.92	1880.28	0.88
Atp2/Atp2	1	11	1308.00	1.97	1.51	1866.20	0.37
Haplotypes (–HU)							
Bantu/Bantu	6*	6.78	2284.33	1.93	2.03	1656.10	0.77
Bantu/Benin	4*	6.68	1815.50	2.09	1.27	1842.43	0.84
Benin/Benin	1	2.1	1222.00	2.09	1.65	2570.42	0.74
Benin/Camer	1	4.8	934.00	1.90	2.00	996.48	0.70
Bantu/Atp1	1	3.2	1287.00	1.95	1.18	2017.61	0.87
Benin/Atp1	0	———	———	———	———	———	———
Benin/Atp2	0	———	———	———	———	———	———
Benin/Atp3	0	———	———	———	———	———	———
Atp2/Atp2	2	7.45	1576.00	1.77	1.45	1248.24	0.32

(+HU): patients treated with HU; (–HU): patients not treated with HU; Camer: Cameroon; Atp: atypical.
*Subgroups subject to statistical comparisons.

expression markers, comparing Bantu/Bantu (–HU) with Bantu/Benin (–HU) and we observed no statistical difference (Table 4). In order to prove the contribution of HU use on these markers, according to haplotypes subgroups, we compared Bantu/Benin (+HU) with Bantu/Benin (–HU) and found an increase in Hb F levels in the treated subgroup ($p < 0.01$) and consequent lipid peroxidation reduction ($p = 0.03$) (Table 5).

The association degree among the studied markers showed that in patients with the same β^S-haplotype (Bantu/Benin), HU promoted an increase of 61.7% in Hb F values (Figure 1A) and a decrease of 41.3% in lipid peroxidation levels (Figure 1B), according to a negative correlation found between these markers ($r = –0.74$, $p = 0.01$) (Figure 1C). The other evaluated biochemical parameters showed no differential expression or association.

Bantu haplotype is associated with the worst clinical outcomes in SCA. Therefore, to better address Bantu haplotype influence on oxidative stress markers and HU usage, we classified the patients into four sample groups:

Table 4 Influence of Bantu and Benin haplotypes on SCA phenotypic expression

Modulators	Bantu/Bantu (–HU) n = 06	Bantu/Benin (–HU) n = 04	P values*
Hb F (%)	6.78 ± 3.60	6.67 ± 6.37	0.9731
TBARS (ng/mL)	2284.33 ± 435.50	1815.50 ± 334.80	0.1074
TEAC (mM)	1.92 ± 0.13	2.08 ± 0.18	0.1465
GST (U/mL)	2.03 ± 1.02	1.27 ± 0.63	0.2245
CAT (U/mL)	1656.10 ± 413.96	1842.42 ± 397.81	0.4993
GSH (μM)	0.77 ± 0.37	0.84 ± 0.62	0.8317

(–HU) patients not treated with HU. Mean ± standard deviation.
* Comparisons were made by Mann–Whitney test.

Table 5 Influence of the HU use in SCA patients with the Bantu/Benin haplotype

Modulators	Bantu/Benin (+HU) n = 06	Bantu/Benin (–HU) n = 04	P values*
Hb F (%)	17.41 ± 3.10	6.67 ± 4.37	0.0069#
TBARS (ng/mL)	1066.26 ± 495.09	1815.50 ± 334.80	0.0303#
TEAC (mM)	2.02 ± 0.16	2.08 ± 0.18	0.6124
GST (U/mL)	1.49 ± 0.62	1.27 ± 0.63	0.5925
CAT (U/mL)	2294.01 ± 297.29	1842.42 ± 397.81	0.0725
GSH (μM)	0.62 ± 0.37	0.84 ± 0.62	0.4981

(–HU) patients not treated with HU. (+HU) patients treated with HU.
*Comparisons were made by Mann–Whitney test.
#Indicates statistical difference ($p < 0.05$).

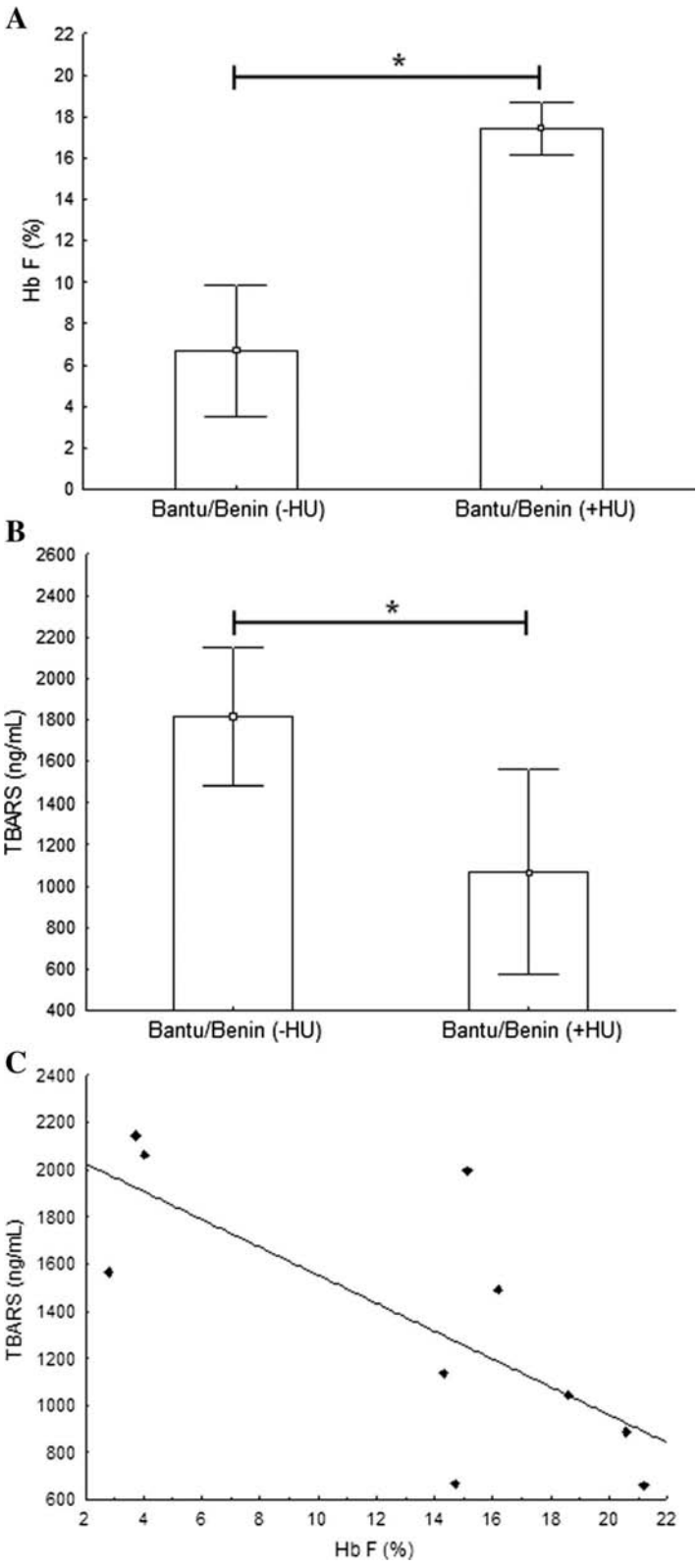

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Hb F and lipid peroxidation levels in SCA patients with Bantu/Benin haplotype. A) Hb F levels were about 2.6 times higher in patients under HU treatment compared to those not treated ($p = 0.0069$; Mann–Whitney test). **B)** Lipid peroxidation levels showed 1.7 times lower in those patients on HU usage ($p = 0.0303$; Mann–Whitney test). **C)** Negative linear correlation between Hb F and lipid peroxidation levels ($r = -0.74$; $p = 0.0156$; Spearman's rank test).

Group I. Patients with Bantu haplotype at least one chromosome without HU treatment. The haplotypes that comprised this group were Bantu/Bantu, Bantu/Benin and Bantu/Atp1;

Group II. Patients with Bantu haplotype at least one chromosome and under HU treatment. The haplotypes were Bantu/Bantu and Bantu/Benin;

Group III. Patients without the Bantu haplotype a HU usage. This group was composed by haplotypes Benin/Benin, Benin/Camer, Atp2/Atp2;

Group IV. Patients without Bantu haplotype in any chromosome, but under HU usage. The haplotypes were Benin/Benin, Benin/Camer, Atp2/Atp2.

Table 6 summarizes obtained results from the comparisons between such groups for all evaluated parameters.

The haplotype sample group analysis also showed significant differences only in the Hb F and lipid peroxidation markers. Bantu haplotype presence was related to the highest lipid peroxidation levels in patients ($p < 0.01$) (Figure 2A), but also, it conferred a differential response to HU treatment, raising Hb F levels in 52.6% ($p = 0.03$) when compared with the group with same molecular profile not treated (Group I). This treatment response was not observed in patients without Bantu haplotype (Figure 2B).

Discussion

Although SCA is one of the first disorders to be clearly defined at molecular level, genetic understanding of the basis for disease expression variability is still unclear [38]. Since β^S-haplotypes discovery as genetic modulators of phenotypic expression in SCA, several studies have been developed to determine haplotypes effect on SCA patients hematological and clinical features [13,27,38-41], but studies associating haplotypes with oxidative stress markers are scarce. This study, to our knowledge, yields a unique opportunity in which both genetic factor (β^S-haplotypes) and oxidative stress markers were simultaneously measured and correlated with Hb F levels and HU use in Brazilian SCA patients.

We found a higher frequency of Bantu haplotype followed by Benin. This distribution of β^S-haplotypes was similar to other studies with Brazilian SCA patients from southeast region [39-44]. The chromosomes majority with β^S gene has one of the five common haplotypes, although in every large series of SCA patients there is a minority of chromosomes ($5 \pm 10\%$) usually referred as "atypical" haplotypes [45]. We found 19.7% of atypical haplotypes, higher frequency than it is expected. None of the identified haplotypes during the study have had presence of *Xmn*I polymorphic site, neither those haplotypes already described in the literature nor the atypical ones. Therefore, other genetic polymorphisms not targeted in this study should be involved in high Hb F levels obtained in SCA patients not treated with HU.

Bantu/Benin haplotype was the most frequent. Therefore in these patients, we confirmed Hb F protective effect provided by HU use. Once increasing Hb F levels resulted in a decrease of the lipid peroxidation levels in accordance with our recent publications [44,46]. The protective effect is due to the increase in Hb F concentration that either inhibits or retards Hb S polymerisation [47], leading to a decreased intravascular sickling and an increasing nitric oxide bioavailability [48]. These alterations result in a decreased oxidative stress with markedly decreased lipid peroxidation and increased activitylevels of antioxidants (SOD, GPx, catalase, and

Table 6 Relationship between the Bantu haplotype and HU treatment on SCA patients

	Sample groups				P values*
	Group I	Group II	Group III	Gruop IV	
	n = 11	n = 08	n = 04	n = 05	
Hb F (%)	6.42 ± 4.45^a	13.55 ± 7.66^b	5.45 ± 5.42^a	6.74 ± 3.46^a	0.0388
TBARS (ng/mL)	2023.18 ± 490.74^a	1099.99 ± 455.70^b	1327.00 ± 463.9^b	1335.57 ± 241.94^b	0.0009
TEAC (mM)	1.99 ± 0.16	2.06 ± 0.15	1.88 ± 0.27	2.09 ± 0.10	0.2296
GST (U/mL)	1.68 ± 0.90	1.44 ± 0.55	1.64 ± 0.53	1.61 ± 0.34	0.8974
CAT (U/mL)	1756.72 ± 385.98	2156.25 ± 540.32	1515.85 ± 759.98	1728.17 ± 446.54	0.1758
GSH (μM)	0.81 ± 0.44	0.79 ± 0.45	0.52 ± 0.24	0.55 ± 0.41	0.4987

*Different letters indicate statistical differences (ANOVA followed by Fisher's post hoc test).
Group I: Bantu (–HU), Group II: Bantu (+ HU), Group III: any haplotype except Bantu (–HU), Group IV: any haplotype except Bantu (+ HU).

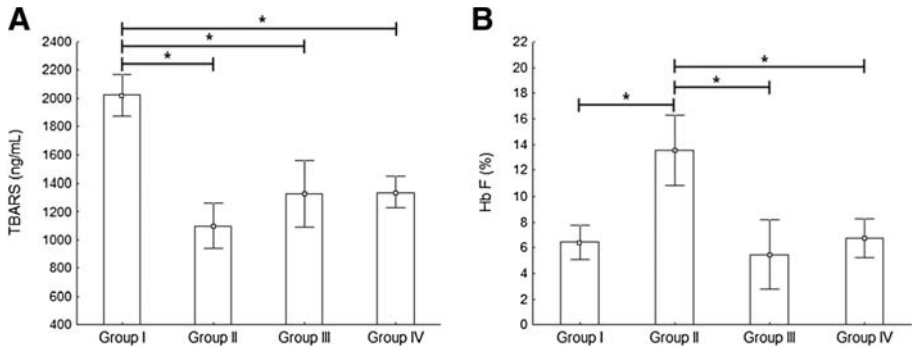

Figure 2 Analysis of Bantu haplotype effect and HU use on SCA phenotypic expression modulators. A) Lipid peroxidation showed its highest mean value in Group I compared to the others groups. **B)** Higher Hb F levels in patients of Group II compared to group with the same molecular profile not treated (Group I) and to the others evaluated groups. *Indicates statistical difference (ANOVA followed by Fisher's post hoc test). Group I: Bantu (−HU), Group II: Bantu (+ HU), Group III: any haplotype except Bantu (−HU), Group IV: any haplotype except Bantu (+ HU).

GSH) [48]. This antioxidant response was not observed though, according to the haplotype profile.

Bantu haplotype presence was related to the highest lipid peroxidation levels in patients, corroborating with the results obtained by Rusanova et al. [49]. The authors showed that SCA patients with Senegal and Indian-Arab alleles had the mild clinical outcomes associated with low oxidative status, whereas high oxidative stress was related to Benin and Bantu haplotypes, consequent severe phenotypes. On the other evaluated parameters (TEAC levels, CAT and GST activities and plasma GSH levels), we have not observed any significant haplotype influence. Thus, oxidative stress biomarkers analysis may be important in clinical condition evaluation of SCA patients, furthermore in therapeutic response monitoring among SCA patients under HU use.

Currently, many researches aimed at identifying inter-individual genetic variations, underlying different pharmacological responses to drug use [50]. In SCA, this paradigm is being applied to elucidate vascular complications pathogenesis and to develop individualized therapies [6]. However, there is no stated relationship in the literature between differential response to HU treatment according to β^S-haplotypes in SCA patients. Vicari et al. [51] showed, in contrast to previous reports [52-54], a significant increase in Hb F levels in SCA patients with Bantu haplotype after HU use, similar HU pharmacological response that we obtained in our studied group. As it is estimated that 40% of the patients do not respond to HU treatment [55] and Bantu haplotype is the most frequent in Brazilian SCA patients, this HU differential response should be carefully interpreted, according to Vicari et al. [51].

We hypothesized that this "haplotype-dependent" pharmacological effect of HU is due to the "highest stress erythropoiesis stimulation" in SCA patients with Bantu haplotype. The presence of Bantu haplotype is associated with a hyperoxidative status and consequent higher hemolytic levels and lower Hb concentrations, characteristics known to increase the circulating erythropoietin concentrations, which in turn stimulates erythropoiesis [56-58]. Based on HU cytotoxic effect which is beneficial in many ways; it targets rapidly dividing cells, which in red cells tend to be those ones with a high Hb S levels and favors the production of red cells with a high Hb F levels, as these levels tend to arise from red cells that divide less rapidly [59]. This way, SCA patients with Bantu haplotype under HU use would have higher erythropoiesis stimulation, favoring production of red cells with a high Hb F levels. This hypothesis agrees with the observations from Gordeuk et al. [60]. The authors confirmed by multiple linear regression that lower hemoglobin concentration was correlated with higher erythropoietin concentration and higher Hb F percentage among sickle cell disease patients. Therefore, even with a small sample size, our results have left perspectives for further studies to better address this hypothesis.

Conclusion

We provided evidence that Bantu haplotype presence seems to be an important predictor factor of oxidative stress and of differential response to HU use in SCA patients. We confirmed a hyperoxidative status among SCA patients. This status should be considered, at least partially, on clinical manifestations variety of these patients. Thus, the use of oxidative stress biomarkers may be important in the evaluation of clinical condition of SCA patients, furthermore in therapeutic response monitoring among SCA patients under HU use. We also suggest that the development of therapies to improve the redox status would be beneficial to reduce the severity of SCA.

Competing interests

The authors declare no competing financial or other relationship with other people or organizations interests.

Authors' contributions

DGHS: data design, data acquisition, data analysis, statistical analysis, data interpretation and manuscript preparation. EBJ: technical assistance on molecular, biochemical and statistical analysis. GCSC: technical assistance in the standardization of molecular biology analysis. LST: technical assistance on biochemical analysis. ORJ: data provision and critical review of manuscript. CLCL: data provision and critical review of manuscript. CRBD: study concept and design and critical review of manuscript. EAA: study concept and design, guidance on standardization of the biochemical methods and critical review of the manuscript. All authors read and approved the final manuscript.

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References

1. Ware RE: **How I use hydroxyurea to treat young patients with sickle cell anemia.** *Blood* 2010, **115:**5300–5311.
2. Leikin SL, Gallagher D, Kinney TR, Sloane D, Klug P, Rida W: **Mortality in children and adolescents with sickle cell disease: cooperative study of sickle cell disease.** *Pediatrics* 1989, **84:**500–508.
3. Serjeant GR, Higgs DR, Hambleton IR: **Elderly survivors with homozygous sickle cell disease.** *N Engl J Med* 2007, **356:**642–643.
4. Kulozik AE, Wainscoat JS, Serjeant GR, Kar BC, Al-Awamy B, Essan GJ, *et al*: **Geographical survey of beta S-globin gene haplotypes: evidence for an independent Asian origin of the sickle-cell mutation.** *Am J Hum Genet* 1986, **39:**239–244.
5. Steinberg MH, Adewoye AH: **Modifier genes and sickle cell anemia.** *Curr Opin Hematol* 2006, **13:**131–136.
6. Steinberg MH: **Predicting clinical severity in sickle cell anaemia.** *Br J Haematol* 2005, **129:**465–481.
7. Fertrin KY, Costa FF: **Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment.** *Expert Rev Hematol* 2010, **3:**443–458.
8. Steinberg MH: **Management of sickle cell disease.** *N Engl J Med* 1999, **340:**1021–1030.
9. Conran N, Franco-Penteado CF, Costa FF: **Newer aspects of the pathophysiology of sickle cell disease vaso-occlusion.** *Hemoglobin* 2009, **33:**1–16.
10. Fibach E, Rachmilewitz E: **The role of oxidative stress in hemolytic anemia.** *Curr Mol Med* 2008, **8:**609–619.
11. Nagel RL, Fleming AF: **Genetic epidemiology of the beta s gene.** *Baillieres Clin Haematol* 1992, **5:**331–365.
12. Pagnier J, Mears JG, Dunda-Belkhodja O, Schaefer-Rego KE, Beldjord C, Nagel RL, *et al*: **Evidence for the multicentric origin of the sickle cell hemoglobin gene in Africa.** *Proc Natl Acad Sci U S A* 1984, **81:**1771–1773.
13. Powars DR, Chan L, Schroeder WA: **Beta S-gene-cluster haplotypes in sickle cell anemia: clinical implications.** *Am J Pediatr Hematol Oncol* 1990, **12:**367–374.
14. Nagel RL, Fabry ME, Pagnier J, Zohoun I, Wajcman H, Baudin V, *et al*: **Hematologically and genetically distinct forms of sickle cell anemia in Africa: the Senegal type and the Benin type.** *N Engl J Med* 1985, **312:**880–884.
15. Padmos MA, Roberts GT, Sackey K, Kulozik A, Bail S, Morris JS, *et al*: **Two different forms of homozygous sickle cell disease occur in Saudi Arabia.** *Br J Haematol* 1991, **79:**93–98.
16. Aliyu ZY, Tumblin AR, Kato GJ: **Current therapy of sickle cell disease.** *Haematologica* 2006, **91:**7–10.
17. Ware RE, Aygun B: **Advances in the use of hydroxyurea.** *Hematology Am Soc Hematol Educ Program* 2009:62–69.
18. Ware RE, Eggleston B, Redding-Lallinger R, Wang WC, Smith-Whitley K, Daeschner C, *et al*: **Predictors of fetal hemoglobin response in children with sickle cell anemia receiving hydroxyurea therapy.** *Blood* 2002, **99:**10–14.
19. Baliga BS, Pace BS, Chen HH, Shah AK, Yang YM: **Mechanism for fetal hemoglobin induction by hydroxyurea in sickle cell erythroid progenitors.** *Am J Hematol* 2000, **65:**227–233.
20. Letvin NL, Linch DC, Beardsley GP, McIntyre KW, Nathan DG: **Augmentation of fetal-hemoglobin production in anemic monkeys by hydroxyurea.** *N Engl J Med* 1984, **310:**869–873.
21. Papayannopoulou T, De Torrealba RA, Veith R, Knitter G, Stamatoyannopoulos G: **Arabinosylcytosine induces fetal hemoglobin in baboons by perturbing erythroid cell differentiation kinetics.** *Science* 1984, **224:**617–619.
22. Cokic VP, Smith RD, Beleslin-Cokic BB, Njoroge JM, Miller JL, Gladwin MT, *et al*: **Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase.** *J Clin Invest* 2003, **111:**231–239.
23. Lou TF, Singh M, Mackie A, Li W, Pace BS: **Hydroxyurea generates nitric oxide in human erythroid cells: mechanisms for gamma-globin gene activation.** *Exp Biol Med (Maywood)* 2009, **234:**1374–1382.
24. Cokic VP, Andric SA, Stojilkovic SS, Noguchi CT, Schechter AN: **Hydroxyurea nitrosylates and activates soluble guanylyl cyclase in human erythroid cells.** *Blood* 2008, **111:**1117–1123.
25. Keefer JR, Schneidereith TA, Mays A, Purvis SH, Dover GJ, Smith KD: **Role of cyclic nucleotides in fetal hemoglobin induction in cultured CD34+ cells.** *Exp Hematol* 2006, **34:**1151–1161.
26. Walker AL, Steward S, Howard TA, Mortier N, Smeltzer M, Wang YD, *et al*: **Epigenetic and molecular profiles of erythroid cells after hydroxyurea treatment in sickle cell anemia.** *Blood* 2011, **118:**5664–5670.
27. Powars D, Hiti A: **Sickle cell anemia: beta s gene cluster haplotypes as genetic markers for severe disease expression.** *Am J Dis Child* 1993, **147:**1197–1202.
28. Powars DR: **Sickle cell anemia: beta s-gene-cluster haplotypes as prognostic indicators of vital organ failure.** *Semin Hematol* 1991, **28:**202–208.
29. Steinberg MH: **Genetic etiologies for phenotypic diversity in sickle cell anemia.** *ScientificWorldJournal* 2009, **9:**46–67.
30. Bonini-Domingos CR: *Metodologias laboratoriais para o diagnóstico de hemoglobinopatias e talassemias.* Sao Paulo, Brazil: NH; 2006:121.
31. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, *et al*: **Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia.** *Science* 1985, **230:**1350–1354.
32. Sutton M, Bouhassira EE, Nagel RL: **Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes.** *Am J Hematol* 1989, **32:**66–69.
33. Uchiyama M, Mihara M: **Determination of malonaldehyde precursor in tissues by thiobarbituric acid test.** *Anal Biochem* 1978, **86:**271–278.
34. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C: **Antioxidant activity applying an improved ABTS radical cation decolorization assay.** *Free Radic Biol Med* 1999, **26:**1231–1237.
35. Keen JH, Habig WH, Jakoby WB: **Mechanism for several activities of glutathione S-transferases.** *J Biol Chem* 1976, **251:**6183–6188.
36. Beutler E: **Red cell metabolism: a manual of biochemical methods.** *Ann Intern Med* 1975, **83:**919.
37. Rodriguez-Ariza A, Toribio F, Lopezbarea J: **Rapid-determination of glutathione status in fish liver using high-performance liquid-chromatography and electrochemical detection.** *J Chromatogr B Biomed Appl* 1994, **656:**311–318.
38. Inati A, Taher A, Bou AW, Koussa S, Kaspar H, Shbaklo H, *et al*: **Beta-globin gene cluster haplotypes and HbF levels are not the only modulators of sickle cell disease in Lebanon.** *Eur J Haematol* 2003, **70:**79–83.
39. Belisario AR, Martins ML, Brito AM, Rodrigues CV, Silva CM, Viana MB: **Beta-globin gene cluster haplotypes in a cohort of 221 children with sickle cell anemia or Sbeta-thalassemia and their association with clinical and hematological features.** *Acta Haematol* 2010, **124:**162–170.

40. Figueiredo MS, Kerbauy J, Goncalves MS, Arruda VR, Saad ST, Sonati MF, *et al*: **Effect of alpha-thalassemia and beta-globin gene cluster haplotypes on the hematological and clinical features of sickle-cell anemia in Brazil.** *Am J Hematol* 1996, **53:**72–76.
41. Gonçalves MS, Nechtman JF, Figueiredo MS, Kerbauy J, Arruda VR, Sonati MF, *et al*: **Sickle cell disease in a Brazilian population from Sao Paulo: a study of the beta S haplotypes.** *Hum Hered* 1994, **44:**322–327.
42. Figueiredo MS, Silva MC, Guerreiro JF, Souza GP, Pires AC, Zago MA: **The heterogeneity of the beta s cluster haplotypes in Brazil.** *Gene Geogr* 1994, **8:**7–12.
43. Zago MA, Figueiredo MS, Ogo SH: **Bantu beta s cluster haplotype predominates among Brazilian blacks.** *Am J Phys Anthropol* 1992, **88:**295–298.
44. Belini-Junior E, Da Silva DG, De Souza TL, De Almeida EA, Cancado RD, Chiattone C: **Oxidative stress and antioxidant capacity in sickle cell anaemia patients receiving different treatments and medications for different periods of time.** *Ann Hematol* 2012, **91**(4):479–489.
45. Srinivas R, Dunda O, Krishnamoorthy R, Fabry ME, Georges A, Labie D, *et al*: **Atypical haplotypes linked to the beta S gene in Africa are likely to be the product of recombination.** *Am J Hematol* 1988, **29:**60–62.
46. Silva DG, Belini-Junior E, Torres LS, Ricci-Junior, Lobo CC, Bonini-Domingos CR, *et al*: **Relationship between oxidative stress, glutathione *S*-transferase polymorphisms and hydroxyurea treatment in sickle cell anemia.** *Blood Cells Mol Dis* 2011, **47:**23–28.
47. Poillon WN, Kim BC, Rodgers GP, Noguchi CT, Schechter AN: **Sparing effect of hemoglobin F and hemoglobin A2 on the polymerization of hemoglobin S at physiologic ligand saturations.** *Proc Natl Acad Sci USA* 1993, **90:**5039–5043.
48. Dasgupta T, Fabry ME, Kaul DK: **Antisickling property of fetal hemoglobin enhances nitric oxide bioavailability and ameliorates organ oxidative stress in transgenic-knockout sickle mice.** *Am J Physiol Regul Integr Comp Physiol* 2010, **298:**R394–R402.
49. Rusanova I, Escames G, Cossio G, De Borace RG, Moreno B, Chahboune M, *et al*: **Oxidative stress status, clinical outcome, and beta-globin gene cluster haplotypes in pediatric patients with sickle cell disease.** *Eur J Haematol* 2010, **85:**529–537.
50. Collins FS, Green ED, Guttmacher AE, Guyer MS: **A vision for the future of genomics research.** *Nature* 2003, **422:**835–847.
51. Vicari P, De Barretto MA, Figueiredo MS: **Effects of hydroxyurea in a population of Brazilian patients with sickle cell anemia.** *Am J Hematol* 2005, **78:**243–244.
52. Charache S, Dover GJ, Moore RD, Eckert S, Ballas SK, Koshy M, *et al*: **Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia.** *Blood* 1992, **79:**2555–2565.
53. Costa FF, Arruda VR, Goncalves MG, Miranda SR, Carvalho MH, Sonati MF, *et al*: **Beta S-gene-cluster haplotypes in sickle cell anemia patients from two regions of Brazil.** *Am J Hematol* 1994, **45:**96–97.
54. Steinberg MH, Lu ZH, Barton FB, Terrin ML, Charache S, Dover GJ: **Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea: multicenter study of hydroxyurea.** *Blood* 1997, **89:**1078–1088.
55. Schnog JB, Duits AJ, Muskiet FA, Ten CH, Rojer RA, Brandjes DP: **Sickle cell disease: a general overview.** *Neth J Med* 2004, **62:**364–374.
56. Ebert BL, Bunn HF: **Regulation of the erythropoietin gene.** *Blood* 1999, **94:**1864–1877.
57. Lee K, Prehu C, Merault G, Keclard L, Roudot-Thoraval F, Bachir D, *et al*: **Genetic and hematological studies in a group of 114 adult patients with SC sickle cell disease.** *Am J Hematol* 1998, **59:**15–21.
58. Nagel RL, Erlingsson S, Fabry ME, Croizat H, Susuka SM, Lachman H, *et al*: **The Senegal DNA haplotype is associated with the amelioration of anemia in African-American sickle cell anemia patients.** *Blood* 1991, **77:**1371–1375.
59. Wiles N, Howard J: **Role of hydroxycarbamide in prevention of complications in patients with sickle cell disease.** *Ther Clin Risk Manag* 2009, **5:**745–755.
60. Gordeuk VR, Campbell A, Rana S, Nouraie M, Niu X, Minniti CP, *et al*: **Relationship of erythropoietin, fetal hemoglobin, and hydroxyurea treatment to tricuspid regurgitation velocity in children with sickle cell disease.** *Blood* 2009, **19**(114):4639–4644.

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Frequencies of -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms in sickle cell anemia patients from Brazil

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ABSTRACT. Sickle cell anemia is an affection that causes chronic inflammation, with consequences for vaso-occlusion, oxidative stress and cytokine production. Genetic polymorphisms in markers involved in this process can modulate the inflammatory response, including polymorphisms -308G/A of TNFA (tumor necrosis factor alpha) and -509C/T of TGFB1 (transforming growth factor beta 1), reported to increase TNF-α and TGF-β1 production, respectively. Changes in the cytokine balance are important risk factors for clinical events; consequently, we examined the frequencies of these polymorphisms in 240 Brazilian sickle cell anemia patients from southeast Brazil. PCR-RFLP was used to detect these polymorphisms. The -509C/T (TGFB1) polymorphism was more frequent than -308G/A (TNFA), with allelic frequency of 0.3 for the mutant allele T (TGFB) agaist 0.1 for the mutant

allele A (TNFA). These allelic frequencies are similar to those known from populations with ethnicity similar to the Brazilian population. Inheritance of these polymorphisms does not seem to be associated with that of the Hb S mutation; however, this information could be useful in analyses of specific clinical characteristics of sickle cell anemia.

Key words: Allelic frequency; Genetic polymorphisms; PCR-RFLP; Sickle cell disease; SNPs; Hemoglobin S

INTRODUCTION

Sickle cell anemia (SCA) is a hemolytic anemia caused by a mutation in the sixth codon of the beta globin chain (GAG → GTG), resulting in homozygous hemoglobin (Hb) S (Honig and Adams III, 1986; Frenette and Atweh, 2007). The inflammatory process that occurs in SCA patients may play an important role in vaso-occlusion, oxidative stress stimulation and cytokine production, contributing to the disease pathogenesis (Chiang and Frenette, 2005).

Evidence shows the involvement of genetic polymorphisms in the inflammatory response in SCA patients, among them, some association studies suggest that *TNFA* (tumor necrosis factor alpha) and *TGFB1* (transforming growth factor beta 1) genes interfere in the SCA clinical profile (Nolan et al., 2006; Cajado et al., 2010). TNF-α and TGF-β cytokine production may be genetically influenced by polymorphisms that affect gene regulation. The -308G/A (*TNFA*) (rs1800629) and -509C/T (*TGFB1)* (rs1800469) polymorphisms are reported to increase the production of TNF-α (Rodriguez-Rodriguez et al., 2011) and TGF-β1 (Bhayal et al., 2011), respectively.

Changes in cytokine balance in SCA patients are important risk factors for clinical events, and thus, the objective of this study was to determine the frequencies of the -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms in Brazilian SCA patients, and to compare our results with the frequencies reported in some recent studies in the literature.

MATERIAL AND METHODS

We analyzed 240 peripheral blood samples of SCA patients from southeast Brazil. All samples were submitted to a classical Hb diagnostic, including Hb electrophoresis at alkaline and acid pH (Marengo-Rowe, 1965; Vella, 1968) and high-performance liquid chromatography for Hb fraction quantification (VARIANT, Bio-Rad) (Bonini-Domingos, 2006). For molecular analysis, genomic DNA was extracted by the phenol-chloroform method (Sambrook et al., 1989). Amplification of the segment that encodes Hb S was performed using specific primers, and fragments were cleaved with the restriction endonuclease Fast*Dde*I (Fermentas, USA) (Belini et al., 2010). -308G/A (*TNFA*) and -509C/T (*TGFB1*) were determined by amplification of the specific genomic segment, with subsequent treatment with specific restriction enzymes for mutations in the *TNFA* (Fast*Nco*I; Fermentas) and *TGFB1* (Fast*Bsu*36I; Fermentas) genes (Wilson et al., 1992; Silverman et al., 2004).

Statistical analyses were performed for comparing the frequencies obtained against those of other studies and databases, using the Statistica 10.0 software with the chi-square test. The level of significance was set at $P < 0.05$.

RESULTS

The genotypic and allelic frequencies for the polymorphisms evaluated in SCA patients are shown in Table 1. The -509C/T (*TGFB1*) polymorphism was more frequent than -308G/A (*TNFA*), with the frequency of the mutant allele being 0.3 versus 0.1. Table 2 presents the literature frequencies for the -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms obtained by other authors and in databases, as well as a comparison with our data, showing that our data were in accordance with other studies with similar populations.

Table 1. Genotypic and allelic frequency of the -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms in sickle cell anemia patients.

Polymorphism	Genotype	Genotypic frequency [N (%)]	Allelic frequency
TNFA	GG	195 (81.25)	G = 0.90
(-308G/A)	GA	43 (17.92)	A = 0.10
	AA	2 (0.83)	
TGFB1	CC	109 (45.42)	C = 0.70
(-509C/T)	CT	116 (48.33)	T = 0.30
	TT	15 (6.25)	

Table 2. Allelic frequency found to -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms in some recent studies and database, comparing with the present study.

	Population group	N	Allelic frequency		P	References
			G	A		
-308G/A (*TNFA*)	General population (Brazil)	200	0.86	0.14	0.1956	(Cajado et al., 2011)
	SCA (Brazil)	210	0.88	0.12	0.5175	(Cajado et al., 2011)
	SCA (Brazil)	49	0.92	0.08	0.6920	(Vicari et al., 2011)
	General population (India)	216	0.94	0.06	0.1200	(Ghosh et al., 2010)
	HapMap_CEU European	113	0.83	0.17	0.0678	**
	HapMap_YRI African	113	0.91	0.09	0.6712	**
	*SCA (Brazil)	240	0.90	0.10	-	-
-509C/T (*TGFB1*)	General population (Korea)	352	0.52	0.48	<0.0001	(Kim et al., 2010)
	General population (Hungary)	30	0.58	0.42	0.1382	(Rovo et al., 2010)
	Severe asthma population (Brazil)	38	0.60	0.40	0.2419	(de Faria et al., 2008)
	HapMap_CEU European	113	0.71	0.29	0.8507	-
	HapMap_YRI African	113	0.79	0.21	0.0843	-
	*SCA (Brazil)	240	0.70	0.30	-	-

SCA = sickle cell anemia; CEU = Utah residents with ancestry from Northern and Western European; YRI = Yoruba in Ibadan, Nigeria. Statistical test: Chi-square test ($\alpha < 0.05$). *Presente study; **available in dbSNP, NCBI [http://www.ncbi.nlm.nih.gov/projects/SNP].

DISCUSSION

The frequencies found for the -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms are similar to those provided by databases for Caucasian and African populations from NCBI databases (http://www.ncbi.nlm.nih.gov/projects/SNP), reflecting the contribution of these groups to the ethnic composition of the Brazilian population. For the -308G/A (*TNFA*) polymorphism, the frequencies obtained were similar to those results of other SCA studies in Brazil (Cajado et al., 2010; Vicari et al., 2011). Our results also did not differ from those found for the Brazilian population without hemoglobinopathies (Cajado et al., 2011), suggesting that the inheritance of the polymorphisms studied is independent of the β^S gene inheritance. In re-

lation to the Indian population, the frequencies found in our population did not differ (Ghosh et al., 2010), probably due to the ethnic heterogeneity of both.

For the -509C/T (*TGFB1*) polymorphism, our data were similar to findings from a Brazilian study in severe asthma patients (de Faria et al., 2008). Regarding that study, there was no association between presence of polymorphism and risk of developing asthma, which is a chronic inflammatory disease. The similarity with the frequencies obtained in our study is probably due to resemblance of the two populations, but not to the chronic inflammatory process. Regarding a study in Hungary with healthy individuals (Rovo et al., 2010), a population with different ethnic characteristics from the Brazilian one, the absence of a difference can be explained by the small sample number (N = 30), which probably was not representative of the Hungarian population. Differences relative to Korean individuals (Kim et al., 2010), in turn, demonstrate the low influence of this ethnicity in the formation of the Brazilian population.

In summary, the frequencies for the polymorphisms evaluated were not associated with inheritance of Hb S and were similar between populations with the same ethnic characteristics. Furthermore, the polymorphisms do not appear to be associated with other inflammatory diseases.

CONCLUSION

Allelic frequencies found in this study for the -308G/A (*TNFA*) and -509C/T (*TGFB1*) polymorphisms are in agreement with the literature for population groups with similar ethnicity as compared to the Brazilian population. The inheritance of the two polymorphisms does not seem to be associated with the Hb S mutation, but knowledge about them can be useful in future studies involving specific clinical characteristics of SCA, since they are involved in the control of inflammation.

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REFERENCES

Belini JE, Cancado RD and Domingos CR (2010). The XmnI polymorphic site 5' to the gene G (gamma) in a Brazilian patient with sickle cell anaemia - fetal haemoglobin concentration, haematology and clinical features. *Arch. Med. Sci.* 6: 822-825.

Bhayal AC, Prabhakar B, Rao KP, Penchikala A, et al. (2011). Role of transforming growth factor-beta1 -509 C/T promoter polymorphism in gastric cancer in south Indian population. *Tumour. Biol.* 32: 1049-1053.

Bonini-Domingos CR (2006). Metodologias Laboratoriais para o Diagnóstico de Hemoglobinopatias e Talassemias. Inovação Distribuidora de Livros Ltda., São Paulo.

Cajado CS, Cerqueira BAV, Barbosa CG and Lyra IM (2010). IL-8 e TNF-alfa: marcadores imunológicos no prognóstico da anemia falciforme. *Gazeta Med. Bahia* 80: 56-61.

Cajado C, Cerqueira BA, Couto FD, Moura-Neto JP, et al. (2011). TNF-alpha and IL-8: serum levels and gene polymorphisms (-308G>A and -251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia. *Cytokine* 56: 312-317.

Chiang EY and Frenette PS (2005). Sickle cell vaso-occlusion. *Hematol. Oncol. Clin. North Am.* 19: 771-84.
de Faria IC, de Faria EJ, Toro AA, Ribeiro JD, et al. (2008). Association of TGF-beta1, CD14, IL-4, IL-4R and ADAM33 gene polymorphisms with asthma severity in children and adolescents. *J. Pediatr.* 84: 203-210.
Frenette PS and Atweh GF (2007). Sickle cell disease: old discoveries, new concepts, and future promise. *J. Clin. Invest.* 117: 850-858.
Ghosh J, Joshi G, Pradhan S and Mittal B (2010). Investigation of TNFA 308G > A and TNFB 252G > A polymorphisms in genetic susceptibility to migraine. *J. Neurol.* 257: 898-904.
Honig GR and Adams III JG (1986). Human Hemoglobin Genetics. Springer-Verlag Wien New York, New York, 19-36.
Kim JJ, Choi YM, Choung SH, Yoon SH, et al. (2010). Analysis of the transforming growth factor beta1 gene -509 C/T polymorphism in patients with advanced-stage endometriosis. *Fertil. Steril.* 93: 2121-2124.
Marengo-Rowe AJ (1965). Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. *J. Clin. Pathol.* 18: 790-792.
Nolan VG, Adewoye A, Baldwin C, Wang L, et al. (2006). Sickle cell leg ulcers: associations with haemolysis and SNPs in Klotho, TEK and genes of the TGF-beta/BMP pathway. *Br. J. Haematol.* 133: 570-578.
Rodriguez-Rodriguez L, Gonzalez-Juanatey C, Palomino-Morales R, Vazquez-Rodriguez TR, et al. (2011). TNFA -308 (rs1800629) polymorphism is associated with a higher risk of cardiovascular disease in patients with rheumatoid arthritis. *Atherosclerosis* 216: 125-130.
Rovo L, Szell M, Bella Z, Korsos A, et al. (2010). The -509 C/T genotype of TGFbeta1 might contribute to the pathogenesis of benign airway stenosis. *Otolaryngol. Head Neck Surg.* 142: 441-443.
Sambrook J, Fritcsh EF and Manatis T (1989). Molecular Cloning: A Laboratory Manual. 2nd edn. Vols. 1, 2 and 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Silverman ES, Palmer LJ, Subramaniam V, Hallock A, et al. (2004). Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. *Am. J. Respir. Crit. Care Med.* 169: 214-219.
Vella F (1968). Acid-agar gel electrophoresis of human hemoglobins. *Am. J. Clin. Pathol.* 49: 440-442.
Vicari P, Silva GS, Nogutti MA, Neto FM, et al. (2011). Absence of association between TNF-alpha polymorphism and cerebral large-vessel abnormalities in adults with sickle cell anemia. *Acta Haematol.* 125: 141-144.
Wilson AG, di Giovine FS, Blakemore AI and Duff GW (1992). Single base polymorphism in the human tumour necrosis factor alpha (TNF alpha) gene detectable by NcoI restriction of PCR product. *Hum. Mol. Genet.* 1: 353.

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Review article

Title: Hemoglobin D-Punjab: Origin, distribution and laboratory diagnosis

Running Head: Hemoglobin D-Punjab: a review

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Abstract

This review discusses about the hemoglobin D-Punjab (Hb D-Punjab), also known as Hb D-Los Angeles, which is one of the most frequent hemoglobin variants in worldwide. It is derived from a point mutation in beta-globin gene (HBB:c.364G>C; rs33946267) prevalent in Punjab region, Indian northwest. Hb D-Punjab can be inherited in heterozygosis with normal Hb A, with no clinical or hematological alterations, or in homozygosis, a rarest form of inheritance commonly not related to clinical symptomatology. This hemoglobin variant can occur in association with others hemoglobinopathies, such as Hb S or thalassemias, but the noticeable clinical severity occur when Hb D-Punjab is associated to Hb S, whose clinical manifestations can be similar to homozygosis for $HBB^{(S)}$ gene. Although Hb D-Punjab is a worldwide frequent variant and has clinical importance, especially in cases of double heterozygosis Hb SD-Punjab, it is still understudied. In Brazil, for example, Hb D-Punjab is the third most common hemoglobin variant, however, poorly studied and understood. Thus, this paper summarizes information about origin, geographic distribution, characterization and occurrence of Hb D-Punjab haplotypes, to assist in the greater knowledge of this variant. Moreover, we provide a compilation about the main methodologies applied for its identification and we emphasize the importance of complementary molecular analysis for accurate diagnosis.

Keywords: Hb D-Los Angeles, Hb D-Punjab, haplotypes, hemoglobin variant, laboratory analysis.

Introduction

Mutations in genes encoding hemoglobin chains are common and are present in about 7% of worldwide population [1]. Such genetic alterations can affect the production rate of globin chains and cause thalassemia, or they can modify molecule structure and generate hemoglobin variants [1,2].

Hemoglobin variants result usually from single amino acid substitutions caused by point mutations in genes encoding globin chain, resulting in a tetramer with different physicochemical characteristics [3]. According to Globin Gene Server database (http://globin.cse.psu.edu/), 1198 hemoglobin variants were described until September 2014. Most of the hemoglobin variants described do not cause symptomatic clinical manifestations; however, in some cases, they can be associated to relevant pathophysiology, e.g., hemoglobin S (Hb S). Hb S is the most frequent hemoglobin variant in the world and its clinical outcome is severe in homozygous or in association with other relatively frequent hemoglobinopathies, such as beta-thalassemia, Hb C or Hb D, for example [3,4]. Contrary to the other hemoglobinopathies, this last one is still poorly studied, especially in Brazil, where there are not many recent studies about its prevalence, diagnosis and clinical aspects. Moreover, Hb D presents considerable geographic distribution and it can be associated with Hb S, forming heterozygous compost with peculiar clinical severity.

Thus, we aimed to gather information about hemoglobins type D available in the literature, more specifically about Hb D-Punjab, in order to draw attention to its importance and to encourage the scientific community interest in the subject, particularly in Brazil, where studies on this variant are scarce.

History

Until the early 50's, only three types of hemoglobin had been described: adult hemoglobin (Hb A) and two variants (Hb S and Hb C). The fourth identified hemoglobin was Hb D and it was first described by Itano (1951). In his study, Itano analyzed a family from Los-Angeles region with multiethnic feature, British, American and Indian ones, and he found a molecule with different characteristics of the other known hemoglobins. This hemoglobin showed electrophoretic mobility similar to Hb S in alkaline pH, however, in acid pH, its migration resembled to Hb A. Furthermore, Hb D exhibited normal solubility when in reduced state and it did not suffer the sickling process [5,6].

Since the discovery of this new variant and the knowledge of its molecular structure, other hemoglobins with similar patterns were discovered in populations from different ethnicities and they were nominated according to the regions where they have been described. In a study performed in 1962 by Baglioni, the author analyzed the molecular structure of five of these hemoglobins, Hb D-Chicago, Hb D-North Carolina, Hb D-Punjab, Hb D-Portugal and Hb D-Oak Ridge and he observed that all of them actually exhibited the same chemical composition of the first discovered one, the Hb D-Los Angeles

[6,7]. The most frequent denominations for this mutant hemoglobin found in the literature are Hb D-Los Angeles or Hb D-Punjab.

Mutation β121 Glu→Gln (GAA→CAA)

The Hb D-Punjab is a variant derived from a point mutation in beta-globin gene (*HBB*) in the first base of the 121 codon (GAA→CAA) giving the substitution of glutamine for glutamic acid (Glu>Gln) in the beta globin chain [8]. According to the Globin Gene Server database, besides Hb D-Punjab, there are seven other types of Hb D, caused by different point mutations in *HBB*. They are Hb D-Agri (HBB:c.29C>A;364G>C), Hb D-Bushman (HBB:c.49G>C), Hb D-Ouled Rabah (HBB:c.60C>A or 60C>G), Hb D-Iran (HBB:c.67G>C), Hb D-Granada (HBB:c.68A>T), Hb D-Ibadan (HBB:c.263C>A) and Hb D-Neath (HBB:c.365A>C).

Origin and geographic distribution

The Hb D-Punjab is one of the most common hemoglobin variants in worldwide, second to Hb S and Hb C, for example. It is prevalent in Punjab region, Indian northwest, which has 2.0% estimated frequency. On the other hand, in west region, more specifically in the Guajarat region, its frequency drops by half [9].

The Hb D-Punjab is also common in countries such as Italy [10], Belgium [11], Austria [12] and Turkey [13]. Hb D-Punjab is the second more common hemoglobin alteration in Turkey and it occurs in 0.2% of population from Denizli province, southeast region, when it represents the most frequent variant. Furthermore, 57.9% of abnormal hemoglobins observed in this province refer to Hb D-Punjab, followed by Hb S, with 21.9% [13]. Similar frequency occurs in Xinjiang province, China northwest, where 55.6% of variant hemoglobins found refers to Hb D-Punjab [14,15].

In Brazil there are no recent studies about the frequency of Hb D-Punjab in population, but in a large survey conducted in 1993 by Bonini-Domingos, which evaluated the prevalence of hemoglobinopathies in about 100,000 Brazilian individuals from all states of the country, Hb D-Punjab was observed to be the third hemoglobin variant more common in the population [16]. Posteriorly, in a screening for hemoglobinopathies performed in hospital-based population from southeast of Brazil by Sonati et al (1996), the authors found hemoglobin alterations in 34.4% of the individuals, of which 0.4% corresponded to Hb D-Punjab. Despite the low frequency, Hb D-Punjab was also the third most frequent variant identified in this population [17].

At first, the geographic distribution of the Hb D-Punjab suggest that this mutation was originated in central region of the Asia, once it is prevalent in Punjab, a region of India, and China Northwest. Then it spread to other nearby countries by migration, presenting currently worldwide distribution [11,18,19]. In contrast, haplotypes analysis of beta-globin cluster provides another insight about the origin of this

hemoglobin. The analysis of polymorphic sites 5'-ε *Hinc*II, γ^G *Hind*III, γ^A *Hind*III, ψβ *Hinc*II, 3'-ψβ *Hinc*II, *5'-β Hinf*I, β *Ava*II, 3'-β *Hinf*I, 3'-β *Bam*HI (Figure 1) showed certain haplotypes associated to Hb D-Punjab, suggesting a multicentric origin for its mutation [19-24].

In the study of Atalay et al (2007), considering seven beta-globin gene polymorphic sites, 5'-ε *Hinc*II, γ^G *Hind*III, γ^A *Hind*III, ψβ *Hinc*II, 3'-ψβ *Hinc*II, β *Ava*II and 3'-β *Hinf*I, three haplotypes are mentioned. One of them is the Mediterranean Haplotype I (+ − − − − + +), that is the most frequent and was also identified in populations from Mexico [22], Italy [10] and Iran [23]. A second pattern recently discovered was associated to Hb D-Punjab cases from Thailand (− + + − + + +) [8] and the third haplotype related was a haplotype from Turkey populations (− + − − + + +) [20].

In 2009, Yavarian et al. analyzed the polymorphic sites 5'-ε *Hinc*II, γ^G *Hind*III, γ^A *Hind*III, ψβ *Hinc*II, 3'-ψβ *Hinc*II, *5'-β Hinf*I and 3'-β *Hinf*I and found four haplotypes patterns associated to Hb D-Punjab in Indian population: the Mediterranean Haplotype I, the most frequent (+ − − − − + +); one haplotype that seems to be a product from Mediterranean Haplotype I variation, originated probably by an independent mutation event (+ − − − − − +); the same haplotype previously found in Turkey (− + − − + + +); and a common haplotype in India and England populations (− + − + + + +) [19].

Considering the same restriction sites assessed by Atalay et al in 2007, also in 2009, Bahadir et al related the occurrence of a new haplotype in Turkey population (− − + − + + +) [20,24]. Due to lack of studies in Chinese and Indian populations, Patel et al (2010) evaluated individuals with Hb D-Punjab from Agharia, India region, and they found another haplotype (− − − − − + +) in addition to haplotypes I (+ − − − − + +) [21]. Table 1 summarizes all these results.

As the described haplotypes in association with Hb D-Punjab are prevalent in different regions, it is suggested that the origin of mutation in codon β121 may be multicentric. According to Yavarian et al (2009), there is also the fact of the mutation site is located in a short palindromic region, where many mutations are described, suggesting that the codon β121 exhibit higher mutation rate than the expected. Therefore, it provides chance for new mutational events giving origin to the same hemoglobin in different regions [19]. However, is not yet possible to assure the origin and distribution of this hemoglobin once at the moment there are not studies about haplotypes in populations from China [20] and from other continents such as North, South and Central America and Africa. In Brazil, no study has been performed for screening of haplotypes associated to Hb D-Punjab.

Inheritance of Hb D-Punjab and associated clinical features

The Hb D-Punjab can be inherited in heterozygosis with normal Hb A, characterizing the heterozygous trait. This condition presents no clinical or hematological alterations. The homozygous component Hb DD, rarest form of inheritance, commonly is not related to symptomatic cases, but eventually the individuals with this profile can develop mild to moderate hemolytic anemia [25,26].

The association of this variant with others abnormal hemoglobins, such as Hb S or thalassemias can also occur. Usually, interactions between Hb D-Punjab and beta thalassemia course with discrete degree of microcytic and hypochromic anemia, but do not present relevant clinical and hematological changes [27].

However, when Hb D-Punjab is associated to Hb S, the double heterozygous Hb SD shows noticeable clinical manifestations of moderate to severe and this association can be clinically similar to homozygous inherence Hb SS [25]. Recent episodes of pain, due to vaso-occlusive events, represent one of the complications more observed in these individuals. Furthermore, stroke and acute chest syndrome can manifest in carriers of this genotype [28,29,30].

Recently, Patel et al (2014) published a study demonstrating HbSD-Punjab patients with moderate to severe clinical symptomatology, similar to Hb SS genotype, evidenced by vaso-occlusion episodes or blood transfusion requirements. Moreover, the authors observed greater vulnerability for red cell lysis in Hb SD-Punjab patients than Hb SS genotype [31]. Clinical severity in individuals Hb SD could be related to the fact that Hb D favors polymerization of Hb S into the erythrocytes [32].

Associations between β^S chains of a tetramer occur by hydrophobic interaction between Val-β6, Phe-β85 and Leu-β88 of adjacent Hb S molecules. Subsequently, these tetramers interact with other β^S tetramers by contact sites, such as β73 e β121, constituting polymers. It is believed that the amino acid changes in the β121 Glu→Gln position encourage the Hb S molecules interaction and consequently its polymerization [33,34]. Adachi et al. (1988) showed that Hb S polymerization speed is higher when in association with Hb D-Punjab. This may explain the clinical severity of sickle cell disease in double heterozygous Hb SD-Punjab, once Hb D-Punjab increases the sickling chance of the erythrocytes in shorter transit time of these cells in the blood capillaries [32].

Hydroxyurea (HU) administration is a potential therapeutic strategy for sickle cell anemia (Hb SS), showing efficacy in increasing HbF levels and consequently reducing the number of painful episodes, acute chest syndrome, transfusions, and hospitalizations. In contrast, HU therapy for Hb SD-Punjab is not established. Patel et al (2014), however following Hb SD-Punjab patients using low doses (10mg/kg/day) of HU noticed an effective reduction in the incidence of vaso-occlusion and frequency of blood transfusion. These results suggest that HU is a promising therapeutic target for clinical symptomatology resulting from Hb SD-Punjab condition [31].

Laboratory diagnosis

There are numerous laboratory methodologies used for hemoglobin variants and thalassemias diagnosis. Here we describe the behavior of samples containing Hb D using methodologies incorporated for Hb D-Punjab characterization in worldwide, but mainly in Brazil.

At first, the hemoglobins can be separated by electrophoresis in cellulose acetate pH 8.6 [35] and electrophoresis in agarose gel pH 6.2 [36]. In the first method, Hb D migration is slower than Hb A and

demonstrates similar position pattern to Hb S, but in acid pH its migration occurs in the same position of Hb A. These electrophoretic patterns, however, are not specific for variants of Hb D group, once there are other hemoglobin variants with the same behavior, such as Hb G and Hb Korle-bu.

Structural characterization of hemoglobin variants by electrophoretic separation of hemoglobin polypeptide chains is used in cases in which the obtained results in initial tests are not conclusive [37,38]. With this method mutant β^D chain presents migration very similar to the β^S chain, being slightly below of this fraction in alkaline pH, while it migrates in the same position of normal β^A chain in acid pH. Figure 2 shows photographs of the electrophoresis methods mentioned above.

It is also possible to separate hemoglobins according to its isoelectric point (pI) by isoelectric focusing (IEF) [38]. In Hb D-Punjab case, its pI is near to pH 7.1, a similar position to other hemoglobin variants such as Hb Korle-Bu (Figure 3). Although it has great resolution, the technique requires specific kits and it is more expensive than conventional electrophoresis.

High liquid performance chromatography (HLPC) methods with high sensitivity and specificity have been developed for hemoglobin fractionation. The system provides an integrated method based on ion-exchange for separation and identification of the relative percent of specific hemoglobins in whole blood samples (Figure 4). Another existent method employed for hemoglobin fractions detection is the capillary electrophoresis (CE) based on the principle of electrokinetic separation. Despite the excellent resolution and reproducibility, the technique is only now coming in some countries, such as Brazil.

Reverse-phase HPLC (RP-HPLC) of human globin chains has been reported as an important tool for detecting hemoglobinopathies. Nevertheless, although it is widely used for the detection, quantification and purification of many proteins from biological materials, its clinical application for hemoglobin is still limited due to long analytical time, complex sample preparation and resolution problems [40,41]. Currently, studies have had success in method optimizing [40,41], but in many laboratories the technique is still employed exclusively for research.

Although all these tests aim to the diagnosis for Hb D-Punjab, they are not considered conclusive, because of the large number of hemoglobin variants, in which patterns may be confused with each other. It is always recommended molecular analysis to genotype confirmation and the PCR-RFLP technique is an alternative for Hb D-Punjab confirmation [42] (Figure 5).

Finally, when the variant Hb D is not identified by abovementioned methodologies, it is recommended sequencing of beta-globin gene or other techniques able to discriminate amino acid changes.

Using abovementioned methodologies, in the least 10 years, we diagnosed in our laboratory (Hemoglobin and Hematological Genetic Diseases Laboratory at Sao Paulo State University (UNESP) - Brazil), a reference center for hemoglobin diagnosis, 70 cases of hemoglobins type D from southeast Brazil. Of these, 66 (94.3%) showed compatible profile to Hb D-Punjab: eight (12.1%) of them compound heterozygous Hb SD-Punjab and 58 (87.9%) heterozygous trait Hb AD-Punjab, all of them confirmed by molecular tests. The other four remaining cases presented electrophoretic and

chromatographic profile compatible with to trait “Hb AD- non Los Angeles”, possibly Hb AD-Iran (data not published).

Conclusion

Although it is not always associated with relevant clinical history, Hb D-Punjab is hemoglobin relatively common worldwide and it represents the third most common hemoglobin variant in Brazil. Greater attention has been given when it is present in interaction with other abnormal hemoglobin or thalassemia and leads to clinical effect. However, this issue is still understudied and the pathophysiological mechanisms caused by Hb D-Punjab are poorly known. Especially in Brazil, there is a lack of studies about the subject. We have not recent information about Hb D-Punjab prevalence in Brazilian population, reports about association with other hemoglobin variants, or characterization of typical haplotypes in our region. Therefore, it is important to conduct studies about this hemoglobin variant, for give it the attention it deserves within the clinical context and for better knowledge of our population.

Declaration of Interest

The authors declare that they have no conflict of interest.

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References

[1] Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ. 2001;79(8):704-712.

[2] Kohne E. Hemoglobinopathies: Clinical manifestations, diagnosis, and treatment. Dtsch Arztebl Int. 2011;108(31-32):532-540.

[3] Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin variants: biochemical properties and clinical correlates. Cold Spring Harb Perspect Med. 2013;3(3):a011858.

[4] Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest. 2007;117(4):850-858.

[5] Itano HA. A third abnormal hemoglobin associated with hereditary hemolytic anemia. PNAS. 1951;37(12):775-784.

[6] Vella F, Lehmann H. Haemoglobin D Punjab (D Los Angeles). J Med Genet 1974;11(4):341-348.

[7] Baglioni C. Abnormal human haemoglobins VII. Chemical studies on haemoglobin D. Biochim Biophys Acta. 1962;59:437-449.

[8] Fucharoen S, Changtrakun Y, Surapot S, Fucharoen G, Sanchaisuriya K. Molecular characterization of Hb D-Punjab [β121(GH4)Glu→Gln] in Thailand. Hemoglobin. 2002;26(3):261-269.

[9] Köseler A, Öztürk O, Atalay A, Atalay EÖ. Allele frequency of VNTR locus D1S80 observed in Hb D-Los Angeles carrires. Mol Biol Rep. 2012;39(12):10747-10750.

[10] Fioretti G, De Angioletti M, Pagano L, Lacerra G, Viola A, de Bonis C et al. DNA polymorphisms associated with Hb D-Los Angeles [b121(GH4)Glu→Gln] in Southern Italy. Hemoglobin 1993;17(1):9-17.

[11] Husquinet H, Parent MT, Schoos-Barbette S, Dodinval-Versie J, Lambotte C, Galacteros F. Hemoglobin D-Los Angeles [β121(GH4)Glu→Gln] in the province of Liege Belgium. Hemoglobin 1986;10(6):587-592.

[12] Lischka A, Pollak A, Bauer K, Aschauer H, Braunitzer G. Hemoglobin D "Los Angeles" in an Austrian family: biochemical identification, clinical aspects, and kindred study. Hemoglobin. 1984;8(4):353-361.

[13] Atalay EÖ, Koyuncu H, Turgut B, Atalay A, Yildiz S, Bahadir A et al. High incidence of Hb D-Los Angeles [β121(GH4) Glu→Gln] in Denizli province Aegean region of Turkey. Hemoglobin. 2005;29(4):307-310.

[14] Li HJ, Zhao XN, Qin F, Li HW, Li L, He XJ et al. Abnormal hemoglobins in the silk road region of China. Hum Genet. 1990;86(2):231-235.

[15] Pandey S, Mishra RM, Pandey S, Shah V, Saxena R. Molecular characterization of hemoglobin D Punjab traits and clinical-hematological profile of the patients. Sao Paulo Med J. 2012;130(4):248-251.

[16] Bonini-Domingos CR. Prevenção das hemoglobinopatias no Brasil – Diversidade Genética e Metodologia Laboratorial. 1993. 144f. Tese (Doutorado em Ciências) – Instituto de Biociências, Letras e Ciência Exatas, Univ. Estadual Paulista – Unesp, São José do Rio Preto-SP.

[17] Sonati MF, Kimura EM, Grotto HZW, Gervasio SA, Costa FF. Hereditary hemoglobinopathies in a population from southeast Brazil. Hemoglobin. 1996;20(2):175-179.

[18] Brittenham GM. Globin gene variants and polymorphisms in India. In: W.P. Winter, Ed. Hemoglobin Variants in Human Populations. Boca Raton. 1987:79–110.

[19] Yavarian M, Karimi M, Paran F, Neven C, Harteveld CL, Giordano PC. Multi Centric origin of Hb D-Punjab [β121(GH4)Glu→Gln,GAA>CAA]. Hemoglobin. 2009;33(6):399-405.

[20] Atalay EÖ, Atalay A, Ustel E, Yildiz S, Oztürk O, Köseler A et al. Genetic origin of Hb D-Los Angeles [β121(GH4)Glu→Gln, GAA→CAA] according to the β-globin gene cluster haplotypes. Hemoglobin. 2007;31(3):387-391.

[21] Patel DK, Mashon RS, Patel S, Dash PM, Das BS. β-globin gene haplotypes linked with the Hb D-Punjab [β121(GH4)Glu→Gln, GAA→CAA] mutation in easter India. Hemoglobin. 2010;34(6):530-537.

[22] Perea FJ, Casas-Castañeda M, Villalobos-Arámbula AR, Barajas H, Alvarez F, Camacho A et al. Hb D-Los Angeles associated with Hb S or β-thalassemia in four Mexican Mestizo families. Hemoglobin. 1999;23(3):231-237.

[23] Rahimi Z, Akramipour R, Nagel RL, Ahmadi AS, Merat A, Bahrehmand F. The β-globin gene haplotypes associated withHb D-Los Angeles β121(GH4)Glu→Gln] in Western Iran. Hemoglobin. 2006;30(1):39-44.

[24] Bahadir A. Hb D-Los Angeles [beta121(GH4)Glu→Gn] and Hb Beograd [beta121(GH4)Glu 1Val]: Implications for their laboratory diagnosis and genetic origins. Turk J Hematol. 2009;26:17-20.

[25] Adekile A, Muah-AlI A, Akar NA. Does elevated hemoglobin F modulate the phenotype in Hb SD-Los Angeles?. Acta Haematol. 2010;123(3):135-139.

[26] Basmanj MT. Co-inheritance of hemoglobin D and β-thalassemia traits in three families: Clinical relevance. Arch Iran Med. 2011;14(1):61-63.

[27] Valeri FF. Hb D/Talassemia beta associada à anemia crônica. Rev Bras Hematol Hemoter. 2002;24(1):51-52.

[28] Ohene-Frempong K, Weiner SJ, Sleeper LA, Miller ST, Embury S, Moohr JW et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood. 1998;91(1):288–294.

[29] Castro O, Brambilla DJ, Thorington B, Reindorf CA, Scott RB, Gillette P et al. The acute chest syndrome in sickle cell disease: incidence and risk factors. The cooperative study of sickle cell disease. Blood. 1994;84(2):643–649.

[30] Hamidieh AA, Jalili M, Khojasteh O, Ghavamzadeh A. First report of successful stem cell transplantation in a patient with sickle cell hemoglobin D disease. J Pediatr Hematol Oncol. 2010;32(5):397-399.

[31] Patel S, Purohit P, Ranjeet SM, Dehury S, Meher S, Sahoo S, et al. The effect of hydroxyurea on compound heterozygotes for sickle cell-hemoglobin D-Punjab—A single centre experience in eastern India. Pediatr Blood Cancer. 2014;61:1341-1346.

[32] Adachi K, Kim J, Ballas S, Surrey S, Asakura T. Facilitation of Hb S polymerization by the substitution of Glu for Gln at β121. JBC. 1988;263(12):5607-5610.

[33] Charache S, Conley CL. Rate of sickle cells during deoxygenation of blood from persons with various sickling disorders. Blood. 1964;24:25-48.

[34] Benesch RE, Kwong S, Benesch R, Edalji R. Location and bond type of intermolecular contacts in the polymerization of haemoglobin S. Nature. 1977;269(5631):772-775.

[35] Marengo-Rowe AJ. Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. J Clin Pathol. 1965;18(6):790-792.

[36] Vella F. Acid-agar gel electrophoresis of human hemoglobins. Am J Clin Pathol. 1968;49(3):440-442.

[37] Schneider RG. Differentiation of electrophoretically hemoglobins-suchas S, D, G and P or A2, C, E, and O- by electrophoresis of the globin chains. Clin Chem. 1974;20(9):1111-1115.

[38] Alter BP, Goff SC, Efremov GD, Gravely ME, Huisman TH. Globin chain electrophoresis: a new approach to the determination of the G/A ratio in fetal haemoglobin and to studies of globin synthesis. Br J Haematol. 1980;44(4):527-532, 1980.

[39] P.C. Naoum. Eletroforese - Técnicas e Diagnósticos. São Paulo: Livraria Santos Editora Ltda. 1998.

[40] Nemati H, Bahrami G, Rahimi Z. Rapid separation of human globin chains in normal and thalassemia patients by RP-HPLC. Mol Biol Rep. 2011;38(5):3213-3218.

[41] Wan JH, Tian PL, Luo WH, Wu BY, Xiong F, Zhou WJ et al. Rapid determination of human globin chains using reversed-phase high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;15(901):53-58.

[42] Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988;239(4839):487-491.

Table 1. Relationship of described haplotypes in the literature in association with Hb D-Punjab and the locations when they were first found.

Polymorphic sites of cluster beta									
5'- ε *Hinc*II	$^{G}\gamma$ *Hind*III	$^{A}\gamma$ *Hind*III	ψβ *Hinc*II	3'- ψβ *Hinc*II	5'-β *Hinf*I	β *Ava*II	3'-β *Hinf*I	3'-β *Bam*HI	Occurrence
+	-	-	-	-	NR	+	NR	+	Mexico [18,20], Italy [10,18], Iran [18,21]
+	-	-	-	-	NR	+	+	NR	Turkey [18,22], India [19]
-	+	+	-	+	NR	+	NR	+	Thailand [8,18]
-	+	+	-	+	NR	+	+	NR	Turkey [18]
-	+	-	-	+	NR	+	+	NR	Turkey [18]
-	-	+	-	+	NR	+	+	NR	Turkey [22]
+	-	-	-	-	+	NR	+	NR	Iran, Dutch, Belgium [17]
+	-	-	-	-	-	NR	+	NR	Iran [17]
-	+	-	-	+	+	NR	+	NR	Iran [17]
-	+	-	+	+	+	NR	+	NR	Iran, India, England [17]

NR: not reported.

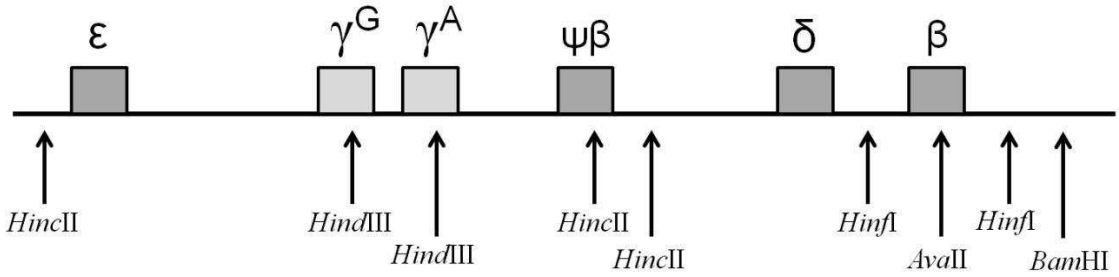

Figure 1. Restriction polymorphic sites in cluster β-globin for haplotypes screening in association with Hb D-Punjab (modified of Yavarian et al., 2009).

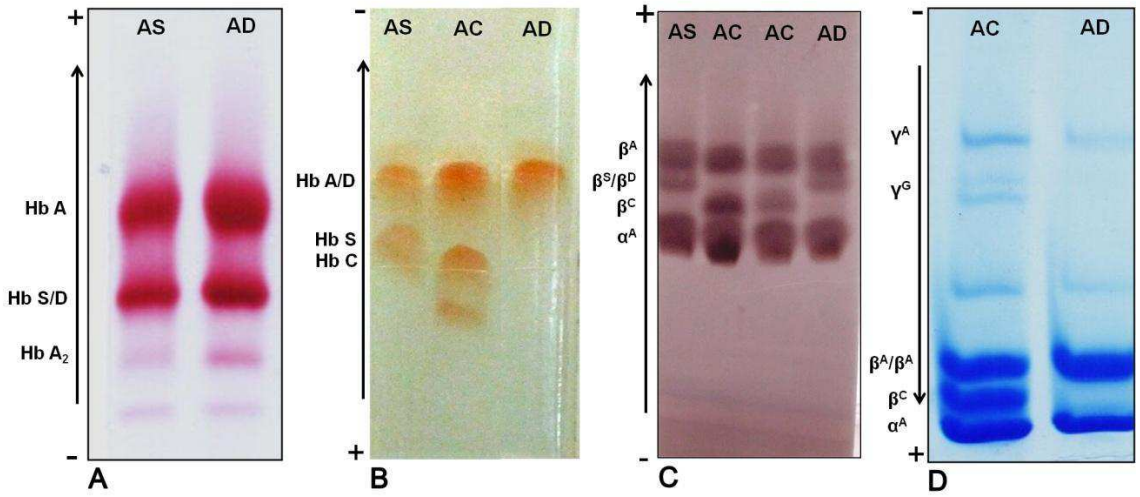

Figure 2. Electrophoretic mobility of Hb D-Punjab comparing to other known hemoglobins, such as Hb A, S and C. Images correspond to hemoglobin electrophoresis in cellulose acetate (A), hemoglobin electrophoresis in agarose gel (B), polypeptide chains electrophoresis in cellulose acetate (C) and polypeptide chains electrophoresis in polyacrylamide gel (D). The genotypes of each sample are at the top of the figure. Current direction and hemoglobins position are beside each method (images of the authors).

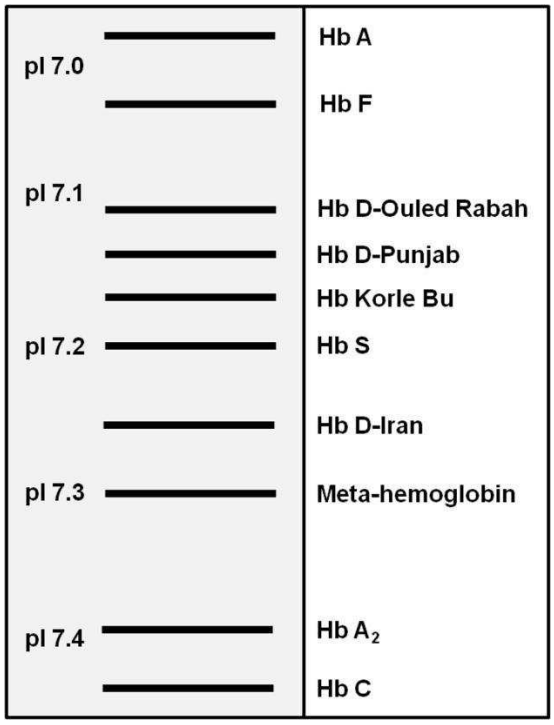

Figure 3. Schematic representation of relative mobilities of different hemoglobins in isoelectric focusing method. Hb D-Punjab is shown in the picture with isoelectric point (pI) between 7.1 and 7.2 (image of the authors).

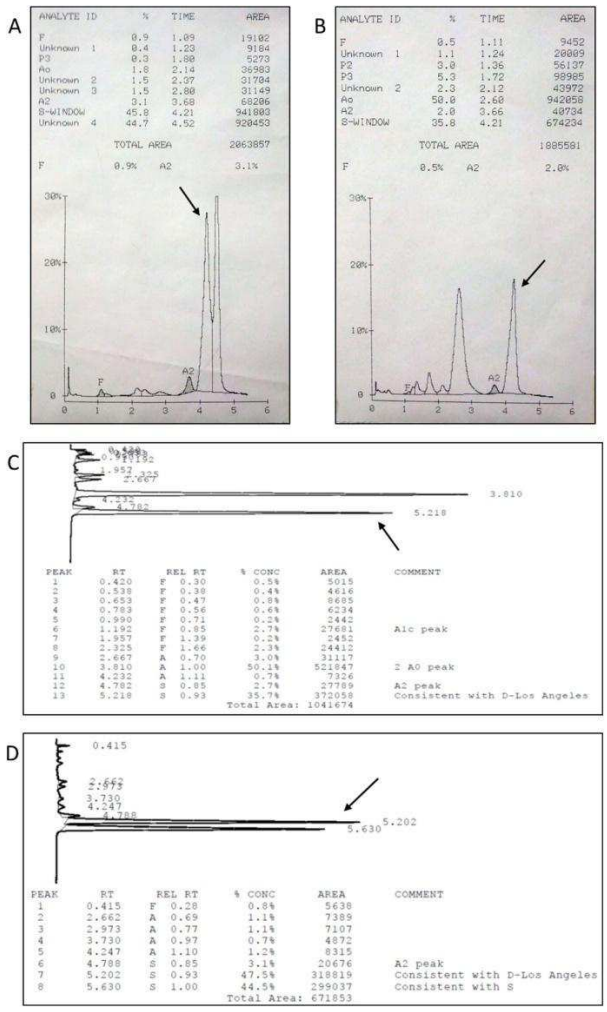

Figure 4. Chromatographic profile of a heterozygous trait for Hb D-Punjab (B and C) and a double heterozygous Hb SD-Los Angeles A and D). A-B) The VARIANT I system chromatogram with heterozygous beta-thalassemia analysis kit. The arrow indicates the pike corresponding to Hb D-Los Angeles with average retention time of 4.1 to 4.3 minutes. It is possible note decreasing in Hb A_2 values, a characteristic of samples with this hemoglobin variant. C-D) The ultra^2 Resolution system chromatogram with Genesys analysis kit. The arrow indicates the Hb D-Punjab pike, with retention time between 0.92 and 0.96 minutes related to Hb S (images of the authors).

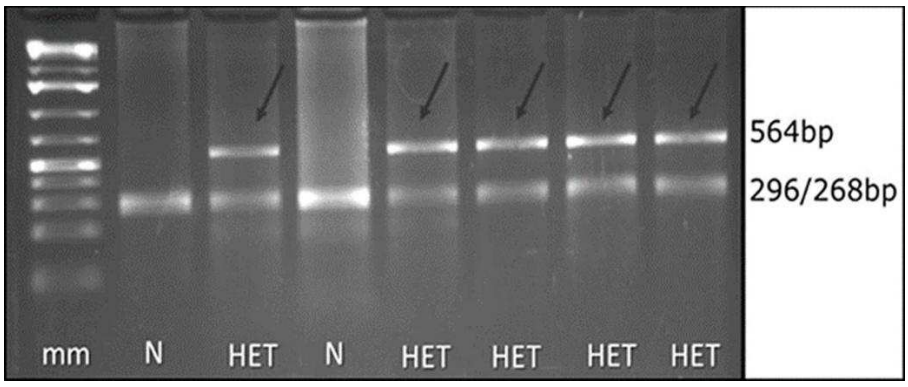

Figure 5. Agarose gel visualization of fragments generated by the PCR-RFLP technique for screening of β121 Glu→Gln (GAA→CAA) mutation. Normal alleles generate two fragments and mutant alleles maintain the 564bp fragment, indicated by the arrows. N = absent mutation; HET = heterozygous for Hb D-Los Angeles; mm = molecular marker of 100bp. Primers: Forward 5'-TGCCTCTTTGCACCATTCTA-3' and Reverse 5'-GACTCCCACATTCCCTTTT-3'. Restriction enzyme: *Eco*RI (G↓AATTC) (image of the authors).

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Severity of Brazilian sickle cell disease patients: Severity scores and feasibility of the Bayesian network model use

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ABSTRACT

The integration of the several clinical and laboratory dimensions and the influence of each parameter on the sickle cell disease (SCD)-related mortality is useful for predicting the phenotype of an individual. This study evaluated the feasibility of the SCD severity calculator use to measure disease severity in Brazilian patients. The study group was composed of 500 SCD patients (440 HbSS and 60 HbSC) diagnosed by molecular biology. We observed a decrease in severity scores in 72 SCD patients assessed before and after the hydroxyurea (HU) use. Furthermore, the HU influenced the increase of mean corpuscular volume (MCV) and HbF concentration, and the decrease of leukocytes and total bilirubin. We found 180 (36.0%) patients with intermediate phenotype, 170 (34.0%) mild phenotype and 150 (30.0%) with severe phenotype. Patients with ages >40 years had higher mean score (0.778 ± 0.177) than patients between 18 and 40 years (0.562 ± 0.152) and patients between 5 and 17 years (0.322 ± 0.145). We observe that there is a tendency of individuals with leg ulcers, avascular necrosis and cardiac complications with increasing age. Correlation analysis showed relations between severity scores with leukocytes, reticulocytes, bilirubin, lactate dehydrogenase, HbS, hemoglobin and hematocrit ($p < 0.05$). Several comparisons involving age groups, SCD genotype and phenotypic classification had satisfactory results and this classification will be used for future studies involving genetic polymorphisms, response to treatment with HU and oxidative stress markers in SCD.

1. Introduction

Sickle cell disease (SCD) is characterized by a very heterogeneous clinical course between patients with the same mutations for sickle hemoglobin (HbS), ranging from patients who have normal life expectancy with relatively few complications. Others can have severe complications such as pulmonary hypertension, priapism, stroke, leg ulceration, recurrent painful episodes, acute chest syndrome (ACS) and avascular necrosis of bone (AVN) [1].

The molecular basis for HbS formation is known, but only the mutation (HBB, glu6val, rs334) is not sufficient to explain the heterogeneous phenotype found in SCD patients, other factors such as HbF levels [2–7], α-thalassemia co-inheritance [8–13], genetic polymorphisms [14–21], hydroxyurea use [22–26] and environmental factors [27] have been identified as modulators of SCD.

Even with improved knowledge of the human genome, development of new genomic tools and identification of single nucleotide polymorphisms (SNPs) associated with sub-phenotypes of SCD by genome-wide association studies (GWAS) [28], there is still a major challenge to combine all these variables and establish potential predictors of the SCD severity. Thus, the knowledge of these determinants may help to unravel the pathophysiological mechanisms underlying the disease process, to identify novel targets for therapeutic interventions, and to enhance patient care [29].

On the other hand, a number of SCD severity classifications have been proposed, aiming at the integration of many clinical and laboratory dimensions and the influence of each parameter on disease-related mortality [29–33]. Therefore, a meaningful single synthetic measure of morbidity and/or risk of death within a given period is clinically useful to understand the relationships among clinical and laboratory measures of disease expression and to identify genetic variants that impact the disease severity [34].

An impediment to this objective has been the inability to integrate the many clinical and laboratory dimensions of the disease into a single measure of disease severity using traditional statistical methods. Furthermore, many results of hematological and biochemical tests are

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usually found outside of normal values hindering the prediction of disease severity.

Sebastiani et al. [35] developed a predictive model of disease severity using a Bayesian network modeling approach in which the 5-year mortality risk was projected as a disease severity score and can be used in other studies involving clinical and laboratory variables. However, the data on the patients were derived from United States residents and the application of this tool in individuals from others populations like in Brazil should be tested.

In this study, we aimed to evaluate the feasibility of the calculator use for disease severity (http://www.bu.edu/sicklecell/downloads/Projects) and to classify the Brazilian SCD patients according to the range of scores established.

2. Methods

2.1. Study design, patients and data collection

An observational study involves 500 SCD patients (237 males and 279 females with a mean age of 25.1 ranging between 5 and 65 years old) receiving medical care at State Institute of Hematology "Arthur de Siqueira Cavalcanti", Rio de Janeiro (HEMORIO). Eligible patients were 5 years old or older at the beginning of the study. Patients were excluded if they were smokers, consumers of alcoholic drinks, pregnant, or if they had a stroke, pain, or a hemolytic crisis in the 4 weeks prior to the date of collection of blood sample for routine laboratory tests. From this date of collection made a retrospective assessment to obtain the clinical events, hydroxyurea (HU) treatment and regular blood transfusions for the severity score calculation. We considered the patients who were using hydroxyurea over 90 days. All patients were under follow-up according to Clinical Protocols and Therapeutic Guidelines for SCD patients of Brazil [36,37].

A group of 72 SCD patients (mean age of 36.4 years old) randomized and adherent to HU treatment from HEMORIO, were studied to assess the HU effect on the severity scores. The severity score was calculated in patients without the HU use and after a mean of 105 days with HU use, the scores were recalculated. The eligible and exclusion criteria, as well as, therapeutic guidelines, followed the same principles mentioned previously.

2.2. Hemoglobin phenotypes and genotypes

Hemoglobin was identified using electrophoresis on cellulose acetate at pH 8.6, and using agar electrophoresis at pH 6.2. Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) with the automated VARIANT^{TM} equipment (Bio-Rad Laboratories, CA, USA). In all samples, the Hb genotype was developed through molecular analysis using PCR–RFLP and PCR–EA [38].

2.3. Sickle cell disease severity calculator

We used the tool "Sickle Cell Disease Severity Calculator", available from http://www.bu.edu/sicklecell/downloads/Projects, for calculation of severity scores and classification of patients into categories by phenotype (mild, intermediate, severe). This tool was developed through a Bayesian network modeling using 25 clinical and laboratory variables to estimate the severity of SCD in a study involving 3380 patients followed in the Cooperative Study of Sickle Cell Disease (CSSCD). The network model calculates the death risk within 5 years and considers this risk as a disease severity score, which ranges from 0 (least severe) to 1 (most severe). The predictive value (ie, accuracy of forecasting death based on a clinical and laboratory profile) of the model was validated in two unrelated sets of patients and showed high specificity and sensitivity [35].

The calculator requires the following variables that are sufficient to compute the score for severity of disease: age, acute chest syndrome (ACS), serum total bilirubin, blood transfusion, lactate dehydrogenase (LDH), mean corpuscular volume (MCV), pain crises, priapism, reticulocyte count, gender, stroke, total leukocyte (WBC), genotype of SCD and avascular necrosis of bone (AVN). Bilirubin, LDH, MCV, reticulocyte, and WBC levels were obtained from the collection date of samples for routine laboratory tests. Blood transfusion was assigned to patients who have regular blood transfusion for more than 1 year before the start of the study. Priapism and ACS were considered as variables present in patients with these frequent events near collection date of blood samples. Painful crises were considered present in patients who had more than 3 episode crises per person per year in the last year before the date of sample collection.

Two parameters required by the calculator "Sepsis" and "systolic blood pressure" could not be used for this study because they were not available in the routine medical records, in Brazil. Even in the absence of any variable (clinical or laboratory) the calculator allows the researcher to enter "non available" for the missing variable. Thus, the calculator fixes a reference value and the changes in the risk for death changes according to the other variables filled.

2.4. Statistical analysis

Statistical analysis was performed using the Statistica 11.0 and Graphpad Prism 5.0 softwares. Data are expressed as mean ± standard deviation and were tested for normal distribution using the Lilliefors test. Data were also tested for homogeneity of variances using Levene's test. Means between the groups were compared by applying the t-test or one-way ANOVA, followed by the post hoc Tukey's test for parametric data, and either the Mann–Whitney test or Kruskal–Wallis test, followed by the post hoc Dunn's test for non-parametric data. To assess association degree between the studied variables, we performed Pearson's correlation for parametric data and Spearman's correlation for non-parametric data. Categorical data were compared by Binomial test for proportion analysis, and Pearson Chi-square test supplemented by Fisher's exact test, for association analysis. A p value of <0.05 was considered statistically significant.

3. Results and discussion

This is the first Brazilian study involving SCD patients that assesses the feasibility of "sickle cell disease severity calculator" to calculate the severity scores of disease and classify patients into phenotypic categories. In this regard, we conducted several comparisons involving age groups, SCD genotype, HU use and phenotype classification.

We separated the patients into three age groups according to calculator (age below 18 years, age between 18 and 40 years, age above 40 years) and evaluated the HU influences on the disease severity, hematological and biochemical data between patients using and not using HU (Table 1). The HU influenced in the increase in mean corpuscular volume (MCV) and HbF concentration. Furthermore, we observed a decrease of leukocytes amount and of total bilirubin levels in the HU-sample groups. The beneficial effects of HU as increased HbF synthesis, increased hemoglobin and VCM, and decreased hemolysis markers and number of leukocytes have been characterized in the literature [25,39–41].

Severity scores in three age groups were not statistically different between patients using and not using HU. This difference was not found because the calculation of the scores was a punctual assessment and we do not calculate the baseline score severity in patients using HU. In this sense, when we evaluated 72 patients before and after the use of HU, we checked the HU influence on various laboratory and clinical data, as shown in Table 2. As expected, we observed an increased HbF synthesis, increased Hb, MCV and hematocrit values, furthermore, decreased of leukocyte number, and decreased reticulocyte and bilirubin levels.

Table 1
Demographics and clinical data and laboratory values in SCD patients separated into age groups with and without hydroxyurea use.

Characteristics	5–17 years		p value	18–40 years		p value	>40 years		p value
	−HU (n = 108)	+HU (n = 82)		−HU (n = 143)	+HU (n = 79)		−HU (n = 43)	+HU (n = 45)	
Hb genotypes (HbSS/HbSC)	89/19	79/03	–	125/17	74/5	–	34/09	39/06	–
Gender (female/male)	48/60	34/48	–	87/56	42/37	–	33/10	29/16	–
Hemoglobin (g/dL)	9.5 ± 6.4	8.6 ± 1.4	0.15	8.8 ± 1.8	9.0 ± 1.7	0.24	8.5 ± 1.9	8.7 ± 1.9	0.7
Hematocrit (%)	25.9 ± 7.8	24.7 ± 4.4	0.38	25.1 ± 5.5	25.7 ± 5.1	0.31	24.5 ± 6.2	24.4 ± 5.8	0.95
MCV (fL)	84.1 ± 9.0	94.0 ± 9.9	<0.001	87.8 ± 8.2	97.4 ± 12.2	<0.001	87.6 ± 9.1	98.1 ± 22.9	<0.001
Leucocytes (/mm^3)	10.7 ± 3.3	9.9 ± 3.2	0.04	10.8 ± 3.4	9.9 ± 5.0	<0.001	10.9 ± 4.4	8.2 ± 3.4	0.03
Reticulocyte (k/uL)	248.2 ± 112.5	240.2 ± 91.4	0.36	252.7 ± 99.9	229.0 ± 93.8	0.09	200.3 ± 102.9	177.2 ± 79.2	0.32
LDH (U/L)	1081.9 ± 567.3	953.0 ± 343.5	0.12	940.3 ± 482.6	873.3 ± 416.5	0.31	927.7 ± 519.1	750.1 ± 79.2	0.12
Total bilirubin (mg/dL)	3.4 ± 2.2	2.7 ± 1.9	0.01	3.7 ± 2.6	2.8 ± 2.2	0.01	3.1 ± 2.2	2.2 ± 1.4	0.03
ALT (U/L)	23.1 ± 15.3	22.6 ± 13.5	0.58	30.4 ± 29.6	33.6 ± 25.8	0.93	25.2 ± 14.3	26.1 ± 16.3	0.98
AST (U/L)	54.1 ± 22.1	52.9 ± 22.1	0.66	54.7 ± 28.4	56.9 ± 46.0	0.47	50.6 ± 21.2	52.9 ± 30.1	0.65
Creatinine level (mg/dL)	0.6 ± 0.1	0.6 ± 0.1	0.92	0.7 ± 0.2	0.8 ± 0.2	0.12	1.2 ± 1.1	1.1 ± 0.5	0.18
HbF (%)	5.6 ± 4.8	9.9 ± 5.5	<0.001	4.8 ± 4.6	8.8 ± 7.1	<0.001	5.7 ± 4.8	11.7 ± 9.3	<0.001
Blood transfusion [n (%)]	39 (36.1%)	45 (54.9%)	0.009	53 (37.1%)	47 (59.5%)	0.013	14 (32.5%)	21 (47.0%)	0.17
Number of sickle cell crises per person per year in the last year [n (%)]									
0–2	81 (75.0%)	50 (61.0%)	0.07	101 (70.6%)	33 (41.8)	<0.001	25 (58.1%)	29 (64.4%)	0.38
3–5	24 (22.2%)	22 (26.8%)	0.62	29 (20.3%)	30 (38.0%)	<0.001	13 (30.2%)	11 (24.4%)	0.63
≥6	03 (2.8%)	10 (12.2%)	0.01	13 (9.1%)	16 (20.2%)	0.02	05 (11.6%)	05 (11.1%)	0.52
Complications of sickle cell disease [n (%)]									
Stroke	22 (20.4%)	16 (19.5%)	0.71	22 (15.4%)	12 (15.2%)	0.92	07 (16.3%)	04 (8.9%)	0.29
Leg ulcers	04 (3.7%)	01 (1.2%)	0.26	30 (21.0%)	22 (27.8%)	0.28	19 (44.2%)	25 (55.5%)	0.22
Avascular necrosis	02 (1.9%)	01 (1.2%)	0.68	11 (7.7%)	08 (10.1%)	0.56	12 (27.3%)	11 (24.4%)	0.83
Acute chest syndrome	47 (43.5%)	58 (70.3%)	<0.001	81 (56.6%)	61 (77.2%)	<0.001	18 (41.0%)	21 (46.7%)	0.51
Cardiac complications	02 (1.9%)	03 (3.6%)	0.48	15 (10.5%)	10 (12.6%)	0.66	12 (27.3%)	12 (27.1%)	0.99
Priapism	04 (7.0%)	10 (20.8%)	0.03	12 (21.4%)	14 (37.8%)	0.04	02 (20.0%)	03 (18.7%)	0.64
Mean severity score	0.320 ± 0.147	0.324 ± 0.144	0.85	0.549 ± 0.145	0.585 ± 0.164	0.09	0.795 ± 0.194	0.762 ± 0.161	0.38

MCV (mean corpuscular volume); LDH (lactate dehydrogenase); ALT (alanine aminotransferase); AST (aspartate aminotransferase); −HU (patients without hydroxyurea use); +HU (patients hydroxyurea use). Cardiac complication (congestive heart failure, cardiomegaly, cardiomyopathy and myocardial infarction).
We considered the patients who were using hydroxyurea over 90 days. Quantitative data comparisons were made by Mann–Whitney test (non-parametric data) and t-test (parametric data). Categorical data comparisons were made by Binomial test.

Significant decrease in severity scores ($p = 0.02$) in patients using HU was possible because there was an improvement of laboratory indices, as well as in the reduction of patients with painful crises and other clinical events (even not statistically significant). The small decrease in scores on the HU influence may be due to association strength in that each variable represents in the network [35]. For example, the variables painful crises and MCV has a low strength of association (odds ratio (OR) of 1.98 and 1.61, respectively) when compared with the stroke that has a higher OR of 3.8. Precisely this variable (stroke) may have influenced the overall mean scores of patients using HU because there was an increase of three patients in this group.

Table 2
Clinical data and laboratory values in SCD patients before and after the hydroxyurea use.

Characteristics	Before HU use	After HU use	P value
Hemoglobin (g/dL)	8.2 ± 1.8	8.8 ± 1.7	0.0017
Hematocrit (%)	23.4 ± 5.6	24.9 ± 5.2	0.0023
MCV (fL)	91.2 ± 13.7	99.6 ± 17.7	0.0008
HbF (%)	6.5 ± 5.6	12.2 ± 7.7	<0.0001
Leucocytes (/mm^3)	10.7 ± 4.0	9.1 ± 4.6	0.0088
Reticulocyte count (%)	12.2 ± 5.2	8.4 ± 4.1	<0.0001
LDH (U/L)	768.2 ± 505.6	746.5 ± 335.3	0.6909
Total bilirubin (mg/dL)	3.5 ± 2.6	2.6 ± 1.9	<0.0001
Complications of sickle cell disease [n (%)]			
Blood transfusion	32 (44.4%)	27 (37.5%)	0.3969
Painful crises (more than 3 episodes in last year)	25 (34.7%)	13 (18.1%)	0.0233
Stroke	7 (9.7%)	10 (13.9%)	0.4385
Priapism	12 (44.4%)	10 (37.0%)	0.6432
Mean severity score	0.641 ± 0.194	0.601 ± 0.223	0.0281
Classes of phenotype [n (%)]			
Mild	07 (9.7%)	16 (20.8%)	0.0406
Intermediate	30 (41.7%)	31 (43.1%)	0.7364
Severe	35 (48.6%)	25 (34.7%)	0.0910

MCV (mean corpuscular volume); LDH (lactate dehydrogenase); ALT (alanine aminotransferase); AST (aspartate aminotransferase). Quantitative data comparisons were made by paired t-test. Categorical data comparisons were made by Binomial test.

According to the severity scores distribution, in the age group between 5 and 17 years, 13.2% of patients had scores greater than 0.5; the age group between 18–40 years, 60.8% of patients were above 0.5 and in the age group >40 years, 90.8% of the patients had risk of death above 0.5 (Fig. 1). Furthermore, independent of HU use, patients age >40 years had the highest mean score (0.778 ± 0.177) followed by patients of age between 18 and 40 years (0.562 ± 0.152) and finally patients age between 5 and 17 years (0.322 ± 0.145) ($p < 0.0001$). These observations demonstrate that age is a factor that influences the disease severity and the calculator was accurate for the age, i.e., the risk of death increases with age and this observation has been well documented once age is related to the natural history of SCD [42–44].

The frequency of phenotypes among the age groups presented statistical difference. We observed that there is a tendency of higher number of individuals with leg ulcers, avascular necrosis and cardiac complications with increasing age. The occurrence of priapism in men and number of painful crisis were lower in subjects between 5 and 17 years compared with other age groups (Table 3). We believe that this gravity might be a consequence of two factors in older patients: one would be the management and diagnosis of the disease that were still in improvement process in Brazil in previous decades, and the other factor would be the less knowledge of the pathophysiology and treatment of the disease which had at that time [45].

Regarding to the genotype of the disease, the mean severity scores were higher in HbSS (0.501 ± 0.218) compared with HbSC (0.395 ± 0.223), ($p < 0.001$). Patients with HbSC disease are usually less severe than individuals with sickle cell anemia and HbS-β^0 thalassemia [46]. On average, individuals with HbSC disease have half the number of painful episodes than sickle cell anemia patients. Stroke is less frequent

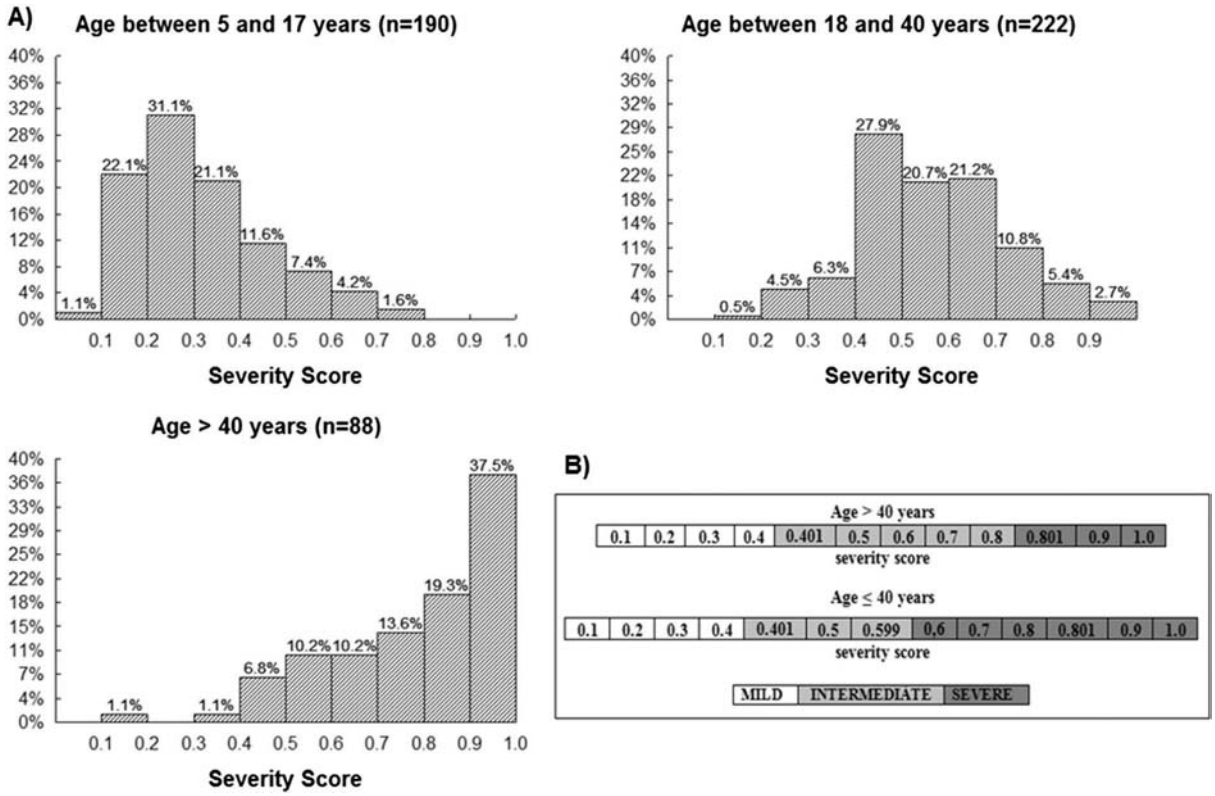

Fig. 1. Distribution of the severity score in sickle cell disease patients and score ranges for phenotypes classification. (A) The histograms show the different distribution of disease severity score in three age groups. In ascending order, the frequency of patients with more severe scores: [age > 40 years] > [age between 18 and 40 years] > [age between 5 and 17 years]. (B) Scores ranges for phenotype classification in mid, intermediate and severe.

and leg ulcers are rare in HbSC patients [30,47,48]. Thus, the calculator showed a sensitivity of severity in the aspect of the SCD genotypes.

In order to classify patients into phenotypes classes (mild, intermediate and severe), we used the score ranges established in the work Sebastiani et al. [49], and adapted for our study. The classification was based on the observation that the severity score has a U shape that changes in age groups. Therefore, the scores ≤0.4 were classified as "mild phenotype" independent of age. The score >0.8 for patients age >40 years and scores ≥0.6 for patients age ≤40 years were considered as "severe phenotype". For patients age >40 years, the score >0.8 was considered for severe phenotype to reduce the risk of misclassification. The scores that do not correspond to "mild" and "severe" phenotype were classified as "intermediate" phenotype (Fig. 1B). After determining the score ranges, we classified the SCD phenotypes and we found that 180 (36.0%) patients had intermediate phenotype, 170 (34.0%) mild phenotype and 150 (30.0%) the severe phenotype.

For the use of phenotypic classification in future analyses, we verified whether the SCD complications were associated with the phenotype class. In Table 4, we found the association of severe phenotype with stroke, leg ulcers, cardiac complications and blood transfusion. Pain crises of 3–5 per person in the last year and avascular necrosis were more frequent in intermediate and severe phenotypes when compared with the mild phenotype. The SCD severity is based on the number of painful episodes, frequency of hospital admissions, priapism, history of stroke, acute chest syndrome or sepsis, chronic leg ulcers, pulmonary hypertension, renal impairment, and avascular necrosis of

Table 3
Complications of sickle cell disease separated according to age groups.

Complications of SCD	Age groups			P value
	5–17 years (n = 190)	18–40 years (n = 222)	>40 years (n = 88)	
Stroke	38 (20.0%)	34 (15.3%)	11 (13.2%)	0.23
Leg ulcers	5 (2.6%)[a]	52 (23.4%)[b]	44 (50.0%)[c]	<0.001
Avascular necrosis	3 (1.6%)[a]	19 (8.6%)[b]	23 (26.5%)[c]	<0.001
Acute chest syndrome	106 (55.8%)	142 (63.9%)	39 (44.3%)	0.107
Cardiac complications	5 (2.6%)[a]	25 (11.3%)[b]	24 (27.3%)[c]	<0.001
Priapism [n/total male]	14/108 (12.9%)[a]	26/93 (27.9%)[b]	5/26 (19.2%)[b]	0.025
Blood transfusion	84 (44.2%)	100 (45.1%)	35 (39.8%)	0.065
*Painful crises [0–2]	82 (43.2%)[a]	134 (60.4%)[b]	54 (61.4%)[b]	<0.001
*Painful crises [3–5]	24 (12.6%)[a]	59 (26.6%)[b]	24 (27.3%)[b]	<0.001
*Painful crises [≥6]	7 (3.7%)[a]	29 (13.1)[b]	10 (11.4%)[b]	0.003

Data were expressed as number of patients with complication (percent of patients). Different letters indicate statistical difference (Pearson Chi-square test supplemented by Fisher's exact test).
* Number of sickle cell crises per person per year in the last year.

Table 4
Complications of sickle cell disease separated according to phenotypes characterized by ranges of severity scores in mild, intermediate and severe.

Complications of SCD	Phenotypes			P value
	Mild (n = 170)	Inter (n = 180)	Severe (n = 150)	
Stroke	21 (12.4%)[a]	25 (13.9%)[a]	37 (24.7%)[b]	<0.01
Leg ulcers	9 (5.3%)[a]	43 (23.9%)[b]	49 (32.7%)[c]	<0.001
Avascular necrosis	4 (2.3%)[a]	23 (12.8%)[b]	18 (12.0%)[b]	0.002
Acute chest syndrome	97 (57.1%)	94 (52.2%)	95 (63.3%)	0.067
Cardiac complications	6 (3.5%)[a]	21 (11.7%)[b]	27 (18.0%)[c]	0.006
Priapism [n/total male]	11/88 (12.5%)	23/83 (27.7%)	11/54 (20.4%)	0.081
Blood transfusion	59 (34.7%)[a]	60 (33.3%)[a]	100 (66.7%)[b]	<0.001
*Painful crises [0–2]	117 (68.8%)	119 (66.1%)	83 (55.3%)	0.241
*Painful crises [3–5]	40 (23.5%)[a]	48 (26.7%)[b]	41 (27.3%)[b]	0.001
*Painful crises [≥6]	13 (7.7%)[a]	13 (7.2)[a]	26 (17.3%)[b]	0.122

Data were expressed as number of patients with complication (percent of patients). Different letters indicate statistical difference (Pearson Chi-square test supplemented by Fisher's exact test).
* Number of sickle cell crises per person per year in the last year.

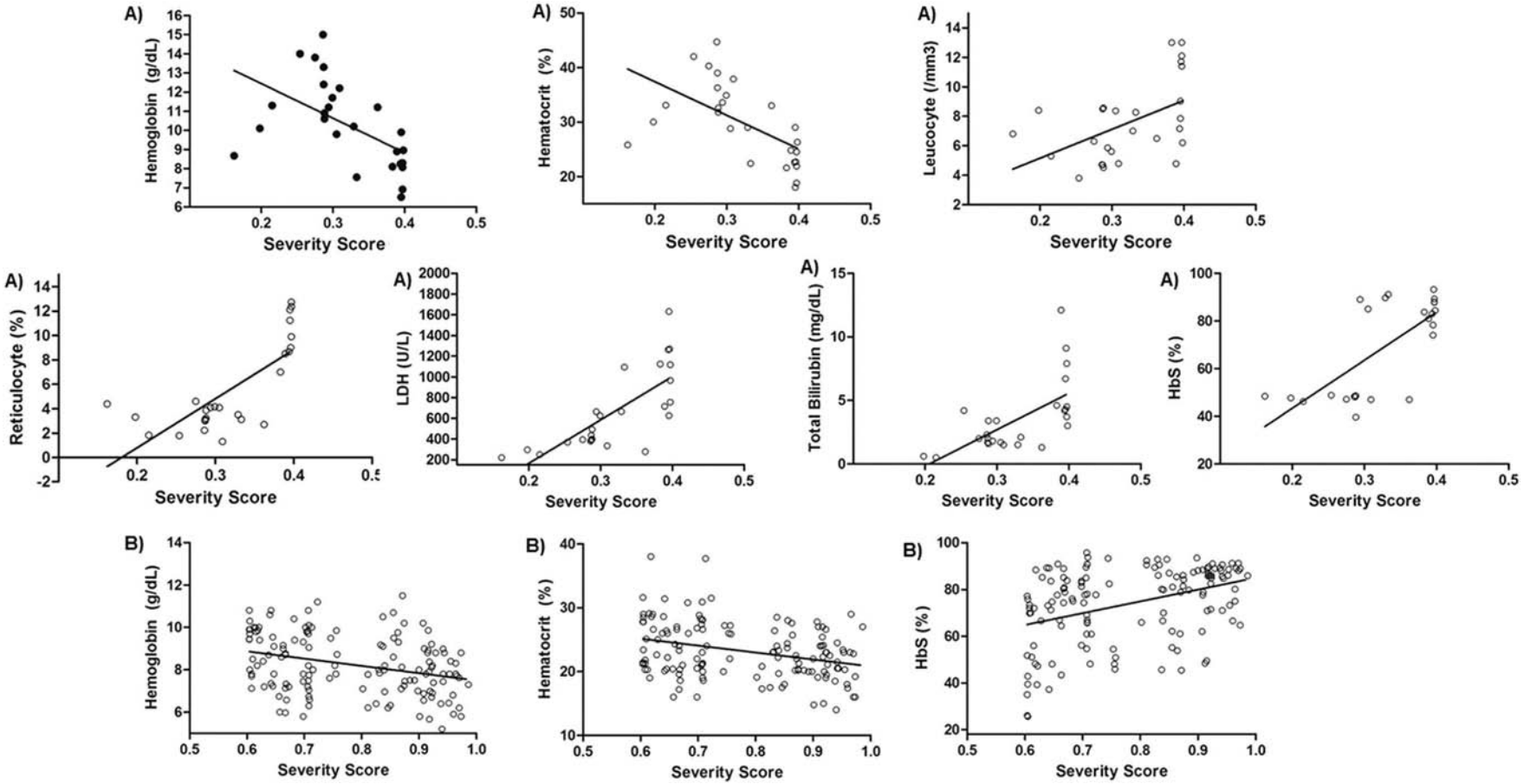

Fig. 2. Correlation analyzes using severity scores and laboratory variables. A) Correlation analysis in mild phenotype without the group 5–17 years. The degree of relation between severity score (ssco) and hemoglobin ($p < 0.001$, $r = -0.65$) and ssco and hematocrit ($p < 0.001$, $r = -0.68$) were significantly negative. The degree of relation between ssco and leucocyte ($p < 0.001$ and $r = 0.54$), ssco and reticulocyte ($p < 0.01$ and $r = 0.64$), ssco and LDH ($p < 0.001$ and $r = 0.76$) ssco and bilirubin ($p < 0.01$ and $r = -.31$) and ssco and hematocrit ($p = <0.01$ and $r = -0.32$) were significantly negative. The degree or relation between ssco and HbS ($p < 0.001$ and $r = 0.34$) was significantly positive. When hemoglobin and hematocrit values decrease the severity score values tended to increase; and when leucocyte, reticulocyte, LHD, bilirubin and HbS values increased the severity score values tended to increase.

bones [50,51]. Therefore, we demonstrated that the disease manifestations were associated with the classes of severe phenotypes.

The few studies associated with disease severity and severity scores have shown lower scores than those found in Brazilian patients. Anoop et al. [52] found in patients >18 years classified phenotypically by clinicians, mean score of 0.182 for the mild phenotype, score of 0.19 for the intermediate and mean score of 0.866 for the severe phenotype. However, these patients also had lower frequencies of individuals with priapism (18%), ACS (14%) and stroke (12%) compared to Brazilian patients >18 years that showed 26% of men with priapism, 58% of patients with ACS and 31% stroke patients. Patients >21 years from Boston Medical Centre showed the following mean severity scores: mild phenotype (0.16), intermediate phenotype (0.28) and severe (0.95) [35]. These results show that regardless of the form of phenotypes categorization, Brazilian patients have higher mean scores, as shown: mild phenotype (0.32), intermediate phenotype (0.54) and severe (0.80).

We evaluated in all patients the relationship of biomarkers with severity scores and the degree of relation between severity score (ssco) and hemoglobin ($p = 0.004$, $r = -0.20$), ssco and hematocrit ($p = 0.001$, $r = -0.21$), ssco and leukocyte ($p = 0.02$, $r = 0.10$), ssco and reticulocyte ($p = 0.009$, $r = 0.11$) were statistically significant. Since there is no validation of severity calculator in patients <18 years [35], we remove the group of 5–17 years and have made other correlation analyses. We found the relation between ssco and hemoglobin ($p < 0.001$, $r = -0.32$), ssco and hematocrit ($p < 0.001$, $r = -0.33$), ssco and leukocyte ($p = 0.03$, $r = 0.26$), ssco and reticulocyte ($p < 0.001$, $r = 0.27$), and ssco and LDH ($p < 0.001$, $r = 0.28$). These results show that when LDH, leucocyte and reticulocyte values increased, the ssco values tended to increase. On the other hand, when hemoglobin and hematocrit values decreased the ssco values tended to increase. In addition, we analyzed the degree correlation between severity scores and laboratory variables within each phenotype class (without the patients aged 5–17 years) and the results are shown in Fig. 2.

The correlation analysis allowed us to verify that the disease severity is associated with decreased hemoglobin and hematocrit. Low hemoglobin is linked generally to poor prognosis in SCD with increased risk of many specific complications [30,53–55]. In the first analysis, the correlation coefficients were not high, but when we removed the group 5–17 years, the correlation coefficients increased and other variables became significant. Furthermore, we found that the SCD severity is associated with increased of leukocytes, reticulocytes, bilirubin, LDH and HbS. All these markers are associated with the severity of the disease, e.g., increased leucocytes have been correlated with increased frequency of pain [51], increased hemorrhagic stroke risk [6] and earlier death [2]; increased LDH, bilirubin and reticulocytes are laboratory indicators of chronic hemolytic anemia [48,56,57], and the increase in HbS concentration provides the polymerization, under specific conditions [58,59].

Currently there are two models of disease severity that have been validated by other studies: one involving pediatric patients [29] and another which we are validating [35]. Thus discussion among different studies involving severity tools is limited, Coelho et al. [34] mentioned that both tools are not yet the effective tools needed for patient stratification in genotype/phenotype relationship analysis as well as in the discovery and validation of prognosis markers of the largely unpredictable SCD clinical course. Anoop et al. [52] agree that scores have high specificity and positive predictive value, but for patients >40 years start off with a high score and if data on one or more severity parameters for such patients are unavailable, the score is likely to be spuriously high.

Importantly, there are already results in the literature showing the relationship between genotype and severity score. Studies have reported the association of several SNPs (for example, rs652785 in complement component 8 gene-C8A) and some biomarkers (tumor necrosis factor-α receptor-1 and vascular cell adhesion molecule-1) with the severe phenotype classified by severity calculator [49,60]. Thereby, demonstrating the applicability of the calculator for patient clinic association with genetic and biochemical markers.

The correlation analysis and comparisons of clinical manifestations in different groups helped to evaluate the feasibility of tool. Furthermore, the group of patients <18 years deserves more attention, because the calculator does not contain clinical and laboratory variables specific to this age which may compromise the generation severity score. Another observation was in patients classified as intermediate phenotype. In this group, we have not found significant correlations as in the other two phenotypes classes (mild and severe). In this group, we found patients with very heterogeneous hematological and biochemical characteristics, which prevented the significant correlations. Maybe a subdivision of the intermediate phenotype could be done in these patients, fulfilling other criteria for classification. Moreover, the incorporation of genetic polymorphisms that participate in relevant pathological events of SCD (e.g., rapid destruction of sickle cells, dense cell formation, and adhesion to endothelium) might improve the utility of the scoring system.

For our study the severity calculator, showed high sensitivity and positive predictive value. Several comparisons involving age groups, SCD genotype, HU use and phenotype classification had satisfactory results and this classification will be used in future studies involving genetic polymorphisms, response to treatment with hydroxyurea and oxidative stress markers in SCD.

Authorship

E.B.J.: data design, data acquisition, data analysis, data interpretation, and manuscript preparation. D.G.H.S.: data interpretation and statistical analysis assistance. L.S.T.: technical and statistical analysis assistance. J.V.O.: technical and statistical analysis assistance. C.L.C.L.: study concept and design and critical review of manuscript. C.R.B.D.: study concept and design and critical review of manuscript.

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References

[1] M.J. Stuart, R.L. Nagel, Sickle-cell disease, Lancet 364 (2004) 1343–1360.
[2] O.S. Platt, D.J. Brambilla, W.F. Rosse, P.F. Milner, O. Castro, M.H. Steinberg, et al., Mortality in sickle cell disease. Life expectancy and risk factors for early death, N. Engl. J. Med. 330 (1994) 1639–1644.
[3] K. Bailey, J.S. Morris, P. Thomas, G.R. Serjeant, Fetal haemoglobin and early manifestations of homozygous sickle cell disease, Arch. Dis. Child. 67 (1992) 517–520.
[4] P.D. Fox, D.T. Dunn, J.S. Morris, G.R. Serjeant, Risk factors for proliferative sickle retinopathy, Br. J. Ophthalmol. 74 (1990) 172–176. http://dx.doi.org/10.1136/bjo.74.3.172.
[5] S. Charache, M.L. Terrin, R.D. Moore, G.J. Dover, F.B. Barton, S.V. Eckert, et al., Effect of hydroxyurea on the frequency of painful crises in sickle-cell-anemia, N. Engl. J. Med. 332 (1995) 1317–1322.
[6] K. Ohene-Frempong, S.J. Weiner, L.A. Sleeper, S.T. Miller, S. Embury, J.W. Moohr, et al., Cerebrovascular accidents in sickle cell disease: rates and risk factors, Blood 91 (1998) 288–294.
[7] G.R. Serjeant, A. Singhal, I.R. Hambleton, Sickle cell disease and age at menarche in Jamaican girls: observations from a cohort study, Arch. Dis. Child. 85 (2001) 375–378.
[8] D.R. Higgs, B.E. Aldridge, J. Lamb, J.B. Clegg, D.J. Weatherall, R.J. Hayes, et al., The interaction of alpha-thalassemia and homozygous sickle-cell disease, N. Engl. J. Med. 306 (1982) 1441–1446.
[9] K. De Ceulaer, G.R. Serjeant, Acute splenic sequestration in Jamaican adults with homozygous sickle cell disease: a role of alpha thalassaemia, Br. J. Haematol. 77 (1991) 563–564.
[10] R. Mouélé, O. Pambou, J. Feingold, F. Galactéros, α-Thalassemia in Bantu population from Congo-Brazzaville: its interaction with sickle cell anemia, Hum. Hered. 50 (2000) 118–125.

[11] L.L. Hsu, S.T. Miller, E. Wright, A. Kutlar, V. McKie, W. Wang, et al., Alpha thalassemia is associated with decreased risk of abnormal transcranial Doppler ultrasonography in children with sickle cell anemia, J. Pediatr. Hematol. Off. J. Am. Soc. Pediatr. Hematol. 25 (2003) 622–628.
[12] M.B. Mukherjee, R. Surve, A. Tamankar, R.R. Gangakhedkar, K. Ghosh, C.Y. Lu, et al., The influence of alpha-thalassaemia on the haematological & clinical expression of sickle cell disease in western India, Indian J. Med. Res. 107 (1998) 178–181.
[13] D.R. Powars, A. Hiti, E. Ramicone, C. Johnson, L. Chan, Outcome in hemoglobin SC disease: a four-decade observational study of clinical, hematologic, and genetic factors, Am. J. Hematol. 70 (2002) 206–215.
[14] C. Hoppe, W. Klitz, S. Cheng, R. Apple, L. Steiner, L. Robles, et al., Gene interactions and stroke risk in children with sickle cell anemia, Blood 103 (2004) 2391–2396.
[15] C. Hoppe, W. Klitz, K. D'Harlingue, S. Cheng, M. Grow, L. Steiner, et al., Confirmation of an association between the TNF(−308) promoter polymorphism and stroke risk in children with sickle cell anemia, Stroke 38 (2007) 2241–2246.
[16] V.G. Nolan, A. Adewoye, C. Baldwin, L. Wang, Q. Ma, D.F. Wyszynski, et al., Sickle cell leg ulcers: associations with haemolysis and SNPs in Klotho, TEK and genes of the TGF-beta/BMP pathway, Br. J. Haematol. 133 (2006) 570–578.
[17] K.J. Sullivan, S.R. Goodwin, J. Evangelist, R.D. Moore, P. Mehta, Nitric oxide successfully used to treat acute chest syndrome of sickle cell disease in a young adolescent, Crit. Care Med. 27 (1999) 2563–2568.
[18] S.A. Zimmerman, R.E. Ware, Inherited DNA mutations contributing to thrombotic complications in patients with sickle cell disease, Am. J. Hematol. 59 (1998) 267–272.
[19] K.Y. Fertrin, F.F. Costa, Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment, Expert. Rev. Hematol. 3 (2010) 443–458.
[20] K. Sharan, S. Surrey, S. Ballas, M. Borowski, M. Devoto, K.F. Wang, et al., Association of T-786C eNOS gene polymorphism with increased susceptibility to acute chest syndrome in females with sickle cell disease, Br. J. Haematol. 124 (2004) 240–243.
[21] J.G. Taylor, D.C. Tang, S.A. Savage, S.F. Leitman, S.I. Heller, G.R. Serjeant, et al., Variants in the VCAM1 gene and risk for symptomatic stroke in sickle cell disease, Blood 100 (2002) 4303–4309.
[22] S. Zimmerman, W. Schultz, J. Davis, C. Pickens, N. Mortier, T. Howard, et al., Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease, Blood 103 (2004) 2039–2045.
[23] R.E. Ware, B. Eggleston, R. Redding-Lallinger, W.C. Wang, K. Smith-Whitley, C. Daeschner, et al., Predictors of Fetal Hemoglobin Response in Children With Sickle Cell Anemia Receiving Hydroxyurea Therapy, 2002.
[24] M.H. Steinberg, F. Barton, O. Castro, C.H. Pegelow, S.K. Ballas, A. Kutlar, et al., Effect of Hydroxyurea on Mortality and Morbidity in Adult Sickle Cell Anemia: Risks and Benefits up to 9 years of Treatment, 2003.
[25] M.H. Steinberg, W.F. McCarthy, O. Castro, S.K. Ballas, F.D. Armstrong, W. Smith, et al., The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: a 17.5 year follow-up, Am. J. Hematol. 85 (2010) 403–408.
[26] S.K. Ballas, F.B. Barton, M.A. Waclawiw, P. Swerdlow, J.R. Eckman, C.H. Pegelow, et al., Hydroxyurea and sickle cell anemia: effect on quality of life, Health Qual. Life Outcomes 4 (2006) 59.
[27] M.H. Steinberg, Genetic etiologies for phenotypic diversity in sickle cell anemia, ScientificWorldJournal 9 (2009) 46–67.
[28] G. Lettre, The search for genetic modifiers of disease severity in the beta-hemoglobinopathies, Cold Spring Harb. Perspect. Med. 2 (2012).
[29] W. van den T, J.H. van der Lee, H. Heijboer, M. Peters, K. Fijnvandraat, Development and validation of a pediatric severity index for sickle cell patients, Am. J. Hematol. 85 (2010) 746–751.
[30] B.F. Cameron, E. Christian, J.S. Lobel, M.H. Gaston, Evaluation of clinical severity in sickle cell disease, J. Natl. Med. Assoc. 75 (1983) 483–487.
[31] P. Joly, C. Pondarré, C. Bardel, A. Francina, C. Martin, The alpha-globin genotype does not influence sickle cell disease severity in a retrospective cross-validation study of the pediatric severity score, Eur. J. Haematol. 88 (2012) 61–67.
[32] S.R. Pearson, A. Alkon, M. Treadwell, B. Wolff, K. Quirolo, W.T. Boyce, Autonomic reactivity and clinical severity in children with sickle cell disease, Clin. Auton. Res. 15 (2005) 400–407.
[33] E. Solomou, P. Kraniotis, A. Kourakli, T. Petsas, Extent of silent cerebral infarcts in adult sickle-cell disease patients on magnetic resonance imaging: is there a correlation with the clinical severity of disease? Neuroradiol. J. 5 (2013) 8–12.
[34] A. Coelho, A. Dias, A. Morais, B. Nunes, P. Faustino, J. Lavinha, Sickle cell disease severity scoring: a yet unsolved problem, Eur. J. Haematol. 89 (2012) 501–502.
[35] P. Sebastiani, V.G. Nolan, C.T. Baldwin, M.M. bad-Grau, L. Wang, A.H. Adewoye, et al., A network model to predict the risk of death in sickle cell disease, Blood 110 (2007) 2727–2735.
[36] BRAZIL, Ministry of Health, Department of Health Care, http://bvsms.saude.gov.br/bvs/saudelegis/sas/2010/prt0055_29_01_2010.html2010.
[37] BRAZIL, Ministry of Health, Department of Health Care, http://bvsms.saude.gov.br/bvs/saudelegis/sas/2013/prt0473_26_04_2013.html2013.
[38] J.E. Belini, R.D. Cancado, C.R. Domingos, The XmnI polymorphic site 5′ to the gene G(gamma) in a Brazilian patient with sickle cell anaemia — fetal haemoglobin concentration, haematology and clinical features, Arch. Med. Sci. 6 (2010) 822–825.
[39] C. Halsey, I.A.G. Roberts, The role of hydroxyurea in sickle cell disease, Br. J. Haematol. 120 (2003) 177–186.
[40] C. Lopes de Castro Lobo, J.F.C. Pinto, E.M. Nascimento, P.G. Moura, G.P. Cardoso, J.S. Hankins, The effect of hydroxcarbamide therapy on survival of children with sickle cell disease, Br. J. Haematol. 161 (2013) 852–860.
[41] M. Nouraie, J.S. Lee, Y. Zhang, T. Kanias, X. Zhao, Z. Xiong, et al., The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe, Haematologica 98 (2013) 464–472.
[42] P.S. Frenette, G.F. Atweh, Sickle cell disease: old discoveries, new concepts, and future promise, J. Clin. Invest. 117 (2007) 850–858.
[43] G.R. Serjeant, Natural history and determinants of clinical severity of sickle cell disease, Curr. Opin. Hematol. 2 (1995) 103–108.
[44] G.R. Serjeant, The natural history of sickle cell disease, Cold Spring Harb. Perspect. Med. 3 (2013) a011783.
[45] G.R. Serjeant, Screening for sickle-cell disease in Brazil, Lancet 356 (2000) 168–169.
[46] D.C. Rees, T.N. Williams, M.T. Gladwin, Sickle-cell disease, Lancet 376 (2010) 2018–2031.
[47] M. Koshy, R. Entsuah, A. Koranda, A.P. Kraus, R. Johnson, R. Bellvue, et al., Leg ulcers in patients with sickle cell disease, Blood 74 (1989) 1403–1408.
[48] M.H. Steinberg, Sickle cell anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches, ScientificWorldJournal 8 (2008) 1295–1324.
[49] P. Sebastiani, N. Solovieff, S.W. Hartley, J.N. Milton, A. Riva, D.A. Dworkis, et al., Genetic modifiers of the severity of sickle cell anemia identified through a genome-wide association study, Am. J. Hematol. 85 (2010) 29–35.
[50] M.C. Walters, M. Patience, W. Leisenring, J.R. Eckman, J.P. Scott, W.C. Mentzer, et al., Bone Marrow Transplantation for Sickle Cell Disease, 1996.
[51] O.S. Platt, B.D. Thorington, D.J. Brambilla, P.F. Milner, W.F. Rosse, E. Vichinsky, et al., Pain in sickle cell disease. Rates and risk factors, N. Engl. J. Med. 325 (1991) 11–16.
[52] P. Anoop, D.H. Bevan, S. Chakrabarti, Usefulness and limitations of Bayesian network model as a mortality risk assessment tool in sickle cell anemia, Am. J. Hematol. 84 (2009) 312–313.
[53] G.J. Kato, V. McGowan, R.F. Machado, J.A. Little, J. Taylor VI, C.R. Morris, et al., Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease, Blood 107 (2006) 2279–2285.
[54] S.K. Ballas, S. Lieff, L.J. Benjamin, C.D. Dampier, M.M. Heeney, C. Hoppe, et al., Definitions of the phenotypic manifestations of sickle cell disease, Am. J. Hematol. 85 (2010) 6–13.
[55] S.K. Ballas, 7 sickle cell disease: clinical management, Baillieres Clin. Haematol. 11 (1998) 185–214.
[56] M.S. West, D. Wethers, J. Smith, M. Steinberg, Laboratory profile of sickle cell disease: a cross-sectional analysis. The cooperative study of sickle cell disease, J. Clin. Epidemiol. 45 (1992) 893–909.
[57] S.K. Ballas, Lactate dehydrogenase and hemolysis in sickle cell disease, Blood 121 (2013) 243–244.
[58] P.G. Vekilov, Sickle-cell haemoglobin polymerization: is it the primary pathogenic event of sickle-cell anaemia? Br. J. Haematol. 139 (2007) 173–184.
[59] G.J. Kato, R.P. Hebbel, M.H. Steinberg, M.T. Gladwin, Vasculopathy in sickle cell disease: biology, pathophysiology, genetics, translational medicine, and new research directions, Am. J. Hematol. 84 (2009) 618–625.
[60] D.A. Dworkis, E.S. Klings, N. Solovieff, G. Li, J.N. Milton, S.W. Hartley, et al., Severe sickle cell anemia is associated with increased plasma levels of TNF-R1 and VCAM-1, Am. J. Hematol. 86 (2011) 220–223. http://dx.doi.org/10.1002/ajh.21928.