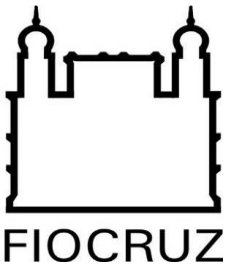

FUNDAÇÃO OSWALDO CRUZ

INSTITUTO GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

EFEITOS DA *N*(ÉPSILON)-(CARBOXIMETIL)LISINA (CML), UM PRODUTO FINAL DE GLICAÇÃO AVANÇADA (PFGA), NA DOENÇA FALCIFORME

UCHE SAMUEL NDIDI

Salvador - Bahia

2018

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Investigativa

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Orientadora: Profa. Dra. Marilda de Souza Gonçalves

Co-Orientador: Profa. Dra. Cynara Gomes Barbosa

Tese apresentada ao curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa para a obtenção do grau de Doutor.

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A minha adorável esposa *Charity Unekwuojo Ndidi*, minha filha, a doce *Chioma Grace Uche-Ndidi* e meu filho amado *Chibuzo Ebenezer Uche-Ndidi*

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NDIDI, Uche Samuel. Efeitos da *N*(épsilon)-(carboximetil)lisina (CML), um produto final de glicação avançada (PFGA), na doença falciforme. 163 f. il. Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) – Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, 2018.

RESUMO

INTRODUÇÃO: A doença falciforme (DF) caracteriza-se pela presença da hemoglobina S, que pode estar em homozigose (HbSS), como na anemia falciforme (AF), bem como em associação com outras hemoglobinas variantes ou talassemias, como na doença SC (HbSC). A HbS forma agregados em estado de desoxigenação, com consequente rigidez eritrocitária, fatores que contribuem para alterações na microvasculatura, resultando em vaso-oclusão, isquemia tecidual, lesão de órgãos, eventos dolorosos e comprometimento crônico de vários orgãos e sistemas. A gravidade da vaso-oclusão e a progressão para isquemia do órgão terminal são potencializadas pelo estresse oxidativo, sendo que os produtos finais de glicação avançada (PFGAs), tais como *N*(épsilon)carboximetilisina (CML), constituem marcadores desse processo de oxidação. - **OBJETIVO**: O objetivo geral do presente estudo foi determinar os níveis de CMLs no soro de pacientes com DF (HbSS e HbSC), bem como associar essas concentrações a biomarcadores laboratoriais e genéticos, bem como ao tratamento com a hidroxiuréia (HU). **MATERIAIS E MÉTODOS**: Os testes hematológicos foram realizados em contador de células automatizado; os marcadores bioquímicos foram mensurados no soro por ensaio de imunoquímica. Os perfiis de hemoglobina foram confirmados por cromatografia líquida de alta eficiência (HPLC). Os haplótipos associados ao grupo de genes da globina beta S (*HBB* S) e genes da globina beta C (*HBB* C) foram investigados por reação em cadeia da polimerase, seguida da análise de polimorfismos de comprimento de fragmentos de restrição (RFLP). Os níveis séricos de CML foram avaliados por ELISA competitivo. As análises estatísticas foram realizadas utilizando-se os softwares SPSS e GraphPad, considerando $p \leq 0,05$ como estatisticamente significante. **RESULTADOS**: O presente estudo demonstra a existência de características laboratoriais distintas nos indivíduos com AF em tratamento com HU em comparação aos sem tratamento. Em resumo, o estudo demonstrou que o tratamento com HU reduziu a anemia hemolítica aguda e as crises vaso-oclusivas. Em relação aos níveis de CML, foi demonstrado que os indivíduos com AF apresentaram concentrações mais elevadas no soro, em comparação aos controles saudáveis (HbAA). Foi identificada a correlação significativa entre alanina aminotransferase (ALT) e CML. Demonstrou ainda que a CML está associada ao haplótipo β^S em pacientes com AF e evidenciou que os níveis de CML estiveram mais elevados nos pacientes com doença SC em comparação com os controles saudáveis e também revelou anemia hemolítica, crises de dor e hipocolesterolemia em pacientes com HbSC em comparação com o controle saudável. **CONCLUSÃO**: Nossos resultados sugerem que a HU parece influenciar a inflamação, hemólise e função hepática dos pacientes com AF. As evidências do presente estudo sugerem a possibilidade de que os CMLs tenham papel na patologia da DF, com associação aos haplótipos

da globina beta, nas complicações presentes na AF, mas que os seus níveis séricos não são influenciados pelo tratamento com HU.

Palavras-chave**:** Doença falciforme, Hidroxiureia, *N*(Épsilon)-(Carboximetil)lisina, HbS, HbSC.

NDIDI, Uche Samuel. Effects of serum level of *N*(epsilon)-(carboxymethyl) lysine (CML), an advanced glycation end products (AGEs) on sickle cell disease patients. 163 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, 2018.

ABSTRACT

INTRODUCTION: Sickle cell disease (SCD) is characterized by the presence of hemoglobin S, which may be homozygous (HbSS), like in sickle cell anemia (SCA), as well as in association with other variant hemoglobins or thalassemias, such as SC (HbSC). HbS forms aggregates in a state of deoxygenation, with consequent erythrocyte rigidity, factor that contribute to changes in the microvasculature, resulting in vaso-occlusion, tissue ischemia, organ damage, pain and chronic compromise of various organs and systems. The severity of the vaso-occlusion and progression to end organ ischemia is potentiated by oxidative stress and advanced glycation end-products (AGEs) such as *N*(epsilon)-carboxymethylisine (CML) have been demonstrated to be markers of oxidative stress. **OBJECTIVE**: The general objective of the present study was to determine serum CML levels in patients with SCD (HbSS and HbSC), as well as to associate their concentrations with laboratory and genetic biomarkers, as well as with hydroxyurea (HU) treatment. **MATERIALS AND METHODS**: Hematological tests were performed in an automated cell counter; the biochemical markers were measured in the serum by immunochemistry assay. Hemoglobin profiles were confirmed by high performance liquid chromatography (HPLC). The haplotypes associated with the beta globin gene cluster (*HBB* S) and beta globin C (*HBB* C) genes were investigated by polymerase chain reaction, followed by restriction fragment length polymorphism (RFLP) analysis. Serum levels of CML were evaluated by competitive ELISA. Statistical analyzes were performed using the software SPSS and GraphPad, considering $p \leq 0.05$ as statistically significant. **RESULTS**: The present study demonstrates the existence of distinct laboratory characteristics in individuals with SCA on HU treatment compared to those without treatment. In summary, the study demonstrated that treatment with HU reduced acute hemolytic anemia and vaso-occlusive crises. Regarding CML levels, individuals with SCA presented higher serum concentrations than healthy controls (HbAA). Significant correlation between alanine aminotransferase (ALT) and CML was identified. It also showed that CML is associated with β^S haplotype in patients with SCA and that CML levels were higher in patients with SC disease compared to healthy controls. Hemolytic anemia, pain crises and hypocholesterolemia were identified in individuals with HbSC compared to healthy control. **CONCLUSION**: Our results suggest that HU plays a significant role in inflammation, hemolysis, and hepatic systems in sickle cell anemia. The evidences of the present study suggest the possibility that CMLs have a role in the pathology of SCD. It also suggests an association between CML and SCA haplotypes, and has shown that CML has a role to play in the complications of SCA that is not influenced by HU treatment.

Keywords**:** Sickle cell disease, Hydroxyurea, Advanced glycation end products, HbS, HbSC**.**

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

A1AT	alfa 1 antitripsina
AF	Anemia falciforme (HbSS)
ALT	Alanina amino transferase
AST	Aspartato amino transferase
AVC	Acidente vascular cerebral
BEN	haplótipo de tipo Benin
CAR	haplótipo de tipo Bantu (República Centro-Africana)
CEL	N^{ε}– (carboxietil)lisina
CML	N^{ε}– (carboximetil) lisina
DF	Doença falciforme
3-DG	3-desoxiglucosona
DNA	Ácido desoxirribonucleico
ERO	Espécies reativas de oxigênio
EUA	Estados Unidos da América
FIOCRUZ	Fundação Oswaldo Cruz
GO	Glioxal
GOLD	Dímero de glioxal-lisina
Hb	Hemoglobina
Hb A	Hemoglobina adulta normal
HBB	Gene beta da globina
Hb F	Hemoglobina fetal
Hb S	Hemoglobina falciforme
HU	Hidroxiureia
IGM	Instituto Gonçalo Moniz
LIGHT	Laboratório de Investigação em Genética e Hematologia Translacional

MGO	Metilglioxal
MOLD	metilglioxal-dímero de lisina
MSH	Estudo multicêntrico de hidroxiureia
NO	óxido nítrico
OMS	Organização Mundial de Saúde
pb	Pares de bases
PCR	Reação em cadeia da polimerase
PFGA	Produtos finais de glicação avançada
PSCR	Retinopatia falciforme proliferativa
RAGE	Receptor para PFGAs
SEN	haplótipo de tipo Senegal
SNP	Polimorfismo de um único nucleótido
UFBA	Universidade Federal d Bahia
β	beta

SUMÁRIO

1 INTRODUÇÃO E JUSTIFICATIVA

A doença falciforme (DF) possui prevalência mundial elevada, sendo mais comumente descrita em indivíduos cujos ancestrais viviam em regiões tropicais e subtropicais, onde a malária é ou era prevalente (KWIATKOWSKI, 2005; PIEL et al., 2010). A DF possui origem genética, com herança autossômica recessiva, sendo caraterizada pela presença do alelo beta S (β^S), proveniente da mutação de ponto (G**A**G>G**T**G) no sexto codon (17º nucleótideo) do gene da globina beta (*HBB*) localizado no cromossomo 11 (11p15.5), dando origem a hemoglobina (Hb) variante S (HbS), onde ocorre a substituição do acido glutâmico por valina na sexta posição da porção N terminal da cadeia da globina beta (WILLIAMS, 2016). Esta mutação produz um domínio hidrofóbico no tetrâmero HbS desoxigenado que resulta na ligação entre cadeias β1 e β2 de duas moléculas de hemoglobina. Esta cristalização produz um núcleo de polímero, que altera a conformação bicôncava das hemácias, cresce e enche os eritrócitos, perturbando a sua arquitetura e flexibilidade. A polimerização é reforçada por desidratação celular e condições de hipóxia, com estresse celular físico e oxidativo (REES et al., 2010). A taxa e a extensão da polimerização da HbS são proporcionais à duração da desoxigenação da hemoglobina, à concentração de HbS intracelular e à presença de hemoglobina fetal (HbF) no eritrócito (REES et al., 2010). As hemácias falcizadas têm sobrevida média reduzida (16 a 20 dias), quando comparada às hemácias normais (80 a 120 dias) (FELIX et al., 2010). Eles formam agregados em estado de desoxigenação, com consequente rigidez eritrocitária, que contribuem para alterações na microvasculatura, resultando em vaso-oclusão, isquemia tecidual, lesões em órgãos, dor, bem como risco elevado de óbito (PIEL et al., 2010).

A DF afeta diferentes órgãos e possui características de doença crônica, que aumentam proporcionalmente com a idade, bem como eventos agudos. As complicações crônicas comuns incluem dano pulmonar falciforme crônico, hipertensão pulmonar, disfunção renal, lesão óssea crônica (necrose avascular), retinopatia e ulceras em membros inferiores (HOWARD e TELFER, 2015). Os indivíduos com a DF possuem dor frequente, responsável por mais de 90% das internações hospitalares agudas e pela morbidade significativa. Outras complicações agudas que podem ocorrer, incluem síndrome torácica aguda, acidente vascular cerebral, sequestro esplênico, priapismo e aumento do risco de infecção (REES et al., 2010).

A DF afeta pessoas originárias da África subsaariana, países árabes, mediterrâneos, subcontinentes indianos, Caribe e América do Sul, bem como afro-americanos e descendentes de imigrantes dos países acima mencionados em outras partes do mundo. A taxa estimada de natalidade global é cerca de 300.000 indivíduos afetados por ano, sendo que cerca de 85% destes bebês (aproximadamente 255.000 casos/ano) nascem na África subsaariana (PIEL et al., 2013; WILLIAMS e OBARO, 2011). Sem tratamento, que raramente está disponível em países com nível socioeconômico baixo, principalmente nos países Africanos, acredita-se que a maioria das crianças nascidas com a doença morre em seus primeiros anos de vida (WILLIAMS e OBARO, 2011). Na verdade, milhões de pessoas em todo o mundo nascem e, infelizmente, morrem devido a esse transtorno sanguíneo hereditário, quando não têm acesso ao diagnóstico precoce e cuidados clínicos adequados (McGANN et al., 2017).

A anemia falciforme (AF) corresponde ao perfil de hemoglobina HbSS, a forma mais grave da DF, que representa cerca de 70% dos casos de DF em todo o mundo (REES et al., 2010). A AF é uma doença prevalente na população da Bahia, estado brasileiro localizado na região nordeste do país.

A doença HbSC, por outro lado, é uma forma menos grave de DF presente em 1 a cada 1.100 afro-americanos. A presença da mutação associada ao alelo β^C, que dá origem a HbC, leva a substituição do ácido glutâmico pela lisina na posição 6 na cadeia beta. As hemácias contêm 50% de HbS e 50% de HbC. A doença HbSC já foi considerada uma manifestação rara no Brasil antes do estabelecimento da triagem neonatal (ZAGO et al., 1983). No entanto, após 2001, o programa de triagem neonatal demonstrou a frequência elevada inesperada da doença HbSC de 1: 3.450 (BRANDELISE et al., 2004; PAIXAO et al., 2001).

Os fatores genéticos que atuam como moduladores clínicos incluem os haplótipos associados ao gene da globina beta (*HBB*), a variações nos níveis de HbF e a coexistência da alfa-talassemia (BANDEIRA et al., 2014). Os haplótipos associados ao gene da HbS, são conhecidos por influenciar a concentração de Hb F e modificar a gravidade clínica da AF através da presença de polimorfismos de um único nucleótido (SNPs) (LAURENTINO et al., 2014). Estes SNPs têm influência reguladora no gene da globina gama (FIGUEIREDO et al., 1996; RUSANOVA et al., 2010). Os haplótipos são classificados de acordo com suas etnias e região geográfica nas quais foram identificados pela primeira vez.

A fisiopatologia da DF está associada à adesão de hemácias às células endoteliais vasculares. Esta interação aumentada de hemácias falciformes com o endotélio retarda o fluxo sanguíneo, aumenta a desoxigenação da hemoglobina, a polimerização da HbS e a falcização, o que leva à vaso-oclusão (GEE e PLATT, 1995; CONNES et al., 2013; ZHANG et al., 2016). A gravidade da vaso-oclusão é potencializada pelo estresse oxidativo causado pelo aumento da produção de espécies reativas de oxigênio (ROS) e diminuição do sistema antioxidante (STEINBERG e BRUGNARA, 2003). A inflamação crônica e o estresse oxidativo local/sistêmico devido a DF podem levar ao aumento na formação e acumulação de níveis sistêmicos de produtos finais de glicação avançada (PFGAs). Os PFGAs são moléculas formadas principamente durante a reação não enzimática entre açúcares redutores e grupos amino de proteínas, lípidios e ácidos nucléicos chamados de reação de Maillard (NUR et al., 2010). Desde a sua descoberta *in vivo*, tem sido sugerido que os PFGAs podem desempenhar papel importante na fisiopatologia de várias doenças (PENG et al., 2011).

Os PFGAs começaram, recentemente, a receber atenção como fatores potenciais no efeito deletério da DF devido à sua associação com inflamação e estresse oxidativo que são características comuns na doença. Estudos anteriores realizados por Somjee e cols. (2004) e Nur e cols. (2010) foram os únicos que relataram a associação entre DF e os níveis plasmáticos de PFGAs. No entanto, enquanto Somjee e cols. (2004) realizaram o seu estudo em crianças, Nur e cols. (2010) estudaram pacientes adultos. Os resultados de Somjee e cols. (2004) sugerem que os níveis circulantes de PFGAs pode desempenhar papel na patologia vascular da AF, uma vez que os níveis plasmáticos de PFGAs e a taxa de acumulação de PFGAs nos eritrócitos foram significativamente mais elevados nos indivíduos com AF, em comparação com indivíduos controles. Nur e cols. (2010) sugerem que os PFGAs também podem estar implicadas na fisiopatologia do sub-fenótipo hemolítico de DF e associaram a existência de níveis aumentados de pentosidina e CML (tipos de PFGAs) em indivíduos com DF durante o estado estável em comparação com controles saudáveis. Há, portanto, a necessidade de se investigar mais sobre a influência das PFGAs nas complicações da DF. Para o nosso melhor conhecimento, não há nenhuma descrição sobre o efeito de PFGAs em pacientes com DF em uso de hidroxiureia HU). Além disso, até o presente momento não encontramos nenhum relato da existência de relação entre o nível de PFGAs e os vários haplótipos ligados ao grupo da globina beta na AF. A investigação referente aos níveis de PFGAs em indivíduos com DF sob o tratamento com HU

pode contribuir para a compreensão do possível mecanismo de ação desse fármaco, bem como o alvo para outras terapias.

A HU, um potente inibidor da ribonucleótideo-redutase, é a única terapia oficialmente aprovada para uso terapêutico em individuos com DF (KOVACIC, 2011). A função principal da HU está associada ao bloqueio da síntese de DNA pela inibição da ribonucleótídeo redutase, mantendo as células na fase S do ciclo celular (BANDEIRA et al., 2004). A eficácia clínica do tratamento da HU varia entre indivíduos, embora a maioria com fenótipos graves possa se beneficiar de seu uso (WARE, 2010). Estudos anteriores sugeriram que a HU é citotóxica para os precursores eritróides tardios que se dividem mais rapidamente, o que resulta no recrutamento de precursores eritróides precoces com capacidade acrescida para produzir hemoglobina fetal (HbF). O estudo de Cokic e cols (2003) aponta o mecanismo de derivados de óxido nítrico (NO) para a indução de HbF pela HU. O estudo de HO e cols (2003) sugere que a ribonucleótido redutase é responsável por este aumento na HbF. Mesmo assim, a HU pode apresentar outros mecanismos associados a geração de HbF. Dessa forma, a HU pode ser benéfica na AF, por razões não relacionadas com a produção de Hb F. Isto inclui a sua capacidade para aumentar o teor de água na hemácia, reduzir a contagem de neutrófilos, e alterar a adesão de hemácias ao endotélio. O presente estudo pode, prospectivamente, embasar o entendimento relativo ao mecanismo de ação do tratamento com HU, que poderia viabilizar ensaios clínicos para outros efeitos associados a HU, contribuindo para o desenvolvimento de futuras estratégias terapêuticas.

2 REVISÃO DE LITERATURA

2.1 DOENÇA FALCIFORME

A hemoglobina (HbS) é uma variante estrutural da hemoglobina normal do adulto (HbA) que tem característica hereditária como traço mendeliano autossômico recessivo. Enquanto indivíduos heterozigotos para o traço falciforme (HbAS) são geralmente assintomáticos, os indivíduos homozigotos sofrem complicações agudas e crônicas ao longo da vida. A denominação doença falciforme (DF) é utilizada para as situações onde ocorre a homozigose para o alelo β^S (HbSS), comumente referida como anemia falciforme (AF) ou a sua heterozigose dupla, resultado da associação do alelo β^S com alelos associados a outras Hb variantes, como a HbC descrita na doença da hemoglobina SC (HbSC) ou em associação com talassêmias, como o descrito na S talassemia beta (HbSBtal) (Tabela 1) (BENDER e DOUTHITT, 2003).

Tabela 1: Diferentes tipos de doença falciforme (DF).

Doença falciforme (DF)	Características
Doença falciformes grave	
Hb S/S (β6Glu>Val/β6Glu>Val); anemia falciforme (AF)	A forma mais comum de doença falciforme
Hb S/B^o Talassemia	Mais prevalente na região do Mediterrâneo Oriental e Índia
Talassemia grave Hb S/B^+	Mais prevalente na região do Mediterrâneo Oriental e Índia; 1-5% de HbA presente
HbS/OArab (β6Glu>Val/β121Glu>Lys)	Reportado no norte da África, no Oriente Médio e nos Balcãs; relativamente raro
HbS/D Punjab (β6Glu>Val/β121Glu>Gln)	Predominante no norte da Índia, mas ocorre em todo o mundo
HbS/C Harlem (β6Glu>Val/β6Glu>Val/β, β73Asp>Asn)	Eletroforese semelhante ao HbSC, mas clinicamente grave; Mutação dupla no gene da β-globina; muito raro
HbC/S Antilles (β6Glu>Lys/β6Glu>Val, β23Val–Ile)	A mutação dupla no gene da β-globina resulta em doença grave das células falciformes quando co-herdada com HbC; muito raro
HbbS/Quebec-CHORI (β6Glu>Val/β87Thr>Ile)	dois casos relatados; Lembra a característica falciforme com técnica analítica padrão
Doença falciformes moderada	
HbS/C (β6Glu>Val/ β6Glu>Lys)	25-30% de casos de doença falciforme em população de origem africana
HbS/β^+ Talassemia moderada	A maioria dos casos na região do Mediterrâneo Oriental; 6-15% de HbA presente
HbA/S Oman (β^+/β6Glu>Val β121Glu>Lys)	Forma dominante da doença falciforme causada por dupla mutação no gene da β-globina; muito raro
Doença falciforme leve	
HbS/ β^+ Talassemia leve	Principalmente em populações de origem africana; Presente HbA 16-30%
HbS/E (β6Glu>Val/ β26Glu>Lys)	HbE predomina no Sudeste Asiático e, portanto, HbSE é incomum, embora a freqüência está aumentando com a migração populacional
HbA/Jamaican Plain (β^+/β6Glu>Val β68LeuPhe)	Forma dominante da doença falciforme; Resultados de mutação dupla em Hb com baixa afinidade de oxigênio
Doença falciforme muito leve	
HbS/HPFH	Grupo de distúrbios causados por grandes deleções do complexo de genes de β –globina; Tipicamente 30% da hemoglobina fetal
HbS/other Hb variants	HbS é co-herdada com muitas outras variantes de Hb, e os sintomas se desenvolvem apenas em hipóxia extrema

(Adaptado de REES et al., 2010)

A HbS é muito frequente nas populações do continente africano, principalmente nas regiões equatoriais, subsaarianas, localizadas ao norte do deserto de Kalahari, e serviu como barreira natural para a expansão do *Plasmodium falciparum*, parasita transmissor da malária

falciparum (Tabela 2). A frequência dessa hemoglobina mutante chega a 25% da população de algumas regiões, como ao redor dos rios Gâmbia e Senegal e na África ocidental do litoral atlântico, como também na região ocidental centro-africana, nas regiões cortadas pelos dois rios, Benin e Níger, além do entorno do rio Congo, na África Central (Ministério da Saúde, 2015).

Tabela 2. Distribuição da doença falciforme em alguns países da África.

País	Total Nascimento	Crianças com Traço %	Crianças com Doença Falciforme
Nigéria	5.362.500	15 a 28	86.000
Congo	2.715.000	15 a 30	43.440
Angola	722.800	11 a 37	21.684
Guiné	361.200	26	8.300
Burundi	294.800	26	6.486
Gana	646.400	10 a 22	6.464
Serra Leoa	240.000	27 a 30	7.920
Burkina Fasso	585.600	2 a 28	5.900
Ruanda	360.800	20	4.693
Camarões	542.500	2 a 24	4.340
Congo Brazzaville	140.800	22	2.253
República Centro - Africana	144.400	20 a 22	2.166
Costa do Marfim	600.100	11	2.040

Fonte: Brasil. Ministério da Saúde (2015)

Acredita-se que a DF afete aproximadamente 3.500 recém-nascidos por ano no Brasil e que 200 mil nasçam com o traço falciforme (LOBO et al., 2003; PINHEIRO et al., 2006; SILVA-PINTO et al., 2013). Na região Nordeste e nos estados de São Paulo, Rio de Janeiro e Minas Gerais, observou-se que os casos novos da DF ocorrem em cada 1000 nascimentos e os portadores do traço falciforme ocorrem em cada 27 nascimentos (Figura 1) (LERVOLINO et al., 2011). O fenótipo clínico da DF é atribuído aos diferentes genótipos acima mencionados (NAGEL e STEINBERG, 2001; STEINBERG, 2001).

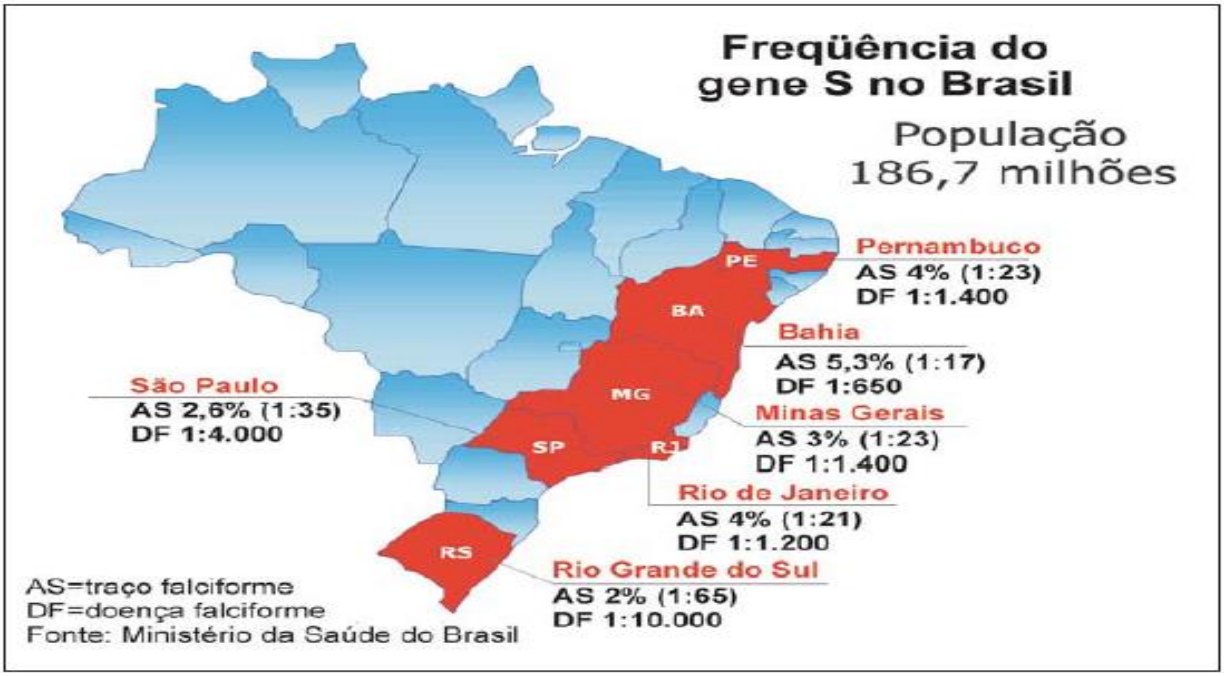

Figura 1. Frequência do alelo beta S nas diferentes regiões do Brasil.

Embora o defeito primário presente na DF seja a presença da HbS, que desencadeia a deformação das hemácias em tensões baixas de oxigênio, as manifestações clínicas da doença não se restringem ao tecido eritróide. A fisiopatologia da DF é complexa e inclui hemólise, ativação da adesão celular, inflamação crônica, leucocitose, aumento do estresse oxidativo e disfunção endotelial (STUART e NAGEL, 2004). Contudo, a principal causa de morbidade e mortalidade associada a DF é a oclusão (micro) vascular e a adesão direta de hemácias à superfície da célula endotelial, que desempenha papel importante neste processo (GEE e PLATT, 1995; SULTANA et al., 1998). Isso resulta em atraso na passagem das hemácias, com aumento concomitante de hemácias falciformes, levando a hipoxia e infarto tecidual (Figura 2) (SETTY e STUART, 1996).

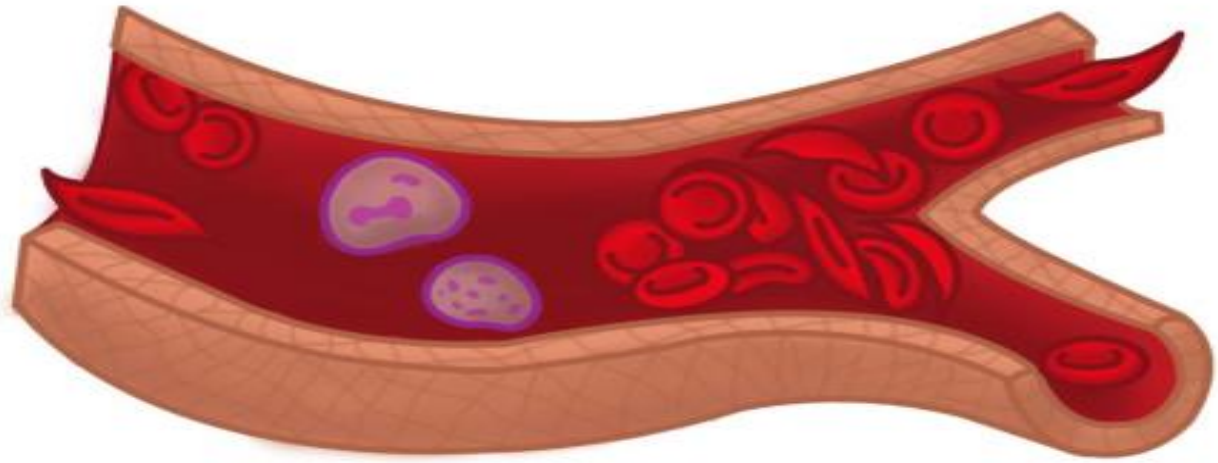

Figura 2: Representação gráfica do fenômeno vaso-oclusivo presente na doença falciforme. Adaptado da GUARDA (2015).

Embora muitos indivíduos afetados pela DF venham a óbito na infância, principalmente nos países em desenvolvimento (CHAKRAVORTY e WILLIAMS, 2015), as taxas de mortalidade em crianças em países de maior renda caíram nas últimas décadas e a maioria das crianças (mais de 95%) agora sobrevive até a idade adulta (QUINN et al., 2010; LANZKRON et al., 2013; GARDNER et al., 2016). O óbito é geralmente causado por insuficiência de órgãos devido ao processo crônico decorrente da falcização (por exemplo, insuficiência renal, hipertensão pulmonar, insuficiência hepática) ou como resultado de evento catastrófico agudo, como acidente vascular cerebral (AVC), síndrome torácica aguda (STA), sequestro esplênico, sepse ou outras complicações. Os óbitos em pediatria são geralmente decorrentes de sepse pneumocócica e hemorragia subaracnóide. Em adultos, as principais causas de morte são AVC, insuficiência multiorgãos, STA, insuficiência renal e causas independentes da DF (NCEPOD, 2008).

As melhorias na qualidade de vida e sobrevida dos pacientes em idade pediátrica estão relacionadas à introdução da triagem neonatal, acesso precoce a cuidados pediátricos abrangentes, profilaxia da penicilina, vacinação para diminuir risco de infecções potencialmente fatais e prevenção do AVC primário com triagem pelo Doppler transcraniano (DTC). Nos adultos, o tratamento baseou-se na identificação de fatores que desencadeiam a crise (incluindo desidratação, infecção e gripe) e tratamento sintomático dos episódios dolorosos agudos. A HU é

atualmente o único tratamento farmacológico licenciado para uso na DF. O uso de hemocomponentes é muitas vezes necessário para tratar complicações agudas ou a longo prazo, para tratar ou prevenir complicações da doença. O transplante de células hematopoéticas é a única opção de tratamento curativo atualmente disponível e é oferecido a crianças com fenótipo de doença grave e que possuam doador compatível com antígeno leucocitário humano (HLA). Outras opções de transplantes de doadores e transplantes de adultos estão atualmente disponíveis apenas no contexto de ensaios clínicos (NEVITT et al., 2017). A terapia genética oferece outro tratamento curativo potencial e, atualmente, está sendo investigada em ensaios clínicos (NEVITT et al., 2017).

2.1.1 Anemia Falciforme (HbSS)

A AF é a desordem hematológica hereditária mais conhecida em humanos, sendo forma homozigótica (HbSS) da DF. A HbS é decorrente da substituição de adenina por timina (G**A**G>G**T**G) no sexto códon do gene da globina beta (*HBB*) localizado no cromossomo 11 (11p15.5), o que leva a substituição do ácido glutâmico (amino ácido polar) pela valina (amino ácido não polar) na sexta posição da cadeia polipeptídica beta (SILVA-PINTO et al., 2013). A HbS é o resultado de um único polimorfismo de nucleotídeos (SNP) (A → HbA/T → HbS; rs334) no gene da globina beta (*HBB*) (WILLIAMS, 2016). O mecanismo fisiopatológico básico baseia-se na polimerização da HbS e na deformação, rigidez e fragilização que levam às principais características da doença, ou seja, anemia hemolítica e eventos vaso-oclusivos (BENKERROU et al., 2002). A vaso-oclusão resulta de interações adesivas entre os eritrócitos falciformes, leucócitos, plaquetas, proteínas solúveis no plasma e a parede vascular (BANDEIRA et al., 2014). Eventos recorrentes de vaso-oclusão, eventos hemolíticos e ativação de células endoteliais induzem a resposta inflamatória contínua na AF (STEINBERG, 2006; MAKIS et al., 2000). Os indivíduos com AF exibem estado inflamatório crônico, que reduzem a sobrevida e a qualidade de vida (CONRAN et al., 2009).

Devido à seleção evolutiva promovida pela malária, as maiores prevalências da AF são observadas em regiões tropicais (PIEL et al., 2010). Tem sido sugerido que 250.000 crianças nascem por ano com AF em todo o mundo (SILVA et al., 2006). No Brasil, a doença é mais

prevalente onde a proporção de afro-descendentes é maior (região Nordeste e os estados de São Paulo, Rio de Janeiro e Minas Gerais) (LERVOLINO et al., 2011).

A adesão das hemácias nas células endoteliais vasculares está aumentada na AF (MOORE et al., 1996). A interação das hemácias falciformes com o endotélio aumenta o tempo de trânsito, desoxigenação da hemoglobina e polimerização, bem como a falcização. Isto leva a vaso-oclusão, um passo fundamental na fisiopatologia de ambas as complicações vasculares agudas e crônicas da AF. No entanto, a AF tem curso clínico heterogêneo, com variabilidade de sintomas entre os indivíduos na mesma ou em diferentes localizações geográficas (POWARS et al., 1990). Os fatores que contribuem para as características clínicas complexas incluem a idade e sexo, bem como fatores genéticos, ambientais e hematológicos (DOVER e PLATT, 1998). Os fatores genéticos que agem como moduladores clínicos incluem haplótipos, a variação no nível de HbF e a coexistência de talassemia alfa. Entre os fatores metabólicos está o estresse oxidativo, enquanto entre os fatores hematológicos incluem os número de glóbulos brancos e de hemácias (ASLAN et al., 2000; OKPALA, 2006; BELCHER et al., 2000).

2.1.2 A doença SC (HbSC)

A DF do tipo HbSC é a segunda hemoglobinopatia mais frequente após a AF (WEATHERALL, 2010). A HbC é decorrente da substituição de guanina por adenina (**G**AG>**A**AG) no sexto códon do gene da globina beta (*HBB*) localizado no cromossomo 11 (11p15.5), o que leva a substituição do ácido glutâmico (carregado negativamente, aminoácido polar) pela lisina (carregado positivamente, aminoácido polar) na sexta posição da cadeia polipeptídica beta. A HbC é o resultado de um único polimorfismo de nucleotídeos (SNP) (G → HbA/A → HbC; rs33930165) no gene *HBB* (WILLIAMS, 2016). A HbC é menos solúvel que a Hb A na hemácia, provavelmente a partir de interações eletrostáticas entre grupos β6-lisina carregados positivamente e grupos carregados negativamente em moléculas adjacentes. A formação de cristais pode resultar no aumento da viscosidade do sangue e rigidez celular, e a redução da sobrevivência das hemácias (WILLIAMS, 2016).

É importante ressaltar que duas complicações específicas podem ocorrer com maior frequência na HbSC do que na HbSS: retinopatia vascular e necrose avascular da cabeça femoral. O genótipo HbSC é um fator de risco bem conhecido para a retinopatia falciforme proliferativa

(PSCR) (MANTOVANI e FIGINI, 2008). Uma análise longitudinal mostrou que os pacientes com HbSC apresentaram probabilidade maior de desenvolverem PSCR grave (fase III-IV) do que pacientes HbSS (LEVEZIEL et al., 2011). A necrose avascular da cabeça femoral também foi relatada com frequência aumentada na HbSC quando comparada a HbSS (PRINDLE e McCURDY, 1970).

Tanto a HbC, como HbS, conferem proteção contra a malária. Um estudo de casos e controles em Burkina Faso encontrou associação forte entre a resistência à malária clínica e a presença da variante HbC (MODIANO et al., 2001). Um estudo longitudinal utilizando análise de associação baseada em família encontrou relação forte entre HbC e proteção contra malária leve, bem como a associação negativa entre HbC e parasitemia (AGARWAL et al., 2000). O estudo realizado em 4 aldeias rurais de Burkina Faso não encontrou evidências de proteção da HbC no estado heterozigótico (odds ratio [OR] para HbAC vs HbAA, 1,49, P = 0,31), mas encontrou proteção maior do que a observada com a HbS nos estados homozigóticos e duplos heterozigóticos (OR para HbCC e HbSC vs HbAA, 0,04; P = 0,002) (MANGANO et al., 2015).

Epidemiologicamente, acredita-se que a HbC tenha se originado como um efeito fundador na África Ocidental. A frequência da HbC é tão alta quanto 40% no norte do Gana, até 50% no norte da Costa do Marfim, até 40% em Burkina Faso (onde 2% da população total tem HbSC), cerca de 20% no Togo e Benin <1% na África Central. HbC é encontrada em indivíduos de descendência africana no Caribe (3,5% de prevalência) e nos EUA (3% de prevalência). Há também incidência significativa de HbC no norte da África (1 a 10% em Marrocos e Argélia) e no sul da Europa (Itália, Turquia). A HbC também foi identificada em indivíduos sem ascendência africana conhecida (GALBRAITH e GREEN, 1960). Estima-se que 54.736 bebês nascem com HbSC a cada ano no mundo (WEATHERALL, 2010). A composição da hemoglobina em eritrócitos HbSC é 50% de HbS e 50% de HbC, em média. Embora individualmente a presença dos heterozigotos da HbS e da HbC não tenha consequência clínica, a HbSC é acompanhada por anomalias clínicas significativas. A principal razão é que HbC aumenta a formação de polímero intracelular de HbS pela desidratação dos eritrócitos (BALLAS et al., 1982; BUNN et al., 1982). Algumas pesquisas afirmam que a HbSC não é uma forma leve da AF (LIONNET et al., 2012; CABAÑAS-PEDRO et al., 2013).

Em populações de origem étnica africana, a AF é tipicamente responsável por 70% dos casos de DF, sendo que a maior parte do restante tem HbSC (NAGEL et al., 2003).

2.2 HIDROXIUREIA

Vários estudos têm sido realizados visando alternativas terapêuticas para a DF, tais como a inibição da polimerização da HbS, prevenção ou reparação da desidratação eritrocitária e a interrupção da interação de eritrócitos falciformes com o endotélio vascular (BRUGNARA e STEINBERG, 2002; TAM et al., 2005). Outros estudos abordam estratégias, como a indução de HbF usando a HU, eritropoietina, 5-azacitidina, butirato, decitabina ou seus derivados; a administração oral de clotrimazol, que é um potente inibidor do canal de Gárdos (canal de potássio independente de voltagem heterotetramérica que é ativado pelo cálcio intracelular); transfusão de hemoderivados e transplante de células hematopoiéticas também são modalidades terapêuticas utilizadas (AMEH et al., 2012; OKPUZOR et al., 2008). Alimentos ricos em tiocianato, suplementos nutricionais, extratos alimentares, fitoquímicos e compostos sintéticos têm sido testados *in vitro* e *in vivo* sobre as suas possíveis funções terapeuticas na DF (OJIAKO et al., 2012).

No entanto, a HU é atualmente o único fármaco eficaz para o tratamento de pacientes com DF, reduzindo assim a morbidade e a mortalidade dos pacientes. A HU foi sintetizada, pela primeira vez em 1869, na Alemanha por Dressler e Stein. Um século mais tarde, os estudos de fase I e II começaram a testar a sua segurança em humanos com tumores sólidos. Em 1967, a FDA aprovou-a para o tratamento de doenças neoplásicas (LATAGLIATA et al., 2012). Atualmente, é também aprovado para o tratamento do melanoma, leucemia mielocítica crônica resistente e recorrente metastática, ou carcinoma do ovário inoperável (BANDEIRA et al., 2004; KOVACIC, 2011). O primeiro estudo multicêntrico randomizado de hidroxiureia (MSH) que provou a eficácia da terapia de HU na AF, foi realizado na década de 1990, e teve grande impacto no manejo da DF (SILVA-PINTO et al., 2013). Ele mostrou que a HU pode reduzir os episódios dolorosos, o tempo de internação e o número de transfusões de hemoderivados e pode proporcionar redução de 50% na ocorrência de novos episódios de STA (STEINBERG et al., 2003).

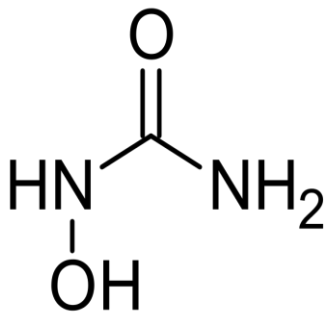

Figura 3: Estrutura de Hidroxiureia

A HU (também conhecido como hidroxicarbamida) (Figura 3) é administrada oralmente e possui como principal efeito o aumento na síntese da HbF, inibindo assim a polimerização da HbS. No entanto, vários estudos indicam que a HU também pode atuar independentemente da sua propriedade indutora de HbF visando moléculas de adesão em células sanguíneas (BARTOLUCCI et al., 2010; CARTRON e ELION, 2008; ODIÈVRE et al., 2008) e células endoteliais (LAURANCE et al., 2010; LAURANCE et al., 2011) e modulando a produção da NO (KING, 2004). Estudos têm mostrado que o aumento da HbF em pacientes com DF ajuda a reduzir o número de crises dolorosas, a hemólise e eventos vaso-oclusivos (FATHALLAH e ATWEH, 2006; STEINBERG et al., 2003). O conteúdo de HbF nos eritrócitos é um dos responsáveis pela heterogeneidade clínica observada nos pacientes com AF, sendo que estudos têm demonstrado a eficácia clínica e a segurança a curto prazo do uso de HU em crianças com AF (HANKINS et al., 2005; WANG et al., 2001; ZIMMERMAN et al., 2004). Entretanto, a resposta a HU, tem sido referida como variável. FATHALLAH e ATWEH (2006) demonstraram que cerca de um terço dos pacientes com AF não responderam ao tratamento com HU. A HU também é conhecida por diminuir a adesão de reticulócitos falciformes ao endotélio em repouso (CHAAR et al., 2014).

2.3 PRODUTOS FINAIS DE GLICAÇÃO AVANÇADA (PFGAS)

Os PFGAs foram reconhecidos pela primeira vez como compostos endógenos que se formaram em excesso em diabetes devido a hiperglicemia (BROWNLEE, 2001). No entanto, estudos confirmaram que os PFGAs podem também ser gerados em condições de aumento do estresse oxidativo, mesmo na ausência de hiperglicemia (URIBARRI et al., 2015). Além disso, evidências crescentes apontam para PFGAs exógenos (derivados principalmente de alimentos e

tabaco) como contribuintes importantes para o grupo PFGA do corpo, onde se tornam indistinguíveis de PFGAs endógenos, tanto em estrutura como em função (CAI et al., 2002).

A produção de moléculas PFGA através da via clássica leva semanas a meses e envolve muitos intermediários reversíveis que finalmente levam à formação de PFGAs irreversíveis (SCHMIDT et al., 1999). Na via clássica, o grupo de carbonilo eletrofílico, tais como glicose (ou outros açúcares redutores, como frutose, pentoses, galactose, manose, ascorbato, xilulose) reagem com um grupo amino livre de diversas moléculas, incluindo proteínas, ácidos nucleicos e lipídios, para formar um composto de aldimina instável, a base de Schiff (AHMED, 2005; GKOGKOLOU e BÖHM, 2012). Por rearranjo, esta base produz uma cetoamina estável, o produto Amadori, uma 1-amino-1-desoxicose, que sofre novas reações que levam à formação de compostos PFGA estáveis e irreversíveis (Figura 4).

Uma vez que esta reação não é enzimática, as variáveis que regulam a reação *in vivo* são as concentrações de açúcares redutores e macromoléculas, a meia-vida das macromoléculas, sua reatividade em termos de grupos amino livres e a permeabilidade dos açúcares redutores.

O produto Amadori também pode, por meio de reações de oxidação e desidratação, degradar-se em uma variedade de compostos de dicarbonilo (glioxal, metilglioxal, desoxiglicossona) que servem como propagadores para PFGAs (Figura 4). Esses propagadores reagem de novo com grupos amino livres e, por meio de reações de oxidação, desidratação e ciclização a formam PFGAs (AHMED, 2005; GKOGKOLOU e BÖHM, 2012). Os compostos de dicarbonilo (oxoaldeídos) como 3-desoxiglucosona (3-DG), metilglioxal (MGO) e glioxal (GO) podem surgir de degradação oxidativa ou autooxidação da base de Schiff, produtos Amadori, intermediários de via glicolítica, peroxidação lipídica, via de poliol e oxidação de aminoácidos (Figura 4) (SINGH et al., 2001; URIBARRI et al., 2015). Os α-dicarbonils (a-oxoaldeídos) podem ser considerados como pontos focais importantes de como a glicose pode continuar a formar PFGAs pela reação clássica de Maillard, a via de poliol e fatores in vivo, como o catabolismo de corpos de cetona e treonina, bem como peroxidação lipídica (THORNALLEY, 1998).

A via para a síntese de PFGA é catalisada por metais de transição redox ativos, oxigênio e ROS (GKOGKOLOU e BÖHM, 2012). No entanto, é inibida pela redução de compostos, tais como ácido ascórbico, superóxido dismutase (SOD), catalase e glutationa.

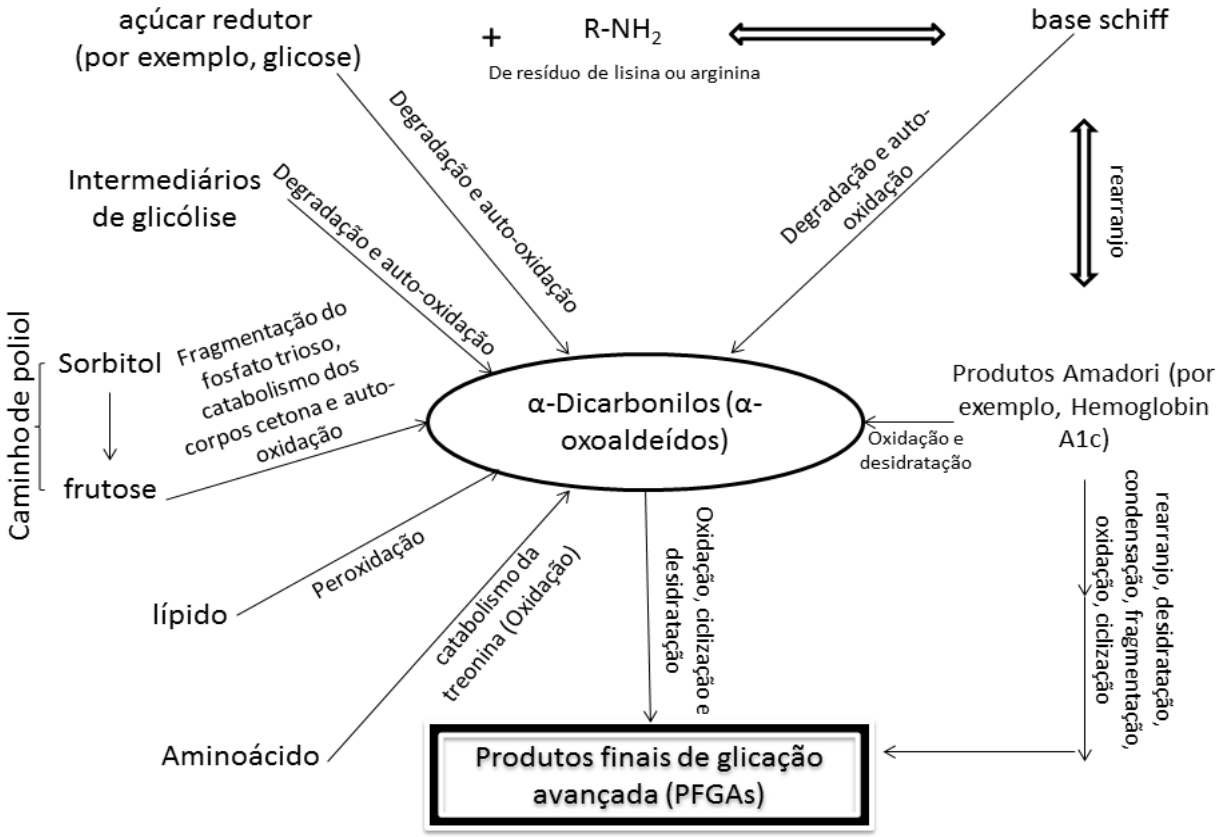

Figura 4. Síntese de produtos finais de glicação avançada endógena (PFGAs). Esta figura representa esquematicamente a via clássica que conduz à síntese de produtos finais de glicação avançada (PFGAs) e também ilustra as diferentes vias que podem levar à formação de PFGAs, mesmo na ausência e redução de açúcar.

Os PFGAs também podem ser formados a partir da peroxidação lipídica (Figura 4) (GOLDBERG et al., 2004; MIYATA et al., 1998) e de uma variedade de outras reações, incluindo a oxidação de açúcares e aminoácidos, para criar oxoaldeídos que se ligam covalentemente a proteínas (URIBARRI et al., 2015). Os PFGAs podem ser formados tanto *in vivo* através do metabolismo normal, e em *in vitro* por aquecimento de açúcares juntamente com as gorduras e proteínas (AHMED, 2005).

Em geral, os PFGAs podem ser PFGAs fluorescentes (como argpirimidina), PFGAs não fluorescentes (como N^{ϵ}-(carboximetil)lisina, CML) e compostos de ligação cruzada fluorescente

(como a pentosidina). Outras PFGAs incluem glucosepanha, carboximetil-hidroxi-lisina, *N*$^{\varepsilon}$-(carboxietil)lisina (CEL), frutose-lisina, hidro-imidazolonas derivadas de metilglioxal e pirralina, que formam aductos de proteínas não fluorescentes, enquanto o dímero de glioxal-lisina (GOLD) e metilglioxal-dímero de lisina (MOLD) formam reticências de proteínas não fluorescentes.

2.3.1 N$^{\varepsilon}$-(carboximetil) lisina (CML)

A N$^{\varepsilon}$- (carboximetil) lisina (CML) (Figura 5) é o produto final de glicação avançado mais abundante e mais pesquisado (TESSIER et al., 2014). É gerado através de várias vias, tais como a clivagem oxidativa do produto Amadori pelo radical hidroxilo (NAGAI et al., 1997) e peroxinitrito (NAGAI et al., 2002). Além dessas vias, a formação de CML também ocorre através do glioxal, que é gerado através da auto-oxidação da glicose (WELLS-KNECHT et al., 1995), clivagem oxidativa de bases de Schiff (GLOMB e MONNIER, 1995) e ácidos graxos poliinsaturados (AGPI) (FU et al., 1996). Uma vez que a formação de CML requer oxidação em todos as vias, pensa-se que a CML é um marcador biológico importante do estresse oxidativo *in vivo* (KOITO et al., 2004). Estudos imunológicos recentes mostraram que a modificação da CML está envolvida no envelhecimento normal (ARAKI et al., 1992), bem como em patogênias de várias doenças associadas ao envelhecimento, como a nefropatia diabética (IMAI et al., 1997), a aterosclerose (KUME et al., 1995), a retinopatia diabética (HAMMES et al., 1996), a hemodiálise associada a amiloidose (MIYATA et al., 1998), insuficiência renal crônica (YAMADA et al., 1994) e doença de Alzheimer (SMITH et al., 1995). A CML é reconhecida pelo receptor para PFGAs (RAGE), e a interação CML-RAGE ativa vias de sinalização celular como NFkB (HEIN et al, 2003).

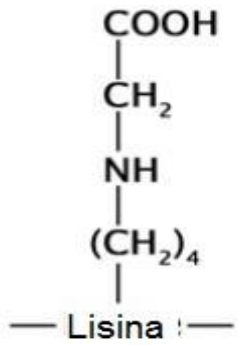

Figura 5: Estrutura de N$^{\varepsilon}$-(carboximetil) lisina (CML).

2.4 HAPLÓTIPOS LIGADOS AO GENE DA GLOBINA BETA S

Os haplotipos ligados ao gene da globina beta influenciam as manifestações clínicas da DF (BANDEIRA et al., 2014; LAURENTINO et al., 2014). Os haplotipos são matrizes de sítios de genes polimórficos que são herdados em conjunto. Os diferentes haplotipos podem influenciar os níveis de HbF e a apresentação clínica de indivíduos com DF. Os haplótipos comuns que estão associados ao alelo β^S são o Bantu (ou Central Africano República, CAR), Benin, Senegal, Camarões, Árabe-Indiano e atípico. Os dados da literatura demonstram que o haplotipo Senegal está associado as maiores concentrações de HbF (> 15%) e, portanto, a uma evolução clínica menos grave da AF e menor ocorrência de dano orgânico (LAURENTINO et al., 2014). A gravidade clínica do haplótipo Benin é intermediária, com concentrações de HbF entre 5% e 15% (FIGUEIREDO et al., 1996; SILVA et al., 2009). O haplótipo Bantu ou CAR está associado à maior gravidade clínica e as menores concentrações de Hb F (<5%). O haplótipo Árabe-Indiano apresenta concentrações elevadas de HbF e curso clínico heterogêneo (NAGEL, 1984; SILVA et al., 2009). Os haplótipos mais comumente encontrados no Brasil são os haplótipos Bantu e Benin (RUSANOVA et al., 2010; GONÇALVES et al., 2003; SILVA and GONÇALVES, 2010; ADORNO et al., 2004). A proteína beta-globina da HbC também tem diferentes haplótipos denominados I, II e III.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito da HU em pacientes com AF e o papel da PFGA N^{ε}-(carboximetil)lisina (CML) nos parâmetros bioquímicos e hematológicos, bem como sua associação com marcadores genéticos clássicos em indivíduos com doença falciforme (DF).

3.2 OBJETIVOS ESPECÍFICOS

I. Avaliar o impacto do tratamento com hidroxiuréia (HU) nos parâmetros laboratoriais hematológicos e bioquímicos em indivíduos com anemia falciforme (HbSS);

II. Determinar os níveis séricos da N^{ε}-(carboximetil)lisina (CML) e sua associação com marcadores bioquímicos, hematológicos e genéticos, bem como, com a utilização de hidroxiuréia (HU) em indivíduos com anemia falciforme (HbSS);

III. Determinar os níveis séricos da N^{ε}-(carboximetil)lisina (CML) e sua associação com marcadores bioquímicos, hematológicos e moleculares em pacientes com doença falciforme SC (HbSC).

4 RESULTADOS

O presente estudo foi distribuído em quatro capítulos, sendo que cada um deles corresponde a um manuscrito, conforme apresentado a seguir:

Capítulo I – Advanced glycation end products: possible role in sickle cell disease.

Capítulo II – Effects of Hydroxyurea on laboratory parameters among sickle cell anemia patients from Bahia, Northeast Brazil: role of leukocytes and lactate dehydrogenase.

Capítulo III – Association of serum level of *N*(epsilon)-(carboxymethyl)lysine with hydroxyurea treatment, laboratory parameters and β^S-globin haplotypes on pediatric sickle cell anemia.

Capítulo IV – Association of serum level of *N*(epsilon)-(carboxymethyl)lysine with hematologic, biochemistry and molecular markers on sickle-hemoglobin SC disease patients.

4.1 CAPÍTULO I

Manuscrito intitulado "*Advanced glycation end products: possible role in sickle cell disease"*

Trata-se de um artigo de revisão que explorou as décadas de pesquisa sobre produtos finais de glicação avançada (PFGAs), realizando um levantamento sobre o que são os PFGAs, como elas são geradas e onde podem ser encontradas. Foi abordado também o papel dos PFGAs em várias doenças, seu mecanismo de ação e procedimentos para determinação laboratorial. Por fim, foi realizada uma breve atualização sobre estudos que exploraram o possível papel dos PFGAs na DF, concluindo-se que existe a necessidade de mais estudos sobre os PFGAs em relação à DF.

Status do manuscrito: Submetido para publicação no periódico "*BioMed Research International*"**.**

Advanced glycation end products: possible role in sickle cell disease

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Abstract

Sickle cell disease (SCD) is the commonest genetic disease in the world. The life-threatening morbidity and early mortality as a result of the disease is well documented. Research on SCD aimed at characterizing the pathophysiology of the disease, finding better ways to manage the disease, prolonging the lifespan of the sufferers and ultimately discovering the cure for the disease has been very intensive in the past few decades. In recent time, advanced glycation end products (AGEs) have received attention as potential factors in the deleterious effect of SCDs due to their association with inflammation and oxidative stress that are common features of SCD. AGEs, a complex and heterogeneous group of compounds formed through non-enzymatic reactions between reducing sugars (or their derivatives) and N-terminal amino groups of lysine and arginine on proteins, lipids, and nucleic acids, have been implicated in several disease complications like diabetic nephropathy, diabetic retinopathy, cardiomyopathy, atherosclerotic disease and peripheral neuropathy. While low levels of AGEs have been reported to be generally harmless, high levels have been linked with the development of many different diseases, such as diabetes, heart disease, kidney failure, Alzheimer's and premature aging. The study of AGEs, therefore, represents a promising area of research as not much is known about their exact roles in sickle cell disease complications.

Keywords: SCD, AGEs, RAGE, Schiff, Amadori, therapy.

SCD = sickle cell disease; AGEs = advanced glycation end products; RAGE = receptor for advanced glycation end products

Introduction

Sickle cell disease (SCD) has an autosomal recessive heritage and is a monogenic hereditary hematological disorder in humans [1]. It occurs as a result of the inheritance of beta S allele (β^S) that originates from a point mutation (GAG>GTG) in the sixth codon (17^{th} nucleotide) of the beta-globin gene (*HBB*) located in chromosome 11 (11p15.5), giving rise to the hemoglobin (Hb)

variant S (HbS) [2]. The mutation leads to the replacement of glutamic acid by valine on the 6th position of the β-globin polypeptide. SCD refers to a heterogeneous group of conditions in which HbS predominates. It can result from the homozygous inheritance of the β^S mutation, a condition most commonly referred to as either HbSS or sickle cell anemia (SCA), which accounts for around 70% of cases worldwide, or from compound heterozygosity for HbS in association with a wide range of other *HBB* gene mutations such as HbSC, HbSβ^o, HbSβ^+, HbSO_{Arab}, etc [3, 4].

Globally, over 300,000 infants are born with the SCD every year [5]; and about 85% of these infants (approximately 255,000 cases/year) are born in sub-Saharan Africa [4]. Without treatment, which is rarely available in low-income high-burden countries mainly in Africa, it is believed that most children born with SCD die in their first years of life [4]. Indeed, millions of persons throughout the world are living with and, sadly, dying from SCD without the benefits of proper diagnosis and appropriate clinical care [6].

The disorder is characterized by high morbidity and mortality in which HbS tends to polymerize under hypoxic conditions otherwise termed deoxygenated state [6]. The polymerization changes the structure of the normally malleable, doughnut-shaped red cell into the characteristic "sickle" shape leading to erythrocyte rigidity and adhesion of the sickle cells to the endothelium. It is well-known that SCD commonly causes vaso-occlusive crises (VOC), tissue ischemia, hemolytic anemia, organ damage, pain, as well as high risk of death [7]. The severity of the vaso-occlusion and progression to end-organ ischemia and pain crisis is potentiated by oxidative stress [8]. Furthermore, the adhesion of the sickle erythrocytes to the endothelial cells is known to increase oxidative stress leading to increased adhesion and diapedesis of circulating leukocytes as well as heightened adhesion of sickle reticulocytes [9, 10, 11]. This activated vascular endothelium expresses adhesion molecules such as vascular cell adhesion molecules 1 (VCAM-1), intracellular adhesion molecules 1 (ICAM-1) and endothelial cell selectin (E-selectin) on their surface which provoke chronic inflammation through the production of proinflammatory cytokines [12, 13]. The chronic inflammation and local/systemic oxidative stress due to SCD may lead to the increased formation and accumulation of systemic levels of advanced glycation end-products (AGEs).

Definition and roles of Advanced glycation end products (AGEs) in the body

Advanced glycation end products (AGEs) are a complex group of compounds formed through non-enzymatic reactions between reducing sugars (or their derivatives like glucose-6-phosphate) and N-terminal amino groups on proteins, lipids, and nucleic acids. They can also form from lipid peroxidation; receiving the name advanced lipoxidation end-products (ALEs) [14, 15] and from a variety of other reactions, including the oxidation of sugars, and amino acids, where reactive oxoaldehydes are first generated before they covalently bind to proteins to form AGEs [16]. The primary targets of the posttranslational modification that generate AGEs are the amino groups of the side chains of arginine and lysine. Glycation, through which AGEs are formed, is

distinguished from glycosylation because the latter involves enzyme-catalyzed attachment of sugars.

AGEs were first recognized as endogenous compounds that formed in excess in diabetes due to hyperglycemia [17]. Today, however, we now know that they can also be generated in conditions of increased oxidative stress, even in the absence of hyperglycemia [16].

AGEs produce one chemical outcome: the formation of covalent cross-links between proteins, which are thought to be one of the central underlying processes by which they cause damage [18]. AGEs may further modify the extracellular matrix (ECM); modify the action of hormones, cytokines, and free radicals via engagement of cell surface receptors; and impact the function of intracellular proteins. They represent one of the most promising areas of research today. Although the initial chemistry behind their formation has been known since the early 1900's, it is only in the last three decades or so that important work has been done to elaborate on them. To this day, however, the chemical processes and pathways that ultimately lead to AGE formation have yet to be fully elucidated.

Classification, Accumulation and Pathophysiology of Advanced Glycation end products (AGEs)

AGEs are end-stage products of glycoxidation and lipoxidation, which can be divided into fluorescent AGEs (such as argpyrimidine), non-fluorescent AGEs (such as N^{ε}-carboxymethyllysine, CML), and fluorescent cross-linking compounds (such as pentosidine). Other AGEs include glucosepane, carboxymethyl-hydroxy-lysine, N^{ε}-carboxyethyl-lysine (CEL), fructose-lysine, methylglyoxal-derived hydroimidazolones and pyrraline, which form non-fluorescent protein adducts, while glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) form non-fluorescent protein crosslinks (Figure 1).

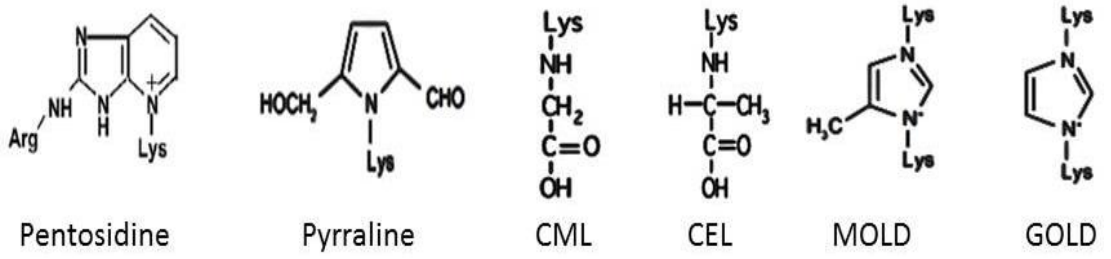

Figure 1. Structures of some advanced glycation endproducts: CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-carboxyethyl-lysine; MOLD, methylglyoxal-lysine dimer; GOLD, glyoxal-lysine dimer.

N^{ε}-(carboxymethyl)lysine (CML) is the most abundant and the most researched advanced glycation end product [19]. It is generated through several pathways, such as oxidative cleavage of the Amadori product by the hydroxyl radical [20] and peroxynitrite [21]. In addition to these pathways, CML formation also takes place through glyoxal, which is generated through autoxidation of glucose [22], oxidative cleavage of Schiff bases [23], and polyunsaturated fatty

acids (PUFAs) [24]. Since CML formation requires oxidation in all pathways, CML is thought to be the important biological marker of oxidative stress in vivo [25]. Immunological studies showed that CML modification is involved in normal aging [26], as well as in the pathogenesis of several age-enhanced diseases such as diabetic nephropathy [27], atherosclerosis [28], diabetic retinopathy [29], hemodialysis-associated amyloidosis [30], chronic renal failure [31], and Alzheimer's disease [32].

CEL is detected in human lens proteins at a concentration similar to that of CML, and its accumulation increases with age in parallel with that of CML [33]. Degenhardt et al [34] have also demonstrated that CEL increases in lens proteins and skin collagen with age.

Pentosidine is composed of an arginine and lysine residue crosslinked to a pentose. It is a fluorescent glycoxidation product and forms protein-protein crosslinks [35]. Studies have shown that pentosidine levels are increased not only in plasma and matrix proteins from diabetic patients but also markedly in non-diabetic hemodialysis patients [30]. It is present in skin collagen, glomerular basement membrane and plasma proteins.

AGE accumulation *in vivo* occurs throughout the body, including the skin, neural, vascular and renal tissue [36]. The accumulation depends largely on sugar concentration and the rate of protein turnover [37]. On the other hand, oxidative stress and inflammatory processes are known to accelerate the formation of AGEs, as seen in rheumatoid arthritis and alcoholism [38], suggesting that oxidative reactions are involved both in the synthesis of AGE as well as in its pathogenic effects. A cohort study of healthy monozygotic and heterozygote twins suggests that the levels of circulating AGEs are genetically determined [39].

Formation and accumulation of AGEs are related to aging as well as to prolonged hyperglycemia and oxidative stress resulting from diabetes mellitus [37, 40]. AGEs are identified to play a role in the development of diabetic complications such as diabetic nephropathy, cardiomyopathy, atherosclerotic disease, peripheral neuropathy, and retinopathy [41, 42]. Elevated AGE levels in women with gestational diabetes mellitus are associated with pregnancy complications such as birth asphyxia, congenital malformations, or stillbirth [43]. A study by Hernebring *et al* [44] revealed a high rate of glycated and oxidized proteins in undifferentiated mouse embryonic stem cells (ESCs) and in mouse blastocyst.

Biochemical Synthesis of Advanced Glycation end products

AGEs are largely generated endogenously through the non-enzymatic reaction of reducing carbohydrates with lysine or arginine side chains (N-terminal amino groups) of macromolecules (proteins, amino acids, phospholipids and nucleic acids) called the Maillard reaction or glycation. It is a slow reaction in which the initial products of glycation can undergo intramolecular rearrangements and reactions of dehydration, condensation, fragmentation, oxidation and cyclization, and finally transform into a stable class of compounds called advanced glycation end products (AGEs) (Figure 2). Production of AGE molecules through the classical pathway takes

weeks to months and involves many reversible intermediates that finally lead to the formation of irreversible AGEs.

In the classical pathway, electrophilic carbonyl group of glucose (or other reducing sugars such as fructose, pentoses, galactose, mannose, ascorbate, xylulose) reacts with a free amino group of several molecules, including proteins, nucleic acids and lipids, to form an unstable aldimine compound, the Schiff base [45, 46] (Figure 2). By rearrangement, this base produces a stable ketoamine, the Amadori product, a 1-amino-1-deoxyketose, which undergoes further complex reactions that involves dehydration, condensation, fragmentation, oxidation and cyclisation leading to the formation of stable and irreversible AGE compounds. Once AGEs are formed, they are nearly irreversible.

Since this reaction is non-enzymatic, the variables that regulate the *in vivo* reaction are the concentrations of reducing sugar and macromolecules, the half-life of macromolecules, their reactivity in terms of free amino groups, and permeability for reducing sugars. Both Schiff bases and Amadori products are reversible reaction products.

The Amadori product can also, through oxidation and dehydration reactions, degrade into a variety of dicarbonyl compounds (glyoxal, methylglyoxal, deoxyglycosone) that serve as propagators (Figure 2). These propagators react again with free amino groups and, through oxidation, dehydration and cyclization reactions, form yellow-brown irreversible and insoluble compounds called advanced glycation end products (AGEs) [45, 46].

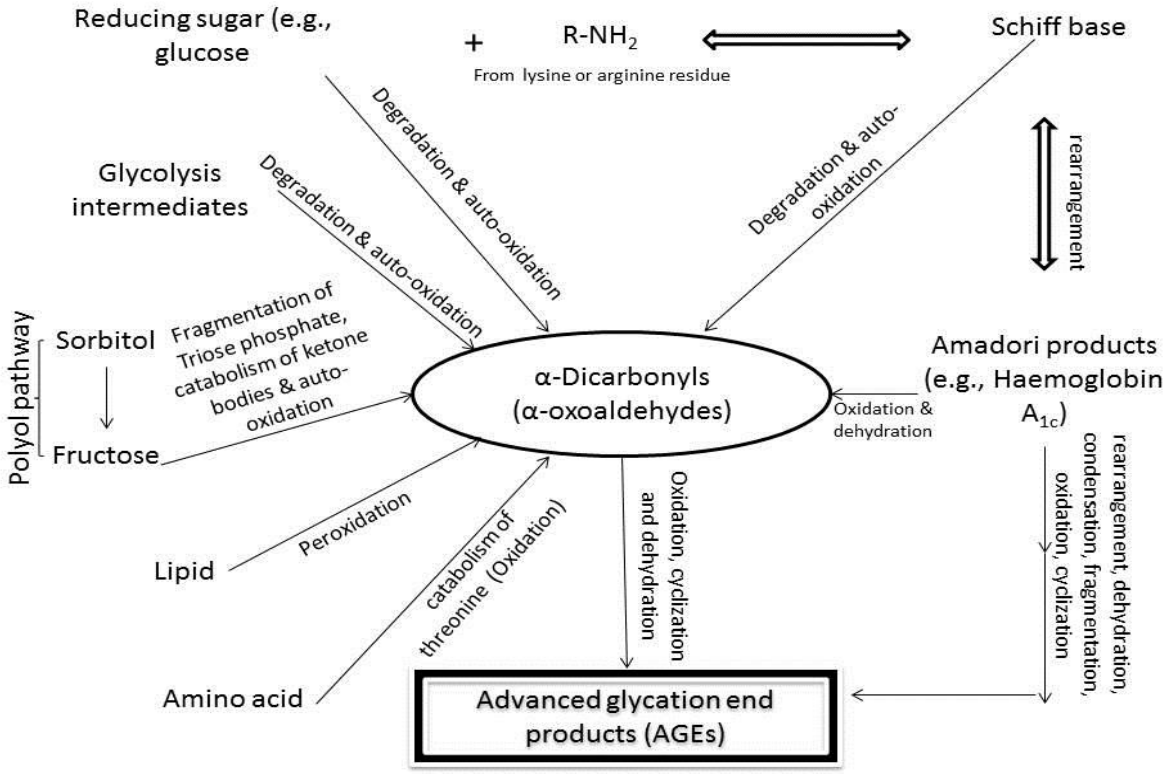

Figure 2. Synthesis of endogenous advanced glycation end products (AGEs). This figure schematically depicts the classical pathway leading to the synthesis of advanced glycation end products (AGEs). The figure also illustrates the many other different pathways that may lead to the formation of AGEs, even in the absence of reducing sugar.

Dicarbonyl (oxoaldehydes) compounds like 3-deoxyglucosone (3-DG), methylglyoxal MGO) and glyoxal (GO) can arise from oxidative degradation or autooxidation of Schiff base, Amadori products, glycolytic pathway intermediates, lipid peroxidation, polyol pathway and amino acids oxidation [16, 18] (Figure 2). 3-DG can be formed from non-oxidative rearrangement and hydrolysis of Amadori adducts and from fructose-3-phosphate, a product of the polyol pathway [18]. Studies showed that methylglyoxal (MG) are generated through the Embden-Meyerhof and polyol pathways, and reacts rapidly with proteins to form MG-derived AGE such as CEL [47]. Methylglyoxal is also formed from non-oxidative mechanisms and from oxidative decomposition of polyunsaturated fatty acids (PUFAs). Furthermore, MGO can be derived from fructose by fragmentation of triose phosphate or the catabolism of ketone bodies and threonine. Methylglyoxal, 3-DG and glyoxal have been proposed to be formed from all stages of the glycation process by degradation of glucose or Schiff bases or from Amadori products [41].

α-Dicarbonyls (α-oxoaldehydes) could be considered as important focal points of how glucose can go on to form AGEs by the classical Maillard reaction, the polyol pathway and from *in vivo* factors such as the catabolism of ketone bodies and threonine as well as lipid peroxidation [48]. They are very reactive molecules (they react with amino, sulfhydryl, and guanidine functional

groups in proteins) leading to protein crosslinks [49]. The accumulation of these reactive dicarbonyl precursors or of glycoxidation or both and lipoxidation products is termed carbonyl stress [50]. This carbonyl stress phenomenon has been observed in both diabetes and uraemia, and has been implicated in the accelerated vascular damage observed in both conditions.

The pathway for the synthesis of AGE is catalyzed by redox active transition metals, oxygen and reactive oxygen species (ROS) [46]. It is, however, inhibited by reducing compounds such as ascorbic acid, superoxide dismutase (SOD), catalase and glutathione.

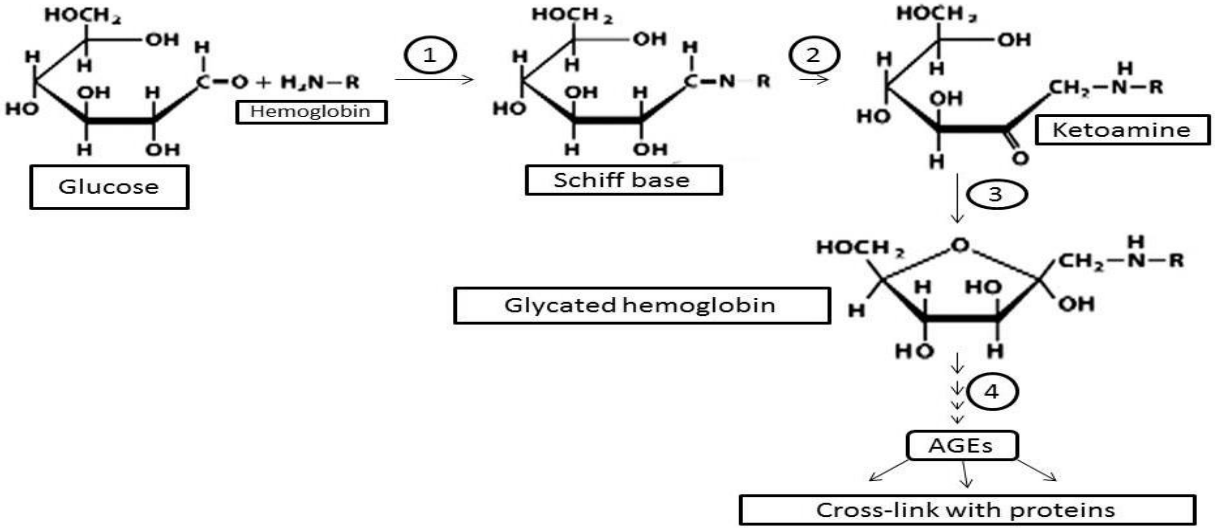

Figure 3. The non-enzymatic reaction of glucose with a primary amino group in hemoglobin A (HbA) begins with (1) the formation of a Schiff base, which (2) undergoes the Amadori rearrangement to generate a stable product; (3) this ketoamine can further cyclize to yield glycated hemoglobin (HbA1c). (4) Subsequent reactions generate advanced glycation end products (AGEs).

Non-enzymatic reaction of glucose with a primary amino group in hemoglobin A (HbA) present in red blood cells (i.e., formation of glycated hemoglobin, HbA1c) occurs in normal individuals (Figure 3). The rate of this process is proportional to the concentration of glucose, so the reaction can be used as the basis for estimating the average blood glucose level over weeks. The estimation of glycated hemoglobin can serve as a marker in the diagnosis of patients with diabetes mellitus whose blood sugar levels are elevated [51].

AGEs can be exogenously ingested (through food consumption), especially foods that have been exposed to high temperatures like grilling, frying, toasting, baking, searing, broiling, sautéing, barbecuing [52]. Certain foods, such as animal foods high in fat and protein are more susceptible to AGE formation during cooking. Foods highest in AGEs include meat (especially red meat), certain cheeses, fried eggs, butter, cream cheese, margarine, mayonnaise, oils and nuts. Cigarette/cigar smoking is also a source of increased AGE accumulation.

Disease Mechanisms of Advanced Glycation end products (AGEs)

AGEs accumulate in long-lasting proteins and cause damage. Recent advances in the understanding of the pathophysiology of AGEs have shed new light on the pathogenesis of atherosclerosis, diabetic nephropathy, dialysis-related amyloidosis, and Alzheimer's disease [30, 45, 53, 54]. AGE-modified proteins are thought to play a role in normal tissue remodeling, i.e., removal and replacement of senescent extracellular matrix components. In pathological conditions, however, accumulation of AGE-modified proteins can cause tissue damage by a variety of mechanisms: by altering the structure and function of tissue proteins [55], by stimulating cellular responses through specific receptors such as receptors for AGEs (RAGE) for the AGE-modified proteins [45, 56, 57], or through the generation of reactive oxygen intermediates [58, 59, 60], i.e., formation of cross-links, interaction with specific cellular receptors, and intracellular glycation [18]. The deleterious actions of AGEs against cellular functions are summarized in two general mechanisms: (1) modifying molecules and (2) forming non-degradable aggregates [25, 40, 61].

AGEs are able to generate ROS, deplete antioxidant molecules and induce the production of various cytokines (mainly tumor necrosis factor alpha (TNF-α), interleukins 1α and 6), VCAM-1 and C-reactive proteins [48, 62] (Figure 4).

RAGE is a multiligand member of the immunoglobulin superfamily of cell surface molecules encoded by a gene on chromosome 6 near the major histocompatibility complex III, which interacts with several ligands, including AGE, amyloids, S-100/calgranulins, β-sheet fibrils and amphoterin [63]. They were identified in various cell systems: monocytes/ macrophages, T lymphocytes, fibroblasts, smooth muscle cells, neurons, red blood cells, and mesangial cells. The binding of AGE on RAGE T lymphocytes stimulates the production of γ-interferon, with consequent tissue damage; binding of AGE to RAGE monocytes/macrophages induces production of cytokines (interleukin 1β, TNF-α, IGF-1, PDGF) and growth factors [64]. AGE-RAGE interactions stimulate a broad spectrum of intracellular cell signaling pathways such as $p21^{ras}$, mitogen-activated protein kinases (MAPKs), Rho GTPases, phosphatidyl-inositol 3 kinase (PI3K), extracellular signal-regulated kinase (ERK) 1 and 2, stress-activated protein kinase/ c-Jun-N-terminal kinase, the janus kinases and NF-κB translocation [65, 66].

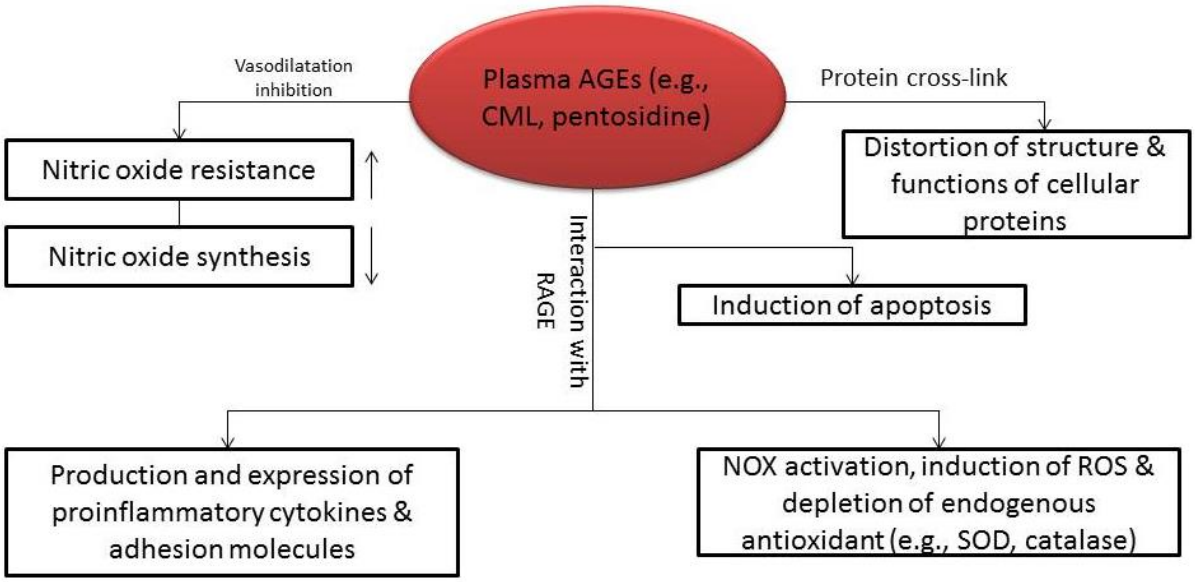

Figure 4. Schematic representation of the pathophysiological mechanism of Advanced glycation end products (AGEs). CML, N^{ε}-(carboxymethyl)lysine; NOX, Nicotinamide adenine dinucleotide phosphate oxidase; SOD, Superoxide dismutase; NO, Nitric oxide; AGEs, Advanced glycation end products; RAGE, Receptor for Advanced glycation end products. (↑, increase; ↓, decrease).

RAGE stimulation results in activation of nuclear factor kappa B, a transcription factor, which stimulates the production of pro-inflammatory cytokines by macrophages and induces changes in gene expression [45, 57]. This results in the upregulation of many inflammatory molecules including VCAM-1, ICAM-1, E-selectin, vascular endothelial growth factor (VEGF), TFN-α, IL-1α, IL-6, and heme oxygenase in response to injury thereby effecting localized tissue repair and reinstating homeostasis [55, 63, 65]. RAGE stimulation can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), which directly induces the generation of ROS, decrease activity of endogenous antioxidants such as superoxide dismutase (SOD) and catalase [65, 67] suggesting that there seems to be a constant supremacy tussle between NOX and endogenous antioxidants such as SOD and catalase since they inhibit the synthesis of AGEs. The AGE-RAGE interaction indirectly reduces cellular antioxidant defenses like reduced glutathione and ascorbic acid [46]. However, the relationship between AGEs with the receptor and the following signaling pathways is still unclear, and is a subject of ongoing research.

AGE Measurement

Currently, there is no universally accepted method to detect AGEs and this could be attributed to the nature of the compounds; their complexity and heterogeneity. AGE accumulation can be measured in blood and in tissue. In blood, employing the plasma or serum, the preferred technique for determination of CML and CEL is stable-isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) [68]. Gas chromatography mass spectrometry could be applied [64]. It can also be carried out in the blood using enzyme-linked immunosorbent assay

(ELISA) technique involving mainly competitive ELISA [69]. For determination of pentosidine in blood, a rapid and sensitive high-performance liquid chromatography (HPLC) method with fluorimetric detection is considered the preferred technique [70]. In skin tissue, AGE accumulation can be measured at the volar side of the lower arm, simple and non-invasively with a skin auto-fluorescence (AF) reader (AGE-reader) [71].

Potential targets for drug action

Due to the deleterious activities of AGEs, they have been marked as target for drug action; especially their biosynthetic pathways. The level of AGEs in the system is defined by the rate of their formation and also by the rate of their removal. As a result, low levels of AGEs have been reported to be generally harmless as they can be readily removed in a functioning system [46]. Many cells have developed intrinsic detoxifying pathways against accumulation of AGEs [72]. Glyoxalase system comprising of glyoxalase (Glo) I and II, play a key role in the defense against glycation through the use of glutathione (GSH) to catalyze the conversion of glyoxal, methylglyoxal and other α-oxoaldehydes to the less toxic D-lactate [73]. Other enzymatic systems include fructosyl-amine oxidases (FAOXs) and fructosamine kinases [74, 75]. These classes of enzymes recognize and break Amadori products. However, FAOXs also known as *amadoriases* have been found to be expressed only in bacteria, yeast and fungi but not in mammals [46].

A variety of different compounds that inhibit AGEs have been identified; though most of them have yet to receive clinical trials. Aminoguanidine, a nucleophilic hydrazine compound, has been reported to prevent AGE formation by reacting mostly with derivatives of early glycation products that are not bound to proteins, like 3-DG. Aminoguanidine treatment increases arterial elasticity and attenuates the effects of diabetes on large arteries [76]. It reduces ECM accumulation of both fibronectin and laminin in streptozotocin-induced diabetic rats with diabetic nephropathy [77]. Aminoguanidine, however, is also a NOS inhibitor, which may impair some of its benefits as an AGE inhibitor [78].

N-(2-Acetamidoethyl)hydrazine carboximidamide hydrochloride (ALT-946) has been shown to be an effective inhibitor of AGE-induced cross-links and like aminoguanidine, ALT-946 also inhibits NOS [79]. 4,5-Dimethyl-3-phenacylthiazolium chloride (ALT-711), N-phenacyl thiazolium and N-phenacyl-4,5-dimethylthiazolium are compounds that break the cross-links of AGEs [80]. Diabetic rats treated for a period of 4 months with ALT-711 show reduced collagen III, increased collagen solubility, reduced RAGE and AGE-R3 mRNA expression compared with placebo [81]. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers have been shown to decrease the production of reactive carbonyl precursors [82]. A Study shows that ACE inhibitor, Ramipril, attenuated AGE accumulation in diabetic animals [83].

Pyridoxamine, a naturally occurring vitamin B_6 isoform, is effective at inhibiting AGEs at 3 different levels [84]. It traps reactive carbonyl intermediates, scavenges ROS and prevents the degradation of post-Amadori intermediates to AGE formation [85]. Pyridoxamine has shown promising results in phase II clinical trial against nephropathy [80]. Zucker rats treated with pyridoxamine have reduced plasma levels of glyoxal, methylglyoxal, and AGEs in collagen [86]. Benfotiamine, a lipid-soluble thiamine derivative, inhibits AGE formation pathway [87].

Association of Advanced glycation end products (AGEs) with sickle cell disease (SCD)

Notwithstanding the fact that we have over a century period since research on SCD began, we are still not where we should be in terms of research into clinical management and cure of the disease. It is even more baffling when we consider the fact that for about 4 decades, AGEs have been implicated in a number of diseases yet little research has been carried out to discover if they are in any way associated with SCD-related complications even when such discovery can go a long way in the clinical management of the disease.

Studies by Somjee *et al* [88] and Nur *et al* [89] were the only known ones to report the association between SCD and plasma levels of AGEs. However, while Somjee *et al* [88] carried out their study in children, Nur *et al* [89] studied adult patients. One major discovery in both studies is that oxidative stress increases significantly with increasing AGEs even as endogenous antioxidants are depleted concomitantly (Figure 5). It appears as though oxidative stress potentiates AGE levels. The results of Somjee *et al* [88] suggest that circulating levels of AGEs may play a role in the vascular pathology of SCA since plasma levels of AGEs and the rate of accumulation of AGEs in RBC were significantly higher in patients with SCD compared to controls.

The research of Nur *et al* [89] suggests that AGEs may also be implicated in the pathophysiology of the hemolytic phenotype of SCD and could be implicated in hemolysis-related organ complications such as priapism, leg ulcer, ischemic strokes and pulmonary hypertension (Figure 5). The suggestion by Nur *et al* [89] is based on their findings, which showed increased levels of pentosidine and CML (AGE types) with increasing hemolytic rate in patients with SCD during steady state compared to healthy controls. It was also discovered that increasing AGEs resulted in increase in the concentration of lactate dehydrogenase and a decrease in HbF [89] (Figure 5).

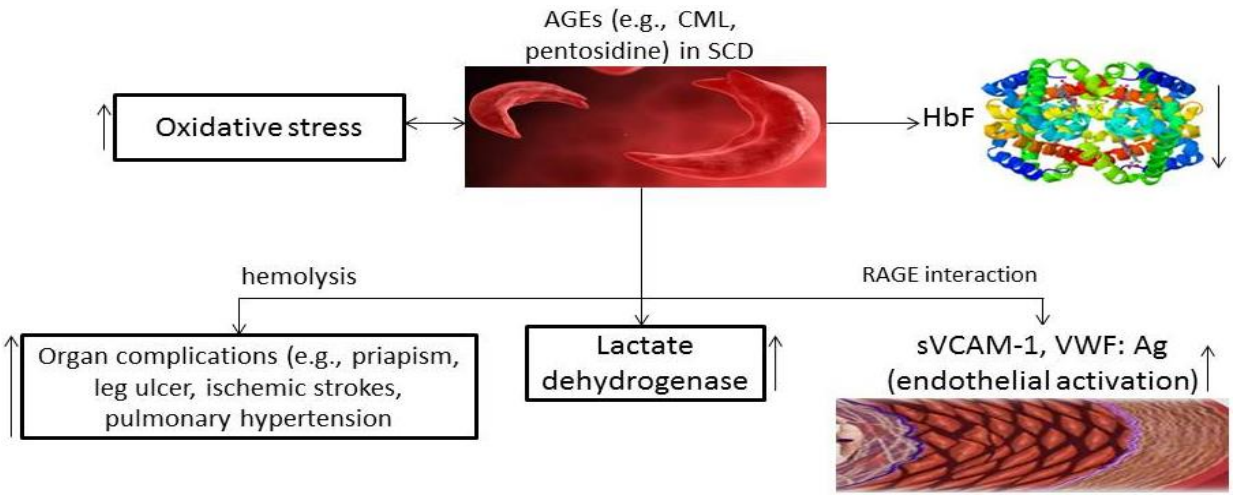

Figure 5. Proposed mechanism of AGEs in SCD. AGEs, Advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; sVCAM-1, soluble vascular adhesion molecule 1; VWF: Ag, von Willebrand factor antigen; RAGE, Receptor for Advanced glycation end products. (↑, increase; ↓, decrease).

Conclusion

From the foregoing, therefore, there is no doubt that we have a dearth of research in the area of AGEs as it relates to SCD. We, therefore, hope that this review will spur more scientists to undertake this important task.

Conflict of Interest Statement

The authors declare no conflict of interest.

References

[1] F. A. Ferrone, "Sickle cell disease: Its molecular mechanism and the one drug that treats it," *International Journal of Biological Macromolecules*, vol. 93, pp. 1168-1173, 2016.

[2] T. N. Williams, "Sickle cell disease in sub-Saharan Africa," *Hematology/Oncology Clinics of North America*, vol. 30, pp. 343-358, 2016.

[3] D. C. Rees, T. N. Williams, and M. T. Gladwin, "Sickle-cell disease," *Lancet*, vol. 376, pp. 2018–2031, 2010.

[4] T. N. Willaims, and S. K. Obaro, "Sickle cell disease and malaria morbidity: a tale with two tails," *Trends in Parasitology*, vol. 27, pp. 315-320, 2011.

[5] F. B. Piel, A.P. Patil, R. E. Howes, O. A. Nyangiri, P. W. Gething, M. Dewi, W. H. Temperley, T. N. Williams, D. J. Weatherall, and S. I. Hay, "Global epidemiology of sickle haemoglobinin neonates: a contemporary geostatistical model-based map and population estimates," *Lancet*, vol. 381, pp. 142–151, 2013.

[6] P. T. McGann, A. G. Hernandez, and R. E. Ware, "Sickle cell anemia in sub-Saharan Africa: advancing the clinical paradigm through partnerships and research," *Blood,* vol. 129, pp. 155-161, 2017.

[7] J. Howard, and P. Telfer, "*Sickle Cell Disease in Clinical Practice*," London 2015: Springer-Verlag, 2015.

[8] R. F. Queiroz, and E. S. Lima, "Oxidative stress in sickle cell disease," *Revista Brasileira de Hematologia e Hemoterapia*, vol. 35, pp. 16–17, 2013.

[9] B. E. Gee, and O. S. Platt, "Sickle Reticulocytes Adhere to VCAM-1," *Blood,* vol. 85, pp. 268-274, 1995.

[10] P. Connes, S. Verlhac, and F. Bernaudin, "Advances in understanding the pathogenesis of cerebrovascular vasculopathy in sickle cell anaemia," *British Journal of haematology*, vol. 161, pp. 484–498, 2013.

[11] D. Zhang, C. Xu, D. Manwani, and P. S. Frenette, "Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology," *Blood,* vol. 127, pp. 801–809, 2016.

[12] C. Sultana, Y. Shen, V. Rattan, C. Johnson, and V. K. Kalra, "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*, vol. 92, pp. 3924-3935, 1998.

[13] I. C. Bandeira, L. B. Rocha, M. C. Barbosa, D. B. Elias, J. A. Querioz, M. V. Freitas, and R. P. Gonçalves, "Chronic inflammatory state in sickle cell anemia patients is associated with HBB(*)S haplotype," *Cytokine*, vol. 65, pp. 217-221, 2014.

[14] T. Goldberg, W. Cai, M. Peppa, V. Dardaine, B. S. Baliga, J. Uribarri, and H. Vlassara, "Advanced Glycoxidation End Products in Commonly Consumed Foods," *Journal of the American Dietetic Association,* vol. 104, pp. 1287-1291, 2004.

[15] T. Miyata, A. Saito, K. Kurokawa, and C. van Ypersele de Strihou, "Advanced glycation and lipoxidation end products: Role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism," *Journal of the American Society of Nephrology,* vol. 11, pp. 1744-1752, 2000.

[16] J. Uribarri, M. D. del Castillo, M. P. de la Maza, R. Filip, A. Gugliucci, C. L. Contreras, M. H. Macías-Cervantes, D. H. M. Bastos, A. Medrano, T. Menini, M. Portero-Otin, A. Rojas, G. R. Sampaio, K. Wrobel, K. Wrobel, and M. E. Garay-Sevilla, "Dietary Advanced Glycation End Products and Their Role in Health and Disease," *Advances in Nutrition*, vol. 6, pp. 461–473, 2015.

[17] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature,* vol. 414, pp. 813–820, 2001.

[18] R. Singh, A. Barden, T. Mori, and L. Beilin, "Advanced glycation end-products: A review," *Diabetologia*, vol. 44, pp. 129 – 146, 2001.

[19] F. J. Tessier, P. Jacolot, and C. Niquet-Léridon, "Open questions around the carboxymethyllysine," *IMARS Highlights*, vol. 9, pp. 14–20, 2014.

[20] R. Nagai, K. Ikeda, T. Higashi, H. Sano, Y. Jinnouchi, T. Araki, and S. Horiuchi, "Hydroxyl radical mediates Nε-(carboxymethyl)lysine formation from Amadori product," *Biochemical and Biophysical Research Communications*, vol. 234, pp. 167–172, 1997.

[21] R. Nagai, Y. Unno, M. C. Hayashi, S. Masuda, F. Hayase, N. Kinae, and S. Horiuchi, "Peroxynitrite induces formation of Nε-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite," *Diabetes*, vol. 51, pp. 2833–2889, 2002.

[22] K. J. Wells-Knecht, D. V. Zyzak, J. E. Litchfield, S. R. Thorpe, and J. W. Baynes, "Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose," *Biochemistry*, vol. 34, pp. 3702–3709, 1995.

[23] M. A. Glomb, and V. M. Monnier, "Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction," *The Journal of Biological Chemistry*, vol. 270, pp. 10017–10026, 1995.

[24] D. B. Jump, "The biochemistry of n-3 polyunsaturated fatty acids," *The Journal of Biological Chemistry*, vol. 277, pp. 8755–8758, 2002.

[25] K. A. Howes, Y. Liu, J. L. Dunaief, A. Milam, J. M. Frederick, A. Marks, and W. Baehr, "Receptor for Advanced Glycation End Products and Age-Related Macular Degeneration," *Investigative Ophthalmology and Visual Science*, vol. 45, pp. 3713–3720, 2004.

[26] N. Araki, N. Ueno, B. Chakrabarti, Y. Morino, and S. Horiuchi, "Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging," *The Journal of Biological Chemistry*, vol. 267, pp. 10211–10214, 1992.

[27] N. Imai, S. Nishi, Y. Suzuki, R. Karasawa, M. Ueno, H. Shimada, S. Kawashima, T. Nakamaru, Y. Miyakawa, N. Araki, S. Horiuchi, F. Gejyo, and M. Arakawa, "Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy," *Nephron*, vol. 76, pp. 153–160, 1997.

[28] S. Kume, M. Takeya, T. Mori, N. Araki, H. Suzuki, S. Horiuchi, T. Kodama, Y. Miyauchi, and K. Takahashi, "Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody," *American Journal of Pathology*, vol. 147, pp. 654–667, 1995.

[29] H. P. Hammes, A. Weiss, S. Hess, N. Araki, S. Horiuchi, M. Brownlee, and K. T. Preissner, "Modification of vitronectin by advanced glycation alters functional properties *in vitro* and in the diabetic retina," *Laboratory Investigation*, vol. 75, pp. 325–338, 1996.

[30] T. Miyata, N. Ishiguro, Y. Yasuda, T. Ito, M. Nangaku, H. Iwata, and K. Kurokawa, "Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers," *Biochemical and Biophysical Research Communications*, vol. 244, pp. 45-49, 1998.

[31] K. Yamada, Y. Miyahara, K. Hamaguchi, M. Nakayama, H. Nakano, O. Nozaki, Y. Miura, S. Suzuki, H. Tuchida, N. Mimura, et al. "Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure," *Clinical Nephrology*, vol. 42, pp. 354–361, 1994.

[32] M. A. Smith, S. Taneda, P. L. Richey, S. Miyata, S. D. Yan, D. Stern, L. M. Sayre, V. M. Monnier, and G. Perry, "Advanced Maillard reaction end products are associated with Alzheimer disease pathology," *Proceedings of the National Academy of Sciences*, vol. 91, pp. 5710–5714, 1995.

[33] M. U. Ahmed, E. B. Frye, T. P. Degenhardt, S. R. Thorpe, and J. W. Baynes, "Nε-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochemical Journal*, vol. 324, pp. 565–570, 1997.

[34] T. P. Degenhardt, S. R. Thorpe, and J. W. Baynes, "Chemical modification of proteins by methylglyoxal," *Cellular and Molecular Biology*, vol. **44**, pp. 1139–1145, 1998.

[35] R. G. Paul, and A. J. Bailey, "Glycation of collagen: the basis of its central role in the late complications of ageing and diabetes," *The International Journal of Biochemistry and Cell Biology*, vol. 28, pp. 1297-310, 1996.

[36] C. G. Schalkwijk, A. Baidoshvili, C. D. Stehouwer, H. V. van, and H. W. Niessen, "Increased accumulation of the glycoxidation product Nepsilon-(carboxymethyl)lysine in hearts of diabetic patients: generation and characterisation of a monoclonal anti-CML antibody," *Biochimica et Biophysica Acta*, vol. 1636, pp. 82–89, 2004.

[37] M. Brownlee, "Advanced protein glycosylation in diabetes and aging," *Annual Review of Medicine*, vol. 46, pp. 223–234, 1995.

[38] S. Drinda, S. Franke, C. C. Canet, P. Petrow, R. Bräuer, C. Hüttich, G. Stein, and G. Hein, "Identification of N-carboxymethyllysine in the synovial tissue of patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 61, pp. 488 – 492, 2002.

[39] R. D. Leslie, H. Beyan, P. Sawtell, B. O. Boehm, T. D. Spector, and H. Snieder, "Level of an advanced glycated end product is genetically determined: a study of normal twins," *Diabetes*, vol. 52, pp. 2441-2444, 2003.

[40] E. Haucke, A. N. Santos, A. Simm, C. Henning, M. A. Glomb, J. Gürke, M. Schindler, B. Fischer, and A. N. Santos, "Accumulation of advanced glycation end products in the rabbit blastocyst under maternal diabetes," *Reproduction*, vol. 148, pp. 169-178, 2014.

[41] N. Ahmed, and P. J. Thornalley, "Advanced glycation endproducts: what is their relevance to diabetic complications?" *Diabetes, Obesity & Metabolism*, vol. 9, pp. 233–245, 2007.

[42] N. Nass, B. Bartling, A. N. Santos, R. J. Scheubel, J. Börgermann, R. E. Silber, and A. Simm, "Advanced glycation end products, diabetes and ageing," *Zeitschrift für Gerontologie und Geriatrie*, vol. 40, pp. 349–356, 2007.

[43] L. Guosheng, S. Hongmei, N. Chuan, L. Haiying, Z. Xiaopeng, and L. Xianqiong, "The relationship of serum AGE levels in diabetic mothers with adverse fetal outcome," *Journal of Perinatology*, vol. 29, pp. 483–488, 2009.

[44] M. Hernebring, G. Brolén, H. Aguilaniu, H. Semb, and T. Nyström, "Elimination of damaged proteins during differentiation of embryonic stem cells," *Proceedings of the National Academy of Sciences*, vol. 103, pp. 7700–7705, 2006.

[45] N. Ahmed, "Advanced glycation endproducts--role in pathology of diabetic complications," *Diabetes Research and Clinical Practice*, vol. 67, pp. 3-21, 2005.

[46] P. Gkogkolou, and M. Bohm, "Advanced glycation end products: key players in skin aging," *Dermato endocrinology*, vol. 4, pp. 259-270, 2012.

[47] W. Koito, T. Araki, S. Horiuchi, and R. Nagai, "Conventional Antibody against Nε-(Carboxymethyl)Lysine (CML) Shows Cross-Reaction to Nε-(Carboxyethyl)Lysine (CEL): Immunochemical Quantification of CML with a Specific Antibody," *The Journal of Biochemistry*, vol. 136, pp. 831-837, 2004.

[48] P. J. Thornalley, "Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs," *Cellular and Molecular Biology* (Noisy-le-grand), vol. 44, pp. 1013 – 1023, 1998.

[49] T. W. Lo, M. E. Westwood, A. C. McLellan, T. Selwood, and P. J. Thornalley, "Binding and modification of proteins by methylglyoxal under physiological conditions: a kinetic and mechanistic study with N--acetylarginine, N-α-acetylcysteine, and N-α-acetyllysine, and bovine serum albumin," *The Journal of Biological Chemistry*, vol. 269, pp. 32299–32305, 1994.

[50] T. Miyata, C. van Ypersele de Strihou, K. Kurakawa, and J. W. Baynes, "Alteration in non-enzymatic biochemistry in uraemia: origin and significance of "carbonyl stress" in long term uraemic complications," *Kidney International*, vol. 55, pp. 389-399, 1999.

[51] N. Alqahtani, W. A. G. Khan, M. H. Alhumaidi, and Y. A. A. R. Ahmed, "Use of Glycated Hemoglobin in the Diagnosis of Diabetes Mellitus and Pre-diabetes and Role of

Fasting Plasma Glucose, Oral Glucose Tolerance Test," *International Journal of Preventive Medicine*, vol. 4, pp. 1025-1029, 2013.

[52] J. Uribarri, S. Woodruff, S. Goodman, W. Cai, X. Chen, R. Pyzik, A. Yong, G. E. Striker, and H. Vlassara, "Advanced Glycation End Products in Foods and a Practical Guide to Their Reduction in the Diet," *Journal of the American Dietetic Association*, vol. 110, pp. 911–916, 2010.

[53] M. L. Lieuw-a-fa, V. W. VanHinsbergh, T. Teerlink, R. Barto, J. Twisk, C. D. Stehouwer, and C. G. Schalkwijk, "Increased levels of N(epsilon)-(carboxymethyl)lysine and N(epsilon)-(carboxyethyl)lysine in type 1 diabetic patients with impaired renal function: correlation with markers of endothelial dysfunction," *Nephrology Dialysis Transplantation*, vol. 19, pp. 631-636, 2004.

[54] P. J. Beisswenger, Z. Makita, T. J. Curphey, L. Moore, S. Jean, T. Brinck-Johnsen, R. Bucala, and H. Vlassara, "Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes," *Diabetes*, vol. 44, pp. 824-829, 1995.

[55] A. Goldin, J. A. Beckman, A. M. Schmidt, and M. A. Creager, "Advanced glycation end products: sparking the development of diabetic vascular injury," *Circulation*, vol. 114, pp. 597-605, 2006.

[56] A. Hirose, T. Tanikawa, H. Mori, Y. Okada, and Y. Tanaka, "Advanced glycation end products increase endothelial permeability through the RAGE/Rho signaling pathway," *FEBS Letters*, vol. 584, pp. 61-66, 2010.

[57] J. Liu, S. Zhao, J. Tang, Z. Li, T. Zhong, Y. Liu, D. Chen, M. Zhao, Y. Li, X. Gong, P. Deng, J. H. Wang, and Y. Jiang, "Advanced glycation end products and lipopolysaccharide synergistically stimulate proinflammatory cytokine/chemokine production in endothelial cells via activation of both mitogen-activated protein kinases and nuclear factor-kappaB," *FEBS Journal*, vol. 276, pp. 4598-4606, 2009.

[58] S. Genuth, W. Sun, P. Cleary, D. R. Sell, W. Dahms, J. Malone, W. Sivitz, and V. M. Monnier, "Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes," *Diabetes*, vol. 54, pp. 3103-3111, 2005.

[59] E. G. Gerrits, H. L. Lutgers, N. Kleefstra, R. Graaf, K. H. Groenier, A. J. Smit, R. O. Gans, and H. J. Bilo, "Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications," *Diabetes Care*, vol. 31, pp. 517-521, 2008.

[60] Y. Koyama, Y. Takeishi, T. Arimoto, T. Niizeki, T. Shishido, H. Takahashi, N. Nozaki, O. Hirono, Y. Tsunoda, J. Nitobe, T. Watanabe, and I. Kubota, "High serum level of pentosidine, an advanced glycation end product (AGE), is a risk factor of patients with heart failure," *Journal of Cardiac Failure*, vol. 13, pp. 199-206, 2007.

[61] S. Willemsen, J. W. L. Hartog, M. R. Heiner-Fokkema, D. J. van Veldhuisen, and A. A. Voors, "Advanced glycation end-products, a pathophysiological pathway in the cardiorenal syndrome," *Heart Failure Reviews*, vol. 17, pp. 221–228, 2012.

[62] G. Hein, R. Weigand, G. Lehmann, G. Stein, and S. Franke, "Advanced glycation end-products pentosidine and N-carboxymethyllysine are elevated in serum of patients with osteoporosis," *Rheumatology*, vol. 42, pp. 1242 – 1246, 2003.

[63] L. Hansen, D. Gupta, G. Joseph, D. Weiss, and R. W. Taylor, "The receptor for advanced glycation end products impairs collateral formation in both diabetic and non-diabetic mice," *Laboratory Investigation*, vol. 97, pp. 34-42, 2017.

[64] A. Lapolla, P. Traldi, and D. Fedele, "Importance of measuring products of non-enzymatic glycation of proteins," *Clinical Biochemistry*, vol. 38, pp. 103– 115, 2005.

[65] A. Bierhaus, P. M. Humpert, M. Morcos, T. Wendt, T. Chavakis, B. Arnold, D. M. Stern, and P. P. Nawroth, "Understanding RAGE, the receptor for advanced glycation end products," *Journal of Molecular Medicine*, vol. 83, pp. 876–886, 2005.

[66] A. Rouhiainen, J. Kuja-Panula, S. Tumova, and H. Rauvala, "RAGE-mediated cell signaling," *Methods in Molecular Biology*, vol. 963, pp. 239–263, 2013.

[67] D. T. Loughlin, and C. M. Artlett, "Precursor of advanced glycation end products mediates ER-stress-induced caspase-3 activation of human dermal fibroblasts through NAD(P)H oxidase 4," *PLoS One*, **5**:e11093; PMID: 20559423, 2010.

[68] T. Teerlink, R. Barto, H. J. Ten Brink, and C. G. Schalkwijk, "Measurement of Nepsilon-(carboxymethyl)lysine and Nepsilon- (carboxyethyl)lysine in human plasma protein by stable-isotopedilution tandem mass spectrometry," *Clinical Chemistry*, vol. 50, pp. 1222–1228, 2004.

[69] M. Svrckova, M. Zatloukalova, P. Dvorakova, D. Coufalova, D. Novak, L. Hernychova, and J. Vacek, "Na+/K+ -ATPase interaction with methylglyoxal as reactive metabolic side product," *Free Radical Biology and Medicine*, vol. 108, pp. 146-154, 2017.

[70] J. L. Scheijen, M. P. van de Waarenburg, C. D. Stehouwer, and C. G. Schalkwijk, "Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, pp. 610–614, 2009.

[71] R. Meerwaldt, R. Graaff, P. H. Oomen, T. P. Links, J. J. Jager, N. L. Alderson, S. R. Thorpe, J. W. Baynes, R. O. Gans, and A. J. Smit, "Simple non-invasive assessment of advanced glycation end product accumulation," *Diabetologia*, vol. 47, pp. 1324–1330, 2004.

[72] P. J. Thornalley, "The enzymatic defence against glycation in health, disease and therapeutics: a symposium to examine the concept," *Biochemical Society Transactions*, vol. 31, pp. 1341-1342, 2003.

[73] M. Xue, N. Rabbani, and P. J. Thornalley, "Glyoxalase in ageing," *Seminars in Cell and Developmental Biology*, vol. 22, pp. 293-301, 2011.

[74] X. Wu, and V. M. Monnier, "Enzymatic deglycation of proteins," *Archives of Biochemistry and Biophysics*, vol. 419, pp. 16-24, 2003.

[75] E. Van Schaftingen, F. Collard, E. Wiame, and M. Veiga-daCunha, "Enzymatic repair of Amadori products," *Amino Acids*, vol. 42, pp. 1143-1150, 2012.

[76] B. Corman, M. Duriez, P. Poitevin, D. Heudes, P. Bruneval, A. Tedgui, and B. I. Levy, "Aminoguanidine prevents age-related arterial stiffening and cardiac hypertrophy," *Proceedings of the National Academy of Sciences*, vol. 95, pp. 1301–1306, 1998.

[77] V. Thallas-Bonke, C. Lindschau, B. Rizkalla, L. A. Bach, G. Boner, M. Meier, H. Haller, M. E. Cooper, and J. M. Forbes, "Attenuation of extracellular matrix accumulation in diabetic nephropathy by the advanced glycation end product cross-link breaker ALT-711 via a protein kinase C-dependent pathway," *Diabetes*, vol. 53, pp. 2921–2930, 2004.

[78] R. Bucala, K. J. Tracey, and A. Cerami, "Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes," *Journal of Clinical Investigation*, vol. 87, pp. 432–438, 1991.

[79] J. M. Forbes, T. Soulis, V. Thallas, S. Panagiotopoulos, D. M. Long, S. Vasan, D. Wagle, G. Jerums, and M. E. Cooper, "Renoprotective effects of a novel inhibitor of advanced glycation," *Diabetologia*, vol. 44, pp. 108–114, 2001.

[80] S. Vasan, P. Foiles, and H. Founds, "Therapeutic potential of breakers of advanced glycation end product-protein crosslinks," *Archives of Biochemistry and Biophysics*, vol. 419, pp. 89-96, 2003.

[81] R. Candido, J. M. Forbes, M. C. Thomas, V. Thallas, R. G. Dean, W. C. Burns, C. Tikellis, R. H. Ritchie, S. M. Twigg, M. E. Cooper, and L. M. Burrell, "A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes," *Circulation Research*, vol. 92, pp. 785–792, 2003.

[82] T. Miyata, C. van Ypersele de Strihou, Y. Ueda, K. Ichimori, R. Inagi, H. Onogi, N. Ishikawa, M. Nangaku, and K. Kurokawa, "Angiotensin II receptor antagonists and angiotensin-converting enzyme inhibitors lower in vitro the formation of advanced glycation end products: biochemical mechanisms," *Journal of the American Society of Nephrology*, vol. 13, pp. 2478–2487, 2002.

[83] J. M. Forbes, M. E. Cooper, V. Thallas, W. C. Burns, M. C. Thomas, G. C. Brammar, F. Lee, S. L. Grant, L. A. Burrell, G. Jerums, and T. M. Osicka, "Reduction of the

accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy," *Diabetes*, vol. 51, pp. 3274–3282, 2002.

[84] P. A. Voziyan, R. G. Khalifah, C. Thibaudeau, A. Yildiz, J. Jacob, A. S. Serianni, and B. G. Hudson, "Modification of proteins in vitro by physiological levels of glucose: pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation end-products through binding of redox metal ions," *The Journal of Biological Chemistry*, vol. 278, pp. 46616–44624, 2003.

[85] P. A. Voziyan, and B. G. Hudson, "Pyridoxamine: the many virtues of a Maillard reaction inhibitor," *Annals of the New York Academy of Sciences*, vol. 1043, pp. 807-816, 2005.

[86] N. L. Alderson, M. E. Chachich, N. N. Youssef, R. J. Beattie, M. Nachtigal, S. R. Thorpe, and J. W. Baynes, "The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats," *Kidney International*, vol. 63, pp. 2123-2133, 2003.

[87] H. P. Hammes, X. Du, D. Edelstein, T. Taguchi, T. Matsumura, Q. Ju, J. Lin, A. Bierhaus, P. Nawroth, D. Hannak, M. Neumaier, R. Bergfeld, I. Giardino, and M. Brownlee, "Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy," *Nature Medicine*, vol. 9, pp. 294–299, 2003.

[88] S. S. Somjee, R. P. Warrier, J. L. Thomson, J. Ory-Ascani, and J. M. Hempe, "Advanced glycation end-products in sickle cell anaemia," *British Journal of Haematology*, vol. 128, pp. 112-118, 2004.

[89] E. Nur, D. P. Brandjes, J. J. B. Schnog, H. M. Otten, K. Fijnvandraat, C. G. Schalkwijk, and B. J. Biemond, "Plasma levels of advanced glycation end products are associated with haemolysis-related organ complications in sickle cell patients," *British Journal of Haematology*, vol. 151, pp. 62-69, 2010.

4.2 CAPÍTULO II

Manuscrito intitulado "*Effects of Hydroxyurea on laboratory parameters among sickle cell anemia patients from Bahia, Northeast Brazil: role of leukocytes and lactate dehydrogenase"*

Trata-se de um artigo que descreve o efeito da hidroxiureia em biomarcadores laboratoriais em pacientes com AF de Salvador e a influência da leucócitos e lactato desidrogenase sobre outros biomarcadores.

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Effects of Hydroxyurea on laboratory parameters among sickle cell anemia patients from Bahia, Northeast Brazil: role of leukocytes and lactate dehydrogenase

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Abstract

This research evaluated effect of hydroxyurea (HU) treatment on laboratory biomarkers in sickle cell anemia (SCA). SCA patients (44) on HU (15 – 25 mg/kg/day) (HU^+ SCA), SCA patients (171) without HU (HU^- SCA) and normal (30, HbAA) healthy age- and sex-matched individuals were recruited. Biochemical markers were measured in serum by immunochemistry assay. Hematological parameters were carried out using automated cell counter. Hemoglobin (Hb) profile and HbF levels were investigated by HPLC. HU^+ SCA and HU^- SCA comprised patients with median age of 11 years. Leukocytes ($p = 0.007$), neutrophils ($p = 0.021$) and eosinophils ($p = 0.002$) were significantly reduced ($p < 0.05$) in the HU^+ SCA compared to the HU^- SCA. There were significant increases ($p < 0.05$) in hemoglobin ($p = 0.028$) and hematocrit ($p = 0.037$) in HU^+ SCA. AST ($p = 0.037$), total bilirubin ($p = 0.016$), indirect bilirubin ($p = 0.023$) and LDH ($p = 0.039$) decreased significantly ($p < 0.05$) in HU^+ SCA compared to the HU^- SCA. Ferritin ($p = 0.003$), creatinine ($p = 0.026$) and CRP ($p = 0.003$) showed significant increase in HU^+ SCA compared to the HU^- SCA. Multivariate analyses showed that leukocyte was significantly impacted by HU ($\beta = -0.161$; $p = 0.016$), AST ($\beta = 0.182$; $p = 0.006$) and creatinine ($\beta = -0.172$; $p = 0.011$) after adjusting for parameters that had significant correlation. LDH was negatively

impacted by CRP ($p = 0.017$; β = -0.150), creatinine ($p = 0.013$; β = -0.157), Hb ($p \leq 0.001$; β = -0.230) while AST ($p \leq 0.001$; β = 0.274) had significantly positive impact on LDH after adjusting for similar parameters. These results provide evidence of the effect of HU and the interplay between these markers on SCA on HU.

Keywords: hydroxyurea; sickle cell anemia; multivariate; laboratory parameters.

1. Introduction

Sickle cell anemia (SCA), the best known form of sickle cell disease (SCD), has an autosomal recessive heritage and is a monogenic hereditary hematological disorder in humans [1,2]. It is characterized by the presence of the beta S allele (β^S), originating from a point mutation (GAG>GTG) in the sixth codon of the beta-globin gene (*HBB*) located in chromosome 11, giving rise to the hemoglobin (Hb) variant S (HbS), where valine replaces glutamic acid in the sixth position of the N-terminal portion of the beta-globin chain [2,3,4]. It is the result of a single nucleotide polymorphism (SNP) (A → HbA / T → HbS; rs334) of the *HBB* gene [5]. In SCA, HbS forms aggregates in the state of deoxygenation, with consequent erythrocyte rigidity leading to changes in the microvasculature, resulting in vaso-occlusion, tissue ischemia, hemolytic anemia, organ damage, pain, as well as high risk of death [6]. SCA, however, presents a heterogeneous clinical course according to patient background and geographic region of origin [7].

It has been estimated that 312,000 persons are born with hemoglobin SS (HbSS) annually throughout the world, with most of these births (236,000) happening in sub-Sahara Africa [8]. Migrations have led to further spread of the sickle cell genes in basically all parts of the world that are not malaria endemic [9]. SCD is believed to affect approximately 3,500 newborns per year in Brazil [4] and the frequencies of individuals of African descent with sickle cell trait (HbSA) range from 6.9% to 15.4% [10]. It is more prevalent where the proportion of afro-descendants is higher (Northeast region and the States of São Paulo, Rio de Janeiro and Minas Gerais) [11]. In these regions, it was observed that new cases of SCD occur in every 1000 births and sickle cell hemoglobin carriers occur in every 27 births [11].

Interaction of the erythrocytes of SCA patients with endothelium increases transit time, deoxygenation of Hb and polymerization, and drowsiness [12,13]. This leads to vaso-occlusion, a key step in the pathophysiology of both acute and chronic vascular complications of SCA. Factors contributing to complex clinical features include age and sex, as well as biochemical and hematological factors [14]. Furthermore, the disease severity varies among patients even within the same geographical regions.

Until the last few decades, management of the disease has been mainly preventive or supportive targeted at controlling or ameliorating the symptoms of the disease especially the painful crises. Currently, hydroxyurea (HU), a potent inhibitor of ribonucleotide reductase, is the most commonly used therapy for individuals suffering from SCA [15] and the only agent approved for

the prevention of complications of SCD [16]. It was first synthesized in 1869 in Germany by Dressler and Stein. A century later, phase I and II assays began to test their safety in humans with solid tumors. In 1967, the FDA approved it for the treatment of neoplastic diseases [17]. It is currently also approved for the treatment of melanoma, resistant chronic myelocytic leukemia, and recurrent, metastatic, or inoperable ovarian carcinoma [17,18]. However, the clinical efficacy of HU treatment varies among SCA individuals [19,20]. It can vary based on the geographical region, age, hematological and biochemical factors of SCA patients. Ironically, there has been little research in evaluating the true efficacy of HU in SCA patients in Brazil.

2. Materials and Methods

2.1 Subjects. In this transversal cohort study, two hundred and fifteen SCA patients of both sexes from the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), Salvador, Bahia, Brazil and the Hospital Universitario Professor Edgar Santos (HUPES) of the Universidade Federal da Bahia (UFBA), Brazil were recruited. The subjects were divided into two groups: (i) children treated with hydroxyurea (15 – 25 mg/kg/day) (n = 44; median age 11 years; interquartile range = 9 – 18 years) (HU^+ SCA group); (ii) children not treated with hydroxyurea (n = 171; median age 11 years; interquartile range = 7 - 15 years) (HU^- SCA). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. The study was carried out between August 2015 and August 2017 and the protocol was submitted to and approved by the Fundação Oswaldo Cruz (FIOCRUZ) Ethics research board (ID 1.400.527). All patients with SCA included in the study answer an epidemiological inquiry questionnaire. The response was from the patient or the parents/guardian in the case of children or teenager (less than 18). Patients were in the steady-state of the disease when samples were collected. Steady-state is described as a period without any acute events and no blood transfusion for 120 days prior to blood sampling.

All participants received verbal and written explanation of the study and signed the Informed Consent Form (ICF) before inclusion in the study. Informed consent was obtained from the patient or the patient's parent/guardian. Patients were in the hospital during crisis or in the clinic for a routine visit. Thirty normal healthy age- and sex-matched individuals (HbAA) of both sexes and of African descent were recruited from volunteers from the Clinical Laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (UFBA). Venous blood (8-10 mL) was collected in Vacutainer tubes containing EDTA. Blood samples were processed within 30 minutes of collection.

Overall exclusion criteria: Patients with diabetes mellitus, renal failure, autoimmune inflammatory disease, active infection and pregnancy, smokers and chronic alcoholics were excluded.

2.2 Standard Laboratory Tests. Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain). Serum ferritin was measured by immunoassay using an Access® 2 Immunoassay system X2 (Beckman Coulter,

Fullerton, CA, USA). C-reactive protein (CRP), alpha 1-antitrypsin (A1AT) and antistreptolysin-O (ASO) were measured by immunochemistry (Immage® 800 system, Beckman Coulter, Fullerton, CA, USA). Hematological analyses were carried out using an automated cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC/VARIANT I; BIO-RAD, CA, USA).

3. Statistical Analysis

Descriptive statistics were summarized with median and interquartile range (25% - 75%) for continuous variable. The data for each variable and for each group were tested for normality (Shapiro-Wilk and Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) prior to analysis. The Mann-Whitney (non-parametric) and independent T-test (parametric) were employed for the analysis of two numerical variables, comparing two groups of values within the same variable, taking into account the distribution of each variable. For correlation studies, the Spearman (rs) coefficient was employed and the differences between the groups were considered significant when $P \leq 0.05$ or $P \leq 0.01$. Statistical analyses were performed using the EPI INFO version 6.04 and SPSS version 18.0 (SPSS 18.0, Chicago, IL, USA). Values of $P < 0.05$ were considered significant for the analyses performed.

4. Results

Table 1 shows the hematological characteristics of SCA patients on HU treatment (HU^+ SCA) and the SCA patients without HU treatment (HU^- SCA). Both groups were composed of patients with median age of 11 years and presented similar interquartile values (9 – 18 and 7 – 15 for HU^+ SCA and HU^- SCA respectively). It also shows that the HU^+ SCA patients have reduced reticulocyte count, leukocytes, neutrophils, eosinophils, basophils, typical lymphocytes, monocytes, platelets and HbS as compared to the HU^- SCA patients. However, only leukocytes ($p = 0.007$), neutrophils ($p = 0.021$) and eosinophils ($p = 0.002$) were significantly reduced ($p < 0.05$) in the HU^+ SCA patients compared to the HU^- SCA patients. On the other hand, there were increases in the RBC count, and in Hb, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and fetal hemoglobin (HbF) concentration among HU^+ SCA patients when compared to the HU^- SCA patients. However, only hemoglobin ($p = 0.028$) and hematocrit ($p = 0.037$) increased significantly ($p < 0.05$) among HU^+ SCA patients compared to the HU^- SCA patients.

Table 2 shows the biochemical characteristics of HU^+ SCA patients and HU^- SCA patients. The results also show that there were reductions in the level of total cholesterol, low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), serum iron, total bilirubin, indirect bilirubin, lactate dehydrogenase (LDH), anti-streptolysin O (ASO) and alpha-1 antitrypsin (AAT) in the HU^+ SCA patients as compared to the HU^- SCA patients. However, only AST ($p = 0.037$), total bilirubin (TB) ($p = 0.016$), indirect bilirubin (IB) ($p = 0.023$) and LDH (p

= 0.039) were significantly reduced ($p < 0.05$) in HU^+ SCA compared to HU^- SCA. Table 2 further presented increases in glucose level, ferritin, creatinine, C-reactive protein (CRP) and albumin/globulin (A/G) ratio in HU^+ SCA patients as compared to the HU^- SCA patients. Here, ferritin ($p = 0.003$), creatinine ($p = 0.026$) and CRP ($p = 0.003$) were significantly increased in HU^+ SCA patients as opposed to the HU^- SCA patients ($p < 0.05$).

Table 3 shows the Spearman's rho correlation between the various parameters in HU^+ SCA patients. From the results, AST level was positively correlated to the level of total bilirubin (r = 0.494**) and LDH (r = 0.485**). On the other hand, AST was negatively correlated to the levels of ferritin (r = -0.150*) and creatinine (r = -0.348**). Ferritin level was also positively correlated to the level of CRP-reactive protein (r = 0.199**), but inversely correlated to the levels of TB (r = -0.248**) and LDH (r = -0.216**). Total bilirubin was directly correlated to LDH (r = 0.379**) and inversely related to creatinine (r = -0.323**). We also observed a significant positive correlation between creatinine and CRP (r = 0.146*), and a significant negative correlation between LDH and creatinine (r = -0.352**). Therefore, increase in creatinine level would result in concomitant increase in CRP and conversely between creatinine and LDH. The correlation between CRP and LDH is negatively significant (r = -0.172**).

We carried out a Spearman's rho correlation analysis of all laboratory parameters against leukocyte and LDH as separate dependent variables (data not shown) for HU^+ SCA. The parameters that showed significant differences (ferritin, creatinine, HU, CRP, AST for leukocyte as dependent variable, and ferritin, HU, creatinine, CRP, AST, Hb for LDH as the dependent variable) were subjected to multivariate regression analysis explained below in order to determine if an independent parameter was influenced by another independent parameter with regards to their relationship with a common dependent variable (here leukocyte and LDH).

Table 4 shows a multivariate analysis of the HU^+ SCA patients with leukocyte (white blood cells, WBC) as the dependent variable. Leukocyte was significantly impacted by HU ($p = 0.016$), after adjusting for AST, creatinine, ferritin and CRP. Leukocyte was significantly impacted by AST ($p = 0.006$) after adjusting for HU, ferritin, creatinine, AST and CRP. Leukocyte is also impacted by creatinine ($p = 0.011$) after adjusting for HU, AST, ferritin, CRP. However, its association on both HU ($\beta = -0.161$) and creatinine ($\beta = -0.172$) was negative while it association on AST ($\beta = 0.182$) was positive. Ferritin ($\beta = 0.126$; $p = 0.058$) and CRP ($\beta = 0.115$; $p = 0.082$) does not have significant influence on leukocyte after adjustment.

Table 5 presents further multivariate analysis of HU^+ SCA with LDH as the dependent variable. We discovered that LDH was impacted negatively by CRP ($p = 0.017$; $\beta = -0.150$) after adjusting for, creatinine, HU, AST and ferritin. LDH was impacted negatively by creatinine ($p = 0.013$; $\beta = -0.157$) after adjusting to HU, CRP, AST, Hb and ferritin. LDH was impacted negatively also by Hb ($p \leq 0.001$; $\beta = -0.230$) after adjusting for all the other parameters earlier mentioned. Ferritin and HU did not present significant influence on LDH when adjusted for the aforementioned

parameters. On the other hand, LDH was impacted negatively by AST ($p \leq 0.001$; $\beta = 0.274$) after adjusting for the other parameters mentioned above.

5. Discussion

The present study analyzed the effect of HU on the hematological and biochemical parameters in SCA patients. Studies have shown that children with SCA even in steady state have differences in several biomarkers as compared to the healthy age-matched children [4,21]. However, not much is known about how these biomarkers are affected by HU, the most common chemotherapy in the management of the disease.

Hb and hematocrit are markers of hemolysis. The level of Hb and hematocrit significantly improved in HU^+ patients suggesting a reduction in anemia. This finding agrees with the works of Hankins et al [22] and Silva-Pinto et al [4]. MCV along with MCH and MCHC is part of RBC indices which are measurements and/or calculations for determining hemolysis. Unlike previous report by Charache [23], our work showed no significant increase in the level of MCV. The non-significant increase in the values of MCV, MCH and MCHC in patients on HU could also be due to poor adherence to the therapy by the respondents and/or variation in the length of HU treatment as suggested above.

Watson in 1948 was the first person to suggest that HbF could be responsible for the lack of clinical symptoms in newborns with SCD [24]. Since then, HbF has been recognized as among the most important known modifiers of the clinical course of SCD. Unlike the findings of Silva-Pinto et al [4] (a research carried out in Brazil just like ours but different location), the HbF level did not significantly increase in the HU treated group in our research. However, Davies and Gilmore [25] reported that half of the patients in a Multicenter Study of Hydroxyurea (MSH) had no increase or had negligible increment in HbF while Steinberg et al [26] stated that, like our findings, the increase in HbF was not significant in MSH. Nevertheless, the insignificant increment in HbF in HU treated group could be attributed to irregular adherence to the daily dosage by some of the respondents and/or variation in the length of HU treatment [15,27]. According to Thornburg et al [27] increase in HbF is moderately associated with good adherence. Davies and Gilmore [25] further suggested that the best result for HU happen in patients with good bone marrow reserve and/or patients on Maximum Tolerated Dose (MTD).

Leukocytes, neutrophils, monocytes and eosinophils are known to activate the endothelial cells [15]. Leukocyte, neutrophil and eosinophil levels were significantly reduced in patients receiving HU suggesting a reduction in vaso-occlusive crisis and general pain in the body [28,29]. Our results also agree with the work of Silva-Pinto et al [4] and the report of Davies and Gilmore [25] that stated the HU is involved in myelosuppression. Charache [23] and Charache et al [30] reinforced the suggestions that neutrophils significantly reduce in HU treated sickle cell anemia patients. High neutrophil counts have been reported to be associated with a severe clinical prognosis and early death [31]. The work of Zimmerman et al [32] suggested that neutrophils

adhere to vascular endothelium thereby potentially impairing the smooth flow of the sickle cells. Neutrophil adherence to vascular endothelium is also reported to cause increase in whole blood viscosity and release of cytokines which are known to be involved in inflammatory response including pain pathways [33,34]. Adherence of sickle cells to neutrophils has also been reported [33]. This adherence is reported to be accompanied usually by activation and production of toxic oxygen radicals. However, unlike the result obtained by Silva-Pinto et al [4], there is no significance in the reduction of platelet level in the HU treated group compared to the HU untreated group.

Multivariate analyses in HU^+ SCA with leukocyte as the dependent variable suggest that the presence of other parameters did not alter the significant association between leukocyte and HU, between leukocyte and AST, and between leukocyte and creatinine thereby suggesting that these parameters are markers of reduced leukocytes in SCA patients on HU. This is a very unique area in SCA research as no publication to the best of our knowledge has delved into finding out the interplay between these markers and how they influence each other in HU^+ SCA patients.

Biochemical markers of inflammation investigated included C-reactive protein (CRP), alpha 1-antitrypsin, ferritin and antistreptolysin O. However, HU^+ SCA patients had significant increases in only serum ferritin and CRP concentrations compared to HU^- SCA patients. High concentrations of ferritin and CRP suggest that HU^+ SCA patients have a more pronounced inflammatory response when compared to non-users, and may be a reflection of the leukoreduction that accompanies treatment [36,37,38]. High serum ferritin reliably indicates a replenishment of iron stores. Our correlation result that presented a significant positive relationship between ferritin and C-reactive protein suggests that ferritin increase could be the reason for the increase in C-reactive protein and vice versa.

Chronic liver impairment is also a frequent occurrence in SCA. Markers of liver function (hepatic markers) such as AST, ALT, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, globulin, albumin/globulin ratio, serum iron and lactate dehydrogenase (LDH) were determined. Total bilirubin, direct bilirubin, indirect bilirubin, iron serum and LDH are also markers of hemolysis. We observed significant reduction in LDH, total bilirubin, indirect bilirubin and AST which suggest that significant reduction in these molecules may be associated with a reduction in hemolytic phenomena due to treatment with HU and a reduction in hepatic diseases, mechanisms of which have not yet been fully elucidated. Our results are in agreement with the results of other works that reported improvement in hepatic markers such as bilirubin, LDH, ALT and AST [39,40,41,42]. The insignificant changes in the levels of ALT, total protein, albumin, globulin and albumin/globulin ratio in HU^+ group compared to HU^- group which are further markers of hepatic damage is not well understood. However, Kinney et al [43] reported an insignificant change in the level of ALT in HU^+ SCA patients after 6 months from the baseline; though 12 months later they observed a significant difference in ALT level in HU^+ SCA compared to the baseline. This, perhaps, suggests that variation in the time length of the treatment with the drug could be a reason for the insignificant change in the ALT level in HU^+ SCA

compared to the HU^- SCA. The significant positive relationship between LDH and AST and between LDH and total bilirubin goes to support our findings that suggest that hemolysis is accompanied with liver damage. This was further corroborated by the significant positive relationship between total bilirubin and AST.

The multivariate analyses with LDH as the dependent variable showed an association of LDH with AST, CRP, creatinine and Hb in HU^+ SCA; thereby suggesting that these parameters are markers of reduced LDH in SCA patients on HU treatment. There is no report in this direction to the best of our knowledge.

Current guidelines suggest that reduction in glomerular filtration rate (GFR) which is associated with elevated serum creatinine level is considered as a marker of renal dysfunction [44]. We observed a significant elevation of serum creatinine level in HU^+ SCA patients. Reports have demonstrated that there is a subclinical glomerular hyper filtration in SCD patients [45]. This hyperfiltration, which is associated with decreased tubular secretion of creatinine, causes an elevation in the serum level of creatinine thereby making creatinine an imperfect marker of glomerular filtration rate (GFR). In addition, HU may have triggered a further increase in creatinine level via interaction with inflammatory factors especially considering that C-reactive protein, in our report, presented a significant positive relationship with creatinine.

We did not observe any significant difference in the markers of lipid metabolism (lipoproteins) between the HU^+ group and HU^- group. This could suggest that HU does not play any role in the lipid metabolism of HU^+ SCA. There is no work to the best of our knowledge that related the lipoproteins in HU^+ patients with HU^- patients. However, it has been reported in several works that cholesterol decrease significantly in SCA patients leading to hypocholesterolemia [46]. Our work reported insignificant decrease in HDL-cholesterol, cholesterol and LDL-cholesterol in the HU^+ treated group. Triglyceride and VLDL showed an insignificant increase which is in agreement with the work of Seixas et al [47] that reported increase in these molecules in SCD patients.

Conclusions

Our results suggest that HU plays a significant role in inflammation, hemolysis, and hepatic systems in the sickle cell anemia.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa, Adekunle Adekile and Dalila Luciola Zanette conceived the study design. Rayra Pereira Santiago, Uche Samuel Ndidi, Magda Oliveira Seixas Carvalho, Camylla Vilas Boas Figueiredo, Caroline Conceicao Da

Guarda, Setondji Cocou Modeste Alexandre Yahouedehou and Bartholomew Friday Chukwu collected the samples, carried out the laboratory analysis and interpreted the results. Uche Samuel Ndidi drafted the manuscript and it was revised by Marilda Souza Goncalves, Cynara Gomes Barbosa and Dalila Luciola Zanette. All authors read and approved the final manuscript.

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Table 1. Hematological characteristics of sickle cell anemia patients on hydroxyurea (HU) compared to values obtained from patients without the use of the therapeutic

Hematological variables	HU^- (N = 171) Median (IQR)	HU^+ (N = 44) Median (IQR)	*P** value
RBC, $\times 10^{12}$/L	2.62 (2.36 – 2.95)	2.70 (2.41 – 3.07)	0.310
Hemoglobin, g/Dl	8.10 (7.30 – 8.90)	8.65 (7.73 – 9.40)	**0.028**
Hematocrit, %	23.35 (20.80 – 25.73)	24.60 (21.88 – 27.38)	**0.037**
MCV, Fl	87.70 (83.03 – 93.63)	90.20 (86.10 – 94.98)	0.937
MCH, ρg	30.65 (29.00 – 33.20)	31.65 (29.87 – 34.15)	0.117
MCHC, g/dL	35.00 (34.10 – 36.03)	34.00 (34.03 – 35.68)	0.799
Reticulocyte, %	6.95 (5.20 – 8.40)	6.25 (4.93 – 8.45)	0.644
Leukocytes, $\times 10^6$/L	13000 (10875.00 – 15850.00)	11400 (8622.50 – 14550.00)	**0.007**
Neutrophils, $\times 10^6$/L	5981.00 (4440.75 – 8021.50)	4811.75 (3399.50 – 6724.50)	**0.021**
Eosinophils, $\times 10^6$/L	644.50 (344.75 – 1338.00)	386.00 (253.50 – 749.25)	**0.002**
Basophils, $\times 10^6$/L	110.00 (0.00 – 150.25)	93.00 (00.00 – 153.75)	0.888
Typical lymphocytes, $\times 10^6$/L	4605.00 (3633.25 – 5868.00)	4083.00 (2871.00 – 5934.00)	0.246
Monocytes, $\times 10^6$/L	838.00 (617.50 – 1158.25)	738.00 (539.25 – 1263.00)	0.139
Platelets, $\times 10^3$/mL	437.00 (353.75 – 559.25)	414.50 (298.25 – 524.75)	0.169
Haptoglobin, mg/dL	5.83 (5.83 – 5.83)	5.83 (5.83 – 5.83)	0.692
HbS, %	86.55 (81.40 – 91.00)	84.30 (77.98 – 89.20)	0.120
HbF, %	8.70 (4.70 – 13.30)	9.25 (5.93 – 13.68)	0.620

HU^+: Patients on hydroxyurea; HU^-: Patients with no intake of hydroxyurea; RBC: Red Blood Cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; IQR: Interquartile range (25^{th} – 75^{th});

*P**: significance at ≤ 0.05 using the Mann-Whitney U test.

Table 2. Biochemical characteristics of sickle cell anemia patients on hydroxyurea (HU) compared to values obtained from patients without the use of the therapeutic

Biochemical variables	HU^- (N = 171) Median (IQR)	HU^+ (N = 44) Median (IQR)	*P** value
Glucose, mg/dL	73.00 (67.00 – 78.00)	75.00 (69.00 – 79.75)	0.171
Total Cholesterol, mg/dL	127.50 (110.00 – 145.00)	117.50 (99.50 – 132.25)	0.073
HDL-C, mg/dL	30.00 (26.00 – 35.25)	28.50 (25.00 – 36.75)	0.383
LDL-C, mg/dL	74.20 (63.00 – 91.00)	65.50 (55.25 – 84.50)	0.128
VLDL-C, mg/dL	17.00 (13.00 – 23.55)	18.00 (13.00 – 23.50)	0.777
Triglyceride, mg/dL	87.00 (64.00 – 117.25)	89.00 (64.75 – 116.50)	0.758
ALT, U/L	22.00 (16.00 – 28.00)	17.00 (13.25 – 28.00)	0.101
AST, U/L	53.00 (41.00 – 67.25)	45.50 (32.25 – 61.75)	**0.037**
Serum Iron, μg/dL	90.00 (67.75 – 121.25)	85.00 (64.50 – 107.75)	0.421
Ferritin, ng,/mL	185.00 (94.53 – 382.73)	303.60 (191.88 – 460.38)	**0.003**
Total Bilirubin, mg/dL	2.20 (1.70 – 2.90)	1.90 (1.43 – 2.58)	**0.016**
Direct Bilirubin, mg/dL	0.50 (0.40 – 0.70)	0.05 (0.40 – 0.70)	0.214
Indirect Bilirubin, mg/dL	1.63 (1.10 – 2.30)	1.30 (0.90 – 1.70)	**0.023**
Total Protein, g/dL	7.90 (7.50 – 8.50)	7.70 (7.43 – 8.18)	0.129
Albumin, g/dL	4.40 (4.18 – 4.70)	4.45 (4.03 – 4.60)	0.468
Globulin, g/dL	3.60 (3.10 – 4.03)	3.45 (2.90 – 3.90)	0.361
Uric Acid, mg/dL	4.00 (3.20 – 4.90)	3.95 (3.40 – 4.90)	0.747
Urea, mg/dL	15.00 (12.00 – 19.00)	15.00 (12.00 – 20.00)	0.922
Creatinine, mg/dL	0.40 (0.30 – 0.50)	0.45 (0.40 – 0.60)	**0.026**
C-reactive protein, mg/L	4.22 (2.82 – 6.98)	7.06 (3.96 – 13.25)	**0.003**
LDH, U/L	1099.50 (831.25 – 1404.75)	917.00 (696.00 – 1262.75)	**0.039**
A/G Ratio	1.20 (1.10 – 1.40)	1.25 (1.03 – 1.60)	0.573
AAT, mg/dL	170.00 (139.75 – 191.00)	164.50 (143.00 – 187.00)	0.842
ASO, UI/mL	79.50 (33.35 – 158.75)	71.50 (25.00 – 149.25)	0.463

HU^+: Patients on hydroxyurea; HU^-: Patients with no intake of hydroxyurea; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; VLDL-C: Very low-density lipoprotein-cholesterol; ALT: Alanine transaminase; AST: Aspartate transaminase; LDH: Lactate dehydrogenase; A/G Ratio: Albumin / globulin ratio; AAT: Alpha 1-antitrypsin; ASO: Antistreptolysin O; IQR: Interquartile range (25^{th} – 75^{th});

*P**: significance at ≤ 0.05 using the Mann-Whitney U test.

Table 3. Correlation analysis of sickle cell anemia (SCA) patients on hydroxyurea (HU) treatment on some biochemical parameters that showed significant difference between patients on HU and patients without HU (Table 2 refer)

	AST	Ferritin	T. Bilirubin	Creatinine	C-reactive protein	LDH
AST	1.000	-0.150*	0.494**	-0.348**	-0.021	0.485**
Ferritin		1.000	-0.248**	0.101	0.199**	-0.216**
T. Bilirubin			1.000	-0.323**	0.096	0.379**
Creatinine				1.000	0.146*	-0.352**
C-reactive protein					1.000	-0.172**
LDH						1.000

*. Correlation is significant at ≤ 0.05 level (2-tailed)

**. Correlation is significant at ≤ 0.01 level (2-tailed)

Table 4. Multivariate analyses of sickle cell anemia (SCA) patients on hydroxyurea (HU) treatment with leukocyte as the dependent variable

Independent variable	Coefficient (β)	*P** value	Dependent variable	R^2	*P* value
Hydroxyurea	-0.161	0.016	Leukocyte (/mL)	0.128	< 0.001
Aspartate transaminase (U/L)	0.182	0.006			
C-reactive protein (mg/L)	0.115	0.082			
Ferritin (hg/mL)	0.126	0.058			
Creatinine (mg/dL)	-0.172	0.011			

P values are significant at ≤ 0.05

Table 5. Multivariate analyses of sickle cell anemia (SCA) patients on hydroxyurea (HU) treatment with lactate dehydrogenase as the dependent variable

Independent variable	Coefficient (β)	*P* value	Dependent variable	R^2	*P* value
Hydroxyurea	-0.041	0.517	Lactate dehydrogenase (U/L)	0.223	< 0.001
Hemoglobin (g/dL)	-0.230	0.001			
Aspartate transaminase (U/L)	0.274	0.001			
C-reactive protein (mg/L)	-0.150	0.017			
Ferritin (hg/mL)	-0.074	0.236			
Creatinine (mg/dL)	-0.157	0.013			

P values are significant at ≤ 0.05

References

[1] D. Weatherall, K. Hofman, G. Rodgers, J. Ruffin, and S. Hrynkow, "A case for developing North-South partnerships for research in sickle cell disease," *Blood*, vol. 105, pp. 921–23, 2005.

[2] F. A. Ferrone, "Sickle cell disease: Its molecular mechanism and the one drug that treats it," *International Journal of Biological Macromolecules*, vol. 93, pp. 1168–1173, 2016.

[3] D. L. Nelson and M. M. Cox, Lehninger Principles of Biochemistry, 5th edition. W. H. Freeman and Co. N. Y., 2008.

[4] A. N. Silva-Pinto, I. L. Angulo, D. M. Brunetta, F. I. R. Neves, S. C. Bassi, G. C. De Santis, D. T. Covas, "Clinical and hematological effects of hydroxyurea therapy in sickle cell patients: a single-center experience in Brazil," *Sao Paulo Medical Journal*, vol. 131, pp. 238–243, 2013.

[5] T. N. Williams, "Sickle cell disease in sub-Saharan Africa," *Hematology/Oncology Clinics of North America*, vol. 30, no. 8, pp. 343 – 358, 2016.

[6]. J. Howard, and P. Telfer, "Sickle Cell Disease in Clinical Practice," London 2015: Springer-Verlag, 2015.

[7] S. H. Embury, R. P. Hebbel, N. Mohandas, and M. H. Steinberg, "Sickle Cell Disease: Basic Principles and Clinical Practice," Raven Press, New York, 1994.

[8] F. B. Piel, A. P. Patil, R. E. Howes, O. A. Nyangiri, P. W. Getting, M. Dewi, W. H. Temperley, T. N. Williams, D. J. Weatherall, and S. I. Hay, "Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates," *Lancet*, vol. 381, pp. 142–51, 2013.

[9] S. L. Saraf, R. E. Molokie, M. Nouraie, C. A. Sable, L. Luchtman-Jones, G. J. Ensing, A. D. Campbell, S. R. Rana, X. M. Niu, R. F. Machado, M. T. Gladwin, and V. R. Gordeuk, "Differences in the clinical and genotypic presentation of sickle cell disease around the world," *Paediatric Respiratory Reviews*, vol. 15, pp. 4–12, 2014.

[10] C. M. Gonçalves, G. C. Bomfim, E. Maciel, I. Cerqueira, I. Lyra, A. Zanette, G. Bomfim, E. V. Adorno, A. L. Albuquerque, A. Pontes, M. F. Dupuit, G. B. Fernandes, and M. G. dos Reis, "ßS-Haplotypes in sickle cell anemia patients from Salvador, Bahia, Northeastern Brazil," *Brazilian Journal of Medical and Biological Research*, vol. 36, pp. 1283 – 1288, 2003.

[11] L. G. Lervolino, P. E. Baldin, S. M. Picado, K. B. Calil, A. A. Viel, and L. A. Campos, "Prevalence of sickle cell disease and sickle cell trait in national neonatal screening studies," *Revista Brasileira de Hematologia e Hemoterapia*, vol. 33, no. 1, pp. 49-54, 2011.

[12] C. M. Moore, M. Ehlayel, L. E. Leiva, and R. U. Sorensen, "New concepts in the immunology of sickle cell disease," *Annals of Allergy, Asthma and Immunology*, vol. 76, pp. 385–400, 1996.

[13] I. C. J. Bandeira, L. B. S. Rocha, M. C. Barbosa, D. B. D. Elias, J. A. N. Querioz, M. V. C. Freitas, and R. P. Gonçalves, "Chronic inflammatory state in sickle cell anemia patients is associated with HBB*S haplotype," *Cytokine*, vol. 65, pp. 217-221, 2014.

[14] G. J. Dover, and O. S. Platt, "Sickle cell disease. In: Nathan DG, Orkin SH, editors. Nathan and Oiski's hematology of infancy and childhood," Philadelphia: WB Saunders Co., pp. 762-810, 1998.

[15] S. J. Nevitt, A. P. Jones, and J. Howard, "Hydroxyurea (hydroxycarbamide) for sickle cell disease," *Cochrane Database of Systematic Reviews*, issue 4, Art. No.: CD002202, 2017.

[16] O. S. Platt, "Hydroxyurea for the treatment of sickle cell anemia," *New England Journal of Medicine*, vol. 358, pp. 1362–1369, 2008.

[17] J. L. Spivak, and H. Hasselbalch, "Hydroxycarbamide: a user's guide for chronic myeloproliferative disorders," *Expert Review of Anticancer Therapy*, vol. 11, no. 3, pp. 403-414, 2011.

[18] J. G. Taylor, D. S. Darari, I. Maric, Z. McIver, and D. C. Arthur, "Therapy-Related Acute Myelogenous Leukemia in a Hydroxyurea-Treated Patient With Sickle Cell Anemia," *Annals of Internal Medicine*, vol. 155, no. 10, pp. 722-724, 2011.

[19] H. Fathallah, and G. F. Atweh, "Induction of fetal hemoglobin in the treatment of sickle cell disease," *Hematology*, vol. 2006, no. 1, pp. 58-62, 2006.

[20] D. K. Shome, A. Al Ajmi, A. A. Radhi, E. J. Mansoor, and K. S. Majed, "The Effect of Hydroxyurea Therapy in Bahraini Sickle Cell Disease Patients," *Indian Journal of Hematology and Blood Transfusion*, vol. 32, no. 1, pp. 104-109, 2016.

[21] U. P. Isichei, "Liver function and the diagnosis significance of biochemical changes in the blood of African children with sickle cell disease," *Journal of Clinical Pathology*, vol. 33, pp. 626-630, 1980.

[22] J. S. Hankins, R. E. Ware, Z. R. Rogers, L. W. Wynn, P. A. Lane, J. P. Scott, and W. C. Wang, "Long-term hydroxyurea therapy for infants with sickle cell anemia: the HUSOFT extension study," *Blood*, vol. 106, no. 7, pp. 2269 – 2275, 2005.

[23] S. Charache, "Experimental therapy of sickle cell disease; use of hydroxyurea," *The American Journal of Pediatric Hematololgy/Oncology*, vol. 16, pp. 62–66, 1994.

[24] J. Watson, "The significance of the paucity of sickle cells in newborn Negro infants," *The American Journal of the Medical Sciences*, vol. 215, pp. 419–423, 1948.

[25] S. C. Davies and A. Gilmore, "The role of hydroxyurea in the management of sickle cell disease," *Blood Reviews*, vol. 17, pp. 99–109, 2003.

[26] M. H. Steinberg, Z. H. Lu, F. B. Barton, M. L. Terrin, S. Charache, and G. J. Dover, "Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea," *Blood*, vol. 89, no. 3, pp. 1078–1088, 1997.

[27] C. D. Thornburg, A. Calatroni, M. Telen, and A. R. Kemper, "Adherence to Hydroxyurea Therapy in Children with sickle cell anemia," *Journal of Pediatrics*, vol. 156, pp. 415–419, 2010.

[28] S. Charache, M. L. Terrin, R. D. Moore, G. J. Dover, F. B. Barton, S. V. Eckert, R. P. Mcmahon, and D. R. Bonds, "Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. investigators of the multicenter study of hydroxyurea in sickle cell anemia," *New England Journal of Medicine*, vol. 332, no. 20, pp. 1317-1322, 1995.

[29] A. Ferster, C. Vermylen, M. Buyse, F. Corazza, C. Devalck, P. Fondu, M. Toppet, and E. Sariban, "Hydroxyurea for treatment of severe sickle cell anemia: a pediatric clinical trial," *Blood*, vol. 88, no. 6, pp. 1960-1964, 1996.

[30] S. Charache, F. B. Barton, R. D. Moore, M. L. Terrin, M. H. Steinberg, G. J. Dover, S. K. Ballas, R. P. McMahon, O. Castro, and E. P. Orringer, "Hydroxyurea and sickle cell anemia. Clinical utility of a myelosuppressive ''switching'' agent," The Multicenter Study of Hydroxyurea in Sickle Cell Anemia, *Medicine*, vol. 75, no. 6, pp. 300 – 326, 1996.

[31] O. S. Platt, D. J. Brambilla, W. F. Rosse, P. F. Milner, O. Castro, M. H. Steinberg, and P. P. Klug, "Mortality in sickle cell disease. Life expectancy and risk factors for early death," *New England Journal of Medicine*, vol. 330, pp. 1639–1644, 1994.

[32] G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre, "Endothelial cell interactions with granulocytes: tethering and signalling molecules," *Immunology Today*, vol. 13, pp. 93–100, 1992.

[33] J. S. Warren and P. A. Ward, The inflammatory response. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, eds. Williams Hematology, sixth ed. New York: Mc Graw-Hill; 67–75, 2001.

[34] G. Weissmann, J. E. Smolen, and S. Hoffstein, "Polymorphonuclear leukocytes as secretory organs of inflammation," *Journal of Investigative Dermatology*, vol.71, pp. 95–99, 1978.

[35] T. C. Hofstra, V. K Kalra, B. J. Meiselman, and T. D. Coates, "Sickle erythrocytes adhere to polymorphonuclear neutrophils and activate the neutrophil respiratory burst," *Blood*, vol. 87, pp. 4440–4447, 1996.

[36] K. Larade and K. B. Storey, "Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate," *Journal of Experimental Biology*, vol. 207, pp. 1353-1360, 2004.

[37] D. Lau, H. Mollnau, J. P. Eiserich, B. A. Freeman, A. Daiber, U. M. Gehling, J. Brümmer, V. Rudolph, T. Münzel, T. Heitzer, T. Meinertz, and S. Baldus, "Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins," *Proceedings of the National Academy of Science* U S A, vol. 102, pp. 431-436, 2005.

[38] E. Nur, E. J. van Beers, S. Martina, I. Cuccovillo, H. M. Otten, J. J. Schnog, J. C. Meijers, A. Mantovani, D. P. Brandjes, B. Bottazzi, and B. J. Biemond, "Plasma levels of pentraxin-3, an acute phase protein, are increased during sickle cell painful crisis," *Blood Cells, Molecules and Diseases*, vol. 46, no. 3, pp. 189-94, 2011.

[39] F. Traina, S. G. Jorge, A. Yamanaka, L. R. de Meirelles, F. F. Costa, and S. T. Saad, "Chronic liver abnormalities in sickle cell disease: a clinicopathological study in 70 living patients," *Acta Haematologica*, vol. 118, no. 3, pp. 129–135, 2007.

[40] H. Ahn, C. S. Li, and W. Wang, "Sickle cell hepatopathy: clinical presentation, treatment, and outcome in pediatric and adult patients," *Pediatric Blood & Cancer*, vol. 45, no. 2, pp. 184–190, 2005.

[41] J. Koskinas, E. K. Manesis, G. H. Zacharakis, N. Galiatsatos, N. Sevastos, and A. J. Archimandritis, "Liver involvement in acute vaso-occlusive crisis of sickle cell disease: prevalence and predisposing factors," *Scandinavian Journal of Gastroenterology*, vol.42, no. 4, pp. 499–507, 2007.

[42] E. Voskaridou, D. Christoulas, A. Bilalis, E. Plata, K. Varvagiannis, G. Stamatopoulos, K. Sinopoulou, A. Balassopoulou, D. Loukopoulos, and E. Terpos, "The effect of prolonged administration of hydroxyurea on morbidity and mortality in adult patients with sickle cell syndromes: results of a 17-year, single-center trial (LaSHS)," *Blood*, vol. 115, pp. 2354–2363, 2010.

[43] T. R. Kinney, R. W. Helms, E. E. O'Branski, K. Ohene-Frempong, W. Wang, C. Daeschner, E. Vichinsky, R. Redding-Lallinger, B. Gee, O. S. Platt, and R. E. Ware, "Safety of Hydroxyurea in Children With Sickle Cell Anemia: Results of the HUG-KIDS Study, a Phase I/II Trial," *Blood*, vol. 94, no. 5, 1550 –1554, 1999.

[44] M. Samra, and A. C. Abcar, "False estimates of elevated creatinine," *The Permanente Journal*, vol. 16, no. 2, 51 – 52, 2012.

[45] R. P. Paula, A. F. Nascimento, S. M. B. Souza, P. R. V. Bastos, and A. A. L. Barbosa, "Glomerular filtration rate is altered in children with sickle cell disease: a comparison between Hb SS and Hb SC," *Revista Brasileira de Hematologia e Hemoterapia*, vol. 35, no. 5, 349 – 351, 2013.

[46] K. I. Ataga, A. Hinderliter, J. E. Brittain, S. Jones, H. Xu, J. Cai, S. Kim, K. A. Pritchard Jr, and C. A. Hillery, "Association of Pro-Inflammatory High Density Lipoprotein Cholesterol with Clinical and Laboratory Variables in Sickle Cell Disease," *Hematology*, vol. 20, no. 5, pp. 289–296, 2015.

[47] M. O. Seixas, L. C. Rocha, M. B. Carvalho, J. F. Menezes, I. M. Lyra, V. M. L. Nascimento, R. D. Couto, A. M. Atta, M. G. Reis, and M. S. Goncalves, "Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease," *Lipids in Health and Disease*, vol. 9, pp. 1–9, 2010.

4.3 CAPÍTULO III

Manuscrito intitulado "*Association of serum level of N(epsilon)-(carboxymethyl)lysine with hydroxyurea treatment, laboratory parameters and β^S-globin haplotypes on pediatric sickle cell anemia"*

O trabalho descreve o efeito da HU em pacientes com AF e o papel da CML no tratamento com HU, bem como sua relação/associação com parâmetros laboratoriais e haplótipos ligados ao gene da globina β^S.

Status do manuscrito: Submetido para publicação no periódico "*British Journal of Haematology*".

Association of serum level of *N*(epsilon)-(carboxymethyl)lysine with hydroxyurea treatment, laboratory parameters and β^S-globin haplotypes on pediatric sickle cell anemia

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Summary

Advanced glycation end products (AGEs), such as N^{ε}-carboxymethyllysine (CML), are well known markers of oxidative stress, which are common occurrence in sickle cell anemia (SCA). The aim of the present study was to determine the association of CML with laboratory parameters and β^S globin gene cluster haplotypes on pediatrics SCA patients treated or not treated with hydroxyurea (HU). Children with SCA (SCA_{total}, n=55) on HU treatment (HU^+ SCA, n = 27) and without HU treatment (HU^- SCA, n = 28) were recruited. Laboratory characteristics were determined using standard measurements. The β^S globin gene haplotypes were determined by RFLP-PCR while CML was measured using competitive ELISA technique. Our results show that MCV (p = 0.003) and MCH (p = 002) increased significantly in HU^+ SCA compared to HU^- SCA. There were significant decreases in leukocytes (p = 0.014), eosinophils (p = 0.017), basophils (p = 0.041), atypical lymphocytes (p = 0.014) lymphocytes (p = 0.033) and monocytes (p = 0.041) in HU^+ SCA compared to HU^- SCA. Aspartate transaminase (p = 0.001) and lactate dehydrogenase ($p < 0.001$) were significantly lowered in the HU^+ SCA group compared to the HU^- SCA while HU^+ SCA has significantly higher level of creatinine compared to HU^- SCA (p = 0.004). CML levels were significantly higher in both HU^+ SCA ($p < 0.0001$) and HU^- SCA ($p <$ 0.0001) compared to the healthy control. Our results also show that HU^+ SCA (r = -0.6126; p = 0.0007) and SCA_{total} (r = -0.3484; p = 0.0092) have significant inverse correlation between CML and alanine transaminase. The p value (0.048) for χ^2 analyses suggests an association between CML and haplotypes. It further showed that CML has a role to play in SCA complications that is not influenced by HU treatment.

Keywords: N^{ε}-(carboxymethyl)lysine (CML), sickle cell anemia (SCA), alanine transaminase, haplotype.

Introduction

Sickle cell anemia (SCA) is a monogenic hematological disorder caused by an autosomal recessive inheritance of two variant β^S alleles, where the GAG codon is replaced by GTG (A→T) at the 6th codon of the beta globin gene (*HBB*) located in chromosome 11, giving rise to the hemoglobin S (HbS) (Lettre and Bauer, 2016).

Although all SCA patients have the same genetic origin, they show wide variability in clinical manifestations including variations in its impact on the functions of vital organs as well as frequency and severity of vaso-occlusive crises (Raghupathy *et al*, 2000; Zago & Pinto, 2007). Several factors which could be responsible for the difference in clinical features of the disease include variations in the fetal hemoglobin (HbF) levels, coexistence of alpha (α) thalassemia, haplotypes associated with the β^S globin gene, oxidative stress, features intrinsic to the red blood cell (RBC) and extracellular environment (Figueiredo, 2007; Steinberg & Brugnara, 2003). Studies have shown that increased concentrations of HbF are associated with improved survival, decreased rates of painful crises, acute chest syndrome and osteonecrosis (Castro *et al*, 1994; Milner *et al*, 1991; Platt *et al*, 1991; Platt *et al*, 1994). Study by Rumaney *et al* (2014) among Cameroonian SCA patients showed that co-inheritance with α-thalassemia is associated with improved hematological indexes and lowered consultation rate in the patients. The β^S-globin has different haplotypes based on their origin: the Senegal (SEN), Cameroon (CAM), Bantu or Central African Republic (CAR), Benin (BEN), Arab-Indian (ARAB) and more recently atypical (AT) haplotype. Studies have shown that the Bantu or CAR haplotype is associated with the most severe clinical features (Fleury, 2007; Silva *et al*, 2009).

The clinical manifestations of SCA result from the polymerization of HbS in the deoxygenated state that predisposes erythrocytes to adopting a sickled conformation. The disease is characterized by vaso-occlusion, stroke, hemolytic anemia, increased infection and ischemic organ damage (Belcher *et al*, 2000; Embury *et al*, 1994; Laurentino *et al*, 2014). The most frequent complication of the disease is acute painful episodes otherwise called vaso-occlusive crises and is the major cause of frequent hospitalization. Auto-oxidation of the HbS along with chronic intravascular hemolysis and ischemia is known to increase the generation of reactive oxygen species (ROS) that potentiates oxidative stress (van Beers *et al*, 2008). Steinberg & Brugnara (2003) reported that the severity of vaso-occlusion as well as organ ischemia is mediated by oxidative stress and other features intrinsic to RBC and its extracellular environment. Advanced glycation end products (AGEs), such as N^{ε}-carboxymethyllysine (CML), are well known markers of oxidative stress (Gerrits *et al*, 2008). They have been implicated in the pathophysiology of organ complications in diabetes, atherosclerosis, ageing, Alzheimer's disease and autoimmune inflammatory disease (Baynes, 2001; Nur *et al*, 2010; Peppa *et al*, 2004; Picklo *et al*, 2002).

Advanced glycation end products (AGEs) are a complex group of compounds formed through non-enzymatic reactions between reducing sugars (or their derivatives like glucose-6-phosphate) and N-terminal amino groups of arginine and lysine side chains on proteins, lipids, and nucleic acids. AGEs produce one chemical outcome: the formation of covalent cross-links between proteins that may be one of the central underlying processes by which they cause damage (Singh *et al*, 2001). CML is the most abundant and the most researched advanced glycation product (Tessier et al, 2014). Studies have already shown that CML modification is involved in normal aging, as well as in the pathogeneses of several age-enhanced diseases such as diabetic nephropathy, atherosclerosis, diabetic retinopathy, hemodialysis-associated amyloidosis, chronic renal failure, and Alzheimer's disease (Araki *et al*, 1992; Hammes *et al*, 1996; Imai *et al*, 1997; Kume *et al*, 1995; Miyata *et al*, 1998; Smith *et al*, 1995; Yamada *et al*, 1994). The works of Nur *et al* (2010) and Somjee *et al* (2004) are the only known studies that tried relating AGEs with SCD. Both studies reported increase in AGEs in SCDs compared to the control patients. The results of Somjee *et al* (2004) suggest that circulating levels of AGEs may play a role in the vascular pathology of SCA while the work of Nur *et al* (2010) suggests that AGEs may also play a significant role in the pathophysiology of the hemolytic phenotype of SCD and could be implicated in hemolysis-related organ complications such as priapism, leg ulcer, ischemic strokes and pulmonary hypertension.

Currently, hydroxyurea (HU), the only agent approved for the management of SCD by the Food and Drug Administration (FDA) in 1998, is the most common therapy for SCA patients (Nevitt *et al*, 2017; Platt, 2008). Reports suggest that HU is a relatively well-tolerated cytotoxic drug (ribonucleotide reductase inhibitor) with limited side effect in the short term; though there are concerns over its long-term effect on male fertility (Grigg, 2007). The targets and mechanisms by which HU ameliorates clinical complications of SCD remain subject of research to this day (Hagar & Vichinsky, 2008; Stuart & Nagel, 2004; Steinberg, 2006; Wang, 2007). The efficacy of HU was initially attributed to pharmacological stimulation of HbF, but the fact that clinical benefits occur before its rise suggests that HU could act through other mechanisms (Bridges *et al*, 1996; Green & Barral, 2014). Therefore, understanding the genetic and other factors underlying the variability in the therapeutic effects of HU is critical for prospectively predicting good responders and for designing other effective therapies.

Having established the role of CML in the pathology of several diseases, the ameliorating effect of hydroxyurea on SCA patients as well as the increasing prevalence of the disease due to migration, this work was designed to evaluate the possible association of CML with laboratory parameters and βS haplotypes in sickle cell anemia patients with or without HU treatment.

2. Materials and Methods

2.1 Subjects. In this transversal cohort study, 55 SCA patients (HbSS) of both sexes from the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), Salvador, Bahia, Brazil were recruited. The subjects were divided into two groups: (i) 27 children treated with HU (15 – 25

mg/kg/day) (HU^+ SCA group) and (ii) 28 children not treated with HU (HU^- SCA). In addition to this, 30 normal healthy age- and sex-matched individuals (HbAA) were recruited as volunteers from the Clinical Laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (UFBA). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. The study was carried out between August 2015 and August 2017 and the protocol was submitted to and approved by the Fundação Oswaldo Cruz (FIOCRUZ) Ethics research board (ID 1.400.527). All patients with SCA included in the study answer an epidemiological inquiry questionnaire. The response was from the patient or the parents/guardian in the case of children or teenager (less than 18). Patients were in the steady-state of the disease when samples were collected. Steady state is described as a period without any acute events and no blood transfusion for 120 days prior to blood sampling.

2.2 Standard Laboratory Tests. Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain). Serum ferritin was measured by immunoassay using an Access® 2 Immunoassay system X2 (Beckman Coulter, Fullerton, CA, USA). C-reactive protein (CRP), alpha 1-antitrypsin (AAT) and antistreptolysin-O (ASO) were measured by immunochemistry (Immage® 800 system, Beckman Coulter, Fullerton, CA, USA). Hematological analyses were carried out using an automated cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC/VARIANT I; BIO-RAD, CA, USA). Nitric oxide (NO) was indirectly quantified through nitrite quantification by colorimetric method at 540nm (Grisham *et al*, 1996).

2.3 Immunoanalysis of N^{ε}-carboxymethyllysine (CML). The detection and quantitative estimation of CML was carried out in the blood serum of the patients using an OxiSelect™ CML ELISA Kit according to the manufacturer's instructions (Cell Biolabs, Inc., CA, USA). The amount of CML in ng/mL was determined by comparing its absorbance with that of a known CML-BSA standard curve. The absorbance results were read on a microplate reader (STAT FAX® 2100) at 450nm.

2.4 Haplotypes analysis. First, the DNA was extracted from leukocytes of patients following instructions of the commercial DNA isolation kit [Puregene blood kit (Qiagen, USA)]. The analysis of β^S globin gene cluster haplotypes was performed by restriction fragment length polymorphism – polymerase chain reaction (PCR – RFLP) with analysis of 7 polymorphic restriction sites performed according to the method of Sutton *et al* (1989): 5′γ^{G-}*Xmn* I, γ^{G-}*Hin*d III, γ^{A--}*Hin*d III, ψβ-*Hin*c II, 3′ψβ-*Hin*c II and 5′β–*Hin*f I, 3′ β-*Hpa* I.

3. Statistical Analysis

Descriptive statistics were summarized with median and 25^{th} – 75^{th} percentile for continuous variable. The data for each variable and for each group were tested for normality (Shapiro-Wilk and Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) prior to analysis. The Mann-Whitney (non-parametric) and independent T-test (parametric) were employed for the

analysis of two numerical variables, comparing two groups of values within the same variable, taking into account the distribution of each variable. Analysis of variance (ANOVA) followed by post hoc multiple range comparison where differences in means were observed was applied for more than two numerical (or quantitative) variables. For correlation studies between all laboratory parameters and CML, the Spearman's rank correlation coefficient was employed and the differences between the groups were considered significant when $P < 0.05$ or $P < 0.01$. Linear regression was carried out where a significant difference was detected. Statistical analyses were performed using GraphPad Prism 7.0 and SPSS version 20.0 (SPSS 20.0, Chicago, IL, USA). Values of $P < 0.05$ were considered significant for the analyses performed.

4. Results

This research evaluated the effect of CML on laboratory parameters (hematological and biochemical), β^S globin gene cluster haplotypes and HU treatment in pediatric SCA patients. The hematological and biochemical parameter were presented as median and $25^{th} – 75^{th}$ percentiles of SCA patients on HU treatment (HU^+ SCA) in comparison with SCA patients without HU treatment (HU^- SCA). The median age and $25^{th} – 75^{th}$ percentile for HU^+ SCA were 7 and 5 – 12 years respectively. The median age and $25^{th} – 75^{th}$ percentile for HU^- SCA were 6 and 4 – 9 years respectively. The HU^+ SCA and HU^- SCA have median body mass index (BMI) ($25^{th} – 75^{th}$ percentile) of 15.6 (15 – 17.3) and 14.95 (14.25 – 15.85) respectively. There were no statistically significant differences between the median ages and BMIs of HU^+ SCA compared to HU^- SCA.

Table 1 presents the hematological characteristics of HU^+ SCA and HU^- SCA. Our results showed that there was no statistical significance for about half of the parameters. From the table, increases in the RBC count (*p*-value = 0.533), and concentrations of Hb (*p*-value = 0.337) and hematocrit (*p*-value = 0.522) were not significant between HU^+ SCA and HU^- SCA. There were also no significant differences between mean corpuscular hemoglobin concentration (MCHC) (*p*-value = 0.890), red cell distribution width (RDW) (*p*-value = 0.398), reticulocytes count (*p*-value = 0.728) in HU^+ SCA and HU^- SCA. However, mean corpuscular volume (MCV) (*p*-value = 0.003) and mean corpuscular hemoglobin (MCH) (*p*-value = 0.002) increased significantly in HU^+ SCA compared to HU^- SCA. There were significant decreases in leukocytes count (*p*-value = 0.014), eosinophils (*p*-value = 0.017), basophils (*p*-value = 0.041), atypical lymphocytes (*p*-value = 0.014) lymphocytes (*p*-value = 0.033) and monocytes (*p*-value = 0.041) in HU^+ SCA compared to HU^- SCA. Though we have a decrease in neutrophils (*p*-value = 0.250) in HU^+ SCA group compared to HU^- SCA group, it was not significant. Platelets (*p*-value = 0.610), mean platelet volume (MPV) (*p*-value = 0.856), HbS (*p*-value = 0.286), HbF (*p*-value = 0.072), HbA_2 (*p*-value = 0.078) show no significant difference between HU^+ SCA and HU^- SCA.

Table 2 shows the biochemical characteristics of HU^+ SCA as against HU^- SCA. There is no significant difference between glucose in HU^+ SCA and HU^- SCA (*p*-value = 0.089). The lipid profile [total cholesterol (*p*-value = 0.637), high-density lipoprotein (HDL)-cholesterol (*p*-value = 0.827), low-density lipoprotein (LDL)-cholesterol (*p*-value = 0.479), very low-density lipoprotein

(VLDL)-cholesterol (*p*-value = 0.381), and triglyceride (*p*-value = 0.381)] show no significant differences between HU^+ SCA and HU^- SCA. Total bilirubin (*p*-value = 0.350), direct bilirubin (*p*-value = 0.730), indirect bilirubin (*p*-value = 0.239) as well as total protein (*p*-value = 0.849), albumin (*p*-value = 0.582), globulin (*p*-value = 0.735), urea (*p*-value = 0.468), C-reactive protein (*p*-value = 0.246), albumin-globulin ratio (*p*-value = 0.754), all present no significant differences between HU^+ SCA and HU^- SCA. Aspartate transaminase (AST) (*p*-value = 0.001) and lactate dehydrogenase (LDH) (*p*-value < 0.001) were significantly lowered in the HU^+ SCA group compared to the HU^- SCA. Alanine transaminase (ALT) (*p*-value = 0.873) and AAT (*p*-value = 0.331) have lower values in HU^+ SCA compared to HU^- SCA but the values were not significant. Nitric oxide (NO) (*p*-value = 0.755) and serum iron (*p*-value = 0.065) levels were not significant for HU^+ SCA compared to HU^- SCA while HU^+ SCA has significantly higher level of creatinine compared to HU^- SCA (*p*-value = 0.004).

Figure 1 presents the analysis of variance (ANOVA) comparison of serum levels of carboxymethyllysine (CML) in healthy control (HbAA), HU^+ SCA and HU^- SCA as well as an unpaired T-test comparison of all SCA patients (SCA_{total}) against HbAA. Figure 1A shows that CML levels are significantly higher in both HU^+ SCA (*p*-value < 0.0001) and HU^- SCA (*p*-value < 0.0001) compared to the healthy control. However, there is no significant difference (*p*-value = 0.3104) in the level of CML between HU^+ SCA and HU^- SCA. Since there is no significant difference between HU^+ SCA and HU^- SCA, we pulled the two (2) groups together (SCA_{total}) (Figure 1B) and compared their CML concentration against HbAA. The result shows that CML levels in SCA_{total} was significantly higher ($p < 0.0001$) compared to the healthy control.

We also carried out univariate correlation analyses of all laboratory parameters against CML (data not shown) for HU^+ SCA, HU^- SCA and SCA_{total}. Our findings showed that only ALT has inverse correlation with CML. Figure 2A, B and C showed the linear regression curve of HU^+ SCA, HU^- SCA and SCA_{total} respectively. From the graphs, we found out that HU^+ SCA (*r* = -0.6126; *p*-value = 0.0007) and SCA_{total} (*r* = -0.3484; *p*-value = 0.0092) have significant inverse correlation between CML and ALT while HU^- SCA presents no significant relationship between CML and ALT (*r* = -0.1395; *p*-value = 0.4790).

Table 3A – C shows the chi (χ^2)-square analyses of the various haplotypes of SCA_{total}, HU^+ SCA and HU^- SCA according to whether the patients have CML values ≤ median or CML value > median. We observed an association ($p = 0.048$) between high or low serum CML values with different haplotypes in SCA_{total} group (Table 3A). Table 3A showed that 61.5% of the haplotypes have CML values above the median (239.33 ng/mL). Bantu/Benin (30.8%) haplotypes have significantly higher CML values compared to Bantu/Bantu (15.4%), Benin/Benin (15.4%) and Bantu/Atypical. Table 3A further indicates that the calculated χ^2 for 3 degrees of freedom is 7.909. Table 3B showed that the calculated χ^2 for 2 degrees of freedom is 1.929 while the p value of 0.381 indicates that there is no association between CML and haplotypes for HU^+ SCA. Similarly Table 3C indicates that there is no association ($\chi^2 = 2.619$; $p = 0.454$) between CML and haplotypes for HU^- SCA.

5. Discussion

This research explored the potential effect of CML, an AGE, on SCA patients, evaluating its possible association with laboratories parameters, the potential effect of HU treatment on CML and a possible association with the different types of β^S haplotypes.

Hemoglobin and hematocrit are markers of hemolysis. However, our report showed that the level of Hb and hematocrit did not significantly improve in HU^+ SCA that is in agreement with the work of Voskaridou *et al* (2010). However, our report differ from the reports of Laurentino et al (2014) and Silva-Pinto *et al* (2013) that showed significant difference between the Hb and hematocrit values in HU^+ SCA and HU^- SCA groups even though the values for HU^+ SCA are similar in both reports (our report has 8.70 and 24.9 while theirs has 8.93 g/dL and 25.85% for Hb and hematocrit respectively). The works of Laurentino *et al* (2014) showed no significant change in MCHC, MCV, MCH and platelet unlike our work that showed significant increase in both MCV and MCH but no significant increase in MCHC and platelet. MCV along with MCH and MCHC is part of RBC indices that are measurements and/or calculations for determining hemolysis. A significant increase in these values shows an improvement in the RBC count.

Watson (1948) was the first to suggest that HbF could be responsible for the lack of clinical symptoms in newborns with SCD. Since then, HbF has been recognized as among the most important known modifiers of the clinical course of SCD. However, the HbF level did not significantly increase in HU^+ SCA in our research which is in agreement with the report of Steinberg *et al* (1997) in which the increase in HbF was not significant in a Multicenter Study of Hhydroxyurea (MSH). The findings of Silva-Pinto *et al* (2013) disagree with our findings. Nevertheless, the insignificant increment in HbF in HU^+ SCA could be attributed to irregular adherence to the daily dosage by some of the respondents (Thornburg *et al*, 2010), variation in the length of HU treatment (Nevitt *et al*, 2017), lack of good bone marrow reserve and/or patients on maximum tolerated dose (Davies and Gilmore, 2003).

White blood cell (WBC), neutrophils, monocytes and eosinophils are known to activate the endothelial cells (Nevitt *et al*, 2017). WBC, eosinophils, basophils, monocytes, atypical and typical lymphocytes counts were significantly reduced in patients receiving HU suggesting a reduction in vaso-occlusive crisis and general pain in the body (Charache *et al*, 1995; Ferster *et al*, 1996). Our results also agree with the works of Davies and Gilmore (2003) and Silva-Pinto *et al* (2013). Zimmerman *et al* (2004) suggested that neutrophils adhere to vascular endothelium thereby potentially impairing the smooth flow of the sickle cells. Neutrophils adherence to vascular endothelium has also been reported to cause increase in whole blood viscosity and release of cytokines that are known to be involved in inflammatory response including pain pathways (Warren & Ward, 2001).

Creatinine is the most commonly used endogenous marker of renal function. A reduction in the glomerular filtration rate (GFR) is usually associated with elevation in the serum creatinine level

(Samra & Abcar, 2012). However, in SCD, renal failure is usually preceded by subclinical glomerular hyperfiltration (Paula *et al*, 2013). Therefore, elevated creatinine level, as we observed in HU^+ SCA, is not always representative of a true reduction in GFR due to decreased tubular secretion caused by hyperfiltration (Paula *et al*, 2013). Drugs such as trimethoprim, cimetidine and other H_2-blockers have been reported to inhibit tubular secretion of creatinine thereby causing an increase in serum creatinine level (Berg *et al*, 1989; Kemperman *et al*, 2000) and this could also be the case with HU.

Our results show that there is no significant difference between the lipid profile of the HU^+ SCA and HU^- SCA. This could suggest that HU does not play a role in lipid metabolism of SCA patients. There is no work to the best of our knowledge that compared HU treated SCA group and non-HU treated group. However, in a work by Ataga *et al* (2015), it was reported that there is no statistically significant difference between the levels of HDL in SCD patients and healthy control subjects. The study also reported that the median level of total cholesterol lowered significantly in SCD subjects compared to healthy control subjects (Ataga *et al*, 2015). Seixas *et al* (2010) also reported a significant decrease in total cholesterol but in addition reported a significant decrease in HDL-cholesterol and LDL-cholesterol in SCD patients compared to healthy control and these support earlier reports of incidence of hypocholesterolemia in SCD patients (Shores *et al*, 2003; VanderJagt *et al*, 2002). We found no significant change in the glucose level too and like the lipid profile, little is known about the relationship between glucose in SCD subjects. Report by Morrison *et al* (1979) is the only work that tried relating glucose in diabetic patients to SCD. They found out that there is low prevalence of diabetes mellitus in SCD subjects.

Serum iron, ferritin, ALT, total protein, total bilirubin, indirect bilirubin, globulin, albumin, albumin-globulin ratio, NO, AAT, urea, CRP, all show no significant difference between HU^+ SCA and HU^- SCA subjects. CRP, AAT and ferritin are markers of inflammation. Our findings suggest that HU did not affect the inflammatory response of the patients on HU treatment. This is at variance with previous reports (Okocha *et al*, 2014; Brownell *et al*, 1986). AST, ALT, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, globulin, albumin/globulin ratio, serum iron and LDH are markers of liver function. However, only LDH and AST showed significant decrease in HU^+ SCA compared to HU^- SCA suggesting that HU has the potential to reduce chronic liver damage as well as hemolysis since they are both equally markers of hemolysis. Our results are in agreement with the results of other works that reported improvement in hepatic markers such as LDH and AST (Traina *et al*, 2007; Voskaridou *et al*, 2010).

Decrease in bioavailability of NO has been suggested to play a role in the pathogenesis of pulmonary hypertension in SCA (Wood *et al*, 2008). Hemolysis due to SCA is believed to lead to NO scavenging and degradation of L-arginine, an NO precursor, by plasma arginase (Akinsheye & Klings, 2010). Our result, however, did not present a significant change in the level of NO, measured indirectly by serum nitrite (a storage pool for NO), between HU^+ SCA and HU^- SCA. This suggests that HU did not improve the susceptibility of subjects to pulmonary hypertension.

However, the works of Gladwin *et al* (2002) suggested that NO increased significantly in subjects on HU.

Reports have already shown that high level of circulating CML is involved in the pathogenesis of several age-enhanced diseases such as diabetic nephropathy, atherosclerosis, diabetic retinopathy, hemodialysis-associated amyloidosis, chronic renal failure, and Alzheimer's disease (Araki *et al*, 1992; Hammes *et al*, 1996; Imai *et al*, 1997; Kume *et al*, 1995; Miyata *et al*, 1998). In our study, the CML, a biomarker of oxidative stress, was significantly higher in both HU^+ SCA and HU^- SCA compared to the healthy control group. This report is in agreement with the works of Somjee *et al* (2004) and Nur *et al* (2010) that revealed significant increase in CML in SCA patients compared to healthy control and suggests that CML may also have a role in the chronic vascular pathology of SCA (Somjee *et al*, 2004), especially since it has been severally reported that it has obvious role in the etiology of chronic microvascular complications in diabetes and other diseases (Imai *et al*, 1997; Kume *et al*, 1995). Our results further revealed that there is no significant difference between CML in HU^+ SCA and HU^- SCA. Nur *et al* (2010) also reported no significant difference between HU^+ SCA and HU^- SCA even though the subjects in the report are adults compared to our report that are pediatric subjects. This could mean that HU may play no role (or have no effect) on the level of CML in SCA patients. This is an interesting revelation because CML has already been identified as a marker of oxidative stress (Nur *et al*, 2010; Somjee *et al*, 2004) and oxidative stress markers such as thiobarbituric acid reactive species (TBARS) have been reported to be significantly higher in SCA patients without HU treatment compared to patients on HU treatment (Torres *et al*, 2012). Perhaps the absence of significant difference could be as a result of the age of the subjects (children in this case), irregular adherence to the daily dosage by some of the subjects, variation in the length of HU treatment (Nevitt *et al*, 2017; Thornburg *et al*, 2010), or because they are stable compounds compared to ROS. Age was included here as a factor even though the report of Nur *et al* (2010) was on adults because CML has been reported to increase with increasing age (Hammes et al, 1996; Imai *et al*, 1997).

Another factor that could have influenced the absence of significant difference in CML level between HU^+ SCA and HU^- SCA is the fact that CML as well as other AGEs are known to be influenced by both oxidative and non-oxidative processes (Hammes *et al*, 1996; Baynes, 2001; Somjee *et al*, 2004). Products of glycoxidation (as many AGEs are aptly considered) are also formed by lipid peroxidation, whose products are appropriately called advanced lipoxidation end products (ALEs). Therefore, CML can be formed by either AGE or ALE synthetic pathway (Baynes, 2001). Furthermore, lipid peroxidation is elevated in patients with SCA and is an important source of ROS (Belcher *et al*, 2000).

Elevated ALT and AST levels among other enzymes such as alkaline phosphatase and 5' nucleotidase are associated with liver damage. We carried out an extensive Spearman's rho correlation analysis of all hematological and biochemical parameters against CML but our findings revealed only ALT as having a significant inverse correlation with CML for HU^+ SCA and SCA_{total} subjects. Previous study on mice fed with AGE diet observed that ALT was higher in

mice on regular AGE diet than mice on high AGE diet (Patel *et al*, 2012). This tends to concur with our observation of an inverse correlation between CML and ALT. However, this finding appears strange considering that CML is a marker of oxidative stress that contributes to the initiation and progression of liver injury (Li *et al*, 2015). One possible explanation is that antioxidative mechanisms were activated in HU^+ SCA subjects which fight against the reactive oxygen species generated by high CML. Indeed, it has been reported that AGEs also induce glutathione S-transferase activity to function as an antioxidant (Lindenmeier *et al*, 2002). However, this may require further investigation as there is no study to the best of our knowledge that correlates CML with ALT in patients with SCA.

Our study further carried out a chi-square analysis that investigated the association between CML and haplotypes. The analysis shows that serum CML is significantly associated with differing βS haplotypes in SCA_{total}. To the best of our knowledge, no study investigated the probable influence of AGEs on varying haplotypes. This result, however, suggests that haplotypes could influence response of SCA patients to the pathological role of CML. Our report suggested that 61.5% of the subjects have high CML values (above median level) which further agree with our Figure 1 that suggests that CML increases in SCA patients. Subjects with Bantu/Benin haplotype were most associated with CML values above the median level compared to other haplotypes that we found among our respondents: Bantu/Bantu, Benin/Benin and Bantu/Atypical. We do not have Arab-Indian haplotype among our respondents. Previous report of Brazilian population did not discover Arab-Indian haplotype (Filho *et al*, 2011; Laurentino *et al*, 2014; Gonçalves *et al*, 2003; Camilo-Araújo *et al*, 2014). We also do not have Senegal haplotype among our subjects. Only the works of Gonçalves *et al* (2003) reported a minute presence of Senegal haplotype in Brazilian population.

Conclusion

In summary, CML has a role to play in SCA complications that seems not to be influenced by HU treatment. Furthermore, our report suggests an association between CML and haplotypes and that higher CML concentration tends to be more associated with the Bantu/Benin haplotype.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa and Adekunle Adekile conceived the study design. Rayra Pereira Santiago, Uche Samuel Ndidi, Corynne Stephanie Ahouefa Adanho, Setondji Cocou Modeste Alexandre Yahouedehou, Cleverson Fonseca and Elisângela Vitoria Adorno collected the samples and carried out the laboratory analysis. Uche Samuel Ndidi and Cynara Gomes Barbosa interpreted the results. Uche Samuel Ndidi drafted the

manuscript and it was revised by Marilda Souza Goncalves and Cynara Gomes Barbosa. All authors read and approved the final manuscript.

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References

Akinsheye, I. & Klings, E.S. (2010) Sickle cell anemia and vascular dysfunction: the nitric oxide connection. *Journal of cellular physiology*, **224**, 620 – 625.

Araki, N., Ueno, N., Chakrabarti, B., Y. Morino, Y. & Horiuchi, S. (1992) Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *The Journal of Biological Chemistry*, **267**, 10211–10214.

Ataga, K.I., Hinderliter, A., Brittain, J.E., Jones, S., Xu, H., Cai, J., Kim, S., Pritchard, K.A. Jr, & Cheryl, H.A. (2015) Association of Pro-inflammatory high density lipoprotein cholesterol with Clinical and laboratory variables in sickle cell disease. *Hematology*, **20**(5), 289 – 296.

Baynes, J.W. (2001) The role of AGEs in aging: causation or correlation. *Experimental Gerontology*, **36**, 1527 – 1537.

Belcher, J.D., Marker, P.H., Weber, J.P., Hebbel, R.P. & Vercellotti, G.M. (2000) Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. *Blood*, **96**, 2451 – 2459.

Berg, K.J., Gjellestad, A., Nordby, G., Rootwelt, K., Djoseland, O., Fauchald, P., Mehl, A., Narverud, J. & Talseth, T. (1989) Renal effects of trimethoprim in ciclosporin- and azathioprine-treated kidney allografted patients. *Nephron*, **53**(3), 218 – 222.

Bridges, K.R., Barabino, G.D., Brugnara, C., Cho, M.R., Christoph, G.W., Dover, G., Ewestein, B.M., Golan, D.E., Guttmann, C.R., Hofrichter, J., Mulkern, R.V., Zhang, B. & Eaton, W.A. (1996) A multiparameter analysis of sickle erythrocytes in patients undergoing hydroxyurea therapy. *Blood*, **88**, 4701–4710.

Brownell, A., Lowson, S. & Brozovic, M. (1986) Serum ferritin concentration in sickle cell crises. *Journal of Clinical Pathology*, **39**(3), 253 – 255.

Camilo-Araújo, R.F., Amancio, O.M.S., Figueiredo, M.S., Cabanãs-Pedro, A.C. & Braga, J.A.P. (2014) Molecular analysis and association with clinical and laboratory manifestations in children with sickle cell anemia. *Revista Brasileira de Hematologia e Hemoterapia*, **36**(5), 334 – 339.

Castro, O., Brambilla, D.J., Thorington, B., Reindorf, C.A., Scott, R.B., Gillete, P., Vera, J.C. & Levy, P.S. (1994) The acute chest syndrome in sickle cell disease: incidence and risk factors. The Cooperative Study of Sickle Cell Disease. *Blood*, **84**, 643–649.

Charache, S., Terrin, M.L., Moore, R.D., Dover G.J., Barton F.B., Eckert, S.V., Mcmahon R.P., & Bonds, D.R. (1995) Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. investigators of the multicenter study of hydroxyurea in sickle cell anemia, *The New England Journal of Medicine*, **332(**20), 1317–1322.

Davies, S.C. & Gilmore, A. (2003) The role of hydroxyurea in the management of sickle cell disease. *Blood Review*, **17**, 99–109.

Embury, S.H., Hebbel, R.P., Steinberg, M.H. & Mohandas, N. Pathogenesis of vasoocclusion. In: Embury SH, Hebbel RP, Mohandas N, Steinberg MH, eds. Sickle Cell Disease. New York: Raven Press, 311-326.

Ferster, A., Vermylen, C., Buyse, M., Corazza, F., Devalck, C., Fondu, P., Toppet, M. & Sariban, E. Hydroxyurea for treatment of severe sickle cell anemia: a pediatric clinical trial. *Blood*, **88**(6), 1960–1964.

Figueiredo, M.S. (2007) Fatores moduladores da gravidade da evolução clínica da anemia falciforme. *Revista Brasileira de Hematologia e Hemoterapia*, **29**, 215-217.

Filho, I.L.S., Leite, A.C.C.B., Moura, P.G., Ribeiro, G.S., Cavalcante, A.C., Azevedo, F.C.M., & Andrada-Serpa, M.J. (2011) Genetic polymorphisms and cerebrovascular disease in children with sickle cell anemia from Rio de Janeiro, Brazil. *Arquivos de Neuropsiquiatria*, **69**(3), 431 – 435.

Fleury, M.K. (2007) Haplótipos do cluster da globina beta em pacientes com anemia falciforme no Rio de Janeiro: Aspectos clínicos e laboratoriais. *RBAC*, **39**, 89-93.

Gladwin, M.T., Shelhamer, J.H., Ognibene, F.P., Pease-Fye, M.E., Nichols, J.S., Link, B., Patel, D.B., Jankowski, M.A., Pannell, L.K., Schechter, A.N. & Rodgers, G.P. (2002) Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. *British Journal of Haematology*, **116**, 436 – 444.

Gonçalves, C.M., Bomfim, G.C., Maciel, E., Cerqueira, I., Lyra, I., Zanette, A., Bomfim, G., Adorno, E.V., Albuquerque, A.L., Pontes, A., Dupuit, M.F., Fernandes, G.B., & dos Reis, M.G. (2003) ßS-Haplotypes in sickle cell anemia patients from Salvador, Bahia, Northeastern Brazil. *Brazilian Journal of Medical and Biological Research*, **36**, 1283–1288.

Green, N.S. & Barral, S. (2014) Emerging Science of Hydroxyurea therapy for pediatric sickle cell diseases. *Pediatric Research*, **75(**0), 196 – 204.

Grigg, A. (2007) Effect of hydroxyurea on sperm count, motility and morphology in adult men with sickle cell or myeloproliferative disease. *Internal Medicine Journal*, **37**, 190–192.

Grisham, M.B., Johnson, G.G. & Lancaster, J.R. Jr. (1996) Quantification of nitrate and nitrite in extracellular fluids. *Methods in Enzymology*, **268**, 237 – 246.

Hagar, W. & Vichinsky, E. (2008) Advances in clinical research in sickle cell disease. *British Journal of Haematology*, **141**, 346–356.

Hammes, H.P., Weiss, A., Hess, S., Araki, N., Horiuchi, S., Brownlee, M. & Preissner, K.T. Modification of vitronectin by advanced glycation alters functional properties *in vitro* and in the diabetic retina. *Laboratory Investigation*, **75**, 325–338.

Imai, N., Nishi, S., Suzuki, Y., Karasawa, R., Ueno, M., Shimada, H., Kawashima, S., Nakamaru, T., Miyakawa, Y., Araki, N., Horiuchi, S., Gejyo, F. & Arakawa, M. (1997) Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. *Nephron*, **76**, 153–160.

Kemperman, F.A., Silberbusch, J., Slaats, E.H., Prins, A.M., Krediet, R.T. & Arisz, L. (2000) Follow-up of GFR estimated from plasma creatinine after cimetidine administration in patients with diabetes mellitus type 2. *Clinical Nephrology*, **54**(4), 255 – 260.

Kume, S., Takeya, M., Mori, T., Araki, N., Suzuki, H., Horiuchi, S., Kodama, T., Miyauchi, Y. & Takahashi, K. (1995) Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *American Journal of Pathology*, **147**, 654–667.

Laurentino, M.R., Filho, P.A.M., Barbosa, M.C., Bandeira, I.C.J., Rocha, L.B.S. & Gonçalves, R.P. (2014) Influence of βS-globin haplotypes and hydroxyurea on tumor necrosis factor-alpha levels in sickle cell anemia. *Revista Brasileira de Hematologia e Hemoterapia*, **36**(2), 121 – 125.

Lettre, G. & Bauer, D.E. (2016) Fetal hemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. *Lancet*, **387**(10037), 2554-2564.

Li, S., Tan, H.Y., Wang, N., Zhang, Z.J., Lao, L., Wong, C.W. & Feng, Y. (2015) The role of oxidative stress and antioxidants in liver diseases. *International Journal of Molecular Sciences*, **16**(11), 26087 – 26124.

Lindenmeier, M., Faist, V. & Hofmann, T. (2002) Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing *in vitro* antioxidative and phase I/II enzyme modulating activity. *Journal of Agricultural and Food Chemistry*, **50**, 6997–7006.

Milner, P.F., Kraus, A.P., Sebes, J.I., Sleeper, L.A., Dukes, K.A., Embury, S.H., Bellevue, R., Koshy, M., Moohr, J.W. & Smith, J. (1991) Sickle cell disease as a cause of osteonecrosis of the femoral head. *The New England Journal of Medicine*, **325,** 1476–1481.

Miyata, T., Ishiguro, N., Yasuda, Y., Ito, T., Nangaku, M., Iwata, H. & Kurokawa, K. (1998) Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from

patients with rheumatoid arthritis and its relation with inflammatory markers. *Biochemical and Biophysical Research Communications*, **244**, 45-49.

Morrison, J.C., Schneider, J.M., Kraus, A.P. & Kitabchi, A.E. (1979) The prevalence of Diabetes Mellitus in sickle cell hemoglobinopathies. *The Journal of clinical endocrinology and metabolism*, **48**(2), 192 – 195.

Nevitt, S.J., Jones, A.P. & Howard, J. (2017) Hydroxyurea (hydroxycarbamide) for sickle cell disease, Cochrane Database Syst. Rev. issue 4, Art. No.: CD002202.

Nur, E., Brandjes, D.P., Schnog, J.J.B., Otten, H.M., Fijnvandraat, K., Schalkwijk, C.G. & Biemond, B.J. (2010) Plasma levels of advanced glycation end products are associated with haemolysis-related organ complications in sickle cell patients. *British Journal of Haematology*, **151**, 62 – 69.

Okocha, C.E., Manafa, P.O., Ozomba, J.O., Ulasi, T.O., Chukwuma, G.O. & Aneke, J.C. (2014) C-reactive protein and disease outcome in Nigerian sickle cell disease patients. *Annals of Medical and Health Science Research*, **4**(5), 701 – 705.

Patel, R., Baker, S.S., Liu, W., Desai, S., Alkhouri, R., Kozielaki, R., Mastrandrea, L., Sarfrazi, A., Cai, W., Vlassara, H., Patel, M.S., Baker, R.D. & Zhu, L. (2012) Effect of dietary advanced glycation end products on Mouse liver. *PLoSOne*, **7**(4), e35143.

Paula, R.P., Nascimento, A.F., Sousa, S.M.B., Bastos, P.R.V & Barbosa, A.A.L. (2013) Glomerular filtration rate is altered in children with sickle cell disease: a comparison between Hb SS and Hb SC. *Revista Brasileira de Hematologia e Hemoterapia*, **35**(5), 349 – 351.

Peppa, M., Uribarri, J. & Vlassara, H. (2004) The role of advanced glycation end products in the development of atherosclerosis. *Current Diabetes Reports*, **4**, 31 – 36.

Picklo, M.J., Montine, T.J., Amarnath, V. & Neely, M.D. (2002) Carbonyl toxicology and Alzheimer's diseases. *Toxicology and Applied Pharmacology*, **184**, 187 – 197.

Platt, O.S., Brambilla, D.J., Rosse, W.F., Milner, P.F., Castro, O., Steinberg, M.H. & Klug, P.P. (1994) Mortality in sickle cell disease. Life expectancy and risk factors for early death. The *New England Journal of Medicine*, **330,** 1639–44.

Platt, O.S. (2008) Hydroxyurea for the treatment of sickle cell anemia. *The New England Journal of Medicine*, **358**, 1362–1369.

Platt, O.S., Thorington, B.D., Brambilla, D.J., Milner, P.F., Rosse, W.F., Vichinsky, E. & Kinney, T.R. (1991) Pain in sickle cell disease. Rates and risk factors. *The New England Journal of Medicine*, **325,** 11–16.

Raghupathy, R., Haider, M.Z., Azizieh, F., Souza, T.M.D., Abdelsalam, R. & Adekile, A.D. (2000) Tumor necrosis factor-α is undetectable in the plasma of SS patients with elevated Hb F. *American Journal of Hematology*, **64**, 91-94.

Rumaney, M.B., Bitoungui, V.J.N., Vorster, A.A., Ramesar, R., Kengne, A.P., Ngogang, J. & Wonkam, A. (2014) The Co-Inheritance of Alpha-Thalassemia and Sickle Cell Anemia Is Associated with Better Hematological Indices and Lower Consultations Rate in Cameroonian Patients and Could Improve Their Survival. *PLOSone*, **9**(6) e100516.

Samra, M. & Abcar, A.C. (2012) False estimates of elevated creatinine. *The Permanente Journal*, **16**(2), 51 – 52.

Seixas, M.O., Rocha, L.C., Carvalho, M.B., Menezes, J.F., Lyra, I.M., Nascimento, V.M.L., Couto, R.D., Atta, A.M., Reis, M.G. & Goncalves M.S. (2010) Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease. *Lipids in Health and Disease*, **9**, 1–9.

Shores, J., Peterson, J., Vanderjagt, D.J. & Gless, R.H. (2003) Reduced cholesterol levels in African-American adults with sickle cell disease. *Journal of the National Medical Association*, **95**(9), 813-817.

Silva, L.B., Gonçalves, R.P. & Rabenhorst, S.H. (2009) Análise dos haplótipos da anemia falciforme em Fortaleza revela as origens étnicas da população cearense. *The Jornal Brasileiro de* Patologia *e Medicina Laboratorial*, **45**, 115-118.

Silva-Pinto, A.N., Angulo, I.L., Brunetta, D.M., Neves, F.I.R, Bassi, S.C., De Santis, G.C. & Covas, D.T. (2013) Clinical and hematological effects of hydroxyurea therapy in sickle cell patients: a single-center experience in Brazil. *Sao Paulo Medical Journal*, **131**, 238–243.

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

Smith, M.A., Taneda, S., Richeyn, P.L., Miyata, S., Yan, S.D., Stern, D., Sayre, L.M., Monnier, V.M. & Perry, G. (1995) Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proceedings of the National Academy of Sciences*, **91**, 5710–5714.

Somjee, S.S., Warrier, R.P., Thompson, J.L., Ory-Ascani, J. & Hempe, J.M. (2004) Advanced glycation end-products in sickle cell anemia. *British Journal of Haematology*, **128**, 112 – 118.

Steinberg, M.H. & Brugnara, C. (2003) Pathophysiological-based approaches to treatment of sickle cell disease. *Annual Review of Medicine*, **54**, 89 – 112.

Steinberg, M.H., Lu, Z.H., Barton, F.B., Terrin, M.L., Charache, S. & Dover, G.J. (1997) Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea. *Blood*, **89**(3), 1078–1088.

Steinberg, M.H. (2006) Pathophysiologically based drug treatment of sickle cell disease. *Trends in Pharmacological Sciences*, **27**, 204–210.

Stuart, M.J. & Nagel, R.L. (2004) Sickle cell disease. *Lancet*, **364**, 1343–1360.

Sutton, M., Bouhassira, E.E. & Nagel, R.L. (1989) Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes. *American Journal of Hematology*, **32**(1), 66–99.

Tessier, F.J., Jacolot, P. & Niquet-Léridon, C. (2014) Open questions around the carboxymethyllysine. *IMARS Highlights*, **9**, 14–20.

Thornburg, C.D., Calatroni, A., Telen, M. & Kemper, A.R. (2010) Adherence to Hydroxyurea Therapy in Children with sickle cell anemia. *The Journal of Pediatrics*, **156**, 415–419.

Torres, L.S., Silva, D.G.H., Junior, E.B., Almeida, E.A., Lobo, C.L.C., Cancado, R.D., Ruiz, M.A. & Bonini-Domingos, C.R. (2012) The influence of hydroxyurea on oxidative stress in sickle cell anemia. *Revista Brasileira de Hematologia e Hemoterapia*, **34**(6), 421 – 425.

Traina, F., Jorge, S.G., Yamanaka, A., de Meirelles, L.R., Costa, F.F. & Saad, S.T. (2007) Chronic liver abnormalities in sickle cell disease: a clinicopathological study in 70 living patients. *Acta Haematologica*, **118**(3), 129–135.

van Beers, E.J., van Tujin, C.F., MacGillavry, M.R., van der, G.A., Schnog, J.J.B. & Biemond, B.J. (2008) Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. *Haematologica*, **93**, 757 – 760.

VanderJagt, D.J., Shores, J., Okorodudu, A., Okolo, S.N. & Glew, R.H. (2002) Hypocholesterolemia in Nigerian children with sickle cell disease. *Journal of Tropical Pediatrics*, **48**, 156-161.

Voskaridou, E., Christoulas, D., Bilalis, A., Plata, E., Varvagiannis, K., Stamatopoulos, G., Sinopoulou, K., Balassopoulou, A., Loukopoulos, D. & Terpos, E. (2010) The effect of prolonged administration of hydroxyurea on morbidity and mortality in adult patients with sickle cell syndromes: results of a 17-year, single-center trial (LaSHS). *Blood*, **115**, 2354–2363.

Wang, W.C. (2007) The pathophysiology, prevention and treatment of stroke in sickle cell disease. *Current Opinion in Hematology*, **14**, 191–197.

Warren, J.S. & Ward, P.A. (2001) The inflammatory response. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, eds. Williams Hematology, sixth ed. New York: Mc Graw-Hill; 67–75.

Watson, J. (1948) The significance of the paucity of sickle cells in newborn Negro infants. *The American Journal of the Medical Sciences*, **215**, 419–423.

Wood, K.C., Hsu, L.L. & Gladwin, M.T. (2008) Sickle cell disease vasculopathy: A state of nitric oxide resistance. *Free Radical Biology and Medicine*, **44**, 1506–1528.

Yamada, K., Miyahara, Y., Hamaguchi, K., Nakayama, M., Nakano, H., Nozaki, O., Miura, Y., Suzuki, S., Tuchida, H., & Mimura, N. (1994) Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure. *Clinical Nephrology*, **42**, 354–361.

Zago, M.A. & Pinto, A.C. (2007) Fisiopatologia das doenças falciformes: da mutação genética à insuficiência de múltiplos órgãos. *Revista Brasileira de Hematologia e Hemoterapia*, **29**, 207-214.

Zimmerman, S.A., Schultz, W.H., Davis, J.S., Pickens, C.V., Mortier, N.A., Howard, T.A. & Ware, R.E. (2004) Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. *Blood*, **103**(6), 2039-2045.

Table 1. Hematological characteristics of sickle cell anemia (SCA) patients on hydroxyurea (HU) (HU^+ SCA) treatment and SCA patients without hydroxyurea (HU) treatment (HU^- SCA)

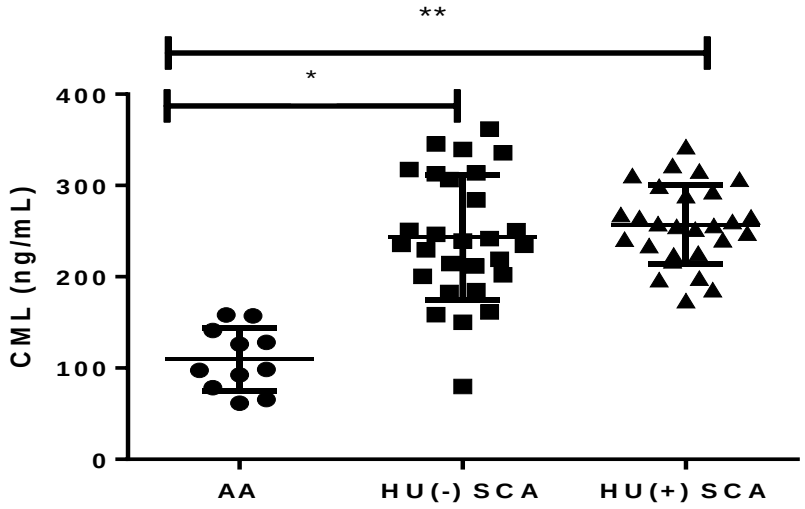
Hematological variables	HU^+ SCA N = 27, Median (25^{th} – 75^{th} percentile)	HU^- SCA N = 28, Median (25^{th} – 75^{th} percentile)	*P**-value
Red Blood Cell, $\times 10^{12}$/L	2.69 (2.15 – 3.18)	2.66 (2.45 – 3.22)	0.533
Hemoglobin, g/dL	8.7 (8 – 10.4)	8.6 (7.9 – 9.3)	0.337
Hematocrit, %	24.9 (22.6 – 30.4)	24.5 (22.2 -27.9)	0.522
Mean Corpuscular Volume (MCV), fL	100.9 (89.7 – 106.7)	92.2 (89.00 – 94.50)	**0.003**
Mean corpuscular hemoglobin (MCH), ρg	34.3 (30.9 – 37.1)	31.6 (29.7 – 32.9)	**0.002**
MCHC, g/dL	34 (33.5 – 35.4)	34.1 (33.3 – 35.6)	0.890
RDW	20.6 (17.3 – 22.8)	21.1 (18.70 – 23.3)	0.398
Reticulocyte count, %	7.1 (5.7 – 8.7)	8.00 (5.4 – 9.6)	0.728
White Blood Cell (WBC) count, $\times 10^6$/L	9335 (5860 – 13100)	12300 (10500 – 14500)	**0.014**
Neutrophils, $\times 10^6$/L	4400 (2602 – 6514)	5280 (4170 – 6380)	0.250
Eosinophils, $\times 10^6$/L	306 (96 – 693)	679 (272 – 1408)	**0.017**
Basophils, $\times 10^6$/L	32 (0 -96)	120 (0 – 158)	**0.041**
Atypical lymphocytes, $\times 10^6$/L	0 (0 – 54)	104 (0 – 380)	**0.014**
Typical lymphocytes, $\times 10^6$/L	3930 (2533 – 5328)	4921 (3770 – 6950)	**0.033**
Monocytes, $\times 10^6$/L	720 (393 – 879)	896 (582 – 1390)	**0.041**
Platelets, $\times 10^3$/mL	374 (275 – 441)	392 (321 – 463)	0.610
Mean Platelet Volume (MPV)	5.8 (5.2 – 7)	5.70 (5.20 – 6.90)	0.856
HbS, %	80.3 (73.2 – 86.95)	85.3 (80.1 – 88.2)	0.286
HbF, %	9.6 (6.8 – 15.9)	8.2 (5.7 – 9.9)	0.072
HbA_2, %	4.4 (3.9 – 5.4)	5.55 (4.15 – 6.90)	0.078

HU^+ SCA, Sickle cell anemia patients on hydroxyurea treatment; HU^- SCA, sickle cell anemia not on hydroxyurea treatment; BMI, Body mass index; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red blood cell Distribution Width. *P**: significance at ≤ 0.05 using the unpaired independent samples T test and Mann-Whitney U test.

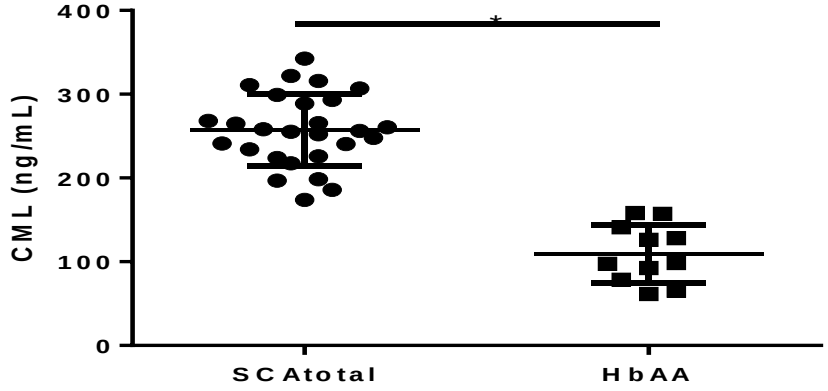
Table 2. Biochemical characteristics of sickle cell anemia (SCA) patients on hydroxyurea (HU) (HU^+ SCA) treatment and SCA patients without hydroxyurea (HU) treatment (HU^- SCA)

Biochemical variables	HU^+ SCA N = 27 Median (25^{th} – 75^{th} percentile)	HU^- SCA N = 28 Median (25^{th} – 75^{th} percentile)	*P**-value
Glucose (fasting), mg/dL	78 (72 – 85)	71 (63 – 85)	0.089
Total Cholesterol, mg/dL	129 (112 – 138)	127.5 (114.5 – 144)	0.637
HDL-C, mg/dL	32 (26 – 45)	32.5 (28 – 42)	0.827
LDL-C, mg/dL	71.6 (57.8 – 91.4)	74.2 (63.1 – 92.5)	0.479
VLDL-C, mg/dL	14.2 (11.8 – 21.2)	16.6 (12.8 – 20)	0.381
Triglyceride, mg/dL	71 (59 – 106)	83 (64 100)	0.381
ALT, U/L	18 (14 – 25)	19.5 (41 – 57)	0.873
AST, U/L	38 (30 -48)	51.5 (41 – 57)	**0.001**
Serum Iron, μg/dL	99.6 (75.3 – 134.3)	74.15 (52.15 – 114.15)	0.065
Ferritin, ng,/mL	265.75 (126.9 – 571.35)	160.25 (122 – 266.65)	0.102
Total Bilirubin, mg/dL	1.9 (1.23 – 3.7)	2.32 (1.53 – 3.67)	0.350
Direct Bilirubin, mg/dL	0.45 (0.32 – 0.59)	0.45 (0.40 – 0.52)	0.730
Indirect Bilirubin, mg/dL	1.29 (0.78 – 3.1)	1.8 (1.07 – 3.27)	0.239
Total Protein, g/dL	7.47 (6.89 – 8)	7.64 (7.03 – 7.83)	0.849
Albumin, g/dL	4.4 (4 – 4.4)	4.4 (4.1 – 4.50)	0.582
Globulin, g/dL	3.3 (2.6 – 3.8)	3.25 (2.5 – 3.55)	0.735
Urea, mg/dL	19 (14 – 24)	17 (15 – 21)	0.468
Creatinine, mg/dL	0.47 (0.41 – 0.56)	0.39 (0.34 – 0.48)	**0.004**
C-reactive protein, mg/L	4.07 (2.69 – 8.3)	3.62 (2.07 – 5.89)	0.246
LDH, U/L	853 (715 – 1128)	1270 (1091 – 1612)	**0.000**
A/G Ratio	1.3 (1.1 – 1.7)	1.30 (1.15 – 1.70)	0.754
A1AT, mg/dL	138.5 (119.5 – 159.5)	154.5 (118 – 174)	0.331
Nitric oxide (NO), μM	20.44 (15.85 – 22.72)	19.02 (14.11 – 25.29)	0.755

HU^+ SCA, Sickle cell anemia patients on hydroxyurea treatment; HU^- SCA, sickle cell anemia not on hydroxyurea treatment; HDL-C, High-density lipoprotein-cholesterol; LDL-C, Low-density lipoprotein-cholesterol; VLDL-C, Very low-density lipoprotein-cholesterol; ALT, Alanine transaminase; AST, Aspartate transaminase; LDH, Lactate dehydrogenase; A/G Ratio, Albumin / globulin ratio; A1AT, Alpha 1-antitrypsin. *P**: significance at ≤ 0.05 using the unpaired independent samples T test and Mann-Whitney U test.



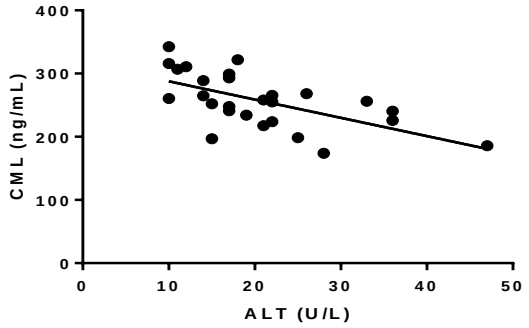
(A)



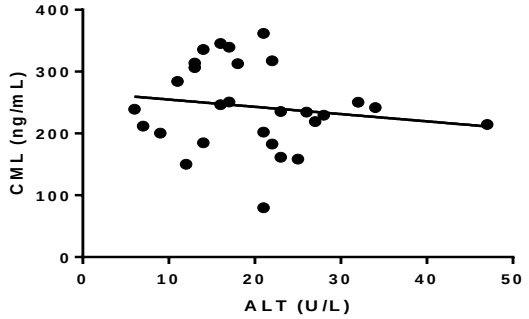
(B)

Figure 1. A comparison analysis of the level of Carboxymethyllysine (CML) in sickle cell anemia patients [HU(-) SCA, HU(+) SCA and SCAtotal] against healthy control (HbAA) (A) Serum levels of CML (ng/mL) in HbAA, HU(+) SCA and HU(-)SCA. CML levels were significantly higher in both SCA patients compared to HbAA. However, there is no significant difference in the level of CML between the HU(+) SCA and HU(-) SCA (*P < 0.0001; **P < 0.0001). (B) Serum levels of CML in HbAA and SCAtotal. CML levels are significantly higher in SCAtotal compared to HbAA (*P < 0.0001). SCA, sickle cell anemia; HU(+) SCA, hydroxyurea treated group; HU(-) SCA, group without hydroxyurea treatment.

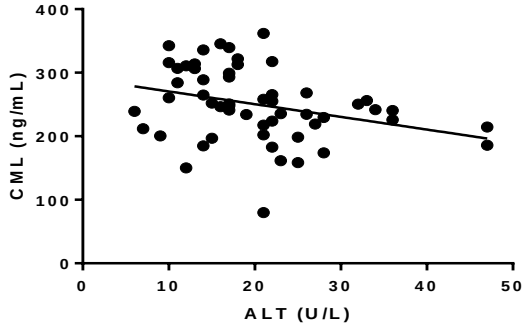
P < 0.05 was considered significant



(A)



(B)



(C)

Figure 2. Linear regression curve of serum CML (ng/mL) against ALT (U/L) (A) is the regression curve for HU^+ SCA. It showed a signficant (r = -0.6126; p-value = 0.0007) inverse relationship between CML (ng/mL) and ALT (U/L). (B) is the regression curve for HU^- SCA. It showed insingificant (r = -0.1395; p-value = 0.4790) inverse relationship between CML (ng/mL) and ALT (U/L). (C) is the regression curve for all SCA without regard to HU treatment (SCA_{total}). It showed a significant (r = -0.3484; p-value = 0.0092) inverse relationship between CML (ng/mL) and ALT (U/L). CML, carboxymethyllysine; ALT, alanine transaminase.

Table 3. Haplotype characterization of all SCA patients (SCA_{total}), SCA on HU treatment (HU^+ SCA) and SCA without HU treatment (HU^- SCA) according to the median level of carboxymethyllysine (CML)

A. (SCA_{total})

	Carboxymethyllysine (CML)				
Molecular characteristic	≤ median*,n (%)	> median*,n (%)	Total, n (%)	χ^2	p
Bantu/Bantu	3 (5.8)	8 (15.4)	11 (21.2)	7.909	0.048**
Bantu/Benin	5 (9.6)	16 (30.8)	21 (40.4)		
Bantu/Atypical	2 (3.8)	0 (0.0)	2 (3.8)		
Benin/Benin	10 (19.2)	8 (15.4)	18 (34.6)		
Total	20 (38.5)	32 (61.5)	52 (100)		

χ^2, Pearson chi-square; *median value = 239.33ng/mL; **Bantu/Bantu versus Bantu/Benin, Bantu/Atypical and Benin/Benin.

B. (HU^+ SCA)

	Carboxymethyllysine (CML)				
Molecular characteristic	≤ median*,n (%)	> median*,n (%)	Total, n (%)	χ^2	p
Bantu/Bantu	1 (4.2)	3 (12.5)	4 (16.7)	1.929	0.381
Bantu/Benin	4 (16.7)	5 (20.8)	9 (37.5)		
Benin/Benin	7 (29.2)	4 (16.7)	11 (45.8)		
Total	12 (50.0)	12 (50.0)	24 (100)		

χ^2, Pearson chi-square; *median value = 256.10 ng/mL.

C. (HU^- SCA)

	Carboxymethyllysine (CML)				
Molecular characteristic	≤ median*,n (%)	> median*,n (%)	Total, n (%)	χ^2	p
Bantu/Bantu	3 (10.7)	4 (14.3)	7 (25.0)	2.619	0.454
Bantu/Benin	5 (17.9)	7 (25.0)	12 (42.9)		
Bantu/Atypical	2 (7.1)	0 (0.0)	2 (7.1)		
Benin/Benin	4 (14.3)	3 (10.7)	7 (25.0)		
Total	14 (50.0)	14 (50.0)	28 (100)		

χ^2, Pearson Chi-square; *median value = 237.39 ng/mL.

4.4 CAPÍTULO IV

Manuscrito intitulado "*Association of serum level of N(epsilon)-(carboxymethyl)lysine with hematologic, biochemistry and molecular markers on sickle-hemoglobin SC disease patients"*

O trabalho descreve o efeito potencial da CML na doença SC e sua relação / associação com marcadores hematológicos, bioquímicos e moleculares nesses pacientes.

Status do manuscrito: Submetido para publicação no periódico "*International Journal of Hematology*".

Association of serum level of *N*(epsilon)-(carboxymethyl)lysine with hematologic, biochemistry and molecular markers on sickle-hemoglobin SC disease patients

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Abstract

This research work was aimed at investigating the effect of *N*(epsilon)-(carboxymethyl)lysine (CML) on sickle hemoglobin SC disease (HbSC). Children with HbSC (n = 20) were recruited along with sex- and age-matched healthy control children (HbAA) (n = 23). Biochemical and hematological markers analyses were measured by immunochemistry assay and automated cell counter respectively. The hemoglobin (Hb) profile was investigated by HPLC while serum CML was determined using competitive ELISA procedure. Haplotypes were measured using RFLP-PCR. Our results show that HbSC subjects have significantly lower median age ($p < 0.0001$) and body mass index ($p = 0.0097$) compared to the healthy control. The HbSC patients also presented significantly lower levels of hemoglobin (Hb) ($p = 0.0001$), hematocrit ($p < 0.0001$), mean corpuscular volume ($p < 0.0001$), total cholesterol ($p = 0.0005$) and HDL-cholesterol ($p < 0.0001$) compared to the healthy control. Mean corpuscular hemoglobin concentration ($p < 0.0001$), red cell distribution width ($p < 0.0001$), reticulocyte ($p < 0.0001$), leukocyte ($p = 0.0479$) showed significantly higher levels in HbSC patients. Serum CML level was significantly higher ($p = 0.0012$) in HbSC compared to the healthy control. Hb ($r = 0.509$; $p = 0.046$), hematocrit ($r = 0.505$; $p = 0.048$), ferritin ($r = -0.518$; $p = 0.023$) and C-reactive protein ($r = -0.505$; $p = 0.028$) have significant relationship with CML. Chi-square (χ^2) analyses of the various haplotypes of the HbSC patients were not significant ($p = 0.48$). Our work suggests that CML has

a role to play in HbSC disease complications but there is no association between CML and haplotypes.

Keywords: Sickle cell disease, HbSC, advanced glycation end products, laboratory parameters, haplotypes.

1. Introduction

Hemoglobin C (HbC) is a structural variant of normal hemoglobin (HbA). Unlike Hemoglobin S (HbS) in which glutamic acid is replaced by valine at position 6 of the β-globin chain (β6Glu → Val), HbC is caused by the replacement of glutamic acid by lysine at position 6 of the β-globin chain (β6Glu → Lys). In HbC autosomal recessive inheritance, GAG codon is replaced by AAG (G→A) at the 6^{th} codon of the beta globin gene (*HBB*) located at chromosome 11. HbC is the second most prevalent variant hemoglobin mutation worldwide after HbS (Piel *et al*, 2013). Its most common double inheritance includes heterozygote HbC normal carriers (CA), homozygote hemoglobin C individuals (CC) and heterozygote sickle hemoglobin (SC).

The HbSC disease is known to cause chronic hemolytic anemia, acute chest syndrome (ACS) and intermittent sickle cell crises, slightly less severe or frequent than SCA (Bain, 2006; Piel *et al*, 2013; Gonçalves *et al*, 2016). The HbSC genotype is a well-known risk factor for proliferative sickle cell retinopathy (PSCR) (Mantovani & Figini, 2008). A longitudinal analysis showed that patients with HbSC were more likely to develop severe PSCR (phase III-IV) than patients with HbSS (Leveziel *et al*, 2011). Osteonecrosis (avascular necrosis) of the femoral head has also been reported with increased frequency in Hb SC than Hb SS (Prindle & Mccurdy, 1970). Clinical features of HbSC have been attributed to the fact that HbC enhances the formation of intracellular polymer of HbS by dehydrating red cells (Ballas *et al*, 1982; Bunn *et al*, 1982). The mechanism through which HbC dehydrates red cell includes binding to the red cell membrane, abnormal cation transport, increased intracellular HbS concentration and deoxygenation (Bunn *et al*, 1982).

Auto-oxidation of the sickle hemoglobin in addition to chronic intravascular hemolysis and ischemia is known to increase the generation of reactive oxygen species (ROS) that potentiates oxidative stress (van Beers *et al*, 2008). Advanced glycation end products (AGEs) such as N^{ε}-carboxymethyllysine (CML) are well known markers of oxidative stress (Gerrits *et al*, 2008). CML, the most abundant and the most researched AGE (Tessier *et al*, 2014), has been implicated in the pathophysiology of organ complications in diabetes, atherosclerosis, ageing, Alzheimer's disease and auto-immune inflammatory disease (Baynes, 2001; Picklo *et al*, 2002; Peppa *et al*, 2004; Nur *et al*, 2010).

There is a dearth of research on HbSC; both on their effect on laboratory parameters and effect of AGEs on HbSC patients. The works of Nur *et al* (2010) and Somjee *et al* (2004) are the only known studies that attempted to explore the possible association of AGEs with SCD. Somjee *et al* (2004) worked only on SCA patients and discovered that the plasma levels of AGEs and the rate of erythrocyte AGE accumulation were significantly higher in patients with SCA compared to

controls. The work of Nur *et al* (2010) had patients with HbSS, HbSβ^0-thalassemia, HbSC and HbSβ$^+$-thalassemia, and their report did not reveal significant change between the CML level in HbSC/HbSβ$^+$ and the control; though there was a significantly higher level of CML in HbSS compared to the healthy control.

HbSC disease accounts for between 25 – 30% of the total cases of sickle cell disease (SCD) worldwide (Lionnet et al, 2012; Weatherall, 2010) yet we have few research works that focused on HbSC disease. Much rarer are works that investigated the role of AGEs such as CML on HbSC disease. Therefore, this study was aimed at investigating the effect of *N*(epsilon)-(carboxymethyl)lysine (CML) on hematologic, biochemistry and molecular markers on sickle-hemoglobin SC disease patients.

2. Materials and Methods

2.1 Subjects. In this transversal cohort study, twenty (20) HbSC patients of both sexes from the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), Salvador, Bahia, Brazil were recruited. In addition to this, 23 normal healthy age- and sex-matched individuals (HbAA) were recruited from volunteers from the Clinical Laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (UFBA). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. The study was carried out between August 2015 and August 2017 and the protocol was submitted to and approved by the Fundação Oswaldo Cruz (FIOCRUZ) Ethics research board (ID 1.400.527).

All participants received verbal and written explanation of the study and signed the Informed Consent Form (ICF) before inclusion in the study. Informed consent was obtained from the patient or the patient's parent/guardian. Patients were in the hospital during crisis or in the clinic for a routine visit. Venous blood (8-10 mL) was collected in Vacutainer tubes containing EDTA. Blood samples were processed within 30 minutes of collection.

2.2 Standard Laboratory Tests. Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain). Serum ferritin was measured by immunoassay using an Access® 2 Immunoassay system X2 (Beckman Coulter, Fullerton, CA, USA). C-reactive protein (CRP) was measured by immunochemistry (Immage® 800 system, Beckman Coulter, Fullerton, CA, USA). Hematological analyses were carried out using an automated cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile was investigated by high performance liquid chromatography (HPLC/VARIANT I; BIO-RAD, CA, USA).

2.3 Immunoanalysis of N^{ε}-carboxymethyllysine (CML). The detection and quantitative estimation of CML was carried out in the blood serum of the patients using an OxiSelect™ CML ELISA Kit (Cell Biolabs, Inc., CA, USA) according to the protocols established by the manufacturer. The amount of CML in ng/mL was determined by comparing its absorbance with that of a known

CML-BSA standard curve. The absorbance results were read on a microplate reader (STAT FAX® 2100) at 450nm.

2.4 Haplotype analysis. First, the DNA was extracted from peripheral blood of patients following instructions in the manual that accompanied the commercial DNA isolation kit [Puregene blood kit (Qiagen, USA)]. The analysis of haplotype cluster βS was performed by restriction fragment length polymorphism – polymerase chain reaction (PCR – RFLP) with analysis of 7 polymorphic restriction sites performed according to the method of Sutton *et al* (1989): 5′γ^{G-}*Xmn* I, γ^{G-}*Hin*d III, γ^{A--}*Hin*d III, ψβ-*Hin*c II, 3′ψβ-*Hin*c II and 5′β–*Hin*f I, 3′ β-*Hpa* I.

3. Statistical Analysis

Descriptive statistics were summarized with median and interquartile range (25% - 75%) for continuous variable. The data for each variable and for each group were tested for normality (Shapiro-Wilk and Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) prior to analysis. The Mann-Whitney (non-parametric) and independent T-test (parametric) were employed for the analysis of two numerical variables, comparing two groups of values within the same variable, taking into account the distribution of each variable. Analysis of variance (ANOVA) followed by post hoc multiple range comparison where differences in means were observed was applied for more than two numerical (or quantitative) variables. For correlation studies between all laboratory parameters and CML, the Spearman's rank correlation coefficient was employed and the differences between the groups were considered significant when $P < 0.05$ or $P < 0.01$. Linear regression was followed where a significant difference was detected. Statistical analyses were performed using GraphPad Prism 6.0 and SPSS version 20.0 (SPSS 20.0, Chicago, IL, USA). Values of $P < 0.05$ were considered significant for the analyses performed.

4. Results

Table 1 shows the descriptive analysis of some clinical and laboratory parameters of HbSC patients. The results were presented as median and $25^{th} – 75^{th}$ percentile.

Our pediatric HbSC patients have insignificantly lower median age ($p = 0.0612$) but significantly lower median body mass index (BMI) ($p = 0.0097$) compared to the healthy control. The HbSC patients also presented significantly lower levels of hemoglobin (Hb) ($p = 0.0001$), hematocrit ($p < 0.0001$), mean corpuscular volume (MCV) ($p < 0.0001$) compared to the healthy control. There were significantly higher levels of mean corpuscular hemoglobin concentration (MCHC) ($p < 0.0001$), red cell distribution width (RDW) ($p < 0.0001$), reticulocytes ($p < 0.0001$), leukocytes ($p = 0.0479$) in HbSC patients compared to healthy control. Total cholesterol ($p = 0.0005$) and high-density lipoprotein (HDL)-cholesterol ($p < 0.0001$) also presented significantly lower values in HbSC patients compared to the healthy control.

Figure 1 shows the dot plot of CML level in the HbSC patients as against the healthy control group. We have shown a significant difference ($p = 0.0012$) in the concentration of CML between the HbSC group and the healthy control group. The HbSC group presented a significantly higher concentration of serum CML compared to the healthy control.

Table 3 shows the Spearman's rho univariate correlation analyses of some clinical and laboratory markers against serum level of CML in children with HbSC disease. There were significant correlations between CML and Hb (Spearman's $r = 0.509$; $p = 0.046$); CML and hematocrit (Spearman's $r = 0.505$; $p = 0.048$); CML and ferritin (Spearman's $r = -0.518$; $p = 0.023$); CML and C-reactive protein (CRP) (Spearman's $r = -0.505$; $p = 0.028$). While ferritin ($y = -0.6488x + 231.0$) and CRP ($y = -0.017x + 5.594$) showed negative (inverse) linear relationship, Hb ($y = 0.0094x + 10.15$) and hematocrit ($y = 0.026x + 29.17$) presented positive (direct) linear relationship with CML (Figure 2A – D).

Table 4 shows the Spearman's rho multivariate regression analysis of some of the biomarkers with emphasis on those that revealed significant correlation with CML at the univariate regression analysis (Hb, hematocrit, ferritin and CRP) in order to determine their association with CML and to determine influence of a parameter on another or others. We did not identify any significant associations for all the parameters [BMI ($\beta = 1.262$; $p = 0.855$), Hb ($\beta = 43.159$; $p = 0.281$), hematocrit ($\beta = -8.205$; $p = 0.521$), total cholesterol ($\beta = 0.973$; $p = 0.176$), serum iron ($\beta = 0.807$; $p = 0.323$), total bilirubin ($\beta = 15.592$; $p = 0.179$), ferritin ($\beta = -0.015$; $p = 0.944$) and CRP ($\beta = -19.011$; $p = 0.077$)] in the model.

Table 5 shows the chi-square (χ^2) analysis of the various haplotypes of the HbSC patients in our study according to whether the patients have CML values ≤ median (186.19 ng/mL) or CML value > median. The results indicate that the calculated χ^2 statistics, for 5 degrees of freedom, is 4.5. It also indicates that significance is more than the usual threshold value of 0.05 suggesting that we accept the null hypothesis that implies that there is no association between CML and the various haplotypes.

5. Discussion

This study evaluated the effect of N^{ε}-(carboxymethl)lysine (CML) on laboratory parameters and haplotypes in sickle HbSC patients. None of the subjects is on hydroxyurea (HU) which naturally could influence much of the laboratory and clinical parameters (Silva-Pinto *et al*, 2013). HbSC disease accounts for between 25 – 30% of the total cases of SCD worldwide yet we have few research works that focused on HbSC disease. Much rarer are works that investigated the role of AGEs such as CML on HbSC disease. Works of Somjee *et al*, (2004) on AGEs focused solely on sickle cell anemia (SCA) while the works of Nur *et al* (2010) stands as the only one that took HbSC into account. Though SCA has been reported to be more severe than all other forms of SCD, HbSC has been reported to have more severe clinical manifestation of proliferative sickle

cell retinopathy (Leveziel *et al*, 2011) and osteonecrosis (Naseer *et al*, 2016). In this report, our clinical and laboratory analyses were compared with normal healthy control group (HbAA).

BMI is a key parameter for assessment of growth development and nutritional status of children. The monitoring of growth and nutritional status in children with SCD helps in early diagnosis of growth failure and nutritional intervention (Odetunde *et al*, 2016). In our study, both groups of subjects (HbSC and HbAA) have similar median ages; however the HbSC group has significantly lower median BMI compared to the healthy control group, which suggests that they have poor growth development and nutritional status. Previous reports suggest that SCD patients are generally underweight and have poor overall growth (Odetunde *et al*, 2016; Singhal *et al*, 1993). However, a study on African American patients with SCD showed that 19% were overweight/obese (Zivot *et al*, 2017).

We reported a lower Hb level in HbSC compared to healthy control that is in agreement with the report of Nur *et al* (2010). Hematocrit was also observed to be significantly lower in HbSC patients. Both Hb and hematocrit are well-known markers of hemolysis and the values suggest that the subjects suffer from hemolysis. MCV, another marker of hemolysis was also found to be significantly lower in HbSC. However, we obtained a significantly higher MCHC and RDW for the HbSC subjects that tend to downplay the significance of the other markers of hemolysis. However, RDW and MCHC are not gold standard for anemia since they can give low or high readings even when the RBC count is normal. The significantly high reticulocytes count is an indication of high production of RBCs due to high turnover of the red cells as a result of the short life span of sickle red cells (~17 days) under anemic condition. Ataga *et al* (2015) also reported significantly higher reticulocytes count in SCDs. The erythropoiesis could also account for the high MCHC and RDW levels. There was significant increase in leukocytes (white blood cell) count. This is in agreement with the works of Nur *et al* (2010) and Ataga *et al* (2015) which reported significant increases in leukocytes. The increase could be in response to inflammation as leukocyte is a marker of inflammation and vaso-occlusion.

Total cholesterol and HDL-cholesterol were discovered to be significantly lower in HbSC compared to HbAA. Previous reports support our findings that cholesterol and HDL-cholesterol levels tend to decrease in patients with SCD (Ataga *et al*, 2015; Seixas et al, 2010; Zorca *et al*, 2010). Indeed SCDs are usually characterized by hypocholesterolemia (Shores *et al*, 2003; VanderJagt *et al*, 2002); however, the works of Ataga *et al* (2015) observed no association between total cholesterol and measures of hemolysis suggesting that hypocholesterolemia in SCD is not solely due to increased hemolysis.

In our research, we discovered that CML was significantly higher in HbSC compared to healthy control. Our report, however, is not in agreement with earlier report by Nur *et al* (2010) in which CML showed no significant difference between HbSC/HbSβ^+ and healthy control. Generally, reports have shown that CMLs are usually higher in sickle cell patients compared to healthy control (Nur *et al*, 2010; Somjee *et al*, 2004). Furthermore, the report of Nur *et al* (2010) and

Somjee *et al* (2004) did not reveal significant difference between patients on asymptomatic state and those on painful crisis. The higher level of CML in SCD patients suggests that intracellular and/or extracellular conditions favor CML synthesis. Since CML has a role in the etiology of microvascular complications in diabetes and many other diseases, it is not out of place to suggest that they may have a role in the pathology of HbSC. The works of Nur *et al* (2010) further suggests that AGEs may play a role in the pathophysiology of chronic hemolysis-associated organ complication; though the mechanism is still unclear.

The Spearman's rho univariate correlation showed that Hb, hematocrit, ferritin and CRP have significant correlations with CML in HbSC patients. Hb and hematocrit are hemolytic markers thereby corroborating the earlier report of Nur *et al* (2010) which suggests that CML may play a role in hemolysis-associated organ complications. The significant correlation of CML with ferritin and CRP suggest a role on inflammation and vaso-occlusion. However, multivariate regression analysis with CML as the dependent variable against Hb, hematocrit, ferritin, CRP and some other biomarkers revealed no significant association between CML and the parameters which suggests that they may play a role on hemolysis- and vascular-associated organ complications in association with other biomarkers and/or factors.

Haplotypes are known to be among several factors that could be responsible for the variable clinical features of SCD (Figueiredo, 2007). The β^S-globin has different haplotypes based on their origin: the Senegal (SEN), Cameroon (CAM), Bantu or Central African Republic (CAR), Benin (BEN), Arab-Indian (ARAB) and more recently atypical (AT) haplotype. Indeed studies have shown that the Bantu haplotype is associated with the most severe clinical features (Fleury, 2007; Silva *et al*, 2009). The β^C-globin also has different haplotypes, namely: I, II and III. The chi-square (χ^2) analysis of the various haplotypes of the HbSC patients in our study was not significant suggesting that there is no association between CML and the various haplotypes. Succinctly put, high or low serum CML were not linked with differing β^S/β^C haplotypes. To our knowledge, this is the first attempt at associating the level of circulating AGE with haplotypes. Perhaps subsequent research would have to be carried out to confirm this report.

The evidence from this work and few other previous works suggest the possibility that AGEs have a role in the pathology of SCD. This means that new therapeutic approaches that target AGEs such as AGE inhibitors (e.g. aminoguanidine, N-(2-Acetamidoethyl)hydrazine carboximidamide hydrochloride (ALT-946) and pyridoxamine, a naturally occurring vitamin B_6 isoform) (Corman *et al*, 1998; Forbes *et al*, 2001) or AGE breakers (e.g. 4,5-Dimethyl-3-phenacylthiazolium chloride (ALT-711) (Thallas-Bonke *et al*, 2004), N-phenacyl thiazolium and N-phenacyl-4,5-dimethylthiazolium) (Voziyan *et al*, 2003) may prove useful in the management of SCD.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa and Adekunle Adekile conceived the study design. Rayra Pereira Santiago, Uche Samuel Ndidi, Corynne Stephanie Ahouefa Adanho, Cynara Gomes Barbosa, Junia Raquel Dutra Ferreira, Isa Menezes Lyra and Caroline Conceicao Da Guarda collected the samples and carried out the laboratory analysis. Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa interpreted the results. Uche Samuel Ndidi drafted the manuscript and it was revised by Marilda Souza Goncalves, Cynara Gomes Barbosa and Adekunle Adekile. All authors read and approved the final manuscript.

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References

Ataga, K.I., Hinderliter, A., Brittain, J.E., Jones, S., Xu, H., Cai, J., Kim, S., Pritchard, K.A. Jr. & Cheryl, H.A. (2015) Association of Pro-inflammatory high density lipoprotein cholesterol with Clinical and laboratory variables in sickle cell disease. *Hematology*, **20**(5), 289 – 296.

Bain, B.J. (2006) Haemoglobinopathy Diagnosis. Second edn, (Blackwell Publishing Ltd).

Ballas, S.K., Lewis, C.N., Noone, A.M., Krasnow, S.H., Kamarulzaman, E. & Burka, E.R. (1982) Clinical, Hematological, and Biochemical Features of Hb SC Disease. *The American Journal of Hematology*, **13**(1), 37 – 51.

Baynes, J.W. (2001) The role of AGEs in aging: causation or correlation. *Experimental Gerontology*, **36**, 1527 – 1537.

Bunn, H.F., Noguchi, C.T., Hofrichter, J., Schechter, G.P., Schechter, A.N. & Eaton, W.A. (1982) Molecular and cellular pathogenesis of hemoglobin SC disease. *PNAS (USA)*, **79**(23), 527 – 531.

Corman, B., Duriez, M., Poitevin, P., Heudes, D., Bruneval, P., Tedgui, A. & Levy, B.I. (1998) Aminoguanidine prevents age-related arterial stiffening and cardiac hypertrophy. *Proceedings of the National Academy of Sciences*, **95**, 1301–1306.

Figueiredo, M.S. (2007) Fatores moduladores da gravidade da evolução clínica da anemia falciforme. *Revista Brasileira de Hematologia e Hemoterapia*, **29**, 215 – 217.

Fleury, M.K. (2007) Haplótipos do cluster da globina beta em pacientes com anemia falciforme no Rio de Janeiro: Aspectos clínicos e laboratoriais. *RBAC*, **39**, 89 – 93.

Forbes, J.M., Soulis, T., Thallas, V., Panagiotopoulos, S., Long, D.M., Vasan, S., Wagle, D., Jerums, G. & Cooper, M.E. (2001) Renoprotective effects of a novel inhibitor of advanced glycation. *Diabetologia*, **44**, 108–114.

Gerrits, E.G., Lutgers, H.L., Kleefstra, N., Graaff, R., Groenier, K.H., Smit, A.J., Gans, R.O. & Bilo, H.J. (2008) Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications. *Diabetes Care*, **31**, 517 – 521.

Gonçalves, B.P., Gupta, S. & Penman, B.S. (2016) Sickle haemoglobin, haemoglobin C and malaria mortality feedbacks. *Malaria Journal*, **15**(26), 1 – 7.

Leveziel, N., Bastuji-garin, S., Lalloum, F., Querques, G., Benlian, P., Binaghi, M., Coscas, G., Soubrane, G., Bachir, D., Galactéros, F. & Souied, E. H. (2011) Clinical and laboratory factors associated with the severity of proliferative sickle cell retinopathy in patients with sickle cell hemoglobin C (SC) and homozygous sickle cell (SS) disease. *Medicine (Baltimore)*, **90**(6), 372 – 378.

Livingstone, F.B. (1985) Frequencies of Hemoglobin Variants: Thalassemia, the Glucose-6-Phosphate Dehydrogenase Deficiency, G6Pd Variants and Ovalocytosis in Human Populations. Oxford University Press, UK.

Lionnet, F., Hammoudi, N., Stojanovic, K.S., Avellino, V., Grateau, G., Girot, R. & Haymann, J-P. (2012) Hemoglobin SC disease complications: a clinical study of 179 cases. *Haematologica*, **97**(8), 1136 – 1141.

Mantovani, A. & Figini, I. (2008) Sickle cell-hemoglobin C retinopathy: transient obstruction of retinal and choroidal circulations and transient drying out of retinal neovessels. *International ophthalmology*, **28**(2), 135-137.

Naseer, Z.A., Bachabi, M., Jones, L.C., Sterling, R.S. & Khanuja, H.S. (2016) Osteonecrosis in sickle cell disease. *Southern medical journal*, **109**(9), 525 – 530.

Nur, E., Brandjes, D.P., Schnog, J.J.B., Otten, H.M., Fijnvandraat, K., Schalkwijk, C.G. & Biemond, B.J. (2010) Plasma levels of advanced glycation end products are associated with haemolysis-related organ complications in sickle cell patients. *British Journal of Haematology*, **151**, 62 – 69.

Odetunde, O.I., Chinawa, J.M., Achigbu, K.I. & Achigbu, E.O. (2016) Body mass index and other anthropometric variables in children with sickle cell anemia. *Pakistan Journal of Medical Sciences*, **32**(2), 341 – 346.

Peppa, M., Uribarri, J. & Vlassara, H. (2004) The role of advanced glycation end products in the development of atherosclerosis. *Current Diabetes Reports*, **4**, 31 – 36.

Picklo, M.J., Montine, T.J., Amarnath, V. & Neely, M.D. (2002) Carbonyl toxicology and Alzheimer's diseases. *Toxicology and Applied Pharmacology*, **184**, 187 – 197.

Piel, F.B., Howes, R.E., Patil, A.P., Nyangiri, O.A., Gething, P.W., Bhatt, S., Williams, T.N., Weatherall, D.J. & Hay, S.I. (2013) The distribution of haemoglobin C and its prevalence in newborns in Africa. *Scientific reports*, **3**(1671), 1 – 8.

Prindle, K.H. Jr. & Mccurdy, P.R. (1970) Red cell lifespan in hemoglobin C disorders (with special reference to hemoglobin C trait). *Blood*, **36**(1), 14-19.

Seixas, M.O., Rocha, L.C., Carvalho, M.B., Menezes, J.F., Lyra, I.M., Nascimento, V.M.L., Couto, R.D., Atta, A.M., Reis, M.G. & Goncalves M.S. (2010) Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease. *Lipids in Health and Disease*, **9**, 1–9.

Shores, J., Peterson, J., Vanderjagt, D.J. & Gless, R.H. (2003) Reduced cholesterol levels in African-American adults with sickle cell disease. *Journal of the National Medical Association*, **95**(9), 813-817.

Silva, L.B., Gonçalves, R.P. & Rabenhorst, S.H. (2009) Análise dos haplótipos da anemia falciforme em Fortaleza revela as origens étnicas da população cearense. *The Jornal Brasileiro de* Patologia *e Medicina Laboratorial*, **45**, 115-118.

Silva-Pinto, A.N., Angulo, I.L., Brunetta, D.M., Neves, F.I.R, Bassi, S.C., De Santis, G.C. & Covas, D.T. (2013) Clinical and hematological effects of hydroxyurea therapy in sickle cell patients: a single-center experience in Brazil. *Sao Paulo Medical Journal*, **131**, 238–243.

Singhal, A., Davies, P., Sahota, A., Thomas, P.W (1993) Serjeant GR. Resting metabolic rate in homozygous sickle cell disease. *The American Journal of Clinical Nutrition*, **57**, 32–34.

Somjee, S.S., Warrier, R.P., Thompson, J.L., Ory-Ascani, J. & Hempe, J.M. (2004) Advanced glycation end-products in sickle cell anemia. *British Journal of Haematology*, **128**, 112 – 118.

Sutton, M., Bouhassira, E.E. & Nagel, R.L. (1989) Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes. *American Journal of Hematology*, **32**(1), 66–99.

Thallas-Bonke, V., Lindschau, C., Rizkalla, B., Bach, L.A., Boner, G., Meier, M., Haller, H., Cooper, M.E. & Forbes, J.M. (2004) Attenuation of extracellular matrix accumulation in diabetic nephropathy by the advanced glycation end product cross-link breaker ALT-711 via a protein kinase C-dependent pathway. *Diabetes*, **53**, 2921–2930.

Tessier, F.J., Jacolot, P. & Niquet-Léridon, C. (2014) Open questions around the carboxymethyllysine. *IMARS Highlights*, **9**, 14–20.

van Beers, E.J., van Tujin, C.F., MacGillavry, M.R., van der Giessen, A., Schnog, J.J.B. & Biemond, B.J. (2008) Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. *Haematologica*, **93**, 757 – 760.

VanderJagt, D.J., Shores, J., Okorodudu, A., Okolo, S.N. & Glew, R.H. (2002) Hypocholesterolemia in Nigerian children with sickle cell disease. *Journal of Tropical Pediatrics*, **48**, 156-161.

Voziyan, P.A., Khalifah, R.G., Thibaudeau, C., Yildiz, A., Jacob, J., Serianni, A.S. & Hudson, B.G. (2003) Modification of proteins in vitro by physiological levels of glucose: pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation end-products through binding of redox metal ions. *The Journal of Biological Chemistry*, **278**, 46616–44624.

Weatherall, D.J. (2010) The inherited diseases of hemoglobin are an emerging global health burden. *Blood*, **115**(22), 4331-4336.

Zivot, A., Apollonsky, N., Gracely, E., Raybagkar, D. (2017). Body mass index and the association with vaso-occlusive crises in pediatric sickle cell disease. *Journal of Pediatric Hematology/Oncology*, **39**(4), 314 – 317.

Zorca, S., Freeman, L., Hildesheim, M., Allen, D., Remaley, A.T., Taylor, J.G. & Kato, G.J. (2010) Lipid levels in sickle-cell disease associated with haemolytic severity, vascular dysfunction and pulmonary hypertension. *British Journal of Haematology*, **149**, 436–445.

Table 1. Comparison of the clinical and laboratory characteristics of sickle HbSC patients against normal healthy control patients

Laboratory parameter	HbSC, N = 20, median (25^{th} – 75^{th} percentile)	HbAA, N = 23, median (25^{th} – 75^{th} percentile)	p-value*
Age, years	5 (3 – 17)**	7 (5 – 14)**	0.0612
BMI, kg/m^2	15 (13.7 – 17.2)	17.23 (15.7 – 19.64)	0.0097
Red Blood Cell, $\times 10^{12}/L$	4.42 (3.99 – 4.82)	4.64 (4.40 – 4.95)	0.0594
Hemoglobin, g/dL	11.8 (11.20 – 12.10)	13.00 (12.30 – 13.30)	0.0001
Hematocrit, %	33.40 (31.80 – 34.70)	39.80 (38.10 – 40.70)	<0.0001
MCV, fL	76.30 (72.20 – 82.40)	85.50 (83.19 – 40.70)	<0.0001
MCH, ρg	26.40 (24.70 – 30.00)	27.89 (27.05 -29.00)	0.2742
MCHC, g/dL	35.00 (34.00 – 35.60)	32.57 (32.18 – 33.08)	<0.0001
RDW, %	16.3 (15.4 – 16.9)	12.8 (12.00 – 13.80)	<0.0001
Reticulocyte, %	3.00 (1.80 – 4.50)	0.6 (0.50 – 1.00)	<0.0001
Leukocytes, $\times 10^6/L$	8370 (7260.00 – 10400.00)	7030 (5306 – 8100)	0.0479
Neutrophils, $\times 10^6/L$	4235.00 (2387.00 – 5498.00)	3402 (1988 – 4447)	0.1558
Eosinophils, $\times 10^6/L$	419.00 (244.00 – 671.00)	236 (179 – 510)	0.2000
Basophils, $\times 10^6/L$	65.00 (0.00 – 151.00)	74 (55 – 97)	0.9618
Typical lymphocytes, $\times 10^6/L$	2977.00 (2416.00 – 3549.00)	2531 (2075 – 2856)	0.1479
Monocytes, $\times 10^6/L$	555.00 (364.00 – 728.00)	561 (419 – 633)	0.8871
Platelets, $\times 10^3/mL$	251.00 (217.00 – 308.00)	286 (243 – 324)	0.2082
Mean Platelet Volume (MPV)	6.7 (6.1 – 7.1)	7.8 (6.70 – 8.14)	0.0694
HbS, %	51.55 (50.00 – 53.50)	-	
HbC, %	41.45 (39.35 – 43.10)	-	
Glucose, mg/dL	81.00 (70.50 – 89.00)	85.50 (82.00 – 88.00)	0.2262
Total Cholesterol, mg/dL	130.00 (119.00 – 153.00)	161.00 (147 – 169)	0.0005
HDL-C, mg/dL	38.00 (34.00 – 42.50)	53 (44 – 60)	<0.0001
LDL-C, mg/dL	77.70 (68.20 – 94.60)	92.60 (75.80 – 106.40)	0.1649
VLDL-C, mg/dL	13.50 (11.00 – 17.50)	15.6 (12.6 – 20.4)	0.1611
Triglyceride, mg/dL	67.50 (55.00 – 87.50)	78.00 (63.00 – 102.00)	0.1468

BMI, Body mass index; MCV, Mean Corpuscular Volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red blood cell Distribution Width; HDL-C, High-density lipoprotein-cholesterol; LDL-C, Low-density lipoprotein-cholesterol; VLDL-C, Very low-density lipoprotein-cholesterol.

*P-value is considered significant at value ≤ 0.05.

** Values in parenthesis presented as minimum and maximum values for age.

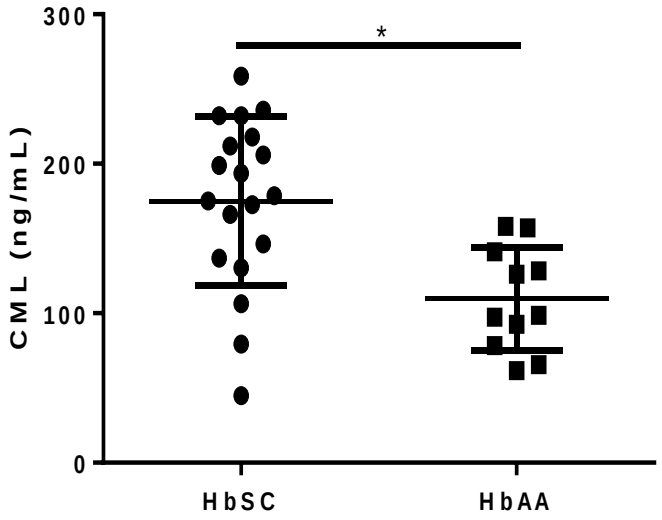


Figure 1. Serum levels of N^{ε}-(carboxymethyl)lysine (CML) in healthy controls (AA) and sickle hemoglobin SC (HbSC) patients. CML levels are significantly higher in HbSC patients compared to the healthy control. * P = 0.0012. We used the Student T-test.

$P < 0.05$ was considered significant.

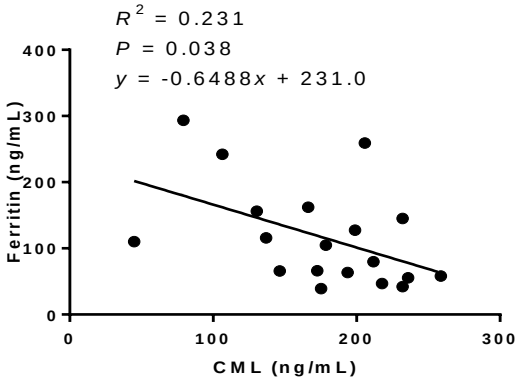
Table 2. Correlation analyses of some biomarkers with serum levels of *N*ε-(Carboxymethyl)lysine in children with sickle HbSC

	CML (ng/mL)
Biomarker	Spearman r (*p*)
Age	-0.237 (0.328)
BMI	-0.389 (0.093)
RBC	0.238 (0.373)
Hb	**0.509* (0.046)**
Hematocrit	**0.505* (0.048)**
MCV	0.332 (0.208)
MCHC	-0.017 (0.941)
Total cholesterol	0.225 (0.354)
HDL-cholesterol	0.038 (0.875)
LDL-cholesterol	0.180 (0.461)
Serum Iron	0.412 (0.080)
Total bilirubin	0.424 (0.071)
Total protein	-0.080 (0.743)
Ferritin	**-0.518* (0.023)**
C-reactive protein	**-0.505* (0.028)**

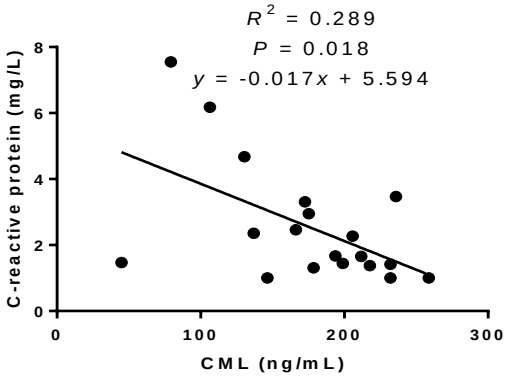
** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

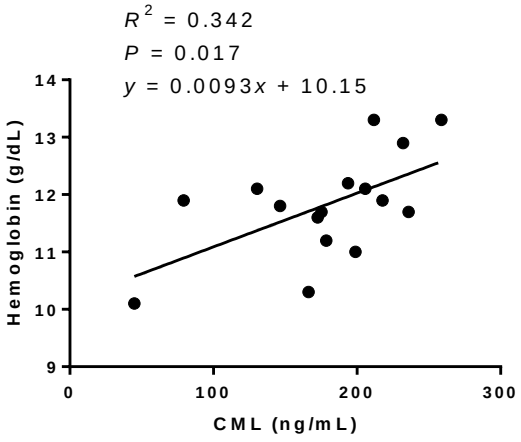
BMI, Body mass index; Hb, Hemoglobin; RBC, red blood cell; MCV, Mean Corpuscular Volume; MCHC, Mean corpuscular hemoglobin concentration; HDL-cholesterol, High-density lipoprotein-cholesterol; LDL-cholesterol, Low-density lipoprotein-cholesterol.


R^2 = 0.231
P = 0.038
y = -0.6488x + 231.0
Ferritin (ng/mL)
400
300
200
100
0
0
100
200
300
CML (ng/mL)

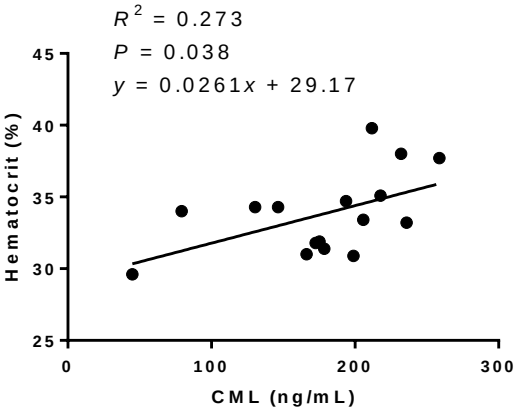
A


R^2 = 0.289
P = 0.018
y = -0.017x + 5.594
C-reactive protein (mg/L)
8
6
4
2
0
0
100
200
300
CML (ng/mL)

B


R^2 = 0.342
P = 0.017
y = 0.0093x + 10.15
Hemoglobin (g/dL)
14
13
12
11
10
9
0
100
200
300
CML (ng/mL)

C



D

Figure 2. Regression curves of some biomarkers that were statistically significant when correlated with carboxymethyllysine (CML). A. Negative linear regression between ferritin (ng/mL) and CML (ng/mL) (r = -0.481; p = 0.038). B. Negative linear regression between C-reactive protein (mg/mL) and CML (ng/mL) (r = -0.538; p = 0.018). C. Positive linear regression between hemoglobin (g/dL) and CML (ng/mL) (r = 0.584; p = 0.017). D. Positive linear regression between hematocrit (%) and CML (ng/mL) (r = 0.522; p = 0.038).

Table 3. Multivariate regression analyses of some biomarkers of sickle hemoglobin SC disease with carboxymethyllysine (CML) as the dependent variable

Independent variable	Coefficient (β)	*P* value	Dependent variable	R^2	*P* value
BMI	1.262	0.855	CML (ng/mL)	0.747	0.114
Hemoglobin	43.159	0.281			
Hematocrit	-8.205	0.521			
Total cholesterol	0.973	0.176			
Serum Iron	0.807	0.323			
Total bilirubin	15.592	0.179			
Ferritin	-0.015	0.944			
C-reactive protein	-19.011	0.077			

BMI signify body mass index; *P* values are significant at ≤ 0.05

Table 4. Haplotype characterization of sickle hemoglobin SC disease according to the median level of carboxymethyllysine (CML)

	Carboxymethyllysine (CML)				
Molecular characteristic	CML ≤ median*, n (%)	CML > median*, n (%)	Total, n (%)	χ^2	p
CAR/I	5 (25)	3 (15)	8 (40)	4.5	0.48
CAR/II	1 (5)	2 (10)	3 (15)		
BEN/I	4 (20)	2 (10)	6 (30)		
BEN/II	0 (0)	1 (5)	1 (5)		
BEN/III	0 (0)	1 (5)	1 (5)		
CAM/II	0 (0)	1 (5)	1 (5)		
Total	10 (50)	10 (50)	20 (100)		

χ^2, Pearson Chi-square; *median value = 186.19ng/mL.

5 DISCUSSÃO

Estudos têm demonstrado que crianças com AF, mesmo em estado estável, apresentam valores alterados de vários biomarcadores, em comparação com valores de referência estabelecidos para a idade (SILVA-PINTO et al., 2013). Entretanto, pouco se sabe sobre como esses biomarcadores são afetados pelo tratamento com HU, que é indicado em casos mais graves da doença.

No presente estudo, as concentrações de Hb e hematócrito foram significativamente mais elevadas nos indivíduos com AF que estavam em uso de HU (AF HU^+), quando comparadas aqueles que não faziam uso dessa terapia (AF HU^-). Esse fato indicou uma melhora no quadro da anemia, o que está de acordo com os trabalhos de Hankins e cols (2005) e Silva-Pinto e cols (2013). Nosso estudo não mostrou aumento significativo nas concentrações de HbF no grupo com AF HU^+, diferentemente do relatado por Silva-Pinto e cols (2013). Entretanto, Steinberg e cols (1997) descreveram que o aumento na HbF não foi significante em um estudo multicêntrico de hidroxiuréia, o que está de acordo com nossos resultados. O aumento não significativo da HbF na AF HU^+ poderia ser atribuído à adesão irregular à dose diária por alguns dos entrevistados e / ou à variação no tempo de tratamento da HU (NEVITT et al., 2017; THORNBURG et al., 2010). As contagens de leucócitos, neutrófilos e eosinófilos foram significativamente mais baixas nos indivíduos com AF que receberam HU, sugerindo a redução nas crises vaso-oclusivas e eventos dolorosos (CHARACHE et al., 1995; FERSTER et al., 1996). Nossos resultados também estão em concordância com os resultados de Silva-Pinto e cols (2013) e o relato de Davies e Gilmore (2003). Concentrações significativamente elevadas de ferritina e PCR sugerem que os indivíduos com AF HU^+ apresentaram resposta inflamatória mais pronunciada quando comparados aos AF HU^-, o que pode ser reflexo da leucorredução que acompanha o tratamento (LARADE e STOREY, 2004; LAU et al., 2005; NUR et al., 2011). A redução significativa na lactato desidrogenase, bilirrubina total e bilirrubina indireta está associada a resposta sistêmica decorrente da redução geral nos fenômenos hemolíticos no grupo AF HU^+. Nosso estudo demonstrou que a AST e a creatinina estiveram diminuídas no grupo AF HU^+. Por outro lado, a AST, PCR, creatinina e Hb foram apontados como possíveis marcadores associados a redução da LDH no grupo AF HU^+. A PCR apresentou a correlação positiva significativa com a creatinina, sugerindo que a lesão renal poderia estimular a resposta inflamatória.

Os PFGAs estão envolvidos na fisiopatologia das doenças cardiovasculares, sarcopenia e doenças renais (BAUMANN, 2012; DALAL et al., 2009). Eles também foram implicados na patogênese da nefropatia e retinopatia diabética, bem como na aterosclerose, amiloidose associada à hemodiálise e doença de Alzheimer (HAMMES et al., 1996; IMAI et al., 1997; KUME et al., 1995; MIYATA et al., 1998; SMITH et al., 1995). Eles demonstraram ser marcadores de estresse oxidativo (NOWOTNY et al., 2015) e promovem na liberação de ROS (BYUN et al., 2017).

A gravidade da vaso-oclusão na DF, a progressão para isquemia de órgãos-alvo e a crise de dor são conhecidas por serem potencializadas pelo estresse oxidativo (QUEIROZ e LIMA, 2013). Isso nos motivou a explorar o papel de PFGAs, como a CML, que são biomarcadores estabelecidos do estresse oxidativo (NUR et al., 2010). No diabetes, níveis mais elevados de PFGAs estão associados ao aumento da glicose plasmática; no entanto, na insuficiência renal, foi sugerido que a excreção renal comprometida e o clearance insuficiente pelas membranas de diálise estão associados a PFGA (VLASSARA et al., 1994; FRIEDLANDER et al., 1995; GERBITZ et al., 1995). Estudos demonstraram que a CML desempenha papel importante nas complicações cardiovasculares do envelhecimento, diabetes e insuficiência renal terminal (MÜNCH et al., 1997), o que implica que níveis elevados de PFGAs podem ocorrer mesmo na ausência de hiperglicemia (URIBARRI et al., 2015).

Cerca de uma década atrás, foi relatado que os níveis circulantes de PFGAs em pacientes com AF desempenham papel na patologia vascular da AF (SOMJEE et al., 2004). Este resultado anterior foi corroborado pelo trabalho de Nur e cols. (2010).

Observamos, em nosso trabalho, elevação da CML em todos os pacientes com DF em comparação aos controles saudáveis. Esses achados estão de acordo com os trabalhos de Somjee e cols. (2004) e Nur e cols. (2010). No entanto, não houve diferença significativa entre o grupo AF HU^+ e AF HU^-, o que sugere que o tratamento com HU não afeta significativamente o nível de PFGAs nos indivíduos com AF. Até o presente momento não encontramos nenhum relato que explorou especificamente o possível efeito de HU no nível de CML na AF. No entanto, Nur e cols. (2010) relataram que alguns de seus sujeitos no tratamento com HU não apresentaram diferença significativa comparados com aqueles sem tratamento com HU. A ausência de diferença significativa entre os grupos AF HU^+ e AF HU^- poderia ser resultado da ingestão

irregular e inconsistente do fármaco pelos pacientes AF HU^{+}, idade ou variação no período de ingestão (THORNBURG et al., 2010).

Nosso estudo mostrou ainda que os pacientes com doença SC também têm níveis significativamente mais elevados de CML em comparação com o grupo controle saudável, ao contrário dos achados de Nur e cols. (2010) em que HbSC assintomático não foi significativamente diferente do controle saudável. Os trabalhos de Nur e cols. (2010) consideraram apenas pacientes adultos com DF e relatos mostraram que o aumento moderado dos PFGAs esteve associado ao envelhecimento normal, onde foi demonstrado que os PFGAs aumentam com a idade (MÜNCH et al., 1997; ODETTI et al., 1994). Portanto, a diferença significativa em nosso relato em comparação com os trabalhos de Nur e cols. (2010) poderia ser devido ao fato de termos considerado apenas crianças com HbSC.

Os haplótipos estão entre vários fatores que poderiam ser responsáveis pelas características clínicas e laboratoriais variáveis da DF (FIGUEIREDO, 2007). De fato, estudos demonstraram que o haplótipo Bantu está associado às características clínicas mais graves (FLEURY, 2007; SILVA et al., 2009). Nossos resultados mostram que níveis elevados ou diminuídos de CML sérica estão significativamente associados a diferentes haplótipos associados a globina β^{S}. Este resultado, no entanto, sugere que os haplótipos poderiam influenciar a resposta dos pacientes com AF quanto ao papel patológico da CML. Nossos resultados revelam, além disso, que a CML elevada ou diminuída não esteve relacionada com diferentes haplótipos β^{S}/β^{C}. Até onde sabemos, nenhum estudo investigou a provável influência de PFGAs em haplótipos variados.

Nossos resultados suscitam perspectivas para estudos futuros de acompanhamento sobre o efeito da HU em pacientes com AF e o papel da CML na resposta ao tratamento com esse fármaco, bem como sua relação/associação com parâmetros laboratoriais e haplótipos ligados ao gene da globina β^{S}. Além disso, estudos adicionais sobre as manifestações clínicas, como crises de vaso-oclusão e de dor, e de outros parâmetros de acompanhamento, associados aos níveis de CML, podem contibuir para a ampliação de conhecimentos acerca dos mecanismos de patogênese da DF.

6 CONCLUSÕES

Nossos resultados sugerem que a HU desempenha papel significativo na inflamação, hemólise e sistemas hepáticos relacionados a patogênese da AF. As evidências deste trabalho sugerem a possibilidade de que a CML tenha influência na fisiopatologia da DF, como tambem, uma possível associação desse PFGA com os haplótipos ligados ao gene da globina β^S. Por fim, sugerimos que a CML pode estar associada a complicações da AF e que seus niveis não são influenciados pelo tratamento com HU.

REFERÊNCIAS

ADORNO, E. V. et al. The beta-globin gene cluster haplotypes in sickle cell anemia patients from Northeast Brazil: A clinical and molecular view. **Hemoglobin**, v. 28, n. 3, p. 267-271, 2004.

AGARWAL, A. et al. Hemoglobin C associated with protection from severe malaria in the Dogon of Mali, a West African population with a low prevalence of hemoglobin S. **Blood**, v. 96, n. 7, p. 2358-2363, 2000.

AHMED, N. Advanced glycation endproducts--role in pathology of diabetic complications. **Diabetes Research and Clinical Practice**, v. 67, n. 1, p. 3-21, 2005.

AMEH, S. J.; TARFA, F. D.; EBESHI, B. U. Traditional herbal management of sickle cell anemia: Lessons from Nigeria. **Anemia**, v. 2012, n. 607436, p. 1-9, 2012.

ARAKI, N. et al. Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. **Journal of Biological Chemistry,** v. 267, p. 10211–10214, 1992.

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

BALLAS, S. K. et al. Clinical, Hematological, and Biochemical Features of Hb SC disease. **American Journal of Hematology,** v. 13, n. 1, p. 37–51, 1982.

BANDEIRA, I. C. J. et al. Chronic inflammatory state in sickle cell anemia patients is associated with HBB*S haplotype. **Cytokine,** v. 65, p. 217-221, 2014.

BANDEIRA, F. M. G. C. et al. Hidroxiuréia em pacientes com síndromes falciformes acompanhados no Hospital Hemope, Recife, Brasil. Hydroxyurea in sickle cell disease patients in Recife, Brazil. **Revista Brasileira de Hematologia e Hemoterapia**, v. 26, n. 3, p. 189-194, 2004.

BARTOLUCCI, P. et al. Decreased sickle red blood cell adhesion to laminin by hydroxyurea is associated with inhibition of Lu/BCAM protein phosphorylation. **Blood**, v. 116, n. 12, p. 2152–2159, 2010.

BAUMANN, M. Role of advanced glycation end products in hypertension and cardiovascular risk: human studies. **Journal of the American Society of Hypertension**, v. 6, n. 6, p. 427–435, 2012.

BELCHER, J. D. et al. Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. **Blood,** v. 96, n. 7, p. 2451-2459, 2000.

BENDER, M. A.; DOUTHITT, S. G. **Sickle Cell Disease**. 2003 Sep 15 [Updated 2014 Oct 23]. In: PAGON, R.A. et al. (Ed.). GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2015. Disponível em: http://www.ncbi.nlm.nih.gov/books/NBK1377/

BENKERROU, M. et al. Hydroxyurea corrects the dysregulated L-selectin expression and increased H_2O_2 production of polymorphonuclear neutrophils from patients with sickle cell anemia. **Blood,** v. 99, n. 7, p. 297-303, 2002.

BRANDELISE, S. et al. Newborn screening for sickle cell disease in Brazil: the Campinas experience. **Clinical and Laboratory Haematology,** v. 26, n. 1, p. 15-9, 2004.

BROWNLEE, M. Biochemistry and molecular cell biology of diabetic complications. **Nature,** v. 414, p. 813–20, 2001.

BRUGNARA, C.; STEINBERG, M. H. Developing treatment for sickle cell disease. **Expert opinion on Investigative Drugs,** v. 11, n. 5, p. 645-659, 2002.

BUNN, H. F. et al. Molecular and cellular pathogenesis of hemoglobin SC disease. **Proceedings of the National Academy Science,** v. 79, n. 23, p. 527–31, 1982.

BYUN, K. et al. Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. **Pharmacology and Therapeutics**, v. 177, p. 44 – 55, 2017.

CABAÑAS-PEDRO, A. C. et al. Hemoglobin sickle cell disease in Brazil. **Haematologica**, v. 98, 2013.

CAI, W. et al. Oxidative stressinducing carbonyl compounds from common foods: novel mediators of cellular dysfunction. **Molecular Medicine**, v. 8, p. 337–46, 2002.

CARTRON, J. P.; ELION, J. Erythroid adhesion molecules in sickle cell disease: effect of hydroxyurea. **Transfusion Clinique et Biologique,** v. 15, n. 1-2, p. 39–50, 2008.

CHAAR, V. et al. Hydoxrycarbamide decreases sickle reticulocyte adhesion to resting endothelium by inhibiting endothelial Lutheran/Basal cell adhesion molecule (Lu/BCAM) through phosphodiesterase 4ª activation. **The Journal of Biological Chemistry,** v. 289, n. 16, p. 11512-11521, 2014.

CHAKRAVORTY, S.; WILLIAMS, T. N. Sickle cell disease: a neglected chronic disease of increasing global health importance. **Archives of Disease in Childhood,** v. 100, n.1, p. 48–53, 2015.

CHARACHE, S. et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. investigators of the multicenter study of hydroxyurea in sickle cell anemia. **New England Journal of Medicine**, v. 332, n. 20, p. 1317-1322, 1995.

COKIC, V. P. et al. Hydroxyurea induces fetal hemoglobin by the nitric oxide–dependent activation of soluble guanylyl cyclase. **The Journal of Clinical Investigation,** v. 111, n. 2, p. 231–239, 2003.

CONNES, P.; VERLHAC, S.; BERNAUDIN, F. Advances in understanding the pathogenesis of cerebrovascular vasculopathy in sickle cell anaemia. **British Journal of haematology**, v. 161, p. 484–498, 2013.

CONRAN, N.; FRANCO-PENTEADO, C. F.; COSTA, F. F. Newer aspects of the pathophysiology of sickle cell disease vaso-occlusion. **Hemoglobin**, v. 33, p. 1-16, 2009.

DALAL, M. et al. Elevated serum advanced glycation end products and poor grip strength in older community-dwelling women. **Journals of Gerontology – Series A Biological Sciences and Medical Sciences**, v. 64, n. 1, p. 132–137, 2009.

DAVIES, S.C.; GILMORE, A. The role of hydroxyurea in the management of sickle cell disease. **Blood Review**, v. 17, p. 99–109, 2003.

DEGENHARDT, T. P.; THORPE, S. R.; BAYNES, J. W. Chemical modification of proteins by methylglyoxal. **Cell and Molecular Biology**, v. 44, p. 1139–1145, 1998.

DOVER, G. J.; PLATT, O. S. Sickle cell disease. In: NATHAN, D.G.; ORKIN, S.H. (Ed.). **Nathan and Oiski's hematology of infancy and childhood.** Philadelphia: WB Saunders Co., 1998. p. 762-810.

FATHALLAH, H.; ATWEH, G. F. Induction of fetal hemoglobin in the treatment of sickle cell disease. **Hematology**, p. 58-62, 2006.

FELIX, A. A.; SOUZA, H. M.; RIBEIRO, S. B. F. Aspectos epidemiológicos e sociais da doença falciforme: Epidemiologic and social aspects of sickle cell disease. **Revista Brasileira de Hematologia e Hemoterapia**, v. 32, n. 3, 203-208, 2010.

FERSTER, A. et al. Hydroxyurea for treatment of severe sickle cell anemia: a pediatric clinical trial. **Blood**, v. 88, n. 6, p. 1960–1964, 1996.

FIGUEIREDO, M. S. Fatores moduladores da gravidade da evolução clínica da anemia falciforme. **Revista Brasileira de Hematologia e Hemoterapia**, v. 29, p. 215-217, 2007.

FIGUEIREDO, M. S. et al. Effects of α–thalassemia and β-globin gene cluster haplotypes on the hematological and clinical features of sickle-cell anemia in Brazil. **American Journal of Hematology**, v. 53, p. 72-76, 1996.

FLEURY, M. K. Haplótipos do cluster da globina beta em pacientes com anemia falciforme no Rio de Janeiro: Aspectos clínicos e laboratoriais. ***RBAC***, v. 39, p. 89 – 93, 2007.

FRIEDLANDER, M. A. et al. Influence of dialysis modality on plasma and tissue concentrations of pentosidine in patients with end-stage renal disease. **American Journal of Kidney Diseases**, v. 25, p. 445-451, 1995.

FU, M.X. et al. The advanced glycation end product, Nε-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. **Journal of Biological Chemistry,** v. 271, p. 9982–9986, 1996.

GALBRAITH, P. A.; GREEN, P. T. Hemoglobin C disease in an Anglo-Saxon family. **The American Journal of Medicine,** v. 28, p. 969-972, 1960.

GARDNER, K. et al. Survival in adults with sickle cell disease in a high income setting. **Blood**, v. 128, n. 10, p. 1436–1438, 2016.

GEE, B. E.; PLATT, O. S. Sickle reticulocytes adhere to VCAM-1. **Blood,** v. 85, n. 1, p. 268-274, 1995.

GERBITZ, K. D. et al. Immunological determination of advanced glycation endproducts in human blood and urine. **Nephrology Dialysis Transplantation**, v. 10, p. 657-664, 1995.

GERRITS, E. G. et al. Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications. **Diabetes Care,** 31, 3, p. 517-521, 2008.

GKOGKOLOU, P.; BÖHM, M. Advanced glycation end products: key players in skin aging. **Dermato endocrinology**, v. 4, p. 259-270, 2012.

GLOMB, M.A.; MONNIER, V.M. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. **Journal of Biological Chemistry**, v. 270, p. 10017–10026, 1995.

GOLDBERG, T. et al. Advanced Glycoxidation End Products in Commonly Consumed Foods. **Journal of the American Dietetic Association**, v. 104, p. 1287-1291, 2004.

GONÇALVES, M. S. et al. Bs-haplotypes in sickle cell anemia patients from Salvador, Bahia, Northeastern Brazil. **Brazilian Journal of Medical and Biological Research**, v. 36, n. 10, p. 1283-1288, 2003.

GUARDA, C. C. Células endoteliais ativadas pelo heme: Avaliação de diferentes abordagens terapêuticas. Dissertação de mestrado não publicada entregou para **Curso de Pós-Graduação em Patologia Humana e Experimenta, Universidade Federal da Bahia**, 2015.

HAMMES, H.P. et al. Modification of vitronectin by advanced glycation alters functional properties in vitro and in the diabetic retina. **Laboratory Investigation**, v. 75, p. 325–338, 1996.

HANKINS, J. S. et al. Long-term hydroxyurea therapy for infants with sickle cell anemia: the HUSOFT extension study. **Blood**, v. 106, n. 7, p. 2269 – 2275, 2005.

HEIN, G. et al. Advanced glycation end-products pentosidine and N-carboxymethyllysine are elevated in serum of patients with osteoporosis. **Rheumatology**, v. 42, n. 10, p. 1242 – 1246, 2003.

HO, J. A. et al. In vitro induction of fetal hemoglobin in human erythroid progenitor cells. **Experimental Hematology**, v. 31, n. 7, p. 586-591, 2003.

HOWARD, J.; TELFER P. **Sickle Cell Disease in Clinical Practice**. London 2015: Springer-Verlag, 2015.

IMAI, N. et al. Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. **Nephron**, v. 76, p. 153–160, 1997.

KING, S. B. Nitric oxide production from hydroxyurea. **Free Radical Biology and Medicine**, v. 37, n. 6, p. 737–744, 2004.

KOITO, W. et al. Conventional Antibody against Nε-(Carboxymethyl)Lysine (CML) Shows Cross-Reaction to Nε-(Carboxyethyl)Lysine (CEL): Immunochemical Quantification of CML with a Specific Antibody. **The Journal of Biochemistry**, v. 136, p. 831-837, 2004.

KOVACIC, P. Hydroxyurea (therapeutics and mechanism): metabolism, carbamoyl nitroso, nitroxyl, radicals, cell signaling and clinical applications. **Medical Hypotheses**, v. 76, n. 1, p. 24-31, 2011.

KUME, S. et al. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. **American Journal of Pathology**, v. 147, p. 654–667, 1995.

KWIATKOWSKI, D. P. How malaria has affected the human genome and what human genetics can teach us about malaria. **American Journal of Human Genetics**, v. 77, p. 171 – 190, 2005.

LANZKRON, S.; CARROLL, C. P.; HAYWOOD JUNIOR, C. Mortality Rates and Age at Death from Sickle Cell Disease: US. 1979-2005. **Public Health Reports**, v. 128, p. 110–116, 2013.

LARADE, K.; STOREY, K. B. Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate. **Journal of Experimental Biology**, v. 207, p. 1353-1360, 2004.

LATAGLIATA, R. et al. Symptomatic mucocutaneous toxicity of hydroxyurea in Philadelphia chromosome-negative myeloproliferative neoplasms: the Mister Hyde face of a safe drug. **Cancer**, v. 118, n. 2, p. 404-409, 2012.

LAU, D. et al. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. **Proceedings of the National Academy of Science U S A**, v. 102, p. 431-436, 2005.

LAURANCE, S. et al. Hydroxycarbamide stimulates the production of proinflammatory cytokines by endothelial cells: relevance to sickle cell disease. **Pharmacogenetics and Genomics**, v. 20, n. 4, p. 257–268, 2010.

LAURANCE, S. et al. Differential modulation of adhesion molecule expression by hydroxycarbamide in human endothelial cells from the micro- and macrocirculation: potential implications in sickle cell disease vasoocclusive events. **Haematologica**, v. 96, p. 534–542, 2011.

LAURENTINO, M. R. et al. Influence of BS-globin haplotypes and hydroxyurea on tumor necrosis factor-alpha levels in sickle cell anemia. **Brazilian Journal of Hematology and Hemotherapy**, v. 36, n. 2, p. 121-125, 2014.

LEVEZIEL, N. V. et al. Clinical and laboratory factors associated with the severity of proliferative sickle cell retinopathy in patients with sickle cell hemoglobin C (SC) and homozygous sickle cell (SS) disease. **Medicine (Baltimore)**, v. 90, n. 6, p. 372-378, 2011.

LERVOLINO, L. G. et al. Prevalence of sickle cell disease and sickle cell trait in national neonatal screening studies. **Revista Brasileira de Hematologia e Hemoterapia**, v. 33, n. 1, p. 49-54, 2011.

LIONNET, F. et al. Hemoglobin sickle cell disease complications: a clinical study of 179 cases. **Haematologica**, v. 97, n. 8, p. 1136-41, 2012.

LOBO, C. L. et al. Triagem neonatal para hemoglobinopatias no Rio de Janeiro, Brasil. **Pan American Journal of Public Health**, v. 13, n. (2/3), p. 154-159, 2003.

MAKIS, A. C.; HATZIMICHAEL, E. C.; BOURANTAS, K. L. The role of cytokines in sickle cell disease. **Annals of Hematology**, v. 79, n. 8, 407-413, 2000.

MANGANO, V. D. et al. Novel insights into the protective role of Hemoglobin S and C against *Plasmodium falciparum* parasitemia. **The Journal of Infectious Diseases**, v. 212, n. 4, p. 626-634, 2015.

MANTOVANI, A.; FIGINI, I. Sickle cell-hemoglobin C retinopathy: transient obstruction of retinal and choroidal circulations and transient drying out of retinal neovessels. **International ophthalmology**, v. 28, n. 2, p. 135-137, 2008.

McGANN, P. T.; HERNANDEZ, A. G.; WARE, R. E. Sickle cell anemia in sub-Saharan Africa: advancing the clinical paradigm through partnerships and research. **Blood**, v. 129, p. 155-161, 2017.

BRASIL. MINISTERIO DA SAUDE. Secretaria de Atenção à Saúde. Departamento de Atenção Especializada e Temática. **Doenca falciforme**: Diretrizes Básicas da linha de cuidado. Brasília, DF, 2015. p. 82.

MIYATA, T. et al. Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. **Biochemical and Biophysical Research Communications**, n. 244, n.1, p. 45-49, 1998.

MODIANO, D. et al. Haemoglobin C protects against clinical Plasmodium falciparum malaria. **Nature**, v. 414, n. 6861, p. 305-308, 2001.

MOORE, C. M. et al. New concepts in the immunology of sickle cell disease. **Annals of Allergy, Asthma and Immunology,** v. 76, p. 385–400, 1996.

MÜNCH, G. et al. Determination of advanced glycation end products in serum by fluorescence spectroscopy and competitive ELISA. **European Journal of Clinical Chemistry and Clinical Biochemistry**, v. 39, n. 9, p. 669 – 677, 1997.

NAGAI, R. et al. Hydroxyl radical mediates Nε-(carboxymethyl)lysine formation from Amadori product. **Biochemical and Biophysical Research Communications**, v. 234, p. 167–172, 1997.

NAGAI, R. et al. Peroxynitrite induces formation of Nε-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. **Diabetes**, v. 51, p. 2833–2889, 2002.

NAGEL, R. L. The origin of the hemoglobin S gene: clinical, genetic and anthropological consequences. **Einstein Quarterly Journal of Biology and Medicine**, v. 2, p. 53-62, 1984.

NAGEL, R. L.; FABRY, M. E.; STEINBERG, M. H. The paradox of hemoglobin SC disease. **Blood Review**, v. 17, p. 167–78, 2003.

NAGEL, R. L.; STEINBERG, M. H. **Hemoglobin SC disease and HbC disorders.** In Disorders of Hemoglobin:Genetics, Pathophysiology, and Clinical Management. STEINBERG, M.H., FORGET, B.G., HIGGS, D.R., and NAGEL, R.L., Eds. Cambridge University Press, Cambridge. pp. 756–785, 2001.

NCEPOD. '**Sickle: A sickle crisis**?' A report of the national confidential enquiry into patient outcome and death. 2008. Disponível em: www.ncepod.org.uk.2008sc.html.

NEVITT, S. J.; JONES, A. P.; HOWARD, J. Hydroxyurea (hydroxycarbamide) for sickle cell disease. **Cochrane Database of Systematic Reviews**, n. 4, Art. n. CD002202, 2017.

NOWOTNY, K. et al. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. **Biomolecules**, 5, n. 1, p. 194 – 222, 2015.

NUR, E. et al. Plasma levels of advanced glycation end products are associated with haemolysis-related organ complications in sickle cell patients. **British Journal of Haematology**, v. 151, n. 1, p. 62-69, 2010.

NUR, E. et al. Plasma levels of pentraxin-3, an acute phase protein, are increased during sickle cell painful crisis. **Blood Cells, Molecules and Diseases**, v. 46, n. 3, p. 189-94, 2011.

ODETTI, P. et al. Relationships between glycation and oxidation related fluorescences in rat collagen during aging - an *in vivo* and *in vitro* study. **Laboratory Investigation**, v. 70, p. 61-67, 1994.

ODIÈVRE, M. H. et al. Modulation of erythroid adhesion receptor expression by hydroxyurea in children with sickle cell disease. **Haematologica**, v. 93, n. 4, p. 502–510, 2008.

OJIAKO, O. A. et al. Evaluation of the amino acid profile and hemoglobin polymerization inhibition potential of some Nigerian legumes. **British Journal of Pharmaceutical Research**, v. 2, n. 2, p. 80 – 88, 2012.

OKPALA, I. Leukocyte adhesion and the pathophysiology of sickle cell disease. **Current Opinion in Hematology**, v. 13, n. 1, p. 40-44, 2006.

OKPUZOR, J. et al. The potential of medicinal plants in sickle cell disease control: A review. **International Journal of Biomedicine and Health Science**, v. 4, n. 2, p. 47-55, 2008.

PAIXAO, M. C. et al. Reliability of isoelectrofocusing for the detection of Hb S, Hb C, and HB D in a pioneering population-based program of newborn screening in Brazil. **Hemoglobin**, v. 25, n. 3, p. 297-303, 2001.

PENG, X. et al. Naturally occurring inhibitors against the formation of advanced glycation end-products. **Food and Function**, v. 2, p. 289-301, 2011.

PIEL, F. B. et al. Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis. **Nature Communcations**, p. 1 – 7, 2010.

PIEL, F. B. et al. Global epidemiology of sickle haemoglobinin neonates: a contemporary geostatistical model-based map and population estimates. **Lancet**, v. 381, n.9861, p. 142–51, 2013.

PINHEIRO, L. S. et al. Prevalência de hemoglobina S em recém-nascidos de Fortaleza: importância da investigação neonatal. **Revista Brasileira de Ginecologia Obstetricia**, v. 28 n. 2, p. 122-125, 2006.

POWARS, D. R.; CHANL, L.; SCHOREDER, W. A. Beta-S-genecluster haplotypes in sickle cell anemia: clinical implications. **The American Journal of Pediatric Hematology/Oncology**, v. 12, p. 367-74, 1990.

PRINDLE JUNIOR, K. H.; McCURDY, P. R. Red cell lifespan in hemoglobin C disorders (with special reference to hemoglobin C trait). **Blood**, v. 36, n. 1, p. 14-19, 1970.

QUEIROZ, R. F.; LIMA, E. S. Oxidative stress in sickle cell disease. **Revista Brasileira de Hematologia e Hemoterapia**, v. 35, p. 16–17, 2013.

QUINN, C. T. et al. Improved survival of children and adolescents with sickle cell disease. **Blood,** v. 115, n. 17, p. 3447–3452, 2010.

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

RUSANOVA, I. et al. Oxidative stress status, clinical outcome, and β-globin gene cluster haplotypes in pedriatic patients with sickle cell disease. **European Journal of Hematology**, v. 85, n. 8, p. 529-537, 2010.

SCHMIDT, A. M. et al. Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. **Circulation Research**, v. 84, p. 489–497, 1999.

SETTY, B. N. Y.; STUART, M. J. Vascular cell adhesion molecule – 1 is involved in mediating Hypoxia-Induced sickle red blood cell adherence to endothelium: Potential role in sickle cell disease. **Blood**, v. 88, n. 6, p. 2311-2320, 1996.

SILVA, L. B.; GONÇALVES, R. P. Características fenotípicas dos pacientes com anemia falciforme de acordo com os haplótipos do gene da β^S-globina em Fortaleza, Ceará. **Revista Brasileira de Hematologia e Hemoterapia**, v. 32, n. 1, p. 40-44, 2010.

SILVA, L.B.; GONÇALVES, R.P.; RABENHORST, S.H. Análise dos haplótipos da anemia falciforme em Fortaleza revela as origens étnicas da população cearense. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 45, p. 115-118, 2009.

SILVA, W. S. et al. Avaliação da cobertura do programa de triagem neonatal de hemoglobinopatias em populações do Recôncavo Baiano, Brasil. **Cadernos de Saúde Pública**, v. 22, n. 12, p. 2561-66, 2006.

SILVA-PINTO, A. N. et al. Clinical and hematological effects of hydroxyurea therapy in sickle cell patients: a single-center experience in Brazil. **Sao Paulo Medical Journal**, v. 131, n. 4, p. 238-243, 2013.

SINGH, R. et al. Advanced glycation end-products: A review. **Diabetologia**, v. 44, n. 2, p. 129 – 146, 2001.

SMITH, M.A. et al. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. **Proceedings of the National Academy of Science of the USA**, v. 91, p. 5710–5714, 1995.

SOMJEE, S. S. et al. Advanced glycation end-products in sickle cell anaemia. **British Journal of Haematology**, v. 128, p. 112-118, 2004.

STEINBERG, M. H. Compound heterozygous and Other Hemoglobinopathies**.** In: STEINBERG, M. H. et al. (Eds.). **Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management**. Cambridge University Press, Cambridge. 2001. p. 786–810.

STEINBERG, M. H. Pathophysiologically based drug treatment of sickle cell disease. **Trends in Pharmacological Sciences**, v. 27, n. 4, p 204-210, 2006.

STEINBERG, M. H. et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. **JAMA**, v. 289, n. 13, p. 1645-1651, 2003.

STEINBERG, M. H.; BRUGNARA, C. Pathophysiological-based approaches to treatment of sickle cell disease. **Annual Review of Medicine**, v. 54, p. 89 – 112, 2003.

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

SULTANA, C. et al. Interactions of sickle erythrocytes with endothelial cells in the presence of endothelial cell conditioned medium induces oxidative stress leading to transendothelial migration of monocytes. **Blood**, v. 92, n. 10, p. 3924-3935, 1998.

TAM, M. F. et al. Enhanced inhibition of polymerization of sickle cell hemoglobin in the presence of recombinant mutants of human fetal hemoglobin with substitutions at position 43 in the gamma-chain. **Biochemistry**, v. 44, n. 36, p. 12188 – 12195, 2005.

TESSIER, F.J.; JACOLOT, P.; NIQUET-LÉRIDON, C. Open questions around the carboxymethyllysine **IMARS Highlights**, v. 9, p. 14–20, 2014.

THORNALLEY, P. J. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. **Cellular and Molecular Biology (Noisy-le-grand)**, v. 44, n. 7, p. 1013 – 1023, 1998.

THORNBURG, C. D. et al. Adherence to Hydroxyurea Therapy in Children with sickle cell anemia. **Journal of Pediatrics**, v. 156, p. 415–419, 2010.

URIBARRI, J. et al. Dietary Advanced Glycation End Products and Their Role in Health and Disease. **Advances in Nutrition**, v. 6, p. 461–473, 2015.

VLASSARA, H.; BUCALA, R.; STRIKER, L. Biology of disease. Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. **Laboratory Investigation**, v. 70, p. 138-151, 1994.

WANG, W. C. et al. A two-year pilot trial of hydroxyurea in very young children with sickle cell anemia. **Journal of pediatrics**, v. 139, p. 790 – 796, 2001.

WARE, R. E. How I use hydroxyurea to treat young patients with sickle cell anemia. **Blood**, v. 115, n. 26, p. 5300-5311, 2010.

WEATHERALL, D. J. The inherited diseases of hemoglobin are an emerging global health burden. **Blood**, v. 115, n. 22, p. 4331–4336, 2010.

WELLS-KNECHT, K. J. et al. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. **Biochemistry**, v. 34, p. 3702–3709, 1995.

WILLIAMS, T. N. Sickle cell diseas in sub-Saharan Africa. **Hematology/Oncology Clinics of North America**, v. 30, n. 8, p. 343-358, 2016.

WILLIAMS, T. N.; OBARO, S. K. Sickle cell disease and malaria morbidity: a tale with two tails. **Trends in Parasitology**, v. 27, n. 7, p. 315 – 320, 2011.

YAMADA, K. et al. Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure. **Clinical Nephrology**, v. 42, p. 354–361, 1994.

ZAGO, M. A. et al. Hereditary hemoglobin disorders in a Brazilian population. **Human Heredity**, v. 33, n. 2, p. 125-9, 1983.

ZHANG, D. et al. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. **Blood**, v. 127, p. 801–809, 2016.

ZIMMERMAN, S. A. et al. Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. **Blood**, v. 103, p. 2039-2045, 2004.

Apêndice I

UFBA

Efeitos da *N*(épsilon)-(carboximetil)lisina (CML), um produto final de glicação avan(
(PFGA), na doença falciforme

FIOCRUZ

QUESTIONÁRIO PARA PACIENTES E CONTROLES

Nome: {NOME} _____ Sigla: {sig} _____ Telefone: ( ) _____

Endereço: _____

Registro: {REG} _____ Nº Pront. HEMOBA: {PRON} _____ Data de Nasc.: ____/____/____

Idade: {I} _____ Gênero: {GENER} ( ) Masculino [0] ( ) Feminino [1]

01. Idade do 1° diagnóstico de Doença Falciforme: {ID} ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2] ( ) 10 - 14anos [3] ( ) 15 - 17anos [4] ( ) > 17 anos [5]

02. Eletroforese de Hb {EHB} ( ) AA[0] ( ) SS[1] ( ) SC[2] ( ) SB+[3] ( ) SB_0[4] ( ) SD[5]

03. Haplótipo {HAPL} ( ) Sen[0] ( ) Car[1] ( ) Ben[2] ( ) Cam[3] ( ) Sau-Ara [4] ( ) Atip[5] ( ) I[6] ( ) II[7] ( ) III[8]

04. Talassemia {TAL} ( ) Negativo[0] ( ) Hetero 3.7[1] ( ) Homo 3.7[2] ( ) Hetero 4.2[3] ( ) Homo 4.2[4]

Mieloperoxidase {MPO} ( ) GG[0] ( ) AG[1] ( ) AA[2]

Alelo mutante Mieloperoxidase ? {MUTMPO} ( ) NÃO [0] ( ) SIM [1]

Alfa 1 antitripsina {A1ATP} ( ) MM[0] ( ) MZ[1] ( ) MS[2] ( ) SZ[3] ( ) SS[4] ( ) ZZ[5]

05. Já esteve internado? {INTER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QINTER} ( ) 1 [0] ( ) 2-5 [1] ( ) 6-10 [2] ( ) 11 ou + [3]

Qual especialidade? {ESPEC} ( ) Cardiologia [0] ( ) Oftalmologia [1] ( ) Neurologia [2]

( ) Infectologia [3] ( ) Pneumologia [4] ( ) Cirurgia [5]

( ) Angiologia [6] ( ) Nefrologia [7] ( ) Clínica da Dor [8]

( ) Outras [9]

06. Já teve pneumonia? {PNEU} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QPNEU} ( ) 1[0] ( ) 2-3[1] ( ) 4-6[2] ( ) 7 ou + [3]

Se SIM, teve febre? {FEBRE} ( ) NÃO [0] ( ) SIM [1]

Anormalidade no RX? {ARX} ( ) NÃO [0] ( ) SIM [1]

Quando internado, usou medicação? {MPNEU} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCMPNEU} _____

07. Teve ou tem esplenomegalia? {ESPLE} ( ) NÃO [0] ( ) SIM [1]

Em que período? {PERIOESPLE} ( ) <6m[0] ( ) 6m-1ano [1] ( ) 2-3a [2] ( ) 4-5a [3] ( ) >6a [4]

Teve crise de seqüestro esplênico? {SEQESPLE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QSEQESPLE} _____

08. Faz uso profilático de Penicilina? {PROP} ( ) NÃO[0] ( ) SIM [1]

Se SIM, qual? {QPEN} ( ) Penicilina V oral [0] ( ) Penicilina benzatina [1]

Se Sim, há quanto tempo? {QTPEN} ( ) até 1 ano [0] ( ) + de 1 ano a 3 anos [1]

( ) + 3 anos a 5 anos [2] ( ) + 5 anos a 7 anos [3]

( ) + de 7 anos [4]

09. Já teve AVC? {AVC} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QAVC} _____

Se SIM, seqüelas do AVC? {SEQAVC} ( ) NÃO [0] ( ) SIM [1]

Já fez ressonância magnética? {RESSOMAG} ( ) NÃO [0] ( ) SIM [1]

Alguma alteração? {ALTRESSOMAG} ( ) NÃO [0] ( ) SIM [1]

Já fez Doppler Transcraniano? {dtc} ( ) NÃO [0] ( ) SIM [1]

Se, SIM, qual resultado? {dtcres} ( ) Normal [0] ( ) Alterado [1]

10. Esplectomizado? {ESPLECTO} ( ) NÃO [0] ( ) SIM [1]

Esplenectomia: {TIPOESPLECTO} ( ) Total [0] ( ) Parcial [1]

11. Apresenta asma? {ASMA} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas crises nos últimos 06 meses? {QASMA} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]

Faz uso regular de nebulização?{NEBU} ( ) [0] NÃO ( ) SIM [1]

12. Tem crises de dor? {CRISDOR} ( ) [0] NÃO ( ) SIM [1]

Se SIM, quantas crises nos últimos 06 meses? {QCRISDOR} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]

Quando foi a última crise? {ULTCRISDOR} ( ) <1mês [0] ( ) 1-3m [1] ( ) 4m ou+[2]

Usa medicação para a dor? {MDOR} ( ) NÃO [0] ( ) SIM [1]

Prescrita por um médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]

Assistido por especialista em dor? {ESPECDOR} ( ) NÃO [0] ( ) SIM [1]

Faz tratamento com hidroxiuréia? {HYDREIA} ( ) NÃO [0] ( ) SIM [1]

13. Faz uso regular de medicamentos? {MEDIC} ( ) NÃO [0] ( ) SIM [1]

Se SIM, qual? {DESCMEDIC} _____

Com que freqüência? {FREQMEDIC} ( ) Diário [0] ( ) Dias alternados[1] ( ) Semanal[2]

( ) Quinzenal[3] ( ) Mensal [4] ( ) Bimestral[5] ( ) Semestral [6]

14. Vaso-Ocusão: {VO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QVO} _____

Fez uso de alguma medicação? {MVO} ( ) NÃO [0] ( ) SIM [1]

15. Retinopatia: {RETIN} ( ) NÃO [0] ( ) SIM [1]

Faz consultas periódicas com oftalmo? {CONSOFTAL} ( ) NÃO [0] ( ) SIM [1]

16. Infecções: {INFEC} ( ) NÃO[0] ( ) SIM [1]

Quais? {DESCINFEC} ( ) Rinite [0] ( ) Sinusite [1] ( ) Otite [2]

( ) Faringite [3] ( ) Amigdalite [4] ( ) Vias aéreas sup. [5]

( ) ITU [6] ( ) Bronquite [7] ( ) ITR [8]

( ) Outros [9]

Fez uso de alguma medicação? {MINFEC} ( ) NÃO [0] ( ) SIM [1]

17. Priapismo: {PRIAP} ( ) NÃO [0] ( ) SIM [1] ( ) NÃO SE APLICA [9]

N° de vezes: {QPRIAP} ( ) Até 4 [0] ( ) 05-09 [1] ( ) 10 ou +[2]

Fez uso de alguma medicação? {MPRIAP} ( ) NÃO [0] ( ) SIM [1]

18. Úlcera maleolar: {ULCMALEO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QULCMALEO} _____

Idade da primeira úlcera: {IDULC} ( ) Até 4 anos [0] ( ) 5 - 9 [1] ( ) 10 - 17 [2]

( ) > 17 [3]

Tratou a úlcera? {TRATULC} ( ) NÃO [0] ( ) SIM [1]

Qual tratamento? {QUALTRAT} _____

19. Síndrome torácica aguda: {SDTOR} ( ) NÃO [0] ( ) SIM [1]

Quantas vezes? {QSDTOR} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

20. Alterações ósseas: {ALTOSSEA} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCALTOSSEA} _____

21. Insuficiência Renal Aguda: {INSRENAG} ( ) NÃO [0] ( ) SIM [1]

Quantas vezes? {QINSRENAG} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

22. Insuficiência Renal Crônica: {INSRENCRO} ( ) NÃO [0] ( ) SIM[1]

Idade diagnóstico: {IDINSRENCRO} ( ) Até 5 anos [0] ( ) 06-11[1] ( ) 12 ou + [2]

33. Alterações cardíacas: {INSCARD} ( ) NÃO [0] ( ) SIM[1]

Qual alteração? {QUALALTCA} _____

Idade diagnóstico: {IDINSCARD} ( ) Até 5 anos [0] ( ) 06-11 [1] ( ) 12 ou + [2]

Fez eletrocardiograma? {ELETRO} ( ) NÃO [0] ( ) SIM [1]

Fez ecocardiograma? {ECOCARD} ( ) NÃO [0] ( ) SIM [1]

24. Seqüestro hepático: {SEQHEP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QSEQHEP} _____

25. Insuficiência respiratória: {INSRESP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QINSRESP} _____

26. Distúrbio do sono? {DISTSONO} ( ) NÃO [0] ( ) SIM [1]

27. Litíase biliar: {LITIBILI} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QLITIBILI} _____

28. Cirurgia: {CIRURG} ( ) NÃO [0] ( ) SIM [1]

Quais? {QUALCIRURG} _____

29. Se SIM, fez uso de profilaxia antibiótica? {PROFANTIB} ( ) NÃO [0] ( ) SIM [1]

30. Completou o calendário vacinal? {CALVAC} ( ) NÃO [0] ( ) SIM [1]

Fez uso das seguintes vacinas? {USOVAC} ( ) 7 valente [0] ( ) 23 valente [1]

( ) Meningo [2] ( ) Haemophilus [3]

31. Fez uso de hemoderivados? {HEMODER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QHEMODER} _____

32. Possui outra patologia? {PATOLOG} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCPATOLOG}	( ) Hipertensão [0]	( ) Diabetes [1]	( ) Obesidade [2]
	( ) Febre Reumática [3]	( ) Hipertensão pulmonar [4]	( ) D. de Chagas [5]
	( ) Osteoporose [6]	( ) Trombose Venosa Profunda [7]	( ) Nefrolitíase [8]
	( ) Outros [9]		

Apêndice II – PCR-RFLP para Haplótipos.

Reagente	Quantidade por amostras
Tampão	5,0 µL
$MgCl_2$ 50 mM	2,5 µL
dNTP 2 mM	5,0 µL
Primers (3 ou 5 ou 6 ou 8 ou 10 ou 12) 25 pmol/ µL	0,5 µL
Primers (4 ou 6 ou 7 ou 9 ou 11 ou 13) 25 pmol/ µL	0,5 µL
Taq 5 U/µL	0,25 µL
DNA	1,5 µL
H_2O qsp 50 µL	34,75 µL

Termociclagem

Desnaturação	94°C, 10 min
35 ciclos	94°C, 45 seg., Temp. Variavel de acordo com a Tab. 1 45 seg 72°C, 1 min 30 seg.
Extensão	72°C, 10 min
	4°C...

RFLP

Reagentes	Quantidade por amostra
Produto de PCR	20 µL
BSA*	0,3 µL
Tampão	3 µL
Enzima de restrição de acordo com tab. 1	
Xmn I	0,2 µL
Hinc II	0,1 µL
Hind III ou Hinf I	0,2 µL
H_2O	Qsp 30 µL

*Verifica as reações que necessitam BSA na tab. 1

Tabela 1. Padrao de banda de PCR e corte

Gene	Primer	Fragmento (pb)	Após digestão	Temp. De pareamento	Enzima
5'γG	3 e 4	650	450 + 200	57°C	Xmn I*
γG/γA	5 e 6	780	440 + 340	60°C	Hind III
γG/γA	6 e 7	760	360 + 400	62°C	Hind III
Ψβ	8 e 9	700	360 + 340	60°C	Hinc II
3'Ψβ	10 e 11	590	470 + 120	57°C	Hinc II
5'β	12 e 13	380	240 + 140	57°C	Hinf I

Apêndice III – Ensaios de padrões de CML.

Reagente	Quantidade por amostra
Anticorpo de Anti-CML 1000×	10 μL
Anticorpo secundário, conjugado de HRP 1000×	20 μL
Diluente de ensaio	50 mL
Tampão de lavagem 10×	100 mL
Solução de substrato	12 mL
Solução de para	12 mL
Padrão CML-BSA (1 mg/mL)	10 mL
Conjugado de CML 1000×	20 μL
Diluente Conjugado 1000×	300 μL
PBS 1×	50 mL

As séries de diluição de padrões de CML-BSA foram preparadas na gama de concentração de 0 a 12,5 μg / mL por diluição dos padrões no diluente de ensaio como mostrado na tabela abaixo.

Tubo padrão	1 mg/mL Padrão CML-BSA (μL)	Diluente de ensaio (μL)	CML-BSA (μg/mL)	CML (ng/mL)
1	5	395	12,5	576
2	200 do tubo #1	200	6,25	288
3	200 do tubo #2	200	3,13	144
4	200 do tubo #3	200	1,56	72
5	200 do tubo #4	200	0,78	36
6	200 do tubo #5	200	0,39	18
7	200 do tubo #6	200	0,20	9
8	200 do tubo #7	200	0,10	4,5
9	200 do tubo #8	200	0,050	2,25
10	0	200	0	0

Apêndice IV

Durante o desenvolvimento deste trabalho, também participamos da pesquisa relacionada ao estudo de PFGAs em pacientes com DF com risco de AVC, com elaboração de mais um manuscrito que está sendo submetido para publicação.

Serum level of *N*(epsilon)-(carboxymethyl)lysine in pediatric patients with sickle cell disease is associated with gender, pain crises and risk of stroke

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Abstract

Background: N^{ε}-(carboxymethyl)lysine (CML) is associated with several diseases and organs complications. This research investigated the association of CML with clinical parameters and risk of stroke in sickle cell disease (SCD).

Method: A total of 72 children with SCD were recruited: 19 were SC and 53 were sickle cell anemia (SCA). CML assay was carried out using the competitive ELISA technique. The transcranial Doppler (TCD) ultrasonography was used to determine stroke risk.

Results: Our result showed that gender is significantly associated with CML in both HbSC ($p = 0.04$) and SCA ($p = 0.039$) individuals. The female gender showed significantly higher mean

CML in both HbSC ($p = 0.015$) and SCA ($p = 0.022$) compared to the male gender. Pain crisis was also significantly associated with CML in both HbSC ($p = 0.037$) and SCA ($p = 0.034$). SCA individuals with pain crises showed significantly higher ($p = 0.03$) mean CML compared to individuals without crises. There was a significantly higher CML in SCA ($p < 0.0001$) and HbSC ($p = 0.0002$) compared to the healthy control (HbAA). SCA individuals have significantly higher CML ($p < 0.0001$) compared to the HbSC group. Based on two (2) stratifications of TCD velocities (normal, < 170 cm/s, and not normal ≥ 170 cm/s), there was a significant association between TCD velocities and CML in SCA.

Conclusion: This study suggests that CML plays a role in SCD complications and is associated with pain crisis and the female gender. It further suggests that CML is associated with increased risk of stroke in SCA.

Keywords: advanced glycation end products, transcranial Doppler, sickle cell disease, clinical parameters.

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive disorder caused by a point mutation at the beta (β)-globin gene (*HBB*) located in chromosome 11. Individuals suffering from SCD inherit at least one HbS in which adenine is replaced by thymine (GAG → GTG) at the 6th codon of the β-globin gene thereby producing valine in place of glutamic acid at the 6th position of the β-globin chain. Sickle cell anemia (SCA) is the most common form of the disease and accounts for about 70% of the cases.[1,2] It is followed by HbSC that accounts for between 25 – 30% of the identified cases.[3,4]

SCD forms aggregates in a state of deoxygenation, with consequent erythrocyte rigidity, resulting in vaso-occlusion, tissue ischemia, organ damage, pain, and high risk of death.[5] The severity of the vaso-occlusion and progression to end organ ischemia is known to be potentiated by oxidative stress.[6]

Advanced glycation end-products (AGEs), which are a complex group of compounds formed through non-enzymatic reactions between reducing sugars and N-terminal amino groups of proteins, lipids and nucleic acids, have been demonstrated to be markers of oxidative stress.[7] Indeed AGE-induced damage has been implicated in the release of reactive oxygen species.[8]

N^{ε}-(carboxymethyl)lysine (CML), commonly generated through pathways such as oxidative cleavage of the Amadori product by the hydroxyl radical[9] and peroxynitrite[10], is one of the most studied AGEs.[11] It is present in both biological systems and heat-processed foods. Previous studies have shown that CML accumulation in the human body is associated with cardiovascular disease, sarcopenia and renal diseases.[12,13] It has also been implicated in the pathogeneses of diabetes nephropathy and retinopathy as well as atherosclerosis, hemodialysis-associated amyloidosis and Alzheimer's disease.[14,15,16,17,18]

The decades of research on AGEs (most especially CML) notwithstanding, only the works of Somjee et al[19] and Nur et al[20] investigated the effect of AGEs on SCD patients. These reports revealed that SCD patients have significantly higher serum AGE levels when compared to healthy control.[19,20] Studies have also shown that higher endogenous antioxidant status were associated with lower plasma AGE levels in SCD[19] corroborating previous reports that AGEs are markers of oxidation.[7]

One of the major causes of morbidity in children with SCD is stroke.[21] Previous stroke has been reported as the strongest predictor of stroke in SCD children.[22,23] One of the major signs that often occur in making predictions about the risk of stroke without a previous event is an abnormal result on an image of cerebral arteries using a transcranial Doppler (TCD) ultrasonography that monitors the cerebral mean blood flow velocities of patients with SCD allowing the identification of those with an increased risk of developing stroke. Abnormal TCD velocities (time-averaged mean maximum velocity (TAMV) $\geq$ 200 cm/s) have been reported to be associated with a 10-fold increase in stroke risk per year.[24,25] SCD children with conditional TCD (time-averaged mean maximum velocity (TAMV) 170 – 199 cm/s) have also been reported to possess increased risk of primary stroke as they can progress quite rapidly, if unchecked, to abnormal TCD, but they have received little attention.[22,26] Interestingly, AGEs have also been linked with increased severity of stroke associated with diabetes.[27,28] But no report has at any time, to the best of our knowledge, investigated a possible link between AGE and stroke in SCD patients.

Therefore, there is need for a further evaluation of the effect of CML and a probe into a possible association with increased risk of stroke in SCD patients.

2. Materials and Methods

2.1 Subjects. In this transversal cohort study, 53 sickle cell anemia (SCA) and 19 HbSC patients of both sexes from the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), Salvador, Bahia, Brazil were recruited. In addition to this, 53 normal healthy age- and sex-matched individuals (HbAA) were recruited from volunteers from the Clinical Laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (UFBA). All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. The study was carried out between August 2015 and August 2017 and the protocol was submitted to and approved by the Fundação Oswaldo Cruz (FIOCRUZ) Ethics research board (ID 1.400.527). The hemoglobin (Hb) profile was investigated by high performance liquid chromatography (HPLC/VARIANT I; BIO-RAD, CA, USA). All SCD patients included in the study answered an epidemiological inquiry questionnaire. The response was from the patient or the parents/guardian in the case of children or teenager (less than 18). Patients were in the steady-state of the disease when samples were collected. Steady-state is described as a period without any acute events and no blood transfusion for 120 days prior to blood sampling.

All participants received verbal and written explanation of the study and signed the Informed Consent Form (ICF) before inclusion in the study. Informed consent was obtained from the patient or the patient's parent/guardian. Patients were in the hospital during crisis or in the clinic for a routine visit. Venous blood (8-10 mL) was collected in Vacutainer tubes containing EDTA. Blood samples were processed within 30 minutes of collection.

Overall exclusion criteria: Patients with diabetes mellitus, renal failure, autoimmune inflammatory disease, active infection and pregnancy, smokers and chronic alcoholics were excluded.

2.2 Immunoanalysis of N$^{\varepsilon}$-carboxymethyllysine (CML). The detection and quantitative estimation of CML was carried out in the blood serum of the patients using an OxiSelect^{TM} CML ELISA Kit according to the manufacturer's manual (Cell Biolabs, Inc., CA, USA). The amount of CML in ng/mL was determined by comparing its absorbance with that of a known CML-BSA standard curve. The absorbance results were read on a microplate reader (STAT FAX$^{®}$ 2100) at 450 nm.

2.3. Transcranial Doppler measurement. A certified TCD examiner performed the TCD in all patients using the same device (Doppler; probe 2 Mhz model, Ezdop, Germany). The TCD was read by a single radiologist. The left and right middle cerebral artery (MCA), distal internal carotid artery (dICA), anterior cerebral artery (ACA) and posterior cerebral artery (PCA) were insonated at 2-mm increments. Maximal systolic and diastolic peak velocities were recorded. The time-averaged mean maximum velocity (TAMV) was used to determine the TCD value of the major intracranial arteries on each side (left and right) as previously described.[29] If TAMV in all arteries was between 70 and 170 cm/s, the examination was considered normal; the TAMV ≥170 cm/s, and < 200 cm/s, in any one artery was considered conditional; the TAMV ≥ 200 cm/s in an artery was considered abnormal, and the TAMV <70 cm/s was considered low. The failure to detect the flux wave during the examination was characterized as inadequate. Patients with a prior overt stroke event, on hydroxyurea therapy, with a simple transfusion in the last 3 months or on chronic blood therapy regimens were not included in the study.

3. Statistical Analysis

Descriptive statistics were summarized with mean/ median and range (minimum and maximum) for continuous variable and the number as well as percentages for categorical variables. The continuous data for each variable and for each group were tested for normality (Shapiro-Wilk and Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) prior to analysis. The Mann-Whitney (non-parametric) and independent T-test (parametric) were employed for the analysis of two numerical variables, comparing two groups of values within the same variable, taking into account the distribution of each variable. Analysis of variance (ANOVA) followed by Tukey's post hoc multiple range comparison where differences in means were observed was applied for more than two numerical (or quantitative) variables. For correlation studies between age and CML as well as body mass index (BMI) and CML, the Spearman's rank correlation

coefficient was employed and relationships between the groups were considered significant when P <0.05 or P < 0.01. Linear regression was followed where a significant difference was detected. Association studies for categorical variables were carried out using the Pearson chi square or Fisher's Exact test where applicable. Statistical analyses were performed using GraphPad Prism 6.0 and SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered significant for the analyses performed.

4. Results

Table 1 shows the association studies between CML and some clinical parameters in SCD patients. To determine the association or relationship between CML and the categorical variables, we divided patients into two (2) CML groups: the low CML group (≤ median value) and the high CML group (> median values) for each SCD type (HbSC median value = 178.66 ng/mL; SCA median value = 247.93 ng/mL). We had a total of 72 SCD patients of which 19 (26.39%) were SC while 53 (73.61%) were SS. Most of the clinical data presented no relationship with CML. However, chi square association study between gender and CML, revealed that HbSC patients with 11 (57.90%) males and 8 (42.10%) females were significantly associated (p = 0.040) with CML while SCA patients with 26 (49.06%) males and 27 (50.94%) females were significantly associated (p = 0.039) with CML. HbSC patients with pain crises, 9 (47.40%), showed significant association (p = 0.037) with CML. SCA patients on pain crises 46 (86.79%) also showed significant association (p = 0.034) with CML.

Figure 1A-B showed mean serum CML in SCD individuals stratified by gender. In SCA individuals, females were discovered to have significantly higher (p = 0.022) CML compared to males (Figure 1A). A similarly significantly higher (p = 0.015) concentration of CML was obtained in females compared to males in HbSC individuals (Figure 1B).

Figure 2A-B showed the result of a comparison of the mean level of CML between individuals with pain crises and individuals without crises. SCA individuals with pain crises showed significantly higher (p = 0.03) mean CML compared to individuals without crises (Figure 2A). However, there was no significant difference (p = 0.121) between the mean CML in HbSC individuals with pain crises and individuals without crises.

In Figure 3A-B, we evaluated the effect of serum CML on SCD patients using ANOVA coupled with Tukey's post hoc test and then T-test for comparison of two groups. There was a significantly higher level of CML (p < 0.0001) in SCA patients compared to the healthy control (HbAA) (Figure 3A). HbSC also showed a significantly higher concentration of CML (p = 0.0002) when compared to healthy control (HbAA) (Figure 3A). A comparison of serum level of CML in SCA against HbSC showed that the SCA group has significantly higher CML (p < 0.0001) compared to the HbSC group (Figure 3A). In Figure 3B, we pulled both the SCA and SC together (SCD) and compared their serum CML level with the healthy control using T-test. Our result showed that SCD patients have significantly higher concentration of CML (p < 0.0001)

compared to the healthy control subjects. Our results showed that there is no significant difference between SCA patients on hydroxyurea (HU) and SCA patients without HU (data not shown).

Table 2A-C showed the Pearson chi-square association studies between serum CML and TCD velocities here defined by time-averaged mean maximum velocity (TAMV). In this study, we have two (2) different stratifications of the TCD velocities. In the first stratification, we divided the velocities into two (2): normal (< 170 cm/s) and not normal (≥ 170 cm/s). This is based on the assumption that any subject who has TAMV above normal value (≥ 170 cm/s) is susceptible to stroke. The second stratification is the commonly known classification of TCD velocities with three (3) strata, namely: normal (< 170 cm/s), conditional (≥ 170 – 199 cm/s), and abnormal (≥ 200 cm/s). Table 2A which has 2 stratifications, showed that TCD velocities in SCD patients are independent of the concentration of CML ($\chi^2 = 0.234$; $p = 0.629$). Table 2B, still based on 2 strata showed that there is a significant association between TCD velocities and CML concentration in SCA patients. Table 2C, with the 3 commonly known strata showed that TCD velocities are independent of the concentrations of CML in SCA subjects. All the HbSC patients fall within the normal group (data not shown).

In Figure 4A-B, we compared mean serum level of CML in SCA patients for each strata described above. Figure 4A showed no significant difference between the CML levels in normal, conditional and abnormal TCD velocities. Figure 4B also showed no significant difference in mean CML between the normal and the not normal groups of SCA.

5. Discussion

Plasma levels of advanced glycation end products (more especially CML) are increased under oxidative condition and are associated with several diseases and organs complications. Over a decade ago, they were associated with SCD, though more specifically with SCA.[19,20] However, not much has been done ever since to discover possible association of AGEs with complications of SCD beside their association with oxidative condition in SCA patients.[19] This research investigated the association of CML with clinical parameters as well as risk of stroke in both SCA and sickle hemoglobin SC disease.

Pain crisis is known to be as a result of the adhesion of RBCs in SCD patients to the vascular endothelial cell membranes.[30] The adhesion tends to increase the transit time of the RBCs. It also increases hemoglobin deoxygenation and polymerization as well as the sickling of the RBCs leading to decreased flexibility of the RBCs, a precursor for vaso-occlusion.[19] The severity of the vaso-occlusion and progression to pain crisis is potentiated by oxidative stress and other features intrinsic to the RBC and its extracellular environment.[31] Our investigation showed that CML is significantly associated with pain crisis. This goes to suggest that CML could be one of the factors that modulate progression to pain crises in SCDs, especially considering that CML is a known biomarker of oxidative stress. Patients with pain crises also showed significantly higher

level of CML compared with patients without pain crises in SCA individuals but this was not observed in HbSC patients. Previous study from Nur et al[20], however, reported that there was no increment in CML concentration during pain crisis which is not in agreement with our report on SCA patients but it is in agreement with our report on HbSC.

Significant gender differences in morbidity and mortality have been reported in SCD.[32,33] Factor adduced for the notable differences include glucose 6-phosphate dehydrogenase deficiency (G6PD), fetal hemoglobin levels and nitric oxide (NO) availability. Our study found an association between CML and gender in SCD individuals. Our report, therefore, suggests that AGEs could well be one of the factors that influence significant gender differences in morbidity and mortality in SCD. To our knowledge, this is the first study that investigated association between CML and gender in SCD patients. Furthermore, Mook-Kanamori et al[34] while investigating AGEs as predictors of cardiovascular risk reported significant gender differences in AGEs. They also went further to report increased AGEs in female than male which tend to support our report that females have significantly higher CML levels compared to males in SCD individuals. The reasons for this significantly higher circulating level of CML in females compared to males are yet unclear. Further research is needed to corroborate this finding.

Our report showed a significantly higher level of CML in both SCA and HbSC compared to healthy control group which is in agreement with previous reports of both Somjee et al[19] and Nur et al[20]. We reported a significantly higher level of CML in SCA compared to that of HbSC which also agree with the report of Nur et al[20]. However, unlike the report of Nur et al[20], there was a significantly higher level of CML in HbSC compared to healthy control group. These all suggest that SCD individuals generally have significantly higher concentration of CML compared to the healthy individuals. Previous reports suggested that only SCA individuals have high level of advanced glycation end products.[19,20] Further research may be needed to confirm this discrepancy. Since there is a consensus that there is an increment in CML level in SCA individuals, we could suggest in line with previous reports that CML play an important role in the pathophysiology of chronic hemolysis-associated organ complications.[20,35,36] CML has also been reported to be associated with endothelial activation as it correlated positively with soluble vascular cell adhesion molecules (sVCAM) and von Willebrand factor antigen (VWF:Ag).[20] Hemolysis, a common feature of SCD, causes increased plasma levels of cell-free heme which intercalates into the plasma membrane of endothelial cells where it releases its iron.[37] The iron is known to modulate endothelial cell activation and damage by catalyzing generation of reactive oxygen species (ROS) and AGEs.[37,38]

Stroke is a common clinical manifestation in pediatric SCD patients of 1 year or older.[39,40,41] AGEs have been reported to contribute to the increased severity of stroke associated with diabetes.[28] However, even though reports show that there are many factors (anemia, leukocytosis, hypertension, silent infarction, and history of acute chest syndrome) responsible for stroke risks in SCD individuals[21,42], no study has, thus far, investigated the possible role of AGEs. The stratification of stroke risk using the TCD is usually determined by measuring TAMV where

values ≥ 200 cm/s are considered high stroke risk (abnormal), values ≥ 170 cm/s and < 200 cm/s are considered conditional whereas values < 170 cm/s are considered low risk (normal). Although children with abnormal TAMV have the highest risk, stroke episodes also occur in those with conditional TAMV.[22] Stroke risk among SCD individuals with conditional TAMV is estimated to be 2 – 5% per year compared to 9% for children with abnormal velocities.[22,25] Therefore, part of our result analysis pulled conditional and abnormal TAMV together. There was a significant association between TCD values and CML level in SCA individuals suggesting that AGEs may contribute to the increased risk of stroke associated with SCA. Reports have shown that stroke incidence is higher among SCA individuals compared to HbSC individuals with SCA having an incidence rate of 0.61/100 patients/year while HbSC has an incidence rate of 0.17/100 patients/year.[43] The fact that there was no such association when HbSC was considered corroborate the report of Ohene-Frempong et al[43] that revealed higher risk of stroke in SCA compared to HbSC and suggests that AGEs may likely not play a significant role in stroke risks in HbSC disease.

Conclusion

In summary, this study suggests that CML plays a role in SCD complications and is associated with pain crisis and the female gender. It further suggests that CML is associated with increased risk of stroke in SCA.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa and Adekunle Adekile conceived the study design. Uche Samuel Ndidi, Corynne Stephanie Ahouefa Adanho, Rayra Pereira Santiago, Cynara Gomes Barbosa, Camilo Vieira, Isa Menezes Lyra, Bartholomew Friday Chukwu collected the samples and carried out the laboratory analysis. Uche Samuel Ndidi, Marilda Souza Goncalves, Cynara Gomes Barbosa interpreted the results. Uche Samuel Ndidi drafted the manuscript and it was revised by Marilda Souza Goncalves, Cynara Gomes Barbosa and Adekunle Adekile. All authors read and approved the final manuscript.

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References

1. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet*. 2010;376:2018–2031.

2. Williams TN, Obaro SK. Sickle cell disease and malaria morbidity: a tale with two tails. *Trends Parasitol*. 2011;27:315-320.

3. Lionnet F, Hammoudi N, Stojanovic KS, Avellino V, Grateau G, Girot R, Haymann J-P. Hemoglobin SC disease complications: a clinical study of 179 cases. *Haematologica*. 2012;97(8):1136 – 1141.

4. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. *Blood*. 2010;115(22):4331-4336.

5. Nevitt SJ, Jones AP, Howard J. Hydroxyurea (hydroxycarbamide) for sickle cell disease. *Cochrane Database Syst Rev*. 2017;issue 4, Art. No.: CD002202.

6. Manwani D, Frenette PS. Vaso-occlusion in sickle cell disease: pathophysiology and novel targeted therapies. *Blood*. 2013;122(24):3892 – 3898.

7. Nowotny K, Jung T, Hohn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. *Biomolecules*, 2015;5(1):194 – 222.

8. Byun K, Yoo YC, Son M, Lee J, Jeong GB, Park YM, Salekdeh GH, Lee B. Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. *Pharmacol Therap*. 2017;177:44 – 55.

9. Nagai R, Ikeda K, Higashi T, Sano H, Jinnouchi Y, Araki T, Horiuchi S. Hydroxyl radical mediates *N*ε-(carboxymethyl)lysine formation from Amadori product. *Biochem Biophys Res Commun.* 1997;234:167–172.

10. Nagai R, Unno Y, Hayashi MC, Masuda S, Hayase F, Kinae N, Horiuchi S. Peroxynitrite induces formation of *N*ε-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes.* 2002;51:2833–2889.

11. Nguyen HT, van der Fels-Klerx HJ, van Boekel MAJS. Kinetics of Ne-(carboxymethyl)lysine formation in aqueous model systems of sugars and casein. *Food Chem*. 2016;192:125 – 133.

12. Baumann M. Role of advanced glycation end products in hypertension and cardiovascular risk: human studies. *J Am Soc Hypertens*. 2012;6(6):427–435.

13. Dalal M, Ferrucci L, Sun K, Beck J, Fried LP, Semba RD. Elevated serum advanced glycation end products and poor grip strength in older community-dwelling women. *J Gerontol – Series A Biol Sci Med Sci*. 2009;64(1):132–137.

14. Imai N, Nishi S, Suzuki Y, Karasawa R, Ueno M, Shimada H, Kawashima S, Nakamaru T, Miyakawa Y, Araki N, Horiuchi S, Gejyo F, Arakawa M. Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. *Nephron.* 1997;76:153–160.

15. Kume S, Takeya M, Mori T, Araki N, Suzuki H, Horiuchi S, Kodama T, Miyauchi Y, Takahashi K. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Am J Pathol.* 1995;147:654–667.

16. Hammes HP, Weiss A, Hess S, Araki N, Horiuchi S, Brownlee M, Preissner KT. Modification of vitronectin by advanced glycation alters functional properties *in vitro* and in the diabetic retina. *Lab Invest.* 1996;75:325–338.

17. Miyata T, Oda O, Inagi R, Iida Y, Araki N, Yamada N, Horiuchi S, Taniguchi N, Maeda K, Kinoshita T. beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. *J Clin Invest.* 1993;92:1243–1252.

18. Smith MA, Taneda S, Richey PL, Miyata S, Yan SD, Stern D, Sayre LM, Monnier VM, Perry G. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc Natl Acad Sci USA.* 1995;91:5710–5714.

19. Somjee SS, Warrier RP, Thompson JL, Ory-Ascani J, Hempe JM. Advanced glycation end-products in sickle cell anemia. *Br J Haematol.* 2004;128:112 – 118.

20. Nur E, Brandjes DP, Schnog JJB, 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.

21. Kassim AA, Galadanci NA, Pruthi S, DeBaun MR. How I treat and manage strokes in sickle cell disease. *Blood*. 2015;125(22):3401 – 3410.

22. Hankins JS, Fortner GL, McCarville MB, et al. The natural history of conditional transcranial Doppler flow velocities in children with sickle cell anaemia. *Br J Haematol*. 2008;142(1):94–99.

23. Brambilla DJ, Miller ST, Adams RJ. Intra-individual variation in blood flow velocities in cerebral arteries of children with sickle cell disease. *Pediatr Blood Cancer*. 2007;49(3):318–322.

24. Adams RJ, McKie VC, Carl EM, et al. Long-term stroke risk in children with sickle cell disease screened with transcranial Doppler. *Ann Neurol*. 1997;42:699–704.

25. Adams RJ, McKie VC, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *New Engl J Med*. 1998;339:5–11.

26. Adams RJ, Brambilla DJ, Granger S, et al. Stroke and conversion to high risk in children screened with transcranial doppler ultrasound during the stop study. *Blood*. 2004;103:3689–3694.

27. Kizer JR, Benkeser D, Arnold AM, et al. Advanced glycation/glycoxidation endproduct carboxymethyllysine and incidence of coronary heart disease and stroke in older adults. *Atherosclerosis*. 2014;235(1):116 – 121.

28. Zimmerman SA, Schultz WH, Burgett S, Mortier NA, Ware RE. Hydroxyurea therapy lowers transcranial Doppler flow velocities in children with sickle cell anemia. *Blood*, 2007;110(3):1043 – 1047.

29. Adams RJ, Nichols FT, Aaslid R, McKie VC, McKie K, Carl E, Stephens S, Thompson WO, Milner P, Figueroa R. Cerebral vessel stenosis in sickle cell disease: criteria for detection by transcranial Doppler. *Am J Pediatr Hematol/Oncol*. 1990;12:277-282.

30. Moore CM, Ehlayel M, Leiva LE, Sorensen RU. New concepts in the immunology of sickle cell disease. *Ann Allergy Asthma Immunol*. 1996;76:385 – 400.

31. Steinberg MH, Brugnara C. Pathophysiological-based approaches to treatment of sickle cell disease. *Ann Rev Med*. 2003;54:89 – 112.

32. Garvin JH. Gender-Specific Aspects of Pediatric Hematology and Oncology. In Legato MJ (Ed.), Principles of Gender-Specific Medicine. (2nd Edn.), Elsevier: Academic Press. 2010;52-53.

33. Kamble M, Chatruvedi P. Epidemiology of sickle cell disease in a rural hospital of central India. *Indian Pediatr*. 2000;37:391-396.

34. Mook-Kanamori MJ, Selim MM, Takiddin AH, et al. Ethnic and gender differences in advanced glycation end products measured by skin auto-fluorescence. *Dermato-endocrinol*. 2013;5(2):325 – 330.

35. Kato GJ, Martyr S, Blackwelder WC, et al. Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction and mortality. *Br J Haematol*. 2005;130:943 – 953.

36. Landburg PP, Teerlink T, Biemond BJ, et al. Plasma asymmetric dimethylarginine concentrations in sickle cell disease are related to the hemolytic phenotype. *Blood Cells Mol Dis*. 2010;44:229 – 232.

37. Jeney V, Balla J, Yachie A, et al. Pro-oxidant and cytotoxic effects of circulating heme. *Blood*, 2002;100:878 – 887.

38. Galaris D, Pantopoulos K. Oxidative stress and iron homeostasis: mechanistic and health aspects. *Clin Rev Clin Lab Sci*. 2008;45:1 – 23.

39. Adams RJ. TCD in sickle cell disease: An important and useful test. *Pediatr Radiol*. 2005;35(3):229–234.

40. Steinberg MH, Rodgers GP. Pathophysiology of sickle cell disease: Role of cellular and genetic modifiers. *Semin Hematol*. 2001;38(4):299–306.

41. Vieira C, de Oliveira CNC, de Figueiredo LAB, et al. Transcranial Doppler in hemoglobin SC disease. *Pediatr Blood Cancer*. 2017; 64:e26342.

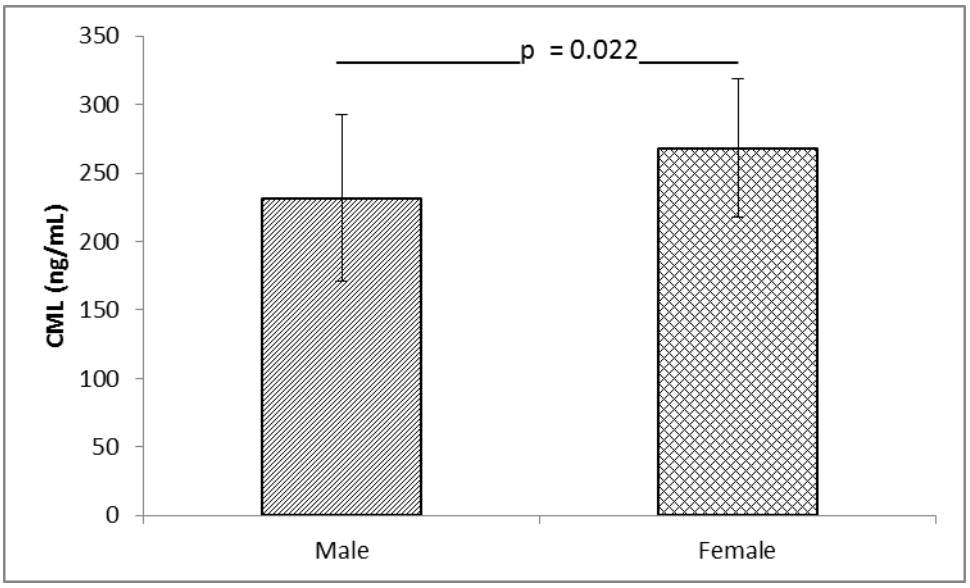
42. Hoppe C. Defining stroke risk in children with sickle cell anemia. *Br J Haematol*. 2004;128:751 – 766.

43. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell disease: Rates and risk factors. *Blood*. 1998;91(1):288–294.

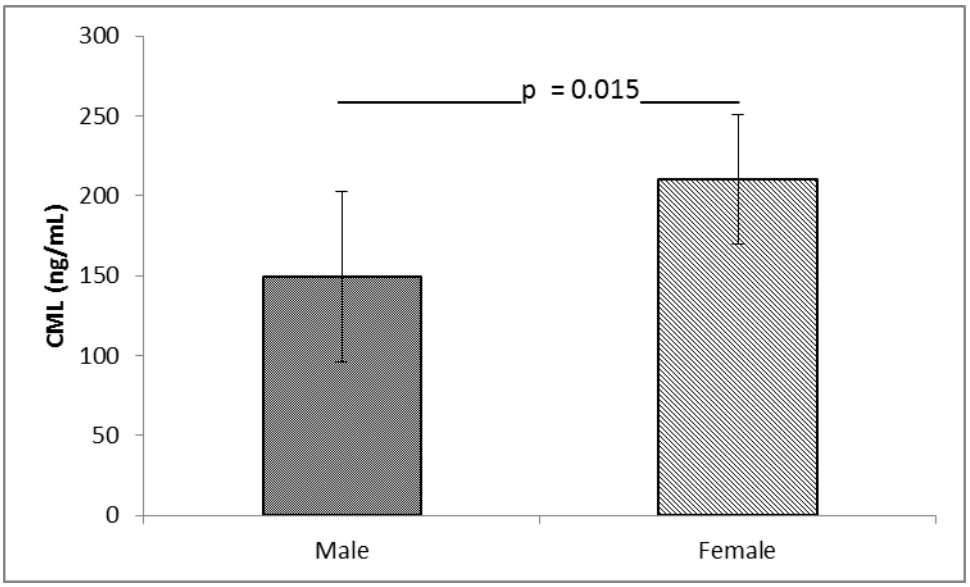
Table 1. Association of N^{ε}-(carboxymethyl)lysine (CML) with clinical data of the sickle cell disease patients with

Parameter	n/N (%)	P* value
SCD Subjects		
SC	19/72 (26.39)	NA
SS	53/72 (73.61)	NA
Gender		
SC : Male/Female	11/19 (57.90)/8/19 (42.10)	0.040
SS: Male/Female	26/53 (49.06)/27/53 (50.94)	0.039
Age: mean (range)		
SC	6.40 (2.00 – 17.00)	0.328
SS	7.25 (2.00 – 18.00)	0.598
BMI: median (range)		
SC	15.10 (13.10 – 19.50)	0.099
SS	15.20 (11.50 – 21.50)	0.093
Pain crises		
SC	9/19 (47.40)	0.037
SS	31/53 (58.49)	0.034
Hospitalization		
SC	12/19 (63.20)	0.109
SS	46/53 (86.79)	0.245
Pneumonia		
SC	5/19 (26.32)	0.114
SS	25/53 (47.17)	0.685
Fever		
SC	5/19 (26.32)	0.153
SS	24/53 (45.28)	0.901
Infection		
SC	7/19 (36.84)	0.764
SS	18/53 (33.96)	0.922
Priapism		
SC	0/19 (0)	NA
SS	4/53 (7.55)	0.317
Acute chest syndrome		
SC	0/19 (0)	NA
SS	8/53 (15.09)	0.478

NA, not applicable. To determine the association between these categorical variables with CML concentration, we divided patients into two (2) CML groups. The low CML group (≤ median value) and the high CML group (> median values) for each SCD type (HbSC median value =; SCA median value = 247.93 ng/mL). *Chi square was used to determine association between each categorical variable and CML while Spearman's rho correlation was carried out for age and body mass index (BMI). N = total count; n = number of subjects in a specific group/parameter.

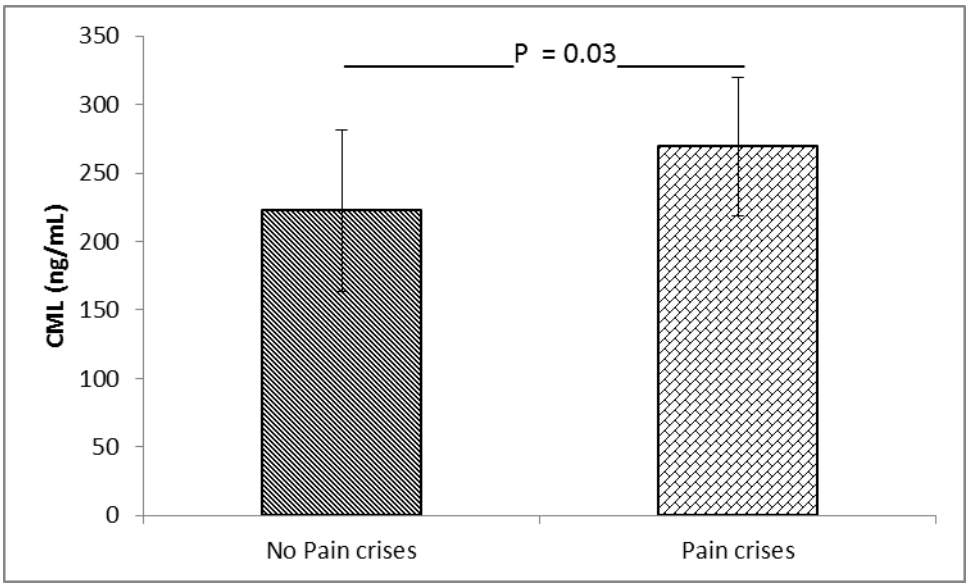


A

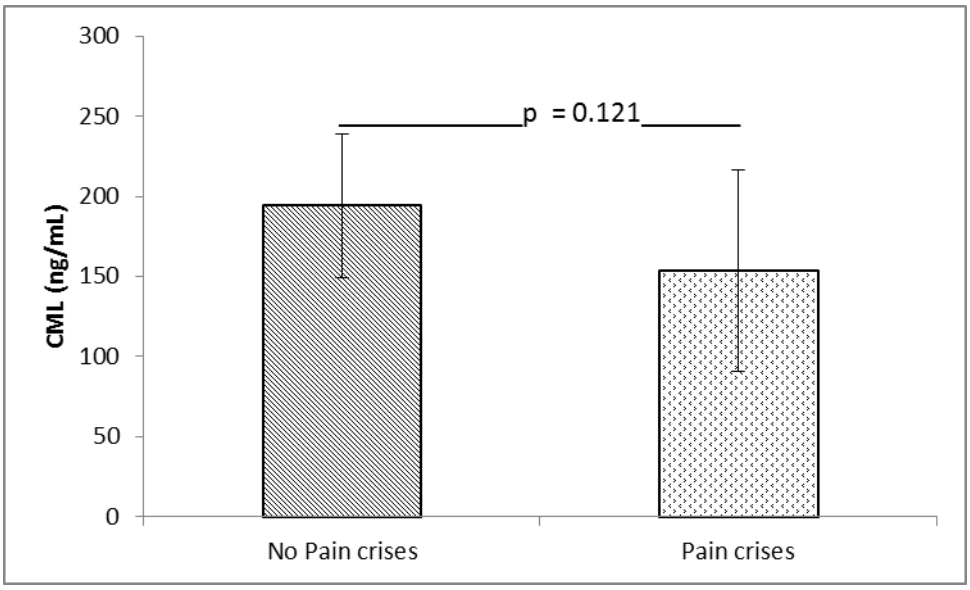


B

Figure 1: Serum N^{ε}-(carboxymethyl)lysine (ng/mL), CML, (mean ± standard deviation) in sickle cell disease individuals stratified by gender. A. The serum level of CML in sickle cell anemia individuals. Females have significantly higher (p = 0.022) CML compared to males. B. The serum level of CML in HbSC individuals. Again females have significantly higher (p = 0.015) CML compared to males. The significance was tested on SPSS 20 using the Independent-samples student T-test for comparison of means. Plots were carried out in Microsoft Excel.

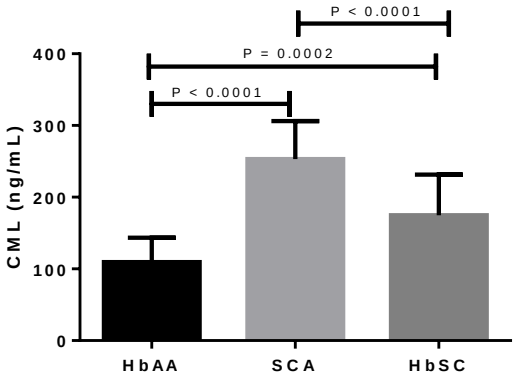


A.

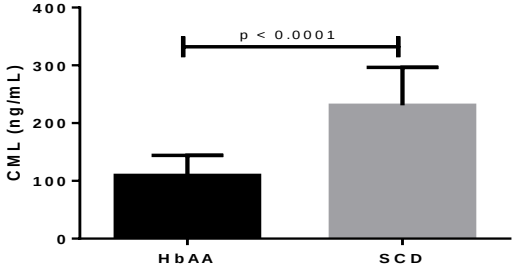


B

Figure 2: Serum N^{ε}-(carboxymethyl)lysine (ng/mL), CML, (mean ± standard deviation) in Sickle cell disease (SCD) patients stratified by pain crises. A. Sickle cell anemia individuals with pain crises show significantly higher ($p = 0.03$) level of CML compared to the patients without crises. B. HbSC individuals with pain crises show no significant difference ($p = 0.121$) in mean when compared with individuals without pain crises. The significance was tested on SPSS 20 using the Independent-samples student T-test for comparison of means. Plots were carried out in Microsoft Excel.



A



B

Figure 3. Serum N^{ε}-(carboxymethyl)lysine (CML) in ng/mL (mean and standard deviation). A. CML in sickle cell anemia (SCA) patients, CML in HbSC and CML in healthy control (HbAA). Tukey's multiple comparison tests showed that there is a significant difference between serum CML level of SCA and HbAA ($p < 0.0001$); between HbSC and HbAA (0.0002); and between SCA and HbSC ($p < 0.0001$). C. CML in all sickle cell disease (SCD) patients (SCA + HbSC). Unpaired t tests showed that SCD had significantly higher CML compared to HbAA ($p < 0.0001$).

Table 2. Time-averaged mean maximum velocity (TAMV) of sickle cell disease (SCD) patients in relation to the serum level of N^{ε}-(carboxymethyl)lysine (CML)

A. *SCD

TAMV (cm/s)	N^{ε}-(Carboxymethyl)lysine (CML)**			χ^2	p
	CML ≤ median, n (%)	CML > median, n (%)	Total, n (%)		
< 170	23 (31.9)	21 (29.2)	44 (61.1)	0.234	0.629
≥ 170	13 (18.1)	15 (20.8)	28 (38.9)		
Total	36 (50.0)	36 (50.0)	72 (100.0)		

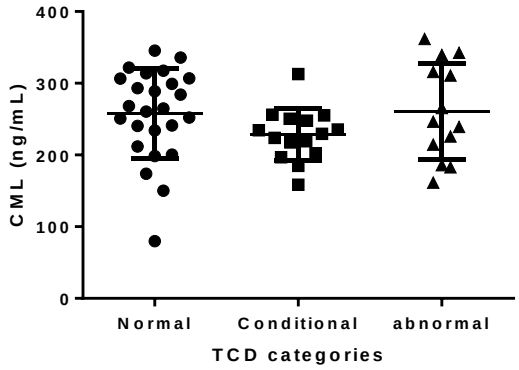
B. [1]SCA

TAMV (cm/s)	N^{ε}-(Carboxymethyl)lysine (CML)***			χ^2	p
	CML ≤ median, n (%)	CML > median, n (%)	Total, n (%)		
< 170	9 (17.0)	16 (30.2)	25 (47.2)	4.228	0.040
≥ 170	18 (34.0)	10 (18.9)	28 (52.8)		
Total	27 (50.9)	26 (49.1)	53 (100.0)		

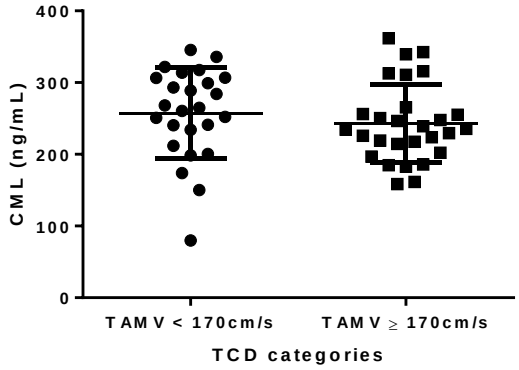
C. [2]SCA

TAMV (cm/s)	N^{ε}-(Carboxymethyl)lysine (CML)***			χ^2	p
	CML ≤ median, n (%)	CML > median, n (%)	Total, n (%)		
Normal (< 170)	9 (17.0)	16 (30.2)	25 (47.2)	4.287	0.071
Conditional (≥ 170 - 199)	11 (20.8)	4 (7.5)	15 (28.3)		
Abnormal (≥ 200)	7 (13.2)	6 (11.3)	13 (24.5)		
Total	27 (50.9)	26 (49.1)	53 (100.0)		

χ^2 = Pearson chi square. SCD, all sickle cell disease patients (SCA and HbSC); SCA, sickle cell anemia patients; *No HbSC patient had conditional time-averaged mean maximum velocity (TAMV) (values ≥ 170 cm/s and < 200 cm/s). **median value for SCD = 233.09 ng/mL ***median value for SCA = 247.93 ng/mL. [1]SCA, sickle cell anemia with two (2) categorizations (normal = < 170cm/s and not normal = ≥ 170 cm/s); [2]SCA, sickle cell anemia based on categorization of TCD by TAMV (normal = < 170cm/s, conditional = ≥ 170cm/s – 199cm/s, abnormal = ≥ 200cm/s).



A.



B.

Figure 4. N^{ϵ}-(carboxymethyl)lysine (CML) concentration (ng/mL) in sickle cell anemia (SCA) patients stratified by their transcranial Doppler (TCD). A. The three (3) TCD categories based on the time-averaged mean maximum velocity (TAMV) against CML (normal, TCD velocity value = < 170cm/s; conditional, TCD velocity = ≥ 170cm/s – 199cm/s; abnormal, TCD velocity value = ≥ 200cm/s). There is no significant difference between the concentrations of CML in all TCD categories. (Normal versus conditional, p = 0.2762; normal versus abnormal, p = 0.9838; conditional versus abnormal p = 0.3032) B. Time-averaged mean maximum velocity (TAMV) against CML (Here the TAMV was divided into two (2): those who are normal, TAMV < 170cm/s and those who are not normal which include conditional and abnormal TCD velocities, TAMV ≥ 170cm/s). There is no significant difference (p = 0.3848) in the concentration of CML between the two groups.

Anexo

Intervalo de referência para algums parâmetros laboratoriais

Variável hematológica	Intervalo de referência*
Hemácia, $\times 10^{12}$/L	3,5 – 6,9
Hemoglobina, g/dL	12, – 18,0
Hematócrito, %	37,0 – 52,0
Volume corpuscular médio (VCM), fL	82,0 – 101,0
Hemoglobina corpuscular media (HCM), ρg	27,0 – 34,0
Concentração de hemoglobina corpuscular media (CHCM), g/dL	31,5 – 36,0
Largura da distribuição das hemácia (LDH)	11,5 – 14,5
Contagem de reticulócitos, %	0,3 – 2,3
Contagem de leucócitos, $\times 10^6$/L	4.300,0 – 10.300,0
Contagem de neutrófilos, $\times 10^6$/L	1.500,0 – 8.000,0
Contagem de linfócitos típicos, $\times 10^6$/L	1.000,0 – 3.000,0
Contagem de monócitos, $\times 10^6$/L	100,0 – 800,0
Contagem de plaquetas, $\times 10^6$/L	140,0 – 440,0
Volume plaquetário medio (VPM), fL	7,8 – 11,0
Variáveis bioquímicas	
Glicose em jejum, mg/dL	70,0 – 99,0
Colesterol total (COLTOT), mg/dL	120,0 – 190,0
Colesterol de lipoproteína de alta densidade (COLHDL), mg/dL	35,0 – 86,0
Colesterol de lipoproteína de baixa densidade (COLLDL), mg/dL	80,0 – 130,0
Triglicéridos, mg/dL	54,0 – 150,0
Alanina transaminase, U/L	10,0 – 40,0
Aspartato aminotransferase, U/L	10,0 – 40,0
Ferro soro, μg/dL	28,0 – 182,0
Ferritina, ηg,/mL	11,0 – 336,0
Bilirrubina total, mg/dL	0,3 – 1,2
Bilirrubina direta, mg/dL	0,0 – 0,4
Proteína total, g/dL	6,0 – 8,0
Albumina, g/dL	3,2 – 5,2
Globulina, g/dL	2,3 – 3,5
Ureia, mg/dL	7,0 – 23,0
Creatinina, mg/dL	0,5 – 1,3
Proteína C reativa, mg/L	1,0 – 3,0
Lactato desidrogenase, U/L	110,0 – 210,0
$Alpha_1$-antitripsina (AAT), mg/dL	88,0 – 174,0

*Valores de referência para ambos os sexos e grupos etários 2 - 55 anos. Fonte: Consenso Brasileiro para Normatização da Determinação Laboratorial do Perfil Lipídico. www.sbpc.org.br/upload/conteudo/consenso_jejum_dez2016_final.pdf e Wallach interpretação de Exames Laboratoriais (nona edicao) por Williamson M. A. e Snyder L. M. (2013).