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UFBA **FIOCRUZ**

Curso de Pós-Graduação em Patologia Humana e Experimental

TESE DE DOUTORADO

DOENÇA FALCIFORME: BIOMARCADORES LABORATORIAIS E INFLAMATÓRIOS ASSOCIADOS A MANIFESTAÇÕES CLÍNICAS E USO DA HIDROXIURÉIA

CAROLINE CONCEIÇÃO DA GUARDA

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"É preciso força pra sonhar e perceber

que a estrada vai além do que se vê"

(Los Hermanos)

Dedico este trabalho aos meus avós Adelino (in memoriam) e Rosa pelo amor, e aos meus sobrinhos Arthur, Lucas, Pedro, João e Maria Beatriz, pela leveza e serenidade.

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RESUMO

INTRODUÇÃO: A doença falciforme (DF) é um grupo de hemoglobinopatias onde a anemia falciforme (AF), caracterizada pela homozigose HbSS, é a forma mais grave, enquanto a hemoglobinopatia SC (HbSC), apesar de menos grave, é a segunda mais prevalente. As manifestações clínicas na DF são heterogêneas, o que faz com que os marcadores laboratoriais e inflamatórios sejam úteis na prática clínica. Diversos mecanismos fisiopatológicos foram propostos ao serem associados as manifestações clínicas e ao envolvimento inflamatório sistêmico da doença. O tratamento da DF consiste, principalmente, no uso da hidroxiureia (HU), que é capaz de melhorar os marcadores laboratoriais e reduzir as manifestações clínicas. **OBJETIVO**: Investigar marcadores inflamatórios e laboratoriais associados a manifestações clínicas na DF, bem como ao uso da HU. **MÉTODOS**: O estudo foi aprovado pelo comitê de ética em pesquisa (CAAE: 52280015.1.0000.0048), as análises laboratoriais foram desenvolvidas por métodos automatizados e os pacientes são acompanhados na Fundação de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA). **RESULTADOS**: O primeiro manuscrito teve a casuística de 181 pacientes com DF (126 HbSS e 55 HbSC), no qual foram comparadas as alterações clínicas e laboratoriais presentes nos dois genótipos. Nossos resultados sugerem que os indivíduos com AF apresentam anemia, hemólise, leucocitose e inflamação de forma mais proeminente, bem como maior frequência de complicações clínicas, enquanto os pacientes HbSC apresentam menos hemólise e complicações clínicas. No segundo manuscrito investigou-se a associação entre as manifestações clínicas e o perfil lipídico em um grupo de 126 pacientes com AF. Observou-se a associação entre o histórico de pneumonia e níveis elevados de colesterol total; úlcera de perna e níveis reduzidos de lipoproteína de baixa densidade (LDL) e crises de dor e níveis elevados de lipoproteína de alta densidade (HDL). Observou-se também a associação entre níveis elevados de colesterol total, LDL e HDL com marcadores de hemólise e concentração de HbF, o que foi confirmado nas análises de correlação entre os indivíduos com pneumonia e crise de dor. No terceiro manuscrito foram estudados 43 indivíduos com AF, sendo investigados os níveis plasmáticos de interleucina-8 (IL-8) e a presença do polimorfismo rs4073 o gene *CXCL8*. Os resultados sugeriram que os indivíduos portadores do alelo A apresentaram níveis elevados de IL-8, que também estiveram associados a marcadores de inflamação e hemólise. Além disso, níveis diminuídos de IL-8 também estiveram associados a ocorrência de esplenomegalia. No quarto e último manuscrito desta tese, investigou-se o efeito do tratamento com HU nos monócitos dos pacientes com AF. Foram incluídos 37 pacientes, dos quais 17 estavam sob tratamento com HU e 20 sem uso da medicação. Os resultados mostraram que a HU foi capaz de reduzir a contagem de monócitos no sangue periférico, reduzir a frequência de monócitos clássicos ($CD14^{++}CD16^{-}$) e aumentar a de monócitos não clássicos

($CD14^{dim}CD16^{+}$). A HU também teve influencia na produção de citocinas, como o TNF-α, IL-1β, IL-6 e de fator tecidual (FT) pelos monócitos, bem como na polifuncionalidade destas células. Os resultados ainda sugeriram a associação entre os monócitos que produzem FT e a ocorrência de vaso-oclusão e que os monócitos clássicos podem ser responsáveis por produzir citocinas e FT na AF. **CONCLUSÕES**: Os resultados obtidos corroboram com estudos prévios sobre os aspectos clínicos e inflamatórios da DF, além de demonstrar associações novas entre os mecanismos fisiopatológicos da doença, marcadores laboratoriais e moléculas pró-inflamatórias.

Palavras-chave: Anemia falciforme, Doença falciforme, Inflamação, Marcadores laboratoriais, Manifestações clínicas.

GUARDA, Caroline Conceição da. Sickle cell disease: Laboratory and inflammatory biomarkers associated with clinical manifestations and hydroxyurea therapy. 2019. 179 f. il. Tese (Doutorado em Patologia) - Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 20191.

ABSTRACT

INTRODUCTION: Sickle cell disease (SCD) is a group of hemoglobinopathies and sickle cell anemia (SCA), characterized by HbSS homozygous, is the most severe form, while SC hemoglobinopathy (HbSC) is milder. Clinical manifestations in SCD are heterogeneous, thus, laboratory and inflammatory biomarkers are very useful in the clinical practice. Several pathophysiological mechanisms were suggested as significant associations were found with clinical manifestations and systemic inflammatory involvement of the disease. Therapy for SCD is based on hydroxyurea (HU), capable to improve laboratory biomarkers as well as to reduce clinical manifestations. **OBJECTIVE**: Investigate laboratory and inflammatory biomarkers associated to clinical manifestations in SCD as well as HU therapy. **METHODS**: This study was approved by the Institutional Research Board (CAAE: 52280015.1.0000.0048), laboratory analyses were carried by automated methods and patients were followed-up at the Bahia Hemotherapy and Hematology Foundation (HEMOBA). **RESULTS**: In the first manuscript we included 181 SCD patients (126 HbSS and 55 HbSC) and we compared the groups based on each genotype. Our results suggest that SCA individuals more prominent exhibit anemia, hemolysis, leukocytosis and inflammation as well as increased frequency of clinical manifestations, while HbSC individuals exhibit less hemolysis and clinical complications. In the second manuscript we investigated among the 126 SCA individuals the association between clinical manifestations and lipid profile. History of pneumonia was found to be associated with higher total cholesterol levels, leg ulcers were associated with decreased low density lipoprotein (LDL) and pain crises were associated with increased high density lipoprotein (HDL). We also found associations between total cholesterol, LDL and HDL levels with hemolysis biomarkers as well as HbF levels, which was confirmed by the correlation analyses between individuals with previous history of pneumonia and pain crises. In the third manuscript 43 SCA patients were included and we evaluated interleukin-8 (IL-8) plasma levels as well as the rs4073 polymorphism. We verified that individuals carriers of the A allele have increased IL-8 levels, which was also associated with biomarkers of inflammation and hemolysis. In addition, lower IL-8 levels were associated with previous history of splenomegaly. In the last manuscript we investigated the effect of HU treatment on monocytes of SCA patients. Thirty seven patients were included, 17 were undergoing HU therapy while 20 were not receiving the medication. Our results suggest that HU was capable to decrease monocyte counts in peripheral blood, decrease classical monocytes ($CD14^{++}CD16^{-}$) frequency and increase non-classical monocytes ($CD14^{dim}CD16^{+}$). HU also decreased TNF-α, IL-1β, IL-6 and tissue factor (TF) production by the monocytes, as well as their polyfunctionality. Monocytes producers of TF were found to be associated with vaso-occlusion, in addition, classical monocytes are responsible for multiple cytokine production. **CONCLUSIONS**: Our data corroborate with previous studies regarding inflammatory and clinical aspects of

SCA, in addition new associations between physiopathological mechanisms, laboratory biomarkers and pro-inflammatory molecules were found.

Key-words: Sickle cell anemia, Sickle cell disease, Inflammation, Laboratory biomarkers, Clinical manifestations.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AAT	Alfa-1 antitripsina
AF	Anemia Falciforme
ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
AVC	Acidente vascular cerebral
CD14	Grupo de diferenciação 14, do inglês *cluster of differentiation 14*
CD16	Grupo de diferenciação 16, do inglês *cluster of differentiation 16*
CHCM	Concentração de hemoglobina corpuscular média
DF	Doença Falciforme
DTC	Doppler transcraniano
eDAMPS	Padrões moleculares eritrocitários associados a dano, do inglês *erythrocyte damage-associated molecular pattern molecules*
eNOS	Óxido nítrico sintase endotelial
FT	Fator tecidual
GGT	Gama glutamiltransferase
GM-CSF	Fator estimulador de colônia granulócito-monócito
Hb	Hemoglobina
HbA	Hemoglobina A
HbC	Hemoglobina C
HbF	Hemoglobina Fetal
HbS	Hemoglobina S
HbSC	Hemoglobinopatia SC
HCM	Hemoglobina corpuscular média
HDL-C	Lipoproteína de alta densidade, do inglês *high density lipoprotein-cholesterol*
HT	Hematócrito
HU	Hidroxiuréia
ICAM-1	Molécula de adesão intercelular-1, do inglês *intercellular adhesion molecule-1*
IL-10	Interleucina-10
IL-1β	Interleucina-1 beta
IL-6	Interleucina-6

IL-8	Interleucina-8
LDH	Lactato desidrogenase
LDL-C	Lipoproteína de baixa densidade, do inglês *low density lipoprotein-cholesterol*
LPS	Lipopolissacarídeo
NF- κB	Fator Nuclear Kappa B
NO	Óxido nítrico
PCR	Proteína C reativa
PDW	Do inglês, *platelet distribution width*
RDW	Do inglês, *red blood cell distribution width*
ROS	Espécies reativas de oxigênio, do inglês *reactive oxygen species*
STA	Síndrome torácica aguda
TNF-α	Fator de Necrose Tumoral-alfa
VCAM-1	Molécula de adesão vascular-1, do inglês *vascular adhesion molecule-1*
VCM	Volume corpuscular médio
VLDL-C	Lipoproteína de muito baixa densidade, do inglês *very low density lipoprotein-cholesterol*
VO	Vaso-oclusão
VPM	Volume plaquetário médio

LISTA DE SÍMBOLOS

α	Alfa
β	Beta
γ	Gama
μ	Mi
κ	Kappa

SUMÁRIO

1 INTRODUÇÃO

A doença falciforme (DF) é causada pela herança de alelos que estão associados a mutação no gene *HBB* (rs334, glu6val β^S), responsável por codificar a globina beta da molécula de hemoglobina. A forma mais comum e mais grave é a homozigose HbSS, denominada anemia falciforme (AF), onde ocorre a formação do tetrâmero de hemoglobina S (HbS) $\alpha_2\beta_2^S$. Outra forma mais comum de DF inclui a heterozigose dupla entre a HbS e a hemoglobina C (HbC), denominada hemoglobinopatia SC (HbSC), sendo que tais hemácias possuem HbS suficiente para causar sua falcização (WARE *et al.*, 2017). Entre os estados do Brasil, a Bahia é aquele onde se observa a maior prevalência da DF (MINISTÉRIO DA SAÚDE, 2014; SANTIAGO*, et al.*, 2017).

A HbS quando submetida a concentrações reduzidas de oxigênio é capaz de formar polímeros longos, que podem acabar lesionando de forma irreversível a membrana das hemácias. As hemácias que carregam os polímeros de HbS são mais suscetíveis a lise, e, portanto, a hemólise constitui um dos mecanismos principais da fisiopatologia da DF. Por outro lado, a obstrução física de micro vasos pelas hemácias falcizadas contribui para a vaso-oclusão, que está relacionada às propriedades aderentes das células presentes no ambiente vascular. Tanto a hemólise quanto a vaso-oclusão são mecanismos fisiopatológicos relacionados ao aparecimento das complicações clínicas na DF (WARE *et al.*, 2017; KATO *et al.*, 2018; WILLIAMS e THEIN, 2018).

Embora o mecanismo genético responsável pela DF seja simples, as manifestações clínicas são extremamente heterogêneas, e diversos autores as classificam como agudas ou crônicas, bem como relacionadas a vaso-oclusão ou hemólise (KATO *et al.*, 2007; WILLIAMS e THEIN, 2018). Acredita-se que a hipertensão pulmonar, priapismo, úlcera de perna e acidente vascular cerebral (AVC) estejam relacionados a marcadores de hemólise e disfunção endotelial, tais como os níveis de lactato desidrogenase (LDH) e a contagem de reticulócitos, enquanto as crises vaso-oclusivas, a síndrome torácica aguda (STA) e a osteonecrose estejam relacionados a marcadores de viscosidade sanguínea, tais como concentrações de Hb e hematócrito (KATO *et al.*, 2007).

Recentemente, estudos também sugeriram associações entre os níveis de marcadores do perfil lipídico com a hipertensão pulmonar, além de associações com marcadores de hemólise e disfunção endotelial (ZORCA *et al.*, 2010; ALELUIA, *et al.*, 2017). No contexto do manejo clínico dos pacientes, o acompanhamento laboratorial é fundamental para investigar os marcadores de hemólise, anemia e inflamação que os pacientes apresentam. Em geral os exames são simples e de acesso fácil, tanto pelos médicos quanto pelos pacientes.

A DF tem como principal característica a resposta inflamatória crônica, mediada por tipos celulares diversos e a diferentes mecanismos moleculares. O envolvimento das hemácias, neutrófilos, monócitos, células endoteliais vasculares e plaquetas é alvo de estudos que tentam elucidar quais mecanismos e quais marcadores estão relacionados com a patogênese das complicações clínicas presentes na DF (ZHANG *et al.*, 2016). Adicionalmente, as citocinas produzidas pelos leucócitos, as moléculas de adesão expressas pelas células endoteliais e os produtos da hemólise também contribuem para a manutenção da resposta inflamatória (VILAS-BOAS *et al.*, 2012; CARVALHO *et al.*, 2018).

O principal tratamento farmacológico para a DF é a hidroxiuréia (HU), também denominada hidroxicarbamida, que tem como principal mecanismo de ação a inibição da enzima ribonucleotídeo redutase. A HU exerce vários efeitos benéficos na DF, incluindo a redução na produção de citocinas pró-inflamatórias, aumento na biodisponibilidade do óxido nítrico (NO), redução na expressão de moléculas de adesão, melhora nos marcadores da coagulação e, sobretudo, aumento nas concentrações de hemoglobina fetal (HbF) (CHARACHE, 1997; YAWN *et al.*, 2014). O aumento da HbF faz com que os pacientes apresentem menos manifestações clínicas e, consequentemente, tenham curso clínico melhor.

Dessa forma, considerando a complexidade dos mecanismos celulares e moleculares envolvidos na hemólise, na vaso-oclusão e na forma com que eles contribuem para a resposta inflamatória crônica e persistente presentes na DF,

salientamos a importância em se investigar marcadores laboratoriais, inflamatórios e manifestações clínicas apresentadas pelos pacientes, bem como a resposta ao tratamento pela HU, de modo a contribuir para a compreensão da fisiopatologia da doença.

2 REVISÃO DE LITERATURA

2.1 DOENÇA FALCIFORME

Em 1910, Herrick foi o primeiro cientista a descrever a doença falciforme (DF), ao observar hemácias “peculiarmente alongadas e em formato de foice” em esfregaço de sangue periférico de um de seus estudantes que sofria anemia (HERRICK, 1910). Mais tarde, outros pesquisadores identificaram o mecanismo molecular responsável pela doença, onde a substituição de um nucleotídeo (GAG → GTG, rs334T) com a troca de aminoácido (substituição de ácido glutâmico por valina) na sexta posição da porção amino terminal da globina β ($\beta^{6Glu\text{-}Val}$), no cromossomo 11 p.15.5, que tem como consequência a síntese da hemoglobina (Hb) variante denominada hemoglobina S (HbS), formada pelo tetrâmero $\alpha_2\beta_2{}^S$. Em 1947, na Bahia, o pesquisador Jessé Acioly propôs o caráter hereditário da DF (PAULING *et al.*, 1949; INGRAM, 1957; GOLDSTEIN *et al.*, 1963; ACCIOLY, 1947). Em função da mutação, a HbS é capaz de formar polímeros longos em condições onde há redução na tensão de oxigênio. Uma vez que as hemácias com HbS são submetidas a condições de hipóxia, os polímeros formados pela HbS são capazes de induzir modificações morfológicas na membrana destes corpúsculos (Figura 1).

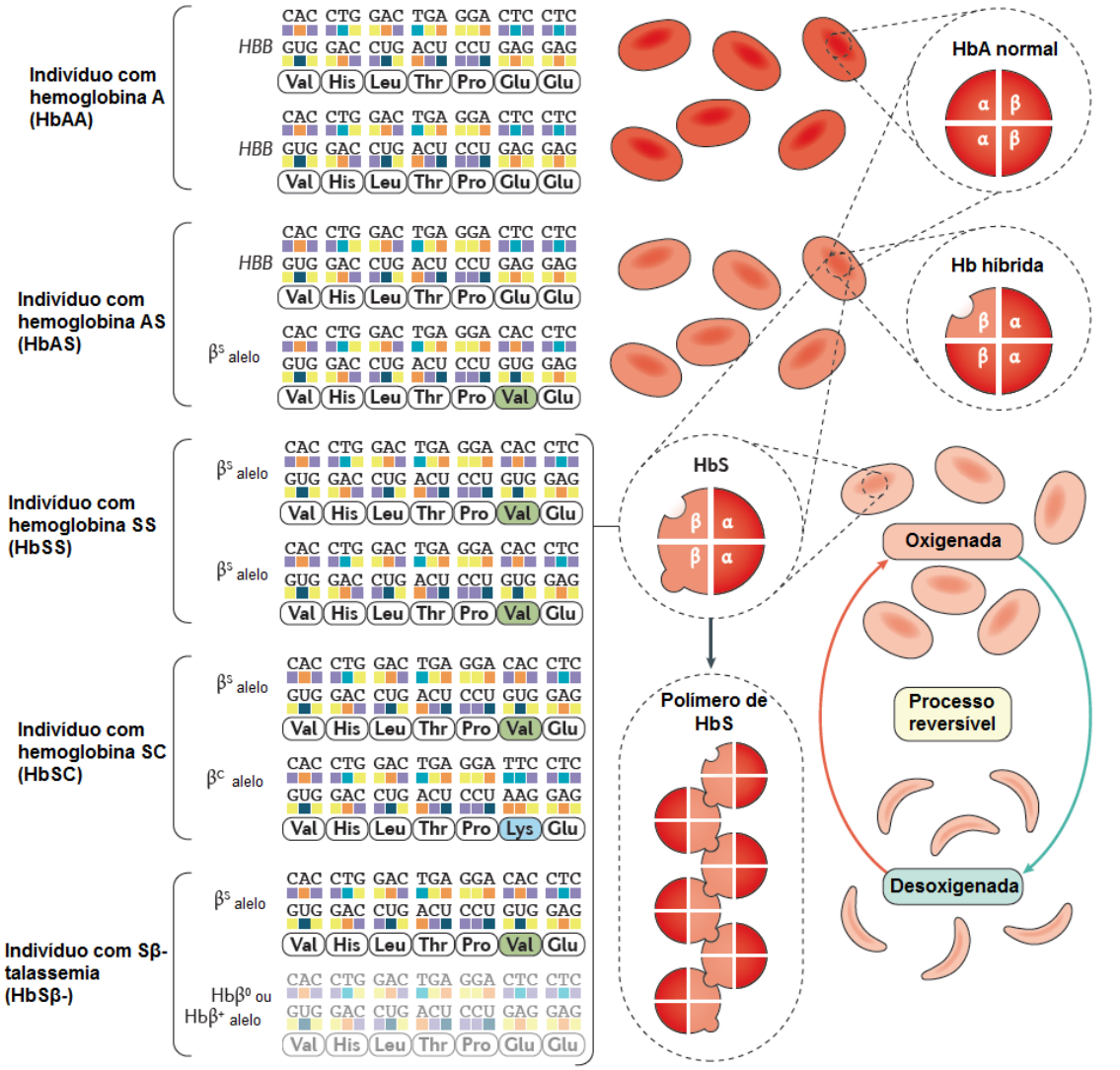

Figura 1. Mecanismos genéticos e moleculares responsáveis pelos diferentes genótipos associados à DF, bem como a sua influência na polimerização da HbS e no fenômeno de falcização. Fonte: adaptado de KATO *et al.*, 2018.

O processo de polimerização da HbS em baixa tensão de oxigênio e o de dissolução dos polímeros no retorno a tensão normal de oxigênio, acontece repetidamente até lesionar de forma definitiva a membrana das hemácias. As hemácias irreversivelmente falcizadas são menos flexíveis e mais frágeis do ponto de vista reológico e podem sofrer lise tanto no meio intravascular quanto no extravascular, onde são removidas pelo sistema reticuloendotelial (WARE *et al.*, 2017).

A DF é caracterizada pela presença da HbS em homozigose (HbSS), também denominada de anemia falciforme (AF), ou em heterozigose, com outra hemoglobina variante (por exemplo, hemoglobina C) ou talassemia (SUNDD *et*

al., 2018). A mutação que ocorre na HbC leva a troca do aminoácido ácido glutâmico por lisina na cadeia beta da globina. Quando herdada em associação com a HbS, o indivíduo apresenta a hemoglobinopatia SC (HbSC), um tipo de DF menos grave que a AF, com concentrações mais elevadas de Hb, o que confere o aumento da viscosidade sanguínea e também menos hemólise e vaso-oclusão (SERJEANT e VICHINSKY, 2018).

No Brasil, acredita-se que a HbS e a HbC tenham sido introduzidas durante o tráfico de escravos vindos da África. Entre os diferentes genótipos de DF, os mais prevalentes no Brasil são a AF e a HbSC, com prevalência heterogênea entre as diferentes regiões geográficas, reflexo da heterogeneidade étnica da população (SANTIAGO, *et al.*, 2017). A incidência da DF entre recém-nascidos varia também entre os diferentes estados brasileiros, sendo que se estima, na Bahia, a incidência de 1 recém-nascido com a DF a cada 650 nascidos vivos; no Rio de Janeiro, a incidência é de 1 a cada 1300 e em Santa Catarina, 1 a cada 13500 (MINISTÉRIO DA SAÚDE, 2014).

A polimerização da HbS e a mudança na morfologia eritrocitária são dois mecanismos fisiopatológicos fundamentais da DF. As anormalidades apresentadas pela membrana das hemácias, tais como expressão aumentada de fosfatidilserina, da proteína de superfície da família das imunoglobulinas (CD47), da proteína banda 3 oxidada, além do aumento na rigidez eritrocitária fazem com que ocorra diminuição significativa no tempo de vida destes corpúsculos, que passa de 120 dias para aproximadamente 31 (YASIN *et al.*, 2003; QUINN *et al.*, 2016).

A obstrução física de micro vasos pelo agregado heterocelular constituído por hemácias, leucócitos, células endoteliais vasculares e plaquetas leva a vaso-oclusão, que é um dos principais fenômenos fisiopatológicos da DF (WARE *et al.*, 2017). Diversas evidências epidemiológicas indicam que a vaso-oclusão é, frequentemente, iniciada por estímulo inflamatório ou ambiental, incluindo infecções, hipóxia, desidratação, acidose ou fatores desconhecidos (SUNDD *et al.*, 2018). Todas as células envolvidas no compartimento vascular na DF costumam apresentar expressão aumentada de moléculas de adesão e produção de citocinas pró-inflamatórias, o que também contribui para a vaso-

oclusão (LARD *et al.*, 1999; ZHANG *et al.*, 2016). A vaso-oclusão induz episódios de isquemia-reperfusão e crises agudas de dor, que constituem manifestações clínicas importantes da DF (HEBBEL, 2014).

Além da vaso-oclusão, outro fenômeno desencadeado pelas hemácias falcizadas é a hemólise crônica que libera o conteúdo eritrocitário no vaso sanguíneo, contribuindo para a manutenção do ambiente oxidante e pró-inflamatório. A arginase eritrocitária consome o aminoácido L-arginina, que serve como substrato para a produção de óxido nítrico (NO) pela enzima óxido nítrico síntase endotelial (eNOS) (KATO *et al.*, 2007). De forma semelhante, a Hb descompartimentalizada também reage diretamente com o NO reduzindo sua disponibilidade (REITER *et al.*, 2002). O NO é um dos mais importantes vasodilatadores e sua falta pode culminar em disfunção endotelial com vasoconstrição (BELHASSEN *et al.*, 2001). A hemólise libera também o heme, que pode atuar como *eDAMP* (do inglês, *erythrocyte damage-associated molecular pattern molecules*, padrões moleculares eritrocitários associados a dano) e ser agonista do receptor do tipo toll, tipo 4 (TLR4), presente, principalmente, em células endoteliais e monócitos, causando a ativação dessas células, no processo denominado inflamação estéril (BELCHER *et al.*, 2014; PITANGA *et al.*, 2016) (Figura 2).

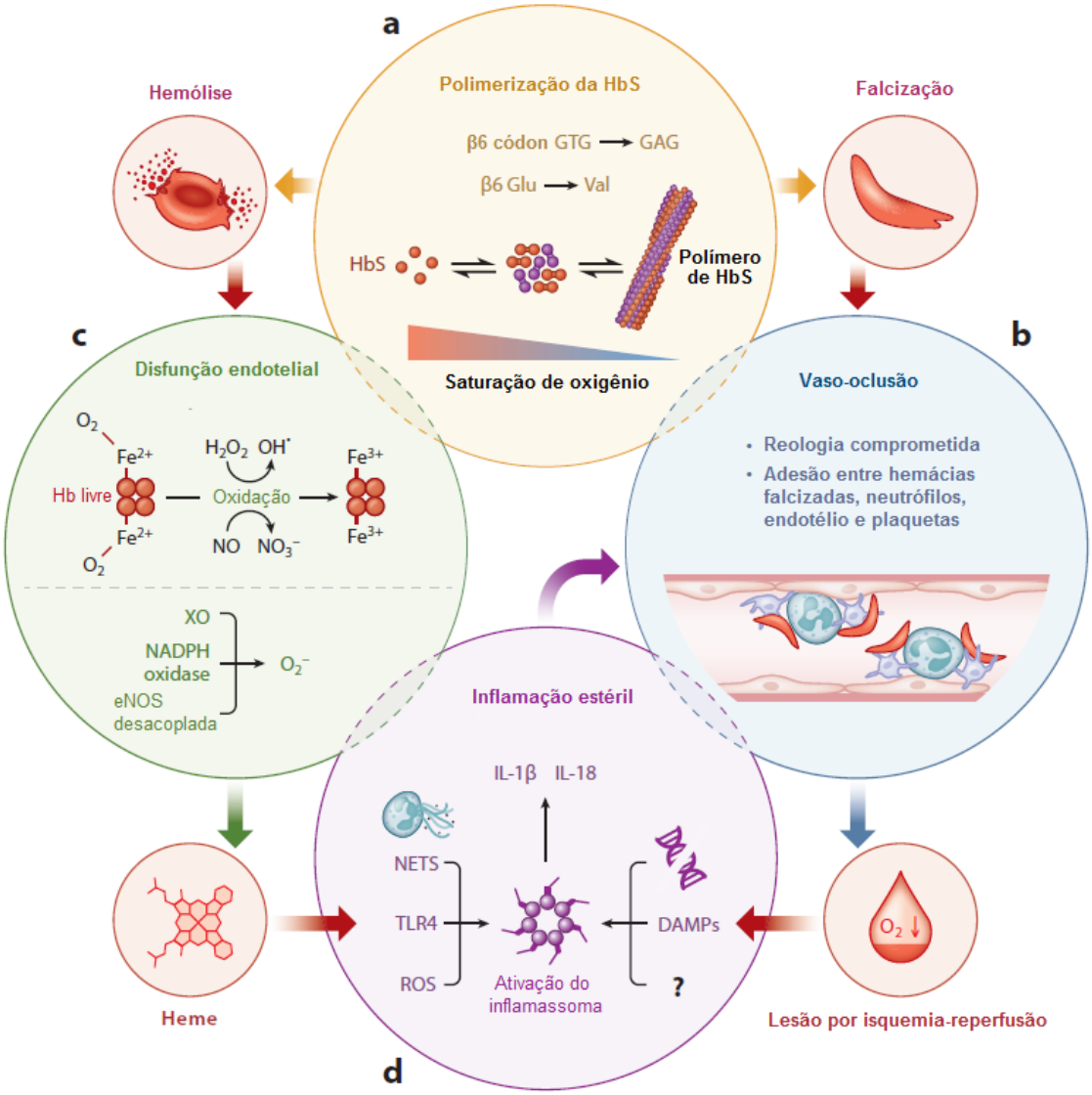

Figura 2. Representação dos mecanismos fisipatológicos responsáveis pela patogenia da DF. a) Polimerização da HbS; b) Vaso-oclusão; c) Disfunção endotelial e d) Inflamação estéril. Fonte: adaptado de: SUNDD *et al*., 2018.

Algumas manifestações clínicas foram atribuídas a esses dois mecanismos fisiopatológicos da DF. Entretanto, acredita-se que o fenótipo hemolítico esteja associado a hipertensão pulmonar, priapismo, úlcera de perna e AVC, enquanto o fenótipo vaso-oclusivo esteja associado a síndrome torácica aguda (STA), osteonecrose e episódios de dor (KATO *et al.*, 2007). Entretanto, é importante ressaltar que os dois fenômenos não ocorrem de forma isolada, uma vez que a vaso-oclusão pode dar origem à hemólise e o contrário também pode ocorrer. Portanto, o acompanhamento clínico dos marcadores associados à vaso-oclusão e à hemólise são importantes para monitorar o curso da doença e a apresentação clínica dos pacientes.

2.2 MANIFESTAÇÕES CLÍNICAS

Na descrição da anemia apresentada pelo estudante de Herrick em 1910, foi possível verificar que o paciente apresentou os sintomas mais marcantes da DF. O indivíduo relatou histórico de diversas úlceras em membros inferiores ao longo da vida, icterícia, mucosas descoradas (compatível com anemia), leucocitose, presença de eritroblastos no sangue periférico e um episódio de hospitalização que durou aproximadamente dois meses em função de crise de dor intensa (HERRICK, 1910). As complicações clínicas são importantes causas de mortalidade entre os pacientes com DF, sendo que em avaliações epidemiológicas sobre a mortalidade, foi observado que no estado da Bahia 42% das mortes ocorrem entre adultos jovens, de 20 a 39 anos de idade (RAMOS *et al.*, 2015).

De modo geral, a DF pode ser organizada em duas fases: a fase assintomática (ou estado-estável) que é o período no qual os pacientes não apresentam crises de dor e sentem-se clinicamente bem, embora a hemólise crônica, o dano aos órgãos e a vaso-oclusão continuem ocorrendo de forma subclínica. Já a segunda fase corresponde à crise, que é o período no qual os pacientes manifestam complicações clínicas associadas aos mecanismos fisiopatológicos de vaso-oclusão e hemólise (WILLIAMS e THEIN, 2018). As complicações clínicas ainda podem ser classificadas em agudas ou crônicas considerando a faixa etária dos indivíduos, apesar de muitas delas ocorrerem em qualquer fase da vida (KATO *et al.*, 2007; WILLIAMS e THEIN, 2018).

Entre as complicações agudas, as crises agudas de dor ocorrem geralmente nas extremidades do corpo, tórax, no abdome e nas costas, sendo desencadeadas por vaso-oclusão, hipóxia e lesão por isquemia e reperfusão (GILL *et al.*, 1995). Embora sejam raras nos primeiros seis meses de vida, nos quais as concentrações de HbF permanecem elevadas, a frequência das crises de dor aumenta até atingir a incidência de aproximadamente 40 a cada 100 indivíduos por ano de observação, após o segundo ano de vida. As crises de dor muitas vezes requerem tratamento com analgésicos potentes, incluindo

opióides (GILL *et al.*, 1995), e são a principal causa de hospitalização na DF (WILLIAMS e THEIN, 2018). Outra complicação aguda bastante comum na AF é a STA, que tem como sintomas tosse, encurtamento da respiração e sinais de hipoxemia que são difíceis de distinguir de uma pneumonia aguda (VICHINSKY *et al.*, 1997). A incidência de AVC é mais elevada entre 2-10 anos de idade, quando ocorre demanda metabólica maior pelo cérebro, o que torna o AVC uma das complicações agudas mais debilitantes da DF (OHENE-FREMPONG *et al.*, 1998; BERNAUDIN *et al.*, 2011). Antes da utilização do doppler transcraniano (DTC) como triagem de pacientes com DF com risco de AVC e do regime de transfusão terapêutica de hemocomponentes nos pacientes com risco, 11% destes tinham evento vascular cerebral até os 20 anos de idade, com risco de 40% de ocorrência de AVC no decorrer de toda a vida (OHENE-FREMPONG *et al.*, 1998; BERNAUDIN *et al.*, 2011).

Por outro lado, entre as complicações crônicas, as úlceras de perna apresentam-se como lesões crônicas na pele, podendo também atingir tecidos mais profundos, sendo mais comumente localizadas na região maleolar. Acredita-se que exista associação entre as úlceras, hemólise e vasculopatia (POWARS *et al.*, 2005). Também associada a hemólise, a colelitíase está relacionada a formação de cálculos biliares e obstrução da vesícula biliar, com frequência crescente em função da idade (POWARS *et al.*, 2005). A colelitíase resulta da taxa de destruição eritrocitária acelerada e crônica em indivíduos com DF. O heme é metabolizado em bilirrubina, que na bile pode formar o bilirrubinato de cálcio que é insolúvel e precipita como pigmentos formando os cálculos biliares (KATO *et al.*, 2018). A esplenomegalia pode ser definida como o aumento anormal do baço (MCKENZIE *et al.*, 2018), que é um dos órgãos mais afetados na AF. Geralmente, o baço está aumentado durante a primeira década de vida em indivíduos com DF; porém, sofre atrofia gradual durante a vida em função das repetidas crises de vaso-oclusão e infartos que levam a auto-esplenectomia. As complicações esplênicas estão associadas ao aumento da morbidade na DF e, em alguns casos, podem levar ao aumento da mortalidade (AL-SALEM, 2011).

2.3 PARÂMETROS LABORATORIAIS: ANEMIA, HEMÓLISE, LIPÍDIOS E INFLAMAÇÃO

Os testes laboratoriais podem avaliar diversos parâmetros sistêmicos, tais como avaliação das concentrações de hemoglobina e contagem de leucócitos. Estes são acessíveis do ponto de vista clínico, sendo que os valores apresentados pelos pacientes durante o estado-estável devem ser acompanhados a cada consulta, principalmente quando não há complicações ou outras doenças associadas (MILLER *et al.*, 2000).

As determinações obtidas através de exames simples, tais como hemograma, contagem de leucócitos, perfil lipídico e perfil inflamatório, estão associadas às complicações clínicas apresentadas pelos pacientes. Tais medidas são úteis, por exemplo, para diferenciar o curso clínico da HbSC e da AF, uma vez que os pacientes com AF apresentam hemólise aumentada, anemia e leucocitose, enquanto os pacientes com HbSC possuem concentrações mais elevadas de colesterol total (ALELUIA, *et al.*, 2017).

Em relação ao perfil hematológico, já foi descrito na DF que a contagem de hemácias, com anisocitose, e de monócitos, bem como as concentrações de Hb, hematócrito, bilirrubina, NO e ferritina estavam associadas ao aumento da velocidade média do fluxo sanguíneo obtida por DTC em pacientes com HbSC (SANTIAGO, *et al.*, 2017), o que sugere a associação entre características laboratoriais e a possível ocorrência de AVC. Já foi observado também que a ocorrência de dactilia, juntamente com concentrações de Hb < 7g/dL, em associação com leucocitose e ausência de infecção, estava associada ao prognóstico grave da DF na infância (MILLER *et al.*, 2000). Alguns marcadores também estão associados a vaso-oclusão na DF, principalmente marcadores relacionados a reologia do sangue, tais como o hematócrito, a viscosidade sanguínea e a deformabilidade dos eritrócitos (SUNDD *et al.*, 2018).

A leucocitose, mesmo na ausência de infecção, constitui parâmetro hematológico importante a ser considerado na DF. Em geral, a contagem de leucócitos está elevada em pacientes com DF e acredita-se que a contagem de maior que 15.000 células/mL esteja associada ao risco aumentado de óbito

entre os pacientes (KASSCHAU *et al.*, 1996). Da mesma forma, pacientes com DF e manifestações clínicas mais graves, em geral, apresentam contagem elevada de neutrófilos quando comparados a indivíduos controles (ANYAEGBU *et al.*, 1998).

A concentração aumentada de HbF também é marcador importante de prognóstico na DF. A HbF tem o efeito de diluir a polimerização da HbS, pois os tetrâmeros híbridos ($\alpha_2\gamma\beta^S$) e a HbF ($\alpha_2\gamma_2$) possuem a capacidade de reduzir a concentração intraeritrocitária de HbS. Além disso, os tetrâmeros de $\alpha_2\gamma\beta^S$ não participam da polimerização da HbS (WILLIAMS e THEIN, 2018). Concentrações elevadas de HbF estão associadas ao curso clínico menos grave da DF, uma vez que pacientes que apresentam esse aumento, possuem menor número de eventos de STA, AVC, úlcera de perna e priapismo (SERJEANT e VICHINSKY, 2018).

Marcadores de hemólise também são importantes na DF, embora a lactato desidrogenase (LDH) seja uma enzima comumente encontrada no organismo humano, acredita-se que a isoenzima liberada pelos eritrócitos durante a hemólise intravascular represente a maior parte da LDH sérica em pacientes com DF. Portanto, a atividade de LDH serve também como marcador sugestivo da quantidade de hemoglobina livre no plasma (STANKOVIC e LIONNET, 2016). A associação entre concentrações elevadas de LDH e hipertensão pulmonar já foi demonstrada (definida pela velocidade tricúspide regurgitante > 2,5 m/s), bem como em úlceras de perna, priapismo e no risco aumentado de óbito. Esses pacientes, em geral, apresentam concentração diminuída de hemoglobina e desenvolvem o fenótipo chamado de “hiper-hemolítico” (TAYLOR *et al.*, 2008). Apesar da LDH ser utilizada para acompanhar a hemólise, também já foi sugerido que a contagem absoluta de reticulócitos é uma medida mais sensível para identificar o tempo de vida média das hemácias, o que estaria associado tanto a hemólise quanto a anemia que os pacientes apresentam (QUINN *et al.*, 2016).

Há cerca de 40 anos foi descrito que indivíduos que apresentam diferentes tipos de anemia, também apresentam anormalidades no perfil lipídico (WESTERMAN, 1975). No contexto da DF, foi demonstrado que os pacientes

em geral apresentam concentrações menores de colesterol total, colesterol de baixa densidade (LDL-C, do inglês *low density lipoprotein-cholesterol*) e colesterol de alta densidade (HDL-C, do inglês *high density lipoprotein cholesterol*) e maiores de triglicerídeos e colesterol de muito baixa densidade (VLDL-C, do inglês *very low density lipoprotein-cholesterol*) em relação a indivíduos HbAA (SEIXAS *et al.*, 2010; ZORCA *et al.*, 2010). Marcadores lipídicos na DF já foram associados a marcadores de hemólise (ALELUIA, *et al.*, 2017), ocorrência de colelitíase (SEIXAS *et al.*, 2010), concentrações do fator de transformação do crescimento beta (do inglês: *transforming growth factor beta,* TGF-β) (FIGUEIREDO *et al.*, 2016), hipertensão pulmonar (ZORCA *et al.*, 2010) e moléculas relacionadas à hemólise, como o heme e hemopexina (VENDRAME *et al.*, 2018). A investigação do perfil lipídico de pacientes com DF também é bastante acessível na prática clínica e, considerando que esses dados sugerem fortemente a associação dos lipídios com outros processos fisiopatológicos da doença, o simples monitoramento dos níveis de colesterol total e frações podem ser úteis no acompanhamento dos pacientes.

Através de exames laboratoriais também é possível avaliar alguns marcadores de inflamação sistêmica, tais como as proteínas de fase aguda proteína C-reativa (PCR) e alfa-1 antitripsina (AAT). Em geral, em pacientes com DF, as concentrações de AAT e PCR estão elevadas mesmo na ausência de processos infecciosos (BOURANTAS *et al.*, 1998; SEIXAS *et al.*, 2010). Na DF, foi observado que os pacientes apresentam concentrações elevadas da AAT mesmo no estado-estável da doença quando comparadas a indivíduos controles (CARVALHO, *et al.*, 2017). Da mesma forma, concentrações elevadas de PCR foram observadas em pacientes com DF durante o estado-estável (BOURANTAS *et al.*, 1998) e o aumento significativo ao longo do período de crise e internação, especialmente em pacientes com AF (SCHIMMEL *et al.*, 2017). Os achados laboratoriais são extremamente importantes para facilitar o manejo clínico dos pacientes e acompanhar a história natural da doença.

2.4 HIDROXIURÉIA

Durante vários anos, os únicos tratamentos disponíveis para a DF eram a transfusão sanguínea e a quelação de ferro, além dos tratamentos analgésicos (YAWN *et al.*, 2014). Com a evolução do conhecimento sobre os mecanismos fisiopatológicos da doença, novas drogas e novos tratamentos têm sido utilizados e investigados.

Até pouquíssimo tempo, a única terapia farmacológica disponível para o tratamento da DF era a hidroxiuréia (HU), também chamada de hidroxicarbamida. Atualmente, a suplementação com L-glutamina também foi aprovada pela agência americana FDA (*Food and Drug Administration*) em função da boa resposta antioxidante e redução das crises de dor (NIIHARA *et al.*, 2018). Alguns protocolos recomendam o tratamento com HU em crianças a partir de 9 meses de idade (YAWN *et al.*, 2014).

A HU é um agente quimioterápico utilizado para tratar malignidades hematológicas que exerce diversos benefícios na DF. Seu mecanismo de ação ocorre através da inibição da enzima ribonucleotídeo redutase, o que reduz o *pool* de nucleotídeos disponíveis para as células que estão em divisão, forçando o ciclo celular a parar na fase S (CHARACHE, 1997). Como consequência, na DF, a HU é capaz de reduzir a contagem de neutrófilos e outros leucócitos, reticulócitos e plaquetas e fazer com que hemácias mais jovens, e com concentrações elevadas de HbF, alcancem o sangue periférico (CHARACHE *et al.*, 1996). A investigação laboratorial dos pacientes que fazem o tratamento com HU reflete aumento nas concentrações de HbF, hemoglobina, hematócrito, volume corpuscular médio (VCM), hemoglobina corpuscular média (HCM), além de redução na contagem de reticulócitos, leucócitos, neutrófilos, eosinófilos e linfócitos (YAHOUEDEHOU *et al.*, 2019).

A HU atua também reduzindo diversas moléculas envolvidas na resposta inflamatória, já foi demonstrado que a HU é capaz de reduzir os níveis de citocinas, tais como do fator de necrose tumoral alfa (TNF-α) e interleucina (IL)-8 (LANARO *et al.*, 2009), IL-1β (KEIKHAEI *et al.*, 2013) e IL-6 (BANDEIRA *et al.*, 2014). Penkert e colaboradores (2018) realizaram um estudo longitudinal

onde foi demonstrado que a HU foi capaz de reduzir, durante o tratamento, os níveis de ligante de CD40 solúvel (sCD40L), IL-7, fator de crescimento endotelial (do inglês: *endotelial growth factor*, EGF) e proteína quimioatraente de monócitos-1 (do inglês: *monocyte chemoattractant protein-1*, MCP-1) (PENKERT *et al.*, 2018).

Do mesmo modo, a HU foi associada a redução de marcadores da coagulação em pacientes com AF sob tratamento, quando comparados aos que não recebiam doses da HU, com redução tanto na expressão gênica quanto nos níveis plasmáticos do fator tecidual (FT), redução plasmática do complexo trombina-antitrombina (TAT), fragmento de protrombina F_{1+2} (F_{1+2}) e trombomodulina (COLELLA *et al.*, 2012). Tais resultados sugerem que a HU melhora os marcadores de hipercoagulabilidade nos pacientes com DF e, possivelmente, exerce efeito positivo nas alterações hemostáticas.

A HU foi capaz de reduzir a expressão de moléculas de adesão, tanto em leucócitos quanto em células endoteliais. Já foi demonstrada a redução nas concentrações de E e P-selectina, molécula de adesão intercelular-1 (do inglês: *intercellular adhesion molecule-1*, I-CAM), molécula de adesão celular endotelial -1 (do inglês: *platelet endothelial cell adhesion molecule,* PECAM-1) e molécula de adesão vascular-1 (do inglês: *vascular cell adhesion protein-1*, VCAM-1) (OKPALA, 2006; LAURANCE *et al.*, 2011), que estão associadas aos processos vaso-oclusivos. Em modelo murino, por exemplo, a redução de E-selectina protegeu os animais de apresentarem infecção exacerbada pela redução da interação entre leucócitos e o endotélio vascular (LEBENSBURGER *et al.*, 2012).

A disfunção endotelial é uma das principais características vasculares da DF. Cokic e colaboradores (2007) sugeriram que a HU consegue proteger de danos oxidativo a isoenzima endotelial óxido nítrico sintase (eNOS), evitando a sua degradação por proteassomas (COKIC *et al.*, 2007). Acredita-se também que a HU é capaz de atuar como doadora de NO. Foi demonstrado que o pico na produção de NO ocorre cerca de 2 horas após a administração da HU, em associação com o aumento plasmático de Hb nitrosilada, nitrato e nitritos (GLADWIN *et al.*, 2002). O NO oriundo da resposta terapêutica à HU também

está relacionado com a via da enzima guanilato ciclase, envolvida na indução da síntese da cadeia gama que compõe o tetrâmero da HbF (COKIC *et al.*, 2008).

Além de todos os mecanismos acima propostos, o principal efeito benéfico do tratamento com HU é o aumento nas concentrações de HbF (CHARACHE *et al.*, 1992). O aumento da HbF esteve diretamente associado à redução das crises de dor na DF, uma vez que a HbF não participa do polímero formado pela HbS, mantendo as hemácias flexíveis, com redução do risco de episódios vaso-oclusivos, com melhora dos eventos hemolíticos (CHARACHE *et al.*, 1995).

Embora muitos aspectos favoráveis sejam atribuídos ao tratamento com HU alguns efeitos colaterais podem ser apresentados pelos pacientes. Entre os mais frequentes destacam-se as alterações dermatológicas, neurológicas, gastrointestinais e hematológicas (GHASEMI *et al.*, 2014). Recentemente tem sido descrito também alterações no processo de espermatogênese em indivíduos com AF tratados com HU por pelo menos 6 meses, com redução da contagem total de espermatozoides (BERTHAUT *et al.*, 2017). No entanto, ainda são necessários novos estudos que busquem identificar os mecanismos farmacocinéticos e farmacodinâmicos da HU, de modo a contribuir com a compreensão do mecanismo de ação do medicamento (YAHOUEDEHOU *et al.*, 2018).

Todos os efeitos positivos do tratamento com HU estão relacionados aos mecanismos fisiopatológicos da DF: redução da polimerização da HbS, redução da vaso-oclusão, redução da hemólise e redução das moléculas inflamatórias, com a consequente melhora clínica do paciente.

2.5 ASPECTOS INFLAMATÓRIOS

A resposta inflamatória crônica ocorre, geralmente, devido a repetidas lesões teciduais decorrentes das crises de falcização. As consequências clínicas são graves e envolvem diversos mecanismos inflamatórios, nos quais as células

estão excessivamente ativadas (PENKERT *et al.*, 2018). Além de influenciar os eventos vaso-oclusivos responsáveis pelos episódios de dor aguda, que são a maior causa de hospitalização na DF, a resposta inflamatória também é componente importante de diversas complicações, tais como auto-esplenectomia, STA, hipertensão pulmonar, úlcera de perna, nefropatia e AVC (BROUSSE *et al.*, 2014; HEBBEL, 2014; MINNITI *et al.*, 2014). É importante ressaltar que tal resposta inflamatória ocorre de maneira estéril, sem necessariamente haver episódio infeccioso que desencadeie a ativação celular. No entanto, diversas células e moléculas presentes no ambiente vascular contribuem para a inflamação sistêmica.

2.5.1 **Componentes celulares e corpusculares**

A polimerização da HbS faz com que a hemácia sofra alterações morfológicas e assuma sua forma característica de foice. As hemácias falcizadas, assim como os reticulócitos, são mais aderentes ao endotélio vascular, especialmente através de interações entre a integrina $\alpha_4\beta_1$ e VCAM-1 nas células endoteliais (BRITTAIN e PARISE, 2008). Como resultado da anemia crônica, a medula óssea torna-se hiperativa, apresenta reticulocitose de estresse e libera reticulócitos que possuem expressão aumentada das moléculas da integrina $\alpha_4\beta_1$ e CD36 (KAUL *et al.*, 2009). As hemácias também são capazes de liberar micropartículas carregadas com heme, o que contribui para alterações na coagulação e, consequentemente, em vaso-oclusão (CAMUS *et al.*, 2015). No entanto, a maior contribuição das hemácias para o processo inflamatório observado na DF são os produtos liberados durante os eventos de hemólise, também chamados de *eDAMPS*, principalmente o heme e a hemoglobina livre que podem promover a ativação do sistema imune inato (BELCHER *et al.*, 2014; PITANGA *et al.*, 2016).

Além das hemácias, os neutrófilos de indivíduos com DF exibem fenótipo ativado, mesmo em estado-estável. A caracterização dos neutrófilos na DF mostrou expressão reduzida de CD62L no estado estável e na crise, quando comparados com indivíduos controle saudáveis. Também se identificou o aumento na expressão de CD66b nos indivíduos em crise quando comparados

aqueles em estado estável. A atividade da lactoferrina e da elastase também está aumentada em indivíduos com DF em crise quando comparados com aqueles em estado estável e controles saudáveis (LARD *et al.*, 1999). Os neutrófilos também são capazes de liberar “armadilhas extracelulares” (do inglês: *neutrophil extracellular traps,* NETs) quando desafiados com heme, o que corrobora o papel pró-inflamatório dessas células, além da contribuição para a vaso-oclusão (CHEN *et al.*, 2014).

Os monócitos também contribuem com a fisiopatologia da DF, principalmente através da interação com células endoteliais. A cultura *in vitro* dos dois tipos celulares mostrou que os monócitos são capazes de aumentar a expressão de moléculas de adesão e citocinas pelas células endoteliais (SAFAYA *et al.*, 2012).

O mecanismo associado a ativação dos monócitos na AF ainda não está completamente elucidado, considerando que diversas moléculas presentes no microambiente podem contribuir para tal ativação. No entanto, três marcadores agonistas de TLR4 têm papel importante nessa ativação: i) heme livre, oriundo da hemólise, ii) proteína de alta mobilidade do grupo 1 (do inglês: *high-mobility group box 1 protein*, HMGB1), liberado durante eventos de isquemia e reperfusão e o iii) heparan sulfato liberado do glicocálice endotelial. A ativação dos monócitos via TLR4 leva ao aumento na produção de TNF-α, que pode dessa forma perpetuar a resposta inflamatória (SOLOVEY *et al.*, 2017). Já foi demonstrado também que monócitos não clássicos ($CD14^{dim}CD16^{+}$) expressam concentrações maiores da enzima hemoxigenase-1 ($HO\text{-}1^{hi}$) e que a interação desses monócitos com células endoteliais, previamente desafiadas com heme, protege da vaso-oclusão (LIU *et al.*, 2018).

O envolvimento dos linfócitos na fisiopatologia da DF até então é pouco conhecido. No entanto, existem evidencias sobre o papel inflamatório dessas células, especialmente, na polarização da resposta do subtipo T auxiliar 17 (Th17, do inglês *T helper 17*) (VILAS-BOAS *et al.*, 2010). Olenscki Gilli e colaboradores demonstraram a redução de células T_{reg} ($Foxp3^{+}CD25^{+}CD4^{+}$) e aumento de células Th17 em indivíduos com DF quando comparados aos sadios; a expressão gênica de *TGF-β*, *IL-6* e *IL-10* foi maior nos indivíduos com

DF que nos saudáveis, e foi identificado que indivíduos aloimunizados com a doença possuíam expressão elevada de IL-8 e IL-10 quando comparados aqueles não-aloimunizados (OLENSCKI GILLI *et al.*, 2016).

As plaquetas desempenham papel importante na homeostase vascular. Na DF, as plaquetas mostram magnitude maior de ativação, e uma vez ativadas, elas são capazes de promover a adesão de hemácias falcizadas ao endotélio vascular através da secreção de trombospondina (BRITTAIN *et al.*, 1993). As plaquetas na DF também são mais inflamatórias, com propriedade aderente elevada e produção maior de IL-1β (PROENCA-FERREIRA *et al.*, 2014). Além disso, também podem contribuir para a ocorrência de trombose e hipertensão pulmonar na DF (VILLAGRA *et al.*, 2007). Assim, é possível verificar o envolvimento de diversos tipos celulares e corpusculares nos processos inflamatórios que são crônicos na DF.

2.5.2 **Componentes moleculares**

O processo inflamatório na DF está diretamente relacionado aos eventos vaso-oclusivos e aos episódios de hemólise, que contribuem para a manutenção da resposta inflamatória através de diversos tipos de moléculas, que possuem diferentes características bioquímicas, tais como proteínas de fase aguda, citocinas, moléculas de adesão, NO e produtos da hemólise (CONRAN e BELCHER, 2018).

Diversos mecanismos moleculares contribuem para o estresse oxidativo na DF, tais como desequilíbrio do estado redox, com excessiva liberação de heme e ferro catalisando a reação de Fenton (CHIRICO e PIALOUX, 2012), lesões frequentes por isquemia e reperfusão que promovem a ativação do sistema xantina-oxidase (OSAROGIAGBON *et al.*, 2000), além da suscetibilidade maior a auto-oxidação da HbS (ASLAN *et al.*, 2000). Vários marcadores de estresse oxidativo foram investigados nas hemácias de indivíduos com DF e comparados com voluntários sadios. Foi observada diferença estatística entre os valores de meta-hemoglobina, substância reativas ao ácido tiobarbitúrico (do inglês: *thiobarbituric acid reactive substances*, TBARS), porcentagem de hemólise, atividade de glicose-6-fosfato desidrogenase (G6PD) e espécies

reativas de oxigênio (HERMANN *et al.*, 2016). Da mesma forma, ao comparar indivíduos com AF, HbSC e HbAA foi identificado que marcadores de estresse oxidativo e a atividade de enzimas antioxidantes estavam aumentados nos grupos AF e HbSC, embora os marcadores de estresse oxidativo estivessem mais alterados nos indivíduos HbSS. Foi verificado também que a hemólise e os metabólitos de NO estavam mais elevados nos indivíduos com AF comparados aos HbSC. Da mesma forma, a função microvascular foi associada com estresse oxidativo e nitrosativo entre os indivíduos com AF, mas não entre aqueles HbSC (MOCKESCH *et al.*, 2017).

O estresse oxidativo está diretamente relacionado ao processo hemolítico crônico que os indivíduos com DF apresentam. Desta forma, a quantificação de heme livre no plasma de indivíduos com AF, HbSC e HbAA mostrou que os indivíduos com AF apresentavam concentrações mais elevadas, seguidos dos HbSC. O mesmo estudo também mostrou concentrações diminuídas de hemopexina e haptoglobina nos indivíduos com DF em relação aos indivíduos HbAA (SANTIAGO *et al.*, 2018). A liberação de Hb livre durante a hemólise possui propriedades pró-inflamatórias, uma vez que é capaz de reagir rápida e irreversivelmente com o NO, dando origem ao nitrato que é inerte. A liberação da Hb intraeritrocitária no plasma, durante a hemólise intravascular, promove a redução do NO e compromete a vasodilatação (REITER *et al.*, 2002; SUNDD *et al.*, 2018). A Hb descompartimentalizada, ou seja, fora da hemácia, também promove a formação de ROS, o que altera o equilíbrio redox vascular do estado-estável produtor de NO para a produção de ROS, com diminuição do equilíbrio entre NO/ROS (SUNDD *et al.*, 2018).

Além do estresse oxidativo mediado pela hemólise, a liberação dos produtos intraeritrocitários também contribui diretamente para a ativação do sistema imune inato. Diversos estudos apontam que o heme é um potencial agonista de TLR4, levando a ativação, principalmente, de células endoteliais e monócitos (BELCHER *et al.*, 2014; DUTRA *et al.*, 2014; PITANGA *et al.*, 2016; GUARDA *et al.*, 2017). O envolvimento da resposta imune inata é evidenciado através de estudos que mostraram que diversos genes associados aos TLRs e a formação do inflamassoma estão super-expressos em monócitos de indivíduos com DF

expostos a sobrecarga de ferro devido ao regime transfusional (VAN BEERS *et al.*, 2015). O mesmo estudo mostrou que o acúmulo de ferro intracelular estava associado a concentrações elevadas de IL-6, PCR e risco de óbito (VAN BEERS *et al.*, 2015). Pitanga e colaboradores (2016) avaliaram células mononucleares do sangue periférico de pacientes com AF e identificaram a expressão gênica elevada de *TLR4*, *TLR5*, *NLRP3* e *IL-1β* quando comparada a células de indivíduos HbAA (PITANGA *et al.*, 2016).

O heme ativa as células endoteliais no ambiente vascular, levando a expressão elevada de moléculas de adesão ICAM-1, VCAM-1 e E-selectina, (WAGENER *et al.*, 1997), sendo capaz de promover a ativação da via do NF-κB, o que leva a mobilização dos corpúsculos de Weibel-Palade e produção elevada de P-selectina e fator de von Willebrand (BELCHER *et al.*, 2014).

Os principais eventos fisiopatológicos da DF ocorrem no ambiente vascular: hemólise e vaso-oclusão. Portanto, a resposta das células endoteliais vasculares é fundamental para a compreensão dos mecanismos associados à doença. Pacientes com DF, em geral, apresentam concentrações elevadas de VCAM-1 e ICAM-1 (VILAS-BOAS *et al.*, 2016). Em estudo experimental realizado em camundongos foi demonstrado que os monócitos foram capazes de promover a ativação endotelial mediante o aumento na expressão de FT e VCAM-1. A ativação endotelial mostrou ser dependente do fator de transcrição NF-κB e mediada através da citocina TNF-α, uma vez que o uso de um agente inibidor do TNF-α mostrou a melhora significativa nos parâmetros inflamatórios avaliados, com redução de VCAM-1, TNF-α e MCP-1 (SOLOVEY *et al.*, 2017). Da mesma forma, o estado de hipercoagulabilidade na DF está frequentemente associado a expressão do FT nas células vasculares, cuja expressão pode ser induzida pelo heme (SETTY *et al.*, 2008). Também já foi demonstrado correlação forte entre marcadores de hemólise e marcadores da ativação da coagulação na DF (SETTY *et al.*, 2012).

As citocinas desempenham papel fundamental na resposta imune presente na DF, com ativação de leucócitos, produção de proteínas de fase aguda, ativação endotelial e manutenção da inflamação (VILAS-BOAS *et al.*, 2012). Baseado nas diferentes fases da doença, estado-estável e crise, já foram demonstradas

concentrações sistêmicas elevadas de IL-1β, IL-6, IL-10 e TNF-α nos pacientes em crise, enquanto as concentrações de IL-12 e IL-8 permaneceram elevadas tanto na crise quanto no estado-estável (CARVALHO *et al.*, 2018). O mesmo estudo mostrou a associação entre TNF-α, IL-1β e IL-10, com poder preditivo elevado para identificar a crise na AF (CARVALHO *et al.*, 2018). Por outro lado, em avaliação de pacientes com DF em crise (priapismo, dor abdominal e musculoesquelética) foram observadas concentrações elevadas de IL-8 em comparação com o grupo fora da crise e também com indivíduos controle. O estudo sugere a IL-8 como marcador de crise vaso-oclusiva na DF (GONCALVES *et al.*, 2001). Além da produção sistêmica, muitos estudos também investigam a expressão gênica de produtos inflamatórios. Entretanto, foi observada a expressão elevada, em sangue total de pacientes com DF, dos genes *TGFβ*, *IL6* e *IL10* quando comparados a indivíduos controles (OLENSCKI GILLI *et al.*, 2016). Já a investigação de citocinas produzidas por tipos corpusculares e celulares específicos, demonstrou, por exemplo, que houve produção aumentada de mediadores inflamatórios produzidos pelas plaquetas de pacientes com DF e mostrou que a secreção de IL-1β, sCD40L e IL-6 foi maior nos pacientes com DF que em indivíduos saudáveis. Por outro lado, o mesmo estudo mostrou concentrações maiores de IL-1β, sCD40L, IL-10, IL-6 e TNF-α em pacientes com DF aloimunizados em comparação com pacientes não aloimunizados e indivíduos saudáveis (DAVILA *et al.*, 2015).

Adicionalmente aos marcadores de estresse oxidativo, produtos da hemólise, moléculas de adesão e citocinas e algumas proteínas específicas produzidas por leucócitos também desempenham papel importante na patogênese da DF. Já foi descrito que concentrações da proteína elastase neutrofílica complexada com seu inibidor, AAT, estiveram alteradas em pacientes com DF durante hospitalização por crise vaso-oclusiva. Os autores identificaram concentrações elevadas em pacientes HbSS e HbSβ^0 em comparação com os HbSC e HbS β^+. Os níveis do complexo elastase neutrofílica em associação com a AAT estavam mais elevados durante o primeiro dia de internação e reduziram após 3 dias. Da mesma forma, os níveis estiveram mais elevados em pacientes que desenvolveram STA em relação ao restante do grupo (SCHIMMEL *et al.*, 2017).

Dessa forma, pode-se observar como a associação complexa entre mecanismos celulares e moleculares contribuem diretamente para o processo inflamatório crônico presente na DF. A grande variabilidade de moléculas e, consequentemente, de células-alvo, pode dar origem a diversas propostas terapêuticas com a finalidade de reduzir não só os processos de polimerização e falcização, mas também os processos inflamatórios e oxidativos envolvidos na DF.

3 JUSTIFICATIVA

A DF é uma doença genética com prevalência elevada na população brasileira, especialmente no estado da Bahia onde a incidência estimada é de 1 indivíduo com DF a cada 650 recém-nascidos (MINISTÉRIO DA SAÚDE, 2014). Em estudos de dados secundários, durante o período de 2008 a 2014, apenas no estado da Bahia, houve registro de 8103 internações por DF, onde 34% correspondia a faixa etária de 4-15 anos de idade. O mesmo estudo estima que durante este período, foi gasto R$ 2 894 556,63 com as internações dos pacientes em todo o estado (MARTINS e TEIXEIRA, 2017). Esses dados sugerem a importância da investigação clínica e do manejo adequado dos pacientes visando reduzir o impacto nos custos da saúde pública.

As principais características da doença são a hemólise e o bloqueio do fluxo sanguíneo nos vasos, o que se reflete na variabilidade dos parâmetros hematológicos e manifestações clínicas. As diferenças entre os fenótipos e apresentações clínicas podem ser explicadas pelos genótipos da doença que resultam da herança da HbS e sua interação com outras hemoglobinas variantes ou talassemias (SERJEANT e VICHINSKY, 2018).

As complicações clínicas são variáveis entre os pacientes com DF, e compreendem desde episódios de dor aguda e úlceras de perna, até complicações mais debilitantes, tais como AVC e hipertensão pulmonar. Como o quadro clínico dos pacientes é bastante heterogêneo, o acompanhamento laboratorial é importante para identificar possíveis processos de hemólise,

leucocitose e inflamação. Os testes laboratoriais são ferramentas úteis e acessíveis ao manejo clínico que permitem distinguir marcadores relacionados às manifestações clínicas.

A inflamação na DF apresenta-se como processo estéril, mediado principalmente por produtos de hemólise, ativação leucocitária e ativação endotelial, que culminam em um ciclo repetitivo de hemólise e vaso-oclusão. Embora a hemólise e a vaso-oclusão estejam limitadas ao ambiente vascular, a resposta inflamatória observada é sistêmica, com o comprometimento de vários órgãos. Nesse contexto, a associação entre monócitos, neutrófilos, plaquetas, citocinas, mediadores lipídicos, ferro, heme livre e Hb descompartimentalizada cria uma rede de mecanismos responsáveis pelo processo inflamatório e, da mesma forma, pelas manifestações clínicas que os pacientes apresentam ao longo da vida (ZHANG *et al.*, 2016; WARE *et al.*, 2017; WILLIAMS e THEIN, 2018).

A principal abordagem terapêutica para a DF é o uso da HU, que tem sido capaz de melhorar os aspectos inflamatórios da doença e, sobretudo, de aumentar as concentrações de HbF e reduzir as complicações clínicas presentes na doença. Portanto, investigar marcadores laboratoriais, manifestações clínicas e suas associações com processos inflamatórios presentes na DF, bem como o efeito da HU em leucócitos, contribui para o conhecimento da fisiopatologia da doença, auxilia as decisões do manejo clínico dos pacientes e pode servir para a identificação de potenciais alvos terapêuticos.

4 HIPÓTESE

Alterações nos parâmetros hematológicos, bioquímicos e celulares estão associadas aos processos de hemólise, dislipidemia e inflamação sistêmica, bem como à ocorrência de manifestações clínicas, as respostas secundárias a terapia pela hidroxiuréia e aos mecanismos fisiopatológicos inflamatórios apresentados pelos indivíduos com doença falciforme.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Investigar e identificar marcadores inflamatórios e laboratoriais associados a ocorrência de manifestações clínicas na doença falciforme, nos seus diferentes genótipos, bem como a resposta celular ao uso de hidroxiuréia.

5.2 OBJETIVOS ESPECÍFICOS

5.2.1 Investigar marcadores hematológicos, bioquímicos e inflamatórios bem como manifestações clínicas associadas aos diferentes genótipos da DF;

5.2.2 Avaliar, em indivíduos com AF, o perfil de marcadores lipídicos (colesterol total, LDL-C e HDL-C), suas associações com manifestações clínicas e outros marcadores laboratoriais utilizados na clínica;

5.2.3 Avaliar, em indivíduos com AF, a associação entre o polimorfismo rs4073 no gene *CXCL8* e os níveis dessa citocina, bem como suas associações com as manifestações clínicas e outros marcadores laboratoriais utilizados na clínica;

5.2.4 Avaliar, em indivíduos com AF, o perfil de monócitos presentes no sangue periférico, as citocinas produzidas por estas células e a influência do tratamento com hidroxiuréia.

6 MANUSCRITOS

6.1 MANUSCRITO 1

Título: Sickle cell disease: a distinction of the two most prevalent genotypes (HbSS and HbSC)

Autores: Caroline Conceição da Guarda, Sétondji Cocou Modeste Alexandre Yahouédéhou, Rayra Pereira Santiago, Joelma Santana dos Santos Neres, Camila Felix de Lima Fernandes, Milena Magalhães Aleluia, Camylla Vilas Boas Figueiredo, Luciana Magalhães Fiuza, Suellen Pinheiro Carvalho, Rodrigo Mota de Oliveira, Cleverson Alves Fonseca, Uche Samuel Ndidi, Valma Maria Lopes Nascimento, Larissa Carneiro Rocha e Marilda Souza Goncalves

Situação: Submetido ao periódico *Plos One*

Objetivos: Investigar marcadores hematológicos, bioquímicos e inflamatórios bem como manifestações clínicas associadas a doença falciforme.

Principais resultados: pacientes com AF tiveram hemólise, leucocitose e inflamação mais proeminentes, enquanto pacientes com HbSC tiveram concentrações elevadas de marcadores lipídicos. A maior causa de hospitalização entre os pacientes foi a crise de dor. Entre as manifestações clínicas, a crise de dor esteve associada a alterações hematológicas em ambos genótipos de DF investigados. A vaso-oclusão esteve associada a anemia na HbSC e a marcadores inflamatórios na AF. O agrupamento dos marcadores laboratoriais em *clusters* demonstrou a presença de grupos de marcadores relacionados a alterações hematológicas, hemólise, disfunção endotelial, inflamação e metabolismo dos lipídios em ambos genótipos.

PLOS ONE

Sickle cell disease: a distinction of two most frequent genotypes (HbSS and HbSC)

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Article Type:	Research Article
Full Title:	Sickle cell disease: a distinction of two most frequent genotypes (HbSS and HbSC)
Short Title:	Sickle cell disease: characterization of HbSS and HbSC genotypes
Corresponding Author:	Marilda Souza Goncalves, Ph.D. FIOCRUZ/UFBA Salvador, BRAZIL
Keywords:	
Abstract:	Sickle cell disease (SCD) consists of a group of hemoglobinopathies in which individuals present highly variable clinical manifestations. Sickle cell anemia (SCA) is the most severe form, while SC hemoglobinopathy (HbSC) is thought to be milder. Thus, we investigated the clinical manifestations and laboratory parameters by comparing each SCD genotype. We designed a cross-sectional study including 126 SCA individuals and 55 HbSC individuals in steady-state. Hematological, biochemical and inflammatory characterization was performed as well as investigation of previous history of clinical events. SCA patients exhibited most prominent anemia, hemolysis, leukocytosis and inflammation, whereas HbSC patients had increased lipid determinations. The main cause of hospitalization was pain crises on both genotype. Vaso-occlusive events and pain crises were associated with hematological, inflammatory and anemia biomarkers on both groups. Cluster analysis reveals hematological, inflammatory, hemolytic, endothelial dysfunction and anemia biomarkers in HbSC disease as well as SCA. The results found herein corroborate with previous studies suggesting that SCA and HbSC, although may be similar from the genetic point of view, exhibit different clinical manifestations and laboratory alterations which are useful to monitor the clinical course of each genotype.
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Opposed Reviewers:	
Additional Information:	
Question	**Response**

Title: Sickle cell disease: a distinction of two most frequent genotypes (HbSS and HbSC)

Short title: Sickle cell disease: characterization of HbSS and HbSC genotypes

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ABSTRACT

Sickle cell disease (SCD) consists of a group of hemoglobinopathies in which individuals present highly variable clinical manifestations. Sickle cell anemia (SCA) is the most severe form, while SC hemoglobinopathy (HbSC) is thought to be milder. Thus, we investigated the clinical manifestations and laboratory parameters by comparing each SCD genotype. We designed a cross-sectional study including 126 SCA individuals and 55 HbSC individuals in steady-state. Hematological, biochemical and inflammatory characterization was performed as well as investigation of previous history of clinical events. SCA patients exhibited most prominent anemia, hemolysis, leukocytosis and inflammation, whereas HbSC patients had increased lipid determinations. The main cause of hospitalization was pain crises on both genotype. Vaso-occlusive events and pain crises were associated with hematological, inflammatory and anemia biomarkers on both groups. Cluster analysis reveals hematological, inflammatory, hemolytic, endothelial dysfunction and anemia biomarkers in HbSC disease as well as SCA. The results found herein corroborate with previous studies suggesting that SCA and HbSC, although may be similar from the genetic point of view, exhibit different clinical manifestations and laboratory alterations which are useful to monitor the clinical course of each genotype.

Key-words: Sickle cell anemia, SC hemoglobinopathy, hematological, biochemical, inflammation, and clinical manifestations.

INTRODUCTION

Sickle cell disease (SCD) consists of a group of hemoglobinopathies in which individuals inherit hemoglobin variants derived from single point mutations, that causes morphological abnormalities in the red blood cells (RBC) (1). Sickle cell anemia (SCA) is characterized by the homozygosity for hemoglobin S (HbS) and is the most frequent and severe form of the disease. The point mutation of GAG to GTG in the sixth codon of the β (beta) globin gene (*HBB*), which replaces the glutamic acid for a valine, leading to HbS formation (2). HbS forms long polymers when the oxygen tension is low, due to the hydrophobic interaction of valine (at 85 position in the globin chain) and phenylalanine (at 88 position in the globin chain) (3). RBC of SCA individuals are less flexible since the polymers lead to rheological and biochemical changes and hence they impair the blood flow causing vaso-occlusion (VO) (1).

In addition to SCA, hemoglobin SC disease (HbSC) is another genotype of SCD. In this case, individuals inherit HbS in association with hemoglobin C (HbC). The molecular basis of HbSC disease is similar to SCA; however, the point mutation is GAG to AAG, which replaces the glutamic acid for lysine, in the globin chain (2). The HbC tends to form amorphous aggregate within the RBC which also leads to morphological modifications (4). In addition, K–Cl cotransporter is also altered in HbSC disease contribute to RBC dehydration, which increases the intracellular hemoglobin concentration, and makes it more dense than HbAA-containing RBC (4).

SCD patients exhibit a wide range of clinical manifestations including acute episodes of pain, pulmonary hypertension (PH), stroke, priapism, leg ulcer, acute chest syndrome (ACS), osteonecrosis and cholelithiasis (1-3). It is thought that PH, leg ulcer and stroke are associated to the chronic hemolytic feature of SCD, while acute pain crises,

osteonecrosis and ACS are associated to VO, which could drive to different subphenotypes (5). However, this dichotomization is not restricted, very often they overlap and may not be useful for SCA and HbSC individually (5-7). Moreover, recently it has been suggested that abnormal lipid homeostasis would be surrogate subphenotype, considering the association with both hemolysis and VO (6). SCA patients usually present clinical events more frequently when compared to HbSC disease, which is considered a milder form of SCD (1, 4, 8). Alternatively, retinopathy is more frequently associated to HbSC disease (9).

In addition to different clinical manifestations, laboratory parameters are also important biomarkers useful for the patients' follow-up due to the possibility to monitor anemia, hemolysis, leukocytosis, endothelial dysfunction and to predict many clinical manifestations (10). In SCA, RBC count and Hb levels are commonly decreased while complete white blood cells (WBC) counts lactate dehydrogenase (LDH) and reticulocyte counts are increased. Regarding HbSC, RBC counts and Hb levels are usually increased whereas mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and red blood cell distribution width (RDW) are decreased (1, 4, 8, 11). Laboratory determinations seem to translate the pathophysiological mechanism underlying SCD. Once the HbS alone or in association with HbC forms the polymer the RBC membrane is also altered (4, 5). Irreversibly sickle RBC are more adherent and can bind to vascular endothelial cells as well as to leukocytes and platelets (12). This heterogeneous multicellular aggregate leads to physical obstruction of the capillaries driving VO, which is a hallmark of SCD (12). VO is even heightened due to persistent intravascular hemolysis releasing free heme, hemoglobin (Hb) and arginase which decrease nitric oxide (NO) bioavailability and is directly responsible for endothelial dysfunction (13).

Hemoglobin variants have a high frequency worldwide (14), likewise, SCD is also distributed in several different countries, especially in Africa (15). Brazilian population bears a heterogeneous genetic background with great admixture, thus SCD prevalence is also diversified through the states, and the incidence of SCD is approximately 1 in 650 newborn babies screened in the state of Bahia (16). Considering the elevated frequency of hemoglobin variants and prevalence of SCD in our population (17, 18), and the peculiarities of SCA and HbSC disease we aimed to investigate the association of classical laboratory parameters and clinical manifestations in each of these SCD genotype.

METHODS

Study design and casuistic

A cross-sectional study was performed in 181 pediatric SCD children residing in the state of Bahia, Brazil, who were seen at Bahia Hemotherapy and Hematology Foundation (HEMOBA), from October 2016 to September 2017. One hundred and twenty-six patients with SCA aged 14.5 ± 3.5 years of whom 60 (47.6%) were female were enrolled in the study, while 55 with HbSC disease aged 14.1 ± 2.8 years of whom 29 (47.2) were female were also included. All patients were in steady state.

Regarding therapy approaches 62 SCA and 9 HbSC individuals were taking hydroxyurea (HU), moreover, all patients were taking folic acid supplementation. This study received approval from the Institutional Research Board (protocol number: 1400535) and was conducted in compliance with the ethical principles established by the revised Declaration of Helsinki. Informed written consent was obtained from each SCD patient's guardian. When applicable, the children's acceptance was also registered.

Clinical data

Data regarding the occurrence and frequency of previous clinical manifestations were collected using a standardized and confidential questionnaire (self-reported or reported by the parents) at the time of the study enrollment and confirmed by the medical records.

Laboratory determinations

Hematological parameters were assessed using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) and blood smears were stained with Wright's stain and examined by light optical microscopy. Reticulocytes were counted after staining supravitally with brilliant cresyl blue dye. Hemoglobin patterns were confirmed by high-performance liquid chromatography employing an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA).

Biochemical determinations, including lipid profile, total bilirubin and fractions, LDH, iron, hepatic metabolism and renal profile were determined in serum samples using an automated A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain). Ferritin levels were determined using Access 2 Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). C-reactive protein (CRP) and alpha-1 antitrypsin (AAT) levels were measured using IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). Determination of NO metabolites (NOm) in serum samples was carried out with the Griess reagent as previously described (19). Laboratory parameters were analyzed at the Clinical Analyses Laboratory of the College of Pharmaceutical Sciences (LACTFAR, Universidade Federal da Bahia).

169

170 *Statistical Analysis*

171 Statistical analyses were performed using the Statistical Package for the Social Sciences
172 (SPSS) version 20.0 software (IBM, Armonk, New York, USA) and GraphPad Prism
173 version 6.0 (Graphpad Software, San Diego, California, USA), which was also used to
174 assemble the graphs. Baseline values of selected variables are expressed as means with
175 their respective standard variation. We tested each variable distribution employing the
176 Shapiro-Wilk test. The Mann-Whitney *U* test and independent t-test were used to
177 compare the groups according to the normality of the distribution for each variable.
178 Parametric ANOVA was used to analyze the means of quantitative or numerical
179 variables with normal distributions, while the nonparametric Kruskal–Wallis test was
180 used for data with non-normal distribution. Fisher's exact test was used to compare
181 categorical variables. Hierarchical clustering of the laboratory parameters was
182 performed using the Ward method and the square Euclidean distance was measured
183 between the variables. All parameters were standardized by the Z score. P values <0.05
184 were considered statistically significant.

185

186 RESULTS

187

188 *Hematological and biochemical parameters are different in SCA and HbSC disease*

189 In order to first distinguish SCA and HbSC individuals we compared laboratory
190 parameters of both groups. We observed that SCA patients had most prominent anemia,
191 hemolysis and increased leukocyte counts. Moreover, SCA patients also presented
192 increased systemic inflammatory mediators. However, HbSC patients exhibited

193 increased lipid profile as well as renal biomarkers, while NOm levels were decreased
194 (Supplementary Table 1).
195
196 *Severe clinical manifestations are more frequent in SCA*
197 We investigated the frequency of clinical manifestations in each group. SCA patients
198 had most cases of hospital admissions, pneumonia, splenomegaly, stroke, painful crises
199 (PC), vaso-occlusive events (VO), infections, leg ulcer, acute chest syndrome (ACS),
200 bone alterations and cholelithiasis (Table 1). The main cause of hospital admission in
201 both groups was acute pain crises (Table 1); although some patients underwent hospital
202 admission for more than one cause. Comparing SCA and HbSC disease, we found
203 statistical significance for PC, VO and cholelithiasis. Considering the
204 physiopathological relevance of both PC and VO for the pathogenesis of SCD we
205 decided to further investigate which laboratory parameters would be associated by
206 comparing the groups who had the clinical manifestation with those who had not.
207

Table 1. Frequency of clinical events in SCA and hemoglobin SC disease patients.

Clinical manifestation	**SCA (N = 126)**	**HbSC (N = 55)**	***P* value**
Hospital admissions	118	40	-
*Causes of hospital admissions**			
Acute pain crises	93	29	
Pneumonia/ACS	36	10	
Infections	32	13	
Surgery	5	-	
Neurology	4	1	
Cardiology	1	-	
Angiology	1	-	
Nephrology	1	-	
Other clinical manifestation	17	12	
Infections	86	31	0.128
Painful crises	78	46	**0.005**
Pneumonia	69	24	0.195

Splenomegaly	59	26	1.00
Vaso-occlusive events	46	9	**0.008**
Cholelithiasis	39	7	**0.014**
Acute chest syndrome	33	8	0.086
Stroke	13	2	0.155
Leg ulcer	12	7	0.600
Bone alterations	10	4	1.000

Bold values indicate significance at $p<0.05$. P-value obtained with Fisher's exact test.
*Of note: some patients underwent hospital admission due to multiple clinical complications.

Hematological parameters are associated with clinical manifestations in HbSC disease

Although HbSC individuals presented less complicated anemia and hemolysis, hematological parameters were associated to clinical manifestations. Patients with HbSC and previous history of PC had decreased mean corpuscular hemoglobin concentration (MCHC) (Fig 1.A). HbSC patients with previous history of VO exhibited decreased RBC counts (Fig 1.B), as well as Hb (Fig 1.C) and Ht (Fig 1.D) levels.

Considering the association between hematological parameters and clinical manifestations in HbSC disease we also performed a multivariate linear regression model with pain crises as dependent variable. Our model has shown that MCHC, Hb and Ht were independently associated with pain crises in HbSC disease (Table 2).

Fig 1. Hematological laboratory parameters are associated to clinical manifestations in HbSC disease. A) Patients with previous history of painful crises (PC) had decreased MCHC; B) Patients with previous history of vaso-occlusion (VO) had decreased red blood cell counts; C) hemoglobin and D) hematocrit levels. p-value obtained using Mann-Whitney *U* test.

Table 2. Multivariate linear regression model of history of pain crises in association with confounding variables in hemoglobin SC disease and SCA patients.

Independent variables	Dependent variable	β	p-value	R^2	p-value of the model
		HbSC			
RBC, 10^6/mL	Pain crises	-0.201	0.343	**0.223**	**.015**
MCHC, %		-1.274	**0.003**		
Hemoglobin, g/dL		4.284	**0.024**		
Hematocrit, %		-4.066	**0.029**		
		SCA			
RBC, 10^6/mL	Pain crises	0.064	0.507	**0.125**	**.012**
Reticulocytes, /mL		0.171	0.073		
CRP, mg/L		0.106	0.249		
AAT, mg/dL		0.120	0.194		
NOm, µM		-0.190	**0.046**		

R^2: coefficient of determination; β: coefficient of regression.

Hematological and inflammatory determinations are associated to clinical manifestations in SCA

SCA patients exhibit the most severe form of SCD. PC was associated to increased RBC (Fig 2.A) and reticulocyte (Fig 2.B) counts; in addition to decreased NOm levels (Fig 2.C). VO also seems to be associated to a chronic inflammatory response since patients with previous history of VO had increased C-RP (Fig 2.D) and AAT (Fig 2.E) levels.

Considering the association between hematological and inflammatory parameters and clinical manifestations in SCA we also performed a multivariate linear regression model with pain crises as dependent variable. Our model has shown that NOm was independently associated with pain crises in SCA (Table 2).

Fig 2. Hematological and inflammatory laboratory parameters are associated to clinical manifestations in SCA. A) Patients with previous history of painful crises (PC) had increased red blood cells and B) increased reticulocyte counts, and C) decreased nitric oxide metabolites (NOm). D) Patients with previous history of vaso-occlusion (VO) had increased C-reactive protein and E) Alpha-1 antitrypsin levels. p-value obtained using Mann-Whitney *U* test.

Cluster analysis reveals different groups of laboratory parameters in SCA and HbSC disease

We also tested which laboratory parameters would be clustered in each genotype. In HbSC disease cluster analysis reveals that in the distance 25 two large groups were formed. In the upper part of the cluster, in the distance 17 two other groups were formed. The upper, in the distance 7, included PDW, MPV, NOm, triglycerides and VLDL-C while the lower, in the distance 15, included AST, ALT, GGT, ferritin, LDH, MCV, MCH, total bilirubin, indirect bilirubin, MCHC and HbF. In the distance 20 two other groups were formed, the first, in the distance 16, included Hb, Ht, RBC, iron, uric acid, creatinine, AAT, total cholesterol, LDL-C, HDL-C, direct bilirubin, basophils and urea; while in the distance 17 a group was formed consisted of reticulocytes, RDW, CRP, eosinophils, alkaline phosphatase, leukocytes, neutrophils, monocytes, platelets, PCT and lymphocytes (Fig 3).

Regarding SCA, cluster analysis reveals that in the distance 25 two large groups were formed. In the distance 19 two other groups were formed, the upper in the distance 13 included total bilirubin, indirect bilirubin, AST, ALT, MCHC, RDW, lymphocytes, direct bilirubin, VLDL-C, triglycerides, NOm and LDH. The other group in the distance 14 included platelets, PCT, reticulocytes, leukocytes, neutrophils, monocytes, eosinophils, basophils, MPV, total cholesterol, LDL-C, urea, CRP, PDW, alkaline

phosphatase, GGT, AAT, HDL-C and ferritin. The lowest cluster in the distance 14 was consisted of MCV, MCH, iron, creatinine, uric acid, Hb, Ht, RBC and HbF (Fig 4).

Fig 3. Cluster analysis of laboratory biomarkers among HbSC disease. Dendogram demonstrating cluster agglomeration of laboratory parameters in the group of patients with HbSC disease. The interval was measured by the square Euclidean distance and measurements were standardized by the Z score.

Fig 4. Cluster analysis of laboratory biomarkers among SCA patients. Dendogram demonstrating cluster agglomeration of laboratory parameters in the group of patients with SCA. The interval was measured by the square Euclidean distance and measurements were standardized by the Z score.

DISCUSSION

Although the molecular basis of each SCD genotype is clear, the mechanisms contributing to clinical manifestations and to the maintenance of inflammation are not fully understood. This study was conducted to perform a wide characterization of SCD assessing the two most frequent genotypes.

Baseline laboratory characteristics of SCA patients are consistent with previous evaluation, revealing anemia, hemolysis, leukocytosis and the increase of systemic inflammation (8, 20, 21). Importantly, total leukocyte counts above 15,000 cells/mL^3 as well as low HbF levels were associated with an increased risk of early death (20). Likewise, intravascular hemolysis is also associated to the severity of clinical outcomes (22). Acute phase proteins, such C-RP and AAT, are produced especially by the liver during infections or inflammatory conditions (23). C-RP and AAT levels were shown to be elevated among SCD patients even during steady-state (24). Altogether, our data reinforce the notion that SCA is the most severe SCD genotype. Laboratory

investigation of HbSC individuals revealed increased lipid, creatinine and uric acid levels as well as decreased NOm. Our findings are in agreement with previous laboratory profile of HbSC disease (25), including increased creatinine levels (26) and increased total cholesterol, HDL-C and LDL-C as well as decreased NOm determinations (8). This lipid profile among HbSC individuals has also been show in other populations (27).

Clinical events in SCD are driven by the pathophysiological mechanism of VO. Indeed, all the clinical manifestations investigated were more prevalent in the SCA group than in HbSC disease. This is in agreement with previous clinical and laboratory characterization of SCA and HbSC disease patients (8), which corroborate that SCA is more severe. An evaluation of a cohort of ten years has also found that the onset of the complications was earlier in SCA compared to HbSC patients, especially for painful crises and acute chest syndrome (28). Acute pain crises are the most common cause of hospitalization among SCD patients. In our population we have found that the most frequent cause of hospital admission was acute pain crises in SCA and HbSC disease, which was also observed in different populations where the major cause of hospital admission was acute painful episodes accounting for 94.6% of the admissions (29). A survey carried out in England has identified that primary diagnoses for admission was sickle cell crises, followed by acute lower respiratory tract infection and asthma (30). In addition, cholelithiasis is a frequent complication in SCD patients due to the ongoing hemolysis which results in the production of large amounts of bilirubin, which is conjugated in the liver and its accumulation, may form calcium bilirubinate gallstones (31). Collectively, these findings suggest that regardless of the SCD genotype, pain crises are the most important clinical event patients have experienced.

PC and VO were statistically different when SCA was compared to HbSC disease, which lead us to examine laboratory parameters in each group. Hematological and inflammatory parameters were shown to be associated with PC and VO in both HbSC disease and SCA.

Reticulocyte and RBC counts as well as MCHC levels were associated with pain crises in our SCA and HbSC patients which allow us to suggest that hemolysis and anemia are thought to contribute to clinical outcome in SCD. Reticulocytosis has been associated to increase in hospitalization during the first three years of life of children with SCA (32). Moreover, an extensive hemolysis evaluation has shown that absolute reticulocyte counts and reticulocyte percentage had a strong inverse correlation with mean RBC survival (33). Correspondingly, HbF levels were shown to be decreased in children with SCA with absolute reticulocyte counts greater than 200 000 cells/mL (34). Altogether, these findings suggest that hemolysis may be measured through routine hematological evaluation, such as reticulocyte counts, which is important to monitor the patient outcome.

Several pain mediators have been described in SCD such as interleukin-1, bradykinin, histamine, substance P and calcitonin gene related peptide (35). Pain crises in SCD may be acute, chronic or a combination of both and is usually secondary to vaso-occlusion (35). Hemolysis leads to endothelial dysfunction since it causes the release of Hb and heme which limits NO bioavailability as well as arginase, which consumes L-arginine, decreasing NO levels even more and contributing to VO (13). Thus, the association of both pathophysiological mechanisms to the triad of factors (VO, inflammation and nociception) may help to initiate the acute painful crises (36). Abnormal lipid homeostasis has also been associated with decreased NOm levels (6).

Chronic inflammatory response is a hallmark of SCD influenced by leukocytes, platelets (37), intravascular hemolysis and innate immune response (13) and increased pro-inflammatory mediators (38). Our cohort of patients with previous history of VO exhibited laboratory parameters associated to anemia and systemic inflammation. Increased AAT levels were found to be associated to infections, gallstones and blood therapy in SCD (39); moreover, C-RP levels were progressively increasing as SCA severity score was higher (40). Our findings are in agreement with the pathophysiological mechanism of VO due to i) heightened ability of sickle RBC to adhere to the vascular endothelium and promote activation of endothelial cells and leukocytes and ii) sickle RBC have the lifespan shortened which also contributes to anemia (41).

Cluster grouping is a very useful approach to identify biomarkers of SCD severity (42). We designed a cluster analysis in order to group the laboratory parameters of each genotype. Cluster analysis among HbSC disease patients has shown 4 different cluster agglomerations with participation of hemolytic and endothelial dysfunction parameters in the two first, as well as hematological and inflammatory parameters in the latter two. Contrarily, cluster analysis among SCA patients has shown 3 different cluster agglomerations with participation of hemolytic parameters in the first cluster, leukocytes, lipid metabolism and inflammatory parameters in the second cluster and markers of iron metabolism and anemia in the last cluster.

HbSC patients are known to exhibit a phenotype with increased viscosity (4, 9, 26) which may be corroborated by our findings of clustering hemolysis and endothelial dysfunction markers in the similar groups in these genotype (8, 10, 25). As they also present less severe anemia, clustering of hematological and inflammatory markers in similar groups is in agreement with the literature (8, 10, 25). SCA patients present the

most severe phenotype of SCD and our cluster analysis demonstrate tree groups: hemolysis, inflammation and anemia. These markers are suggestive of the main underlying pathophysiological mechanisms of the disease that often overlap (1). In the first cluster the association of NOm and hemolytic markers reinforces the role of endothelial dysfunction (13, 43), while in the second the association of leukocytes counts and CRP and AAT highlights the role of inflammation (39, 40) and in the last cluster, grouping of RBC counts along with Hb, Ht and iron levels suggest the importance of anemia (20, 21). Curiously, HbF was differentially clustered in each genotype. In HbSC patients HbF was clustered along with biomarkers of hemolysis (LDH, AST, indirect bilirubin), while in SCA it was clustered along with biomarkers of anemia (RBC counts, Hb, Ht, MCV and MCH levels). HbF levels are one of the most important biomarker for disease prognostic in SCD (10, 34, 44), altogether our results suggests that different mechanisms may be associated with HbF in the different SCD genotypes. The different classification of the same laboratory parameters on HbSC disease and SCA suggests that, indeed, the same measurement obtained with one genotype may have a different relevance when compared with the other genotype of the same disease.

Our data suggest that SCA patients exhibit increased hemolysis and inflammatory parameters as well as more clinical complications. In addition, HbSC patients' exhibit altered lipid metabolism and milder hemolysis. Moreover, laboratory parameters are also important to monitor the disease. Of note, it is important to point that our cohort is composed by pediatric patients and the clinical course is usually more complicated in the greater ages. Nevertheless, our findings support the differences between SCA and HbSC disease that should be taken into account when considered clinical management.

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AUTHOR CONTRIBUTIONS

CCG designed and performed all the experiments and wrote the manuscript. SCMAY, RPS, JSSN, CFLF, MMA, CVBF, LMF, USN, performed the experiments, the hematological and biochemical characterization and helped with the sample collection. SPC, RPS and RMO performed the interviews, collaborated in the study design and reviewed the manuscript. VMLN and LCR assisted the patients and helped with the sample collection. MSG conceptualized and supervised the study as well as co-wrote and critically revised the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article

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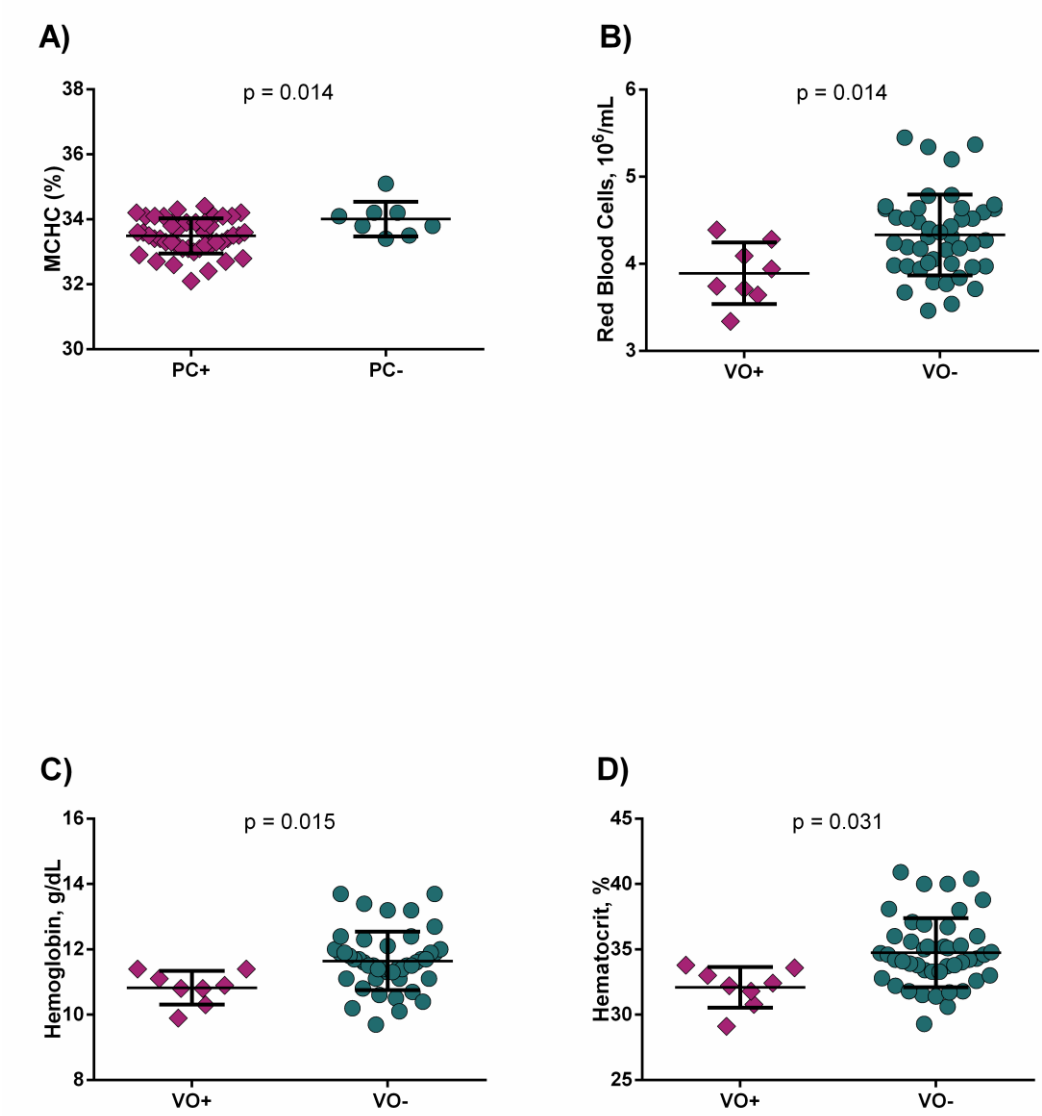
567 **Figures**



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569 Fig 1

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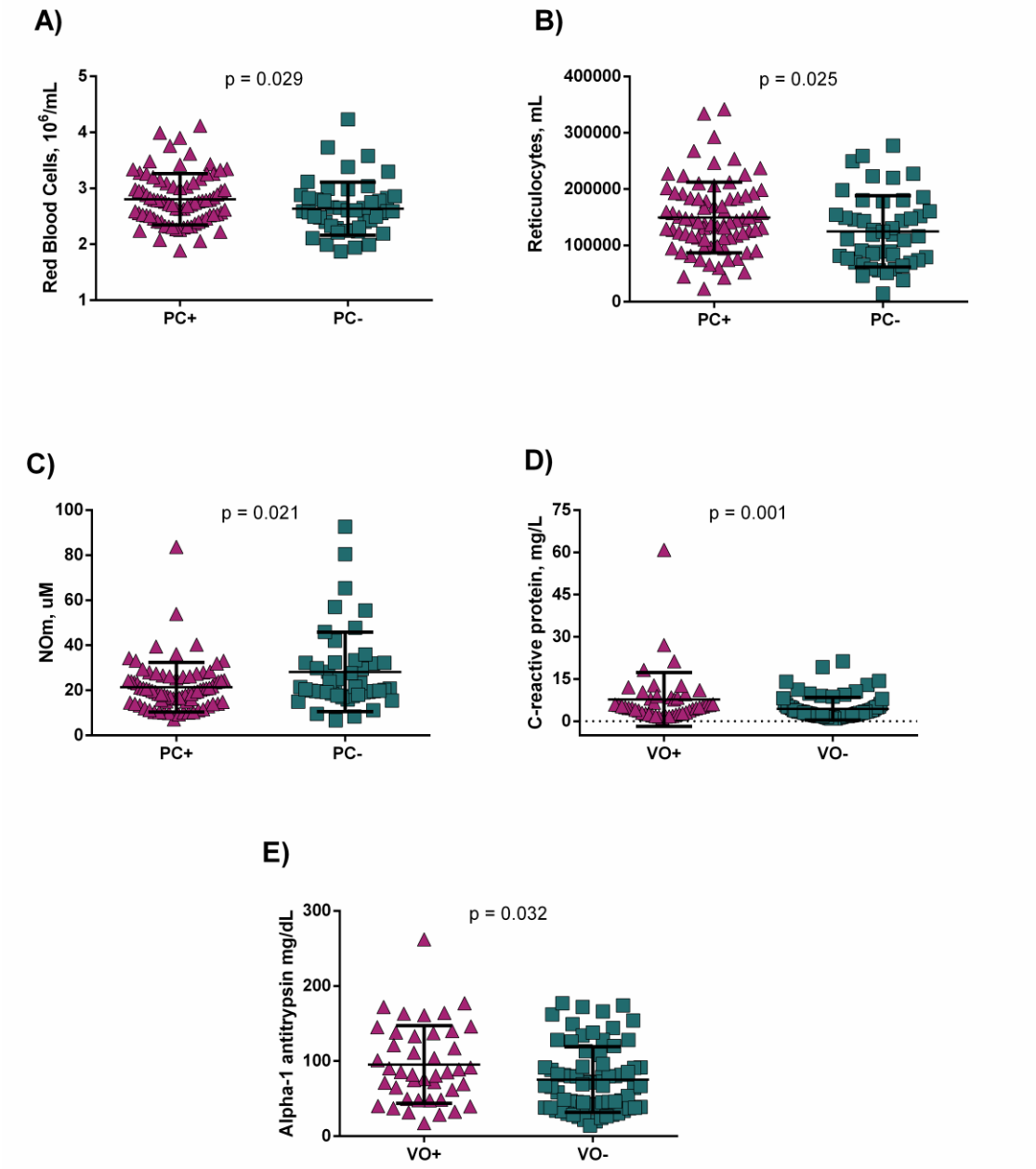

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572 Fig 2

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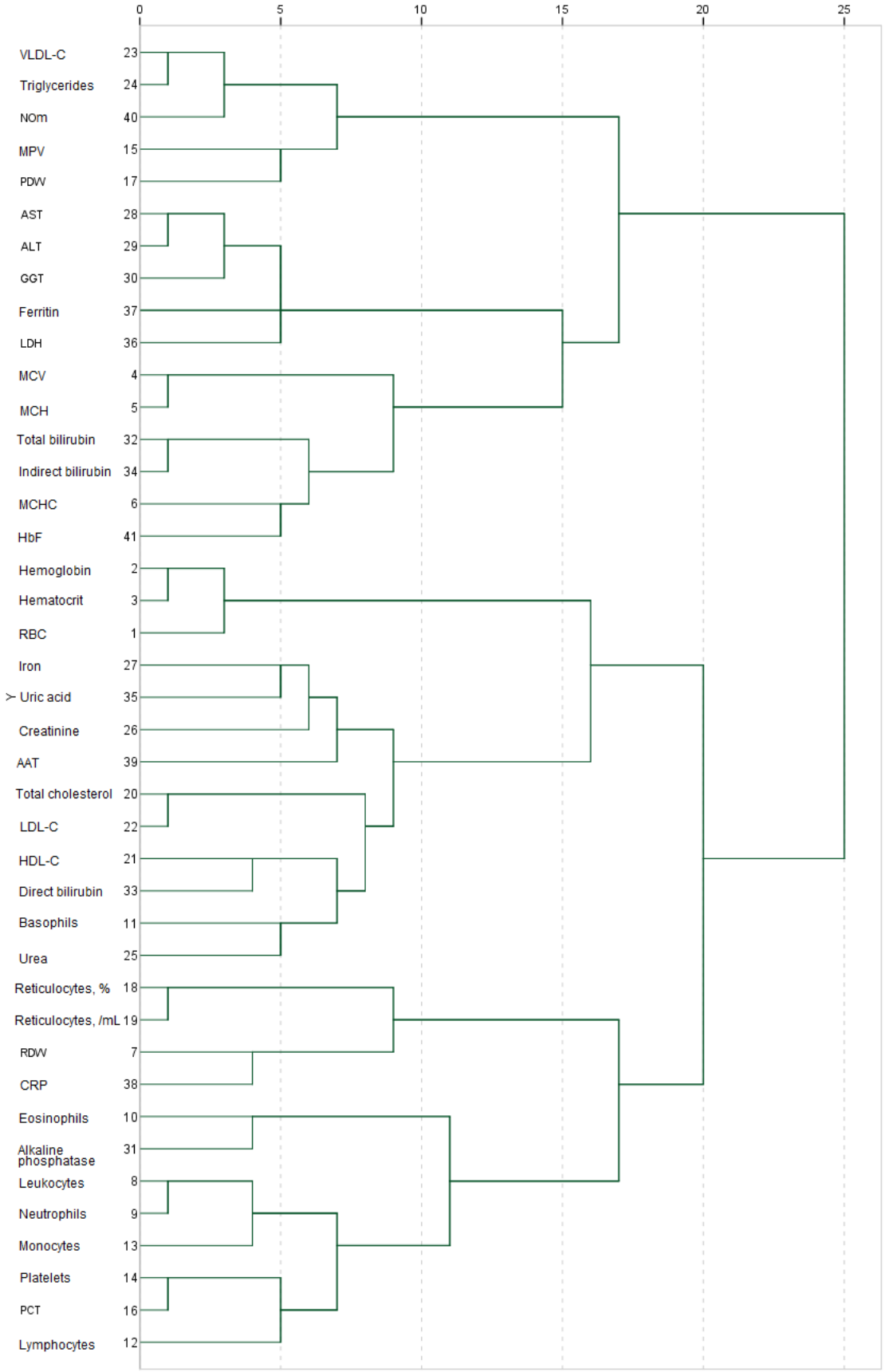

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575 Fig 3

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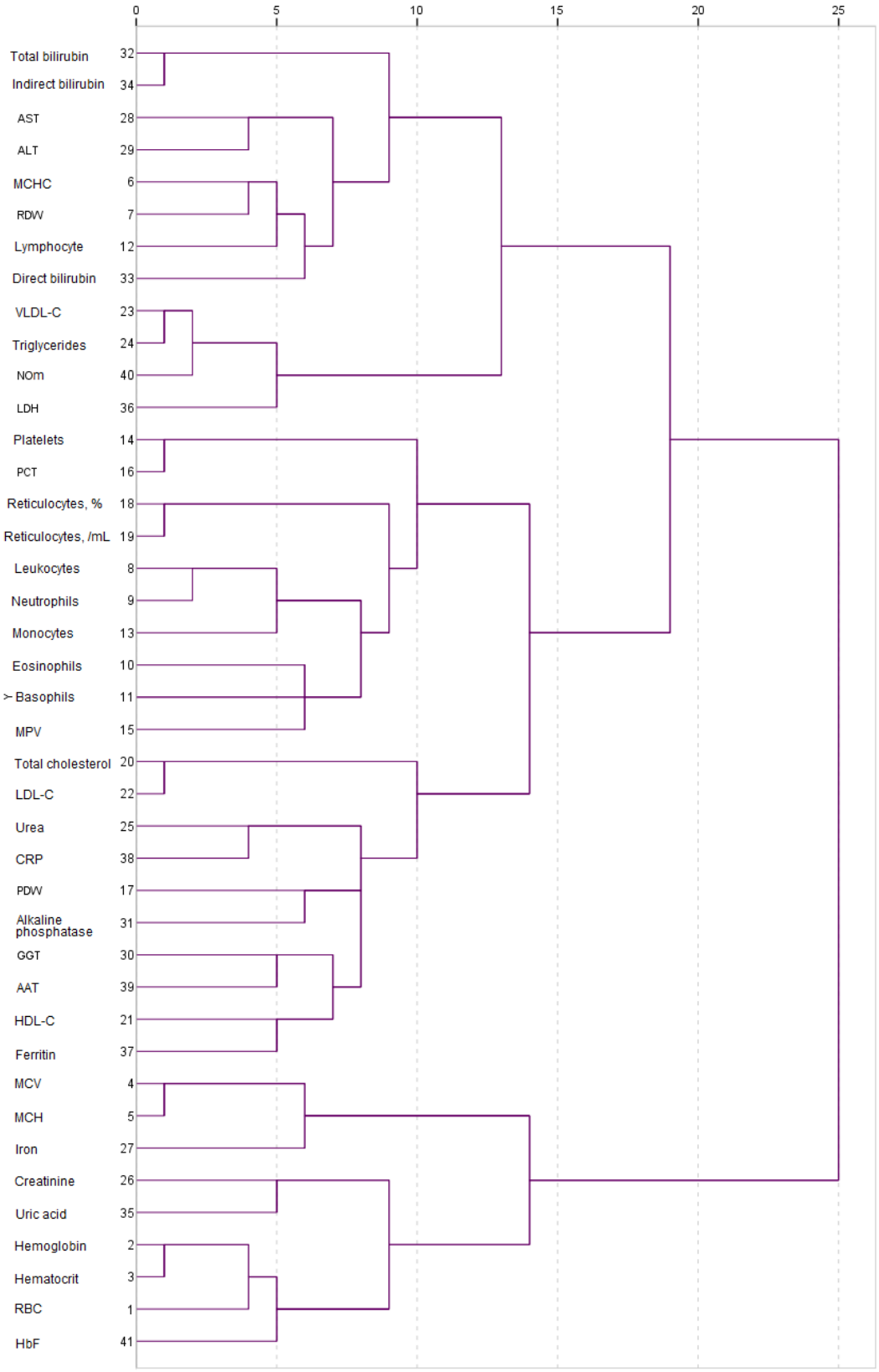

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578 Fig 4

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581 **Supplementary Information**

582

583 Sickle cell disease: a distinction of two most frequent genotypes (HbSS and HbSC)

584

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587 Fernandes[1], Milena Magalhães Aleluia[2], Camylla Vilas Boas Figueiredo[1], Luciana
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Supplementary Table 1. Laboratory characterization of SCA and hemoglobin SC disease patients.

Laboratory parameters	**SCA (N = 126)**	**HbSC (N = 55)**	***P* value**
	Mean ± SD	Mean ± SD	
Sex, % of females	60 (47.6)	29 (47.2)	-
Age, years	14.5 ± 3.5	14.1 ± 2.8	-
Hemolysis markers			
RBC, 10^6/mL	2.74 ± 0.46	4.26 ± 0.47	**0.000**
Hemoglobin, g/dL	8.47 ± 1.03	11.53 ± 0.89	**0.000**
Hematocrit, %	25.15 ± 3.38	33.09 ± 6.99	**0.000***
MCV, fL	92.42 ± 11.63	80.94 ± 5.76	**0.000**
MCH, ρg	31.33 ± 3.97	27.18 ± 2.08	**0.000**
MCHC, g/dL	33.92 ± 1.02	33.56 ± 0.56	**0.004***
Reticulocyte count, %	5.16 ± 2.31	3.34 ± 1.28	**0.000**
Reticulocyte counts, /mL	139781 ± 63905	140882 ± 51713	0.636
RDW, %	22.67 ± 3.77	17.19 ± 2.38	**0.000**
Total bilirubin, mg/dL	3.00 ± 1.67	1.31 ± 0.74	**0.000**
Direct bilirubin, mg/dL	0.41 ± 0.16	0.28 ± 0.11	**0.000**
Indirect bilirubin, mg/dL	2.62 ± 1.63	1.09 ± 0.16	**0.000**
LDH, U/L	1250.72 ± 1292.86	599.33 ± 147.34	**0.000**
Hb pattern			
HbS, %	83.44 ± 10.29	51.53 ± 4.22	-
HbC, %	-	43.37 ± 3.11	-
HbF, %	9.05 ± 5.68	1.87 ± 2.20	**0.000**
Leukocytes			
WBC /mL	11473 ± 3445	9064 ± 3238	**0.000**
Neutrophils /mL	5585 ± 2638	5083 ± 2585	0.124
Monocytes /mL	1098 ± 582	726 ± 350	**0.000**
Eosinophils /mL	492 ± 488	405 ± 324	0.338

Basophils /mL	93 ± 108	49 ± 75	**0.005**
Lymphocytes /mL	4130 ± 1329	2798 ± 1014	**0.000**
Platelets			
Platelet count, $x10^3$/mL	422 ± 137	291 ± 102	**0.000**
MPV, fL	7.93 ± 0.86	7.98 ± 1.84	0.840*****
PCT, %	0.32 ± 0.10	0.22 ± 0.07	**0.000**
PDW, %	16.29 ± 0.64	17.08 ± 0.81	**0.000**
Lipid metabolism			
Total Cholesterol, mg/dL	120.92 ± 24.74	135.00 ± 29.53	**0.002**
HDL-C, mg/dL	35.81 ± 8.72	40.74 ± 11.34	**0.008**
LDL-C, mg/dL	62.10 ± 21.95	72.17 ± 27.64	**0.019**
VLDL-C, mg/dL	22.51 ± 11.26	20.50 ± 6.46	0.984
Triglycerides, mg/dL	109.45 ± 50.48	102.54 ± 32.32	0.905
Iron metabolism			
Iron, mcg/dL	111.89 ± 55.03	91.00 ± 32.46	**0.030**
Ferritin, ηg/mL	259.70 ± 437.89	98.83 ± 100.96	0.287
Renal profile			
Urea, mg/dL	17.54 ± 6.54	18.10 ± 5.76	0.130
Creatinine, mg/dL	0.43 ± 0.14	0.62 ± 0.14	**0.000**
Uric Acid, mg/dL	3.81 ± 1.20	4.23 ± 1.08	**0.014**
Hepatic profile			
AST, U/L	48.10 ± 18.05	26.69 ± 14.16	**0.000**
ALT, U/L	21.22 ± 14.00	14.89 ± 14.52	**0.000**
GGT, U/L	27.30 ± 22.41	23.19 ± 17.81	0.112
Alkaline phosphatase, U/L	135.53 ± 71.10	180.81 ± 101.85	**0.007**
Inflammatory profile			
CRP, mg/L	5.63 ± 6.78	3.87 ± 4.33	**0.001**
AAT, mg/dL	82.49 ± 47.32	69.37 ± 49.32	**0.029**
NOm, µM	23.87 ± 14.22	17.50 ± 7.52	**0.000**

RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; LDH: lactate dehydrogenase; HbS: hemoglobin S; HbF: fetal hemoglobin; WBC: white blood cell; MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet distribution width; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; AST: aspartate amino-transferase; ALT: alanine amino-transferase; GGT: gamma glutamyl-transferase; CRP: C-reactive protein; AAT: alpha-1 antitrypsin. NOm: nitric oxide metabolites. Bold values indicate significance at $p<0.05$; p-value obtained using Mann-Whitney *U* test. * p-value obtained using independent t-test.

6.2 MANUSCRITO 2

Título: Investigation of lipid profile and clinical manifestations in SCA children

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Situação: Submetido ao periódico *Lipids in Health and Disease*

Objetivos: Avaliar o perfil de marcadores lipídicos (colesterol total, LDL-C e HDL-C) em pacientes com anemia falciforme, suas associações com manifestações clínicas e com outros marcadores laboratoriais utilizados na clínica.

Principais resultados: Os níveis de colesterol total foram associados com histórico de pneumonia, marcadores de hemólise e concentrações de HbS, sugerindo um possível papel na gravidade da doença. Os níveis de LDL-C estiveram associados com úlcera de perna e pneumonia, bem como com marcadores de anemia. Os níveis de LDL-C estiveram associados a ocorrência de crise de dor, anisocitose reduzida, hemólise e viscosidade aumentada.

Lipids in Health and Disease

Investigation of lipid profile and clinical manifestations in SCA children

--Manuscript Draft--

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Abstract:	Background Clinical complications in sickle cell anemia (SCA) are heterogeneous and involve several molecules. It has been suggested that SCA individuals present a dyslipidemic phenotype and that lipid parameters are associated with severe clinical complications, such as pulmonary hypertension. We sought to investigate associations between lipid parameters and clinical manifestations, as well as other laboratory parameters in a population of pediatric SCA patients.Methods Our cross-sectional evaluation included 126 SCA patients in steady-state and who were not undergoing lipid-lowering therapy. Hematological and biochemical parameters were characterized and previous clinical manifestations were investigated.Results Total cholesterol and low density lipoprotein-cholesterol (LDL-C) levels were increased in patients with a previous history of pneumonia, which also positively correlated with HbS levels. Decreased LDL-C levels were also associated with leg ulcers and anemia. Elevated high density lipoprotein-cholesterol (HDL-C) levels were associated with pain crises, increased viscosity and decreased hemolysis. Several studies have determined that lipids play a role in the vascular impairment seen in SCA, which was corroborated by our findings.Conclusions In sum, our results suggest that lipid parameters are associated with hemolysis, anemia and, most importantly, with clinical complications related to vasculopathy in SCA.
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Opposed Reviewers:	
Additional Information:	
Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

Investigation of lipid profile and clinical manifestations in SCA children

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ABSTRACT

Background: Clinical complications in sickle cell anemia (SCA) are heterogeneous and involve several molecules. It has been suggested that SCA individuals present a dyslipidemic phenotype and that lipid parameters are associated with severe clinical complications, such as pulmonary hypertension. We sought to investigate associations between lipid parameters and clinical manifestations, as well as other laboratory parameters in a population of pediatric SCA patients. **Methods:** Our cross-sectional evaluation included 126 SCA patients in steady-state and who were not undergoing lipid-lowering therapy. Hematological and biochemical parameters were characterized and previous clinical manifestations were investigated. **Results:** Total cholesterol and low density lipoprotein-cholesterol (LDL-C) levels were increased in patients with a previous history of pneumonia, which also positively correlated with HbS levels. Decreased LDL-C levels were also associated with leg ulcers and anemia. Elevated high density lipoprotein-cholesterol (HDL-C) levels were associated with pain crises, increased viscosity and decreased hemolysis. Several studies have determined that lipids play a role in the vascular impairment seen in SCA, which was corroborated by our findings. **Conclusions:** In sum, our results suggest that lipid parameters are associated with hemolysis, anemia and, most importantly, with clinical complications related to vasculopathy in SCA.

Key words: Lipids, sickle cell anemia, pneumonia, pain crises.

BACKGROUND

The single point mutation in sickle cell anemia (SCA) is responsible for the production of the variant hemoglobin S (HbS) which under low oxygen tension forms long polymers affecting the red cell morphology [1]. HbS polymerization is the first pathophysiological step leading to clinical manifestations in SCA; moreover, several different mechanisms are involved in the pathogenesis of the disease including ischemia reperfusion injury [2], increased adhesiveness of leukocytes, reticulocytes and endothelial cells culminating in vaso-occlusion (VO) [3], and the innate immune system activation with hemolysis products, known as erythrocyte damage-associated molecular pattern molecules (eDAMPs) [4]. Intravascular hemolysis and VO are hallmarks of SCA. Red blood cells lysis release arginase, free heme and hemoglobin which decreases the L-arginine pool, the main source for endothelial cells to produce nitric oxide (NO), and leads to endothelial dysfunction and VO [1]. In addition, many inflammatory molecules have been described in SCA such as cytokines, chemokines, adhesion molecules, NO, heme, reactive oxygen species, adenosine triphosphate (ATP) and lipid mediators [5-7].

Cholesterol is obtained from the diet or may be produced endogenously and is the precursor of steroid hormones, bile acids, cell membranes and blood lipoproteins [8]. It is transported to the tissues packaged with apoliproteins, therefore, generating the blood lipoproteins very low density lipoprotein-cholesterol (VLDL-C), low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) [8, 9]. Abnormal lipid homeostasis is related to different inflammatory diseases, including Alzheimer where, alterations in sphingolipid and cholesterol metabolism results in

accumulation of long-chain ceramides and cholesterol [10]; as well as Psoriasis, since patients present significantly higher cholesterol levels in the VLDL-C and HDL-C fractions [11]. It is thought that LDL-C plays an important pro-inflammatory role in vascular diseases, while HDL-C is thought to be anti-inflammatory depending on the context [12].

Moreover, altered lipid parameters are directly and mostly associated to development of cardiovascular diseases [13], due to the relevance of several cohort studies which have shown that elevations of plasma LDL-C levels in association with decreased HDL-C levels consist an important risk factor for atherosclerosis and other vascular complications [14]. In atherosclerosis, several pathophyisiological mechanisms are similar to SCA vasculopathy such as decreased NO bioavailability, oxidative stress and endothelial dysfunction [15], although, the formation of atheroma plaques is not observed in SCA. Vasculopathy in SCA is closely related to complications such as pulmonary hypertension, leg ulceration, priapism and stroke [16].

It has been over 40 years since the first study suggesting a relationship of lipid determinations and laboratory parameters or clinical manifestations in SCA was published [17]. Since then, several studies attempted to investigate the role of lipids in the pathophysiological mechanism of SCA. It was shown that SCA individuals present decreased total cholesterol levels as well as HDL-C and LDL-C in addition to increased triglycerides and VLDL-C levels [18-20].

Clinical complications in SCA are heterogeneous and often associated to hemolysis such as pulmonary hypertension, leg ulcers and priapism and to VO such as vaso-occlusive pain crises, acute chest syndrome and osteonecrosis [16]. However, pulmonary hypertension, for instance, seems to be related to hemolysis, VO as well as

triglycerides levels in a large cohort of individuals with sickle cell disease (SCD) [20]. In addition, during vaso-occlusive crises patients with SCA presented total cholesterol, triglycerides and LDL-C levels significantly decreased whereas HDL-C levels were increased when compared to steady-state [21]. Also during steady-state, HDL-C levels were found to be associated to NOm and fetal hemoglobin (HbF) levels [18], two of the most important prognostic biomarkers in SCA.

Considering the vascular involvement of both pulmonary hypertension and vaso-occlusive crises, the combined data indicate that lipid parameters may be associated to vasculopathy in SCA. Therefore, we attempted to investigate the association of lipid profile (total cholesterol, LDL-C and HDL-C) with clinical complications and laboratory measurement of hemolysis, anemia, hemoglobin S (HbS) and systemic NO.

METHODS

Study design and patients

A cross-sectional study was performed including 126 SCA individuals (homozygous HbSS genotype), all seen at the Bahia Hemotherapy and Hematology Foundation (HEMOBA), located in Salvador, Bahia-Brazil. The median patient age was 15 years (IQR 12-15 years) and 60 (47.6%) were female. Patients with SCA in steady-state, defined as the absence of acute episodes in the past three months, were recruited to participate during routine clinical visits. All patients were taking folic acid supplementation, 60 were taking hydroxyurea, and none were undergoing therapy with lipid-lowering agents, such as statins. Data regarding the occurrence of previous clinical

manifestations was collected using a standardized and confidential questionnaire (self-reported, or reported by a legal guardian of the patient) at the time of enrollment and subsequently confirmed by medical records. The present study was approved by the Institutional Research Board of the São Rafael Hospital (protocol number: 1400535) and was conducted in compliance with the ethical principles established by the Declaration of Helsinki and its later revisions. All patients were informed regarding the purpose and procedures of this study, and informed written consent was obtained from each SCA patient's legal guardian.

Hematological parameters

Blood samples were collected at the time of enrollment after a 12 hour fast and analyzed immediately. Hematological parameters, including complete blood counts, were examined using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) and blood smears were stained with Wright's stain and examined by optical light microscopy. Reticulocytes were counted after staining supravitally with brilliant cresyl blue dye. Hemoglobin genotyping was performed by high-performance liquid chromatography on an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA) to confirm the presence of HbSS.

Biochemical determinations

LDL-C and VLDL-C levels were determined by the Friedewald equation [22], while total cholesterol, HDL-C and triglycerides, as well as biochemical parameters, including total bilirubin and fractions, lactate dehydrogenase, iron and hepatic and renal markers

were measured in serum samples using an automated A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain). Ferritin levels were determined using an Access 2 Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). NO metabolites (NOm) were quantified in serum samples with Griess reagent employing SoftMaxPro software, as previously described [23]. Laboratory analyses were performed at the Clinical and Toxicological Analysis Laboratory of the College of Pharmaceutical Sciences, Federal University of Bahia (LACTFAR-UFBA).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM, Armonk, New York, USA) and GraphPad Prism version 6.0 (Graphpad Software, San Diego, California, USA), which was also used to assemble graphs. The clinical characteristics of the study participants are expressed as means and respective standard variations. The distribution of each variable was tested by employing the Shapiro-Wilk test. The Mann-Whitney U test and independent t-test were used to compare among groups according to the normality of each variable. Fisher's exact test was used to compare categorical variables. Spearman correlation rank analysis was performed to test correlations between lipid parameters and hematological parameters. P values <0.05 were considered statistically significant.

RESULTS

Investigation of clinical manifestations and lipid parameters in SCA

We first decided to investigate associations between lipid parameters and previous clinical events using the Mann-Whitney U test. We found that patients with a previous history of pneumonia presented increased total-cholesterol levels (Figure 1.A), a previous history of leg ulcers was associated with decreased LDL-C levels (Figure 1.B), and patients with previous history of pain crises had increased HDL-C levels (Figure 1.C).

Associations between laboratory and lipid parameters

In addition to clinical manifestations, we also investigated associations between laboratory and lipid parameters. The patients presented overall median total cholesterol levels of 118 mg/dL (IQR: 103.5 – 135.5 mg/dL), median HDL-C levels of 35 mg/dL (IQR: 31 – 41 mg/dL) and median LDL-C levels of 58.8 mg/dL (IQR: 48.6 – 77.5 mg/dL). The patients were then stratified considering each median lipid parameter value and association analyses were performed. Patients with higher total cholesterol levels (≥118 mg/dL) were also found to present increased LDL-C, VLDL-C, triglycerides and direct bilirubin levels, as well as decreased indirect bilirubin and HbF levels, in addition to basophil counts (Table 1).

Patients with higher HDL-C levels (≥35 mg/dL) also presented decreased red cell distribution width (RDW), mean corpuscular hemoglobin concentration (MCHC) and VLDL-C, triglycerides, uric acid, lactate dehydrogenase (LDH), NOm and HbS levels as well as eosinophil and lymphocyte counts, in addition to increased hemoglobin, hematocrit, urea, iron and ferritin levels (Table 1).

Moreover, patients with higher LDL-C levels (≥58.8 mg/dL) also exhibited decreased mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values (Table 1).

Associations between clinical manifestations and lipid parameters

As some clinical events were previously found to be associated with lipid parameters, we further investigated these associations in patients stratified according to each median lipid variable value. Patients with a previous history of pneumonia were found to exhibit higher total cholesterol (≥118 mg/dL) and LDL-C (≥58.8 mg/dL) levels, whereas patients with a previous history of pain crises exhibited decreased HDL-C (<35 mg/dL) (Table 2).

Correlations between lipid and laboratory parameters

Correlation analysis was performed to investigate associations between laboratory and lipid parameters in patients with a previous history of pneumonia or pain crises. In patients with a previous history of pneumonia, total cholesterol levels were found to be negatively correlated with mean platelet volume (Figure 2.A) and positively correlated with HbS (Figure 2.B) and triglycerides levels (Figure 2.C). In addition, LDL-C levels were also positively correlated with HbS levels (Figure 2.D) and negatively correlated with triglycerides (Figure 2.E).

In patients with a previous history of pain crises, HDL-C levels were found to be positively correlated with hematocrit (Figure 3.A), and negatively correlated with RDW

(Figure 3.B), LDH (Figure 3.C) and triglycerides levels (Figure 3.D). Moreover, total cholesterol levels were also positively correlated with triglyceride levels (Figure 3.E).

DISCUSSION

As has been previously demonstrated by several studies, the significant alterations in lipid parameters presented by SCA patients have been associated with hemolysis, anemia, vaso-occlusive crises, activation of the TGF-β pathway, pulmonary hypertension and other complications [18-21, 24]. Thus, we decided to investigate associations between the lipid profile in SCA individuals and clinical manifestations.

We found that SCA patients with increased total cholesterol levels also had decreased levels of indirect bilirubin, HbF and a previous history of pneumonia; in the pneumonic patients, total cholesterol levels were also correlated with HbS, LDL-C, triglycerides and MPV levels. Pulmonary complications in SCA are mostly associated with vascular impairment and vasoconstriction, leading to VO. The occurrence of pneumonia in SCA patients has been closely linked to an increased frequency of acute chest syndrome, since the presentation of these conditions may overlap, and both are usually associated with pulmonary fat embolism and infectious pathogens [25]. In SCA patients, increasing levels of triglycerides were associated with more frequent episodes of acute chest syndrome [26]. Moreover, in a large cohort of adults, several associations were found between serum cholesterol levels and an increased risk of hospitalization leading to death due to respiratory disease [27]. The present study found a positive correlation between HbS levels and levels of total cholesterol and LDL-C in SCA patients with previous history of pneumonia, which suggests that altered lipid parameters may

indicate a more severe disease phenotype. Additionally, another study investigating lipoproteins in patients with SCD found a positive correlation between total cholesterol levels and hemopexin and hemoglobin, which were also negatively correlated with reticulocyte counts, LDH and bilirubin levels [28]. Together, these findings suggest that total cholesterol levels are related to pulmonary complications and hematological alterations in SCA.

We further observed that SCA individuals with increased HDL-C levels also had increased hemoglobin, hematocrit, iron and ferritin levels, in addition to decreased RDW, uric acid, LDH, NOm and HbS levels. Moreover, increased HDL-C levels were also associated with previous pain crises, and in the patients who reported painful episodes, HDL-C levels were also found to be correlated with markers of hemolysis and anemia as well as total cholesterol and triglycerides levels. These results are consistent with a previous study that associated HDL-C levels with red blood cell counts and hemoglobin and hematocrit levels [29]. Likewise, in a study carried out by Zorca and colleagues that evaluated associations between lipid parameters and pulmonary hypertension, HDL-C levels were negatively correlated with LDH levels [20], similar to what was found in the presently studied patients with a history of pain crises. In the same study, triglycerides levels were correlated with markers of hemolysis, endothelial activation and leukocyte counts [20]. These results reinforce the potential participation of HDL-C in modulating hemolysis and vascular dysfunction [30]. While the clinical presentation of SCA is highly variable, the most widely recognized clinical event is acute pain crisis driven by VO [31]. VO initiates a cascade of events, leading to tissue ischemia, which is responsible for the acute systemic vaso-occlusive crises that frequently necessitate medical care for SCA patients [1]. Increased hematocrit levels are associated with blood rheology and red blood cell deformability, which can also

contribute to VO and pain crises [1, 32]. Correspondingly, although the frequency of acute pain varies among SCA patients, it tends to be more frequent in patients presenting increased hematocrit and reduced HbF levels [33]. Moreover, besides the known anti-inflammatory and vaso-protective properties of HDL-C [14], recent data suggests the participation of HDL-C in the vascular environment with regard to hemolysis and anemia [30], which are important mechanisms underlying pain crises in SCA.

Decreased LDL-C levels were found in SCA individuals with a previous history of leg ulcers, and increased in patients with a previous history of pneumonia, in addition to being associated with decreased MCV and MCH levels. The anemia presented by SCA patients is related to decreased red blood cell survival [34] and intravascular hemolysis [7], which creates a pro-oxidant and proinflammatory vascular milieu that contributes to endothelial dysfunction [35]. In this same vascular environment, LDL-C exerts a strong proinflammatory role [8], which could also contribute to SCA vasculopathy. This is further supported by evidence that LDL-C is also susceptible to oxidative modifications in SCA, based on the observation of increased binding of free heme to LDL fractions, which could favor the production of oxLDL-C [28]. Moreover, multiple vascular mechanisms have been attributed to the pathogenesis of leg ulcers in SCA, such as the physical obstruction caused by irreversibly sickled red blood cells, poor venous recirculation, bacterial infection, anemia, in situ thrombosis and reduced NO bioavailability [36]. Patients with previous history of leg ulcers exhibit elevated hemolytic laboratory parameters, increased uric acid and decreased albumin levels [37]. Since the frequency of leg ulcers was associated with priapism and pulmonary hypertension, venous stasis could justify the causal relationship between pulmonary hypertension and leg ulcers, due to overlapping of pathophysiological mechanisms [37].

Accordingly, the association between anemia and LDL-C levels suggests the vascular involvement of this molecule, which could also contribute to clinical manifestations.

It is important to note that although our study investigated associations between clinical manifestations and laboratory parameters of SCA and serum levels of total cholesterol, HDL-C and LDL-C, the present cohort cannot be characterized as having hypercholesterolemia. Nonetheless, our findings stand in agreement with other studies that also reported decreased total cholesterol, HDL-C and LDL-C levels among SCA individuals in comparison to HbAA individuals [19, 20, 26]. It is thought that the hypocholesterolemia seen in SCA results from the augmented cholesterol utilization in erythropoiesis consequent to anemia and hemolysis. In addition, the occurrence of hypocholesterolemia in patients with non-hemolytic anemia suggests increased erythropoietic activity [17]. Moreover, it is also relevant to point that our investigation was carried out in a pediatric population of SCA patients, which could explain some discrepancies with data in the literatures; however, many of our results are in accordance with previous publications involving patients of a similar age, as well as adults [19, 20]. The cross-sectional design of our study made it difficult to establish any causative roles for lipid parameters with regard to clinical manifestations in SCA, yet the relevant associations found herein will be useful in guiding further evaluations.

CONCLUSIONS

In summary, the present findings serve to affirm and extend the knowledge surrounding the abnormal lipid profile presented by SCA individuals in association with pain crises, leg ulcers and pneumonia, in addition to upholding established correlations with laboratory markers of hemolysis and anemia.

DECLARATIONS

Ethics approval and consent to participate

The present study was approved by the Institutional Research Board of the São Rafael Hospital (protocol number: 1400535) and was conducted in compliance with the ethical principles established by the Declaration of Helsinki and its later revisions. All patients were informed regarding the purpose and procedures of this study, and informed written consent was obtained from each SCA patient's legal guardian.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Competing interests

The authors have not received any funding or benefits from industry companies or otherwise to conduct this study.

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Authors' contributions

CCG designed the project, performed all experiments, statistical analyses and wrote the manuscript. RPS organized the data and helped with statistical analyses. CFLF and JSS performed the hematological measurements and helped with sample collection.

SCMAY, MMA, CVBF and LMF performed biochemical characterizations and helped with sample collection. SPC and RMO supervised the study, helped with sample collection, laboratory characterization and the discussion of results. VMLN and LCR assisted the patients and helped with sample collection. MSG conceived and supervised the study and critically revised the manuscript.

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FIGURE LEGENDS

Figure 1. Associations between total cholesterol, LDL-C, HDL- levels and clinical manifestations in SCA. A) Patients with previous history of pneumonia (N = 69) exhibited increased total cholesterol levels. B) Patients with previous history of leg ulcers (N = 12) presented decreased LDL-C levels. C) Patients with previous history of pain crises (N = 78) had increased HDL-C levels. P-values obtained using the Mann-Whitney *U* test.

Figure 2. Correlations between lipid and hematological parameters in SCA patients with previous history of pneumonia (N = 69). A) Total cholesterol levels were negatively correlated with mean platelet volume (MPV). B) Total cholesterol levels were positively correlated with HbS levels. C) Total cholesterol levels were positively

correlated with triglycerides levels. D) LDL-C levels were positively correlated with HbS levels. E) HDL-C levels were negatively correlated with triglycerides levels. Data comparisons made using Spearman's correlation rank-test.

Figure 3. Correlations between HDL-C and triglycerides levels and hematological parameters in SCA patients with previous history of pain crises (N = 78). A) HDL-C levels were positively correlated with hematocrit levels. B) HDL-C levels were negatively correlated with RDW. C) HDL-C levels were negatively correlated with lactate dehydrogenase (LDH) levels. D) HDL-C levels were negatively correlated with triglycerides levels. E) Total cholesterol levels were negatively correlated with triglycerides. Data were compared using Spearman's correlation rank-test.

LIST OF ABBREVIATIONS

HbF	Fetal Hemoglobin
HbS	Hemoglobin S
HDL-C	High density lipoprotein-cholesterol
LDH	Lactate dehydrogenase
LDL-C	Low density lipoprotein-cholesterol
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
NO	Nitric Oxide
RDW	Red blood cell width
SCA	Sickle cell anemia
VLDL-C	Very low density lipoprotein-cholesterol
VO	Vaso-occlusion

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TABLES

Table 1. Associations between lipid profile and laboratory parameters in SCA patients.

Laboratory parameters	Total cholesterol < 118 mg/dL (N = 64)	Total cholesterol ≥118 mg/dL (N = 62)	p-value
Basophil, mL	111.02 ± 107.40	76.69 ± 106.97	0.035
LDL-C, mg/dL	47.98 ± 11.40	77.44 ± 18.63	0.000
VLDL-C, mg/dL	19.41 ± 9.04	25.67 ± 12.42	0.000
Triglycerides, mg/dL	94.17 ± 39.24	124.97 ± 55.95	0.000
Direct bilirubin, mg/dL	0.38 ± 0.14	0.44 ± 0.16	0.021
Indirect bilirubin, mg/dL	2.91 ± 1.74	2.32 ± 1.47	0.046
HbF, %	10.37 ± 5.97	7.87 ± 5.14	0.015
	HDL-C < 35 mg/dL (N = 61)	HDL-C ≥ 35 mg/dL (N = 65)	
Hemoglobin, g/dL	8.15 ± 1.00	8.79 ± 0.97	0.000
Hematocrit, %	24.04 ± 3.37	26.28 ± 3.03	0.000*
MCHC, %	34.20 ± 1.13	33.62 ± 0.81	0.002*
RDW, %	23.91 ± 3.70	21.40 ± 3.45	0.000*
Eosinophil, mL	619.00 ± 597.22	362.34 ± 295.42	0.002
Lymphocyte, mL	4480.48 ± 1337.12	3757.41 ± 1236.03	0.002
VLDL-C, mg/dL	26.12 ± 12.42	18.72 ± 8.44	0.000
Triglycerides, mg/dL	127.66 ± 59.36	90.34 ± 29.03	0.000
Urea, mg/dL	15.44 ± 4.89	20.02 ± 6.93	0.000
Iron, μg/dL	100.34 ± 48.75	124.00 ± 58.93	0.008
Uric acid, mg/dL	4.12 ± 1.21	3.47 ± 1.10	0.003
LDH, U/L	1471.14 ± 1753.80	1019.46 ± 344.37	0.001
Ferritin, ηg/mL	199.10 ± 461.01	327.55 ± 408.16	0.018
NOm, μM	28.39 ± 17.78	18.97 ± 6.37	0.001
HbS, %	85.50 ± 8.80	81.60 ± 11.05	0.032
	LDL-C < 58.8 mg/dL (N = 65)	LDL-C ≥ 58.8 mg/dL (N = 61)	
MCV, fL	94.63 ± 11.45	90.07 ± 11.44	0.028*
MCH, ρg/mL	32.10 ± 3.91	30.52 ± 3.91	0.025*

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; LDH: lactate dehydrogenase; HbS: hemoglobin S; HbF: fetal hemoglobin; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density

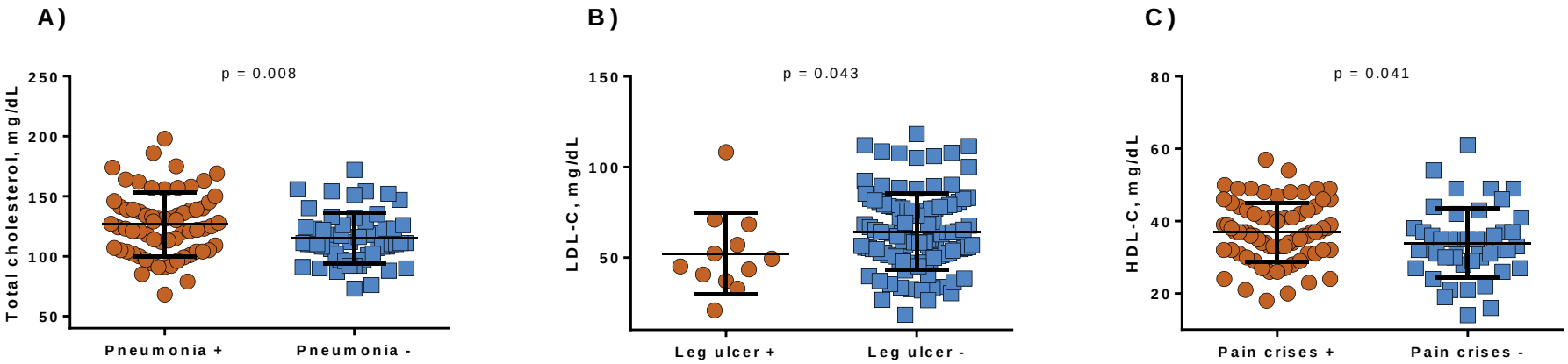
lipoprotein cholesterol; NOm: nitric oxide metabolites. p-value obtained using Mann-Whitney *U* test. * p-value obtained using independent t-test.

Table 2. Frequency of clinical manifestations associated with lipid parameters in SCA patients.

Clinical data	Lipid parameters		p-value
Pneumonia + (N = 69)	Total cholesterol < 118 mg/dL	Total cholesterol ≥ 118 mg/dL	
	27 (39%)	42 (61%)	0.007
Pneumonia + (N = 69)	LDL-C < 58.8 mg/dL	LDL-C ≥ 58.8 mg/dL	
	29 (42%)	40 (58%)	0.048
Pain crises + (N = 78)	HDL-C < 35 mg/dL	HDL-C ≥ 35 mg/dL	
	44 (56%)	34 (44%)	0.026

Data comparisons performed using Fisher's exact test.

FIGURES



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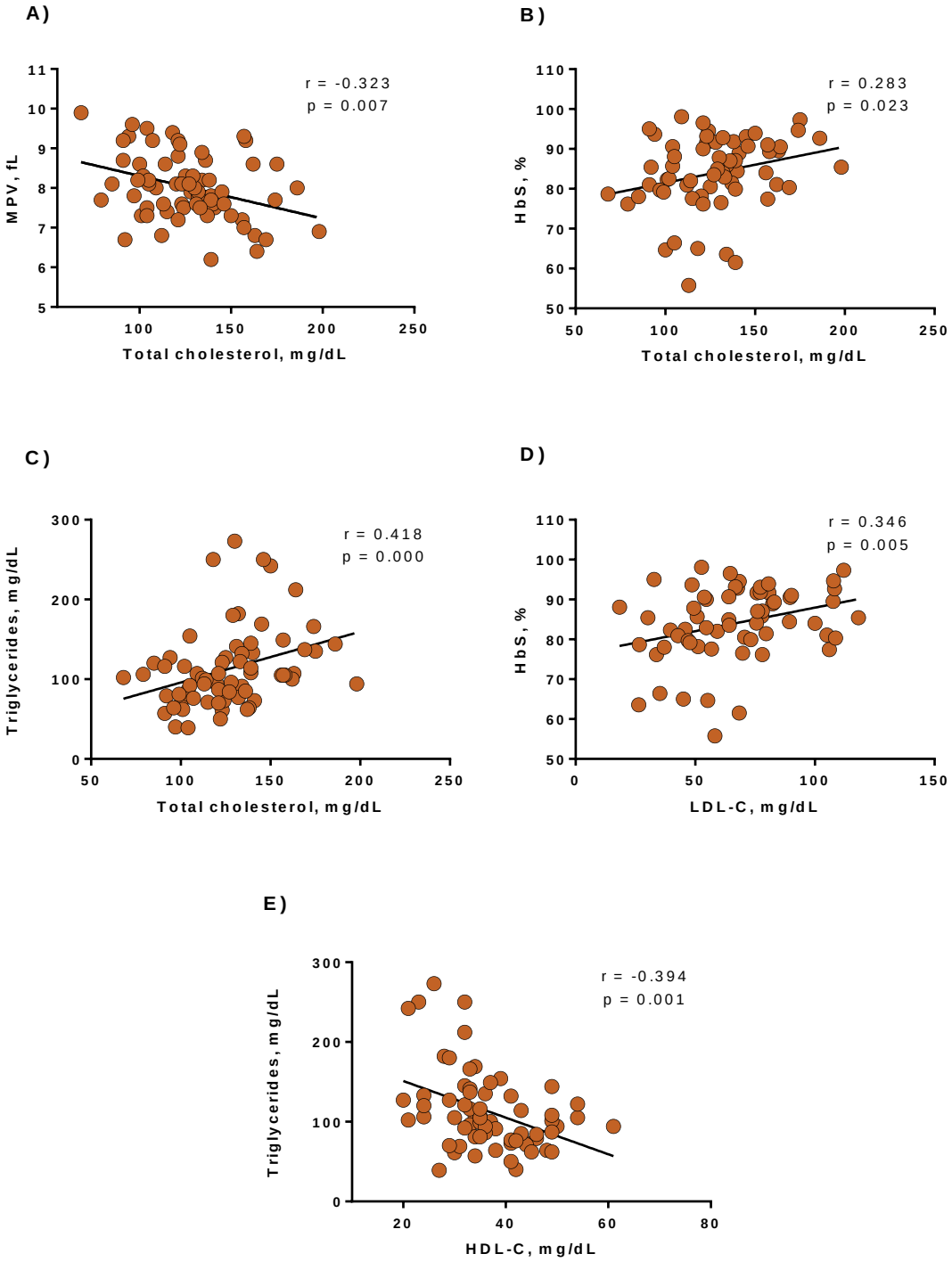

Figure 2. Correlations between lipid and hematological parameters in SCA patients with previous history of pneumonia (N = 69). A) Total cholesterol levels were negatively correlated with mean platelet volume (MPV). B) Total cholesterol levels were positively correlated with HbS levels. C) Total cholesterol levels were positively correlated with triglycerides levels. D) LDL-C levels were positively correlated with HbS levels. E) HDL-C levels were negatively correlated with triglycerides levels. Data comparisons made using Spearman's correlation rank-test.

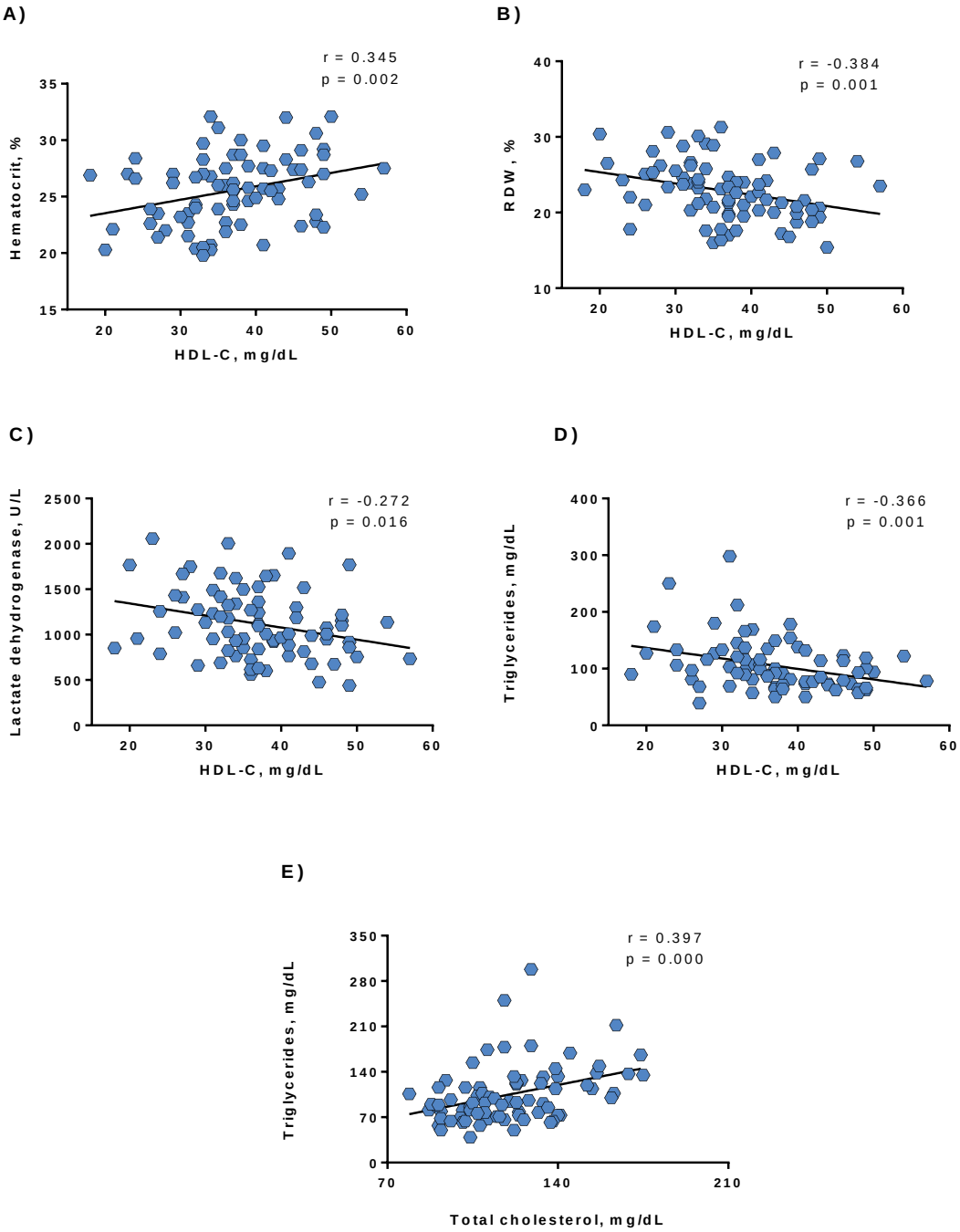

Figure 3. Correlations between HDL-C and triglycerides levels and hematological parameters in SCA patients with previous history of pain crises (N = 78). A) HDL-C levels were positively correlated with hematocrit levels. B) HDL-C levels were negatively correlated with RDW. C) HDL-C levels were negatively correlated with lactate dehydrogenase (LDH) levels. D) HDL-C levels were negatively correlated with triglycerides levels. E) Total cholesterol levels were negatively correlated with triglycerides. Data were compared using Spearman's correlation rank-test.

6.3 MANUSCRITO 3

Título: IL-8 plasma levels and rs4073 *CXCL8*-251A/T polymorphism in sickle cell anemia

Autores: Caroline Conceição da Guarda, Camila Felix de Lima Fernandes, Joelma Santana dos Santos Neres, Sétondji Cocou Modeste Alexandre Yahouédéhou, Rayra Pereira Santiago, Milena Magalhães Aleluia, Camylla Vilas Boas Figueiredo, Luciana Magalhães Fiuza, Suellen Pinheiro Carvalho, Rodrigo Mota de Oliveira, Cleverson Alves Fonseca, Valma Maria Lopes Nascimento, Larissa Carneiro Rocha e Marilda Souza Gonçalves.

Situação: Submetido ao periódico *Mediators of Inflammation*

Objetivos: avaliar a associação entre o polimorfismo rs4073 no gene *CXCL8* e os níveis dessa citocina, bem como suas associações com as manifestações clínicas e com outros marcadores laboratoriais utilizados no mapeamento do perfil do paciente durante o seu acompanhamento clínico.

Principais resultados: A distribuição da frequência dos alelos correspondentes ao polimorfismo rs4073 no gene *CXCL8* estava em equilíbrio de Hardy-Weinberg e a presença do alelo T esteve associada a níveis reduzidos da citocina. Os pacientes com AF que tiveram níveis elevados de IL-8 também apresentaram contagem maior de linfócitos e concentrações menores de alfa-1 antitripsina e contagem menor de reticulócitos. Entre as manifestações clínicas, os pacientes com histórico de esplenogemalia tiveram níveis menores de IL-8, sendo que nesse grupo de pacientes, os níveis de IL-8 estiveram correlacionados positivamente com a contagem de reticulócitos. O modelo proposto pela análise multivariada mostrou que os níveis de IL-8 estiveram associados de forma independente a ocorrência de esplenogemalia nos pacientes.

IL-8 plasma levels and rs4073 CXCL8-251A/T polymorphism in sickle cell anemia

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ABSTRACT

Clinical course of sickle cell anemia (SCA) is highly variable and a chronic inflammatory response is a hallmark of the disease which is also marked by chronic hemolysis. Interleukin-8 (IL-8) is a pro-inflammatory chemokine known to induce a robust chemotactic response of neutrophils. Moreover, polymorphisms in the CXCL8 gene are thought to modulate the chemokine expression. Therefore, considering the intricate inflammatory mechanism underlying SCA we attempted to investigate the association of IL-8 to clinical manifestations and classical laboratory markers. Forty-four SCA individuals were included, all in steady-state. The polymorphism rs4073 CXCL8 -251A/T was investigated as well as plasma levels of IL-8. Our analyses revealed that the presence of the T allele was associated to decreased IL-8 levels. Patients with increased IL-8 levels also presented increased lymphocyte counts and alpha-1 antitrypsin levels, in addition, to decreased reticulocyte counts. Individuals with previous history of splenomegaly had decreased IL-8 levels and, in those patients, reticulocytes counts were positively correlated to IL-8 levels. In a model of multivariate analysis IL-8 was independently associated to splenomegaly. Our findings are in accordance with previous studies of IL-8 levels in SCA individuals. Surprisingly, the association of IL-8 with splenomegaly was found to be related to reticulocyte counts, which suggests a possible role in anemia and hemolysis. Nevertheless, the biological relevance of decreased IL-8 levels and occurrence of splenomegaly in SCA still needs further studies.

Key words: rs4073; sickle cell anemia; IL-8; splenomegaly.

1. INTRODUCTION

The genetic mechanism underlying sickle cell anemia (SCA) is a single point mutation at the sixth codon of the β-globin chain gene, which leads to the synthesis of the hemoglobin S (HbS), a hemoglobin variant that forms long polymers under low oxygen tension. Although the molecular mechanism may seem simple, individuals with SCA usually exhibit heterogeneous clinical events with complex mechanisms associated to them (1). When the red blood cells (RBC) circulate through the vascular beds and are exposed to different oxygen concentrations, repeated cycles of HbS polymerization and depolymerization occurs, hence, RBC morphology is also affected (2). The RBC membrane become irreversibly damaged due to HbS polymerization, in addition to increased expression of phosphatidylserine and adhesive molecules that interact with leukocytes, platelets and the vascular endothelium which can contribute to vaso-occlusion (VO) (3).

Vaso-occlusion and hemolysis are thought to contribute to the pathophysiology as well as to the variability of clinical presentations in SCA. Inflammation induced by hemolysis is a hallmark of SCA, both triggered by deoxy-HbS polymerization (4, 5). As consequence, diverse inflammatory molecules are involved in the pathogenesis of SCA, such as cytokines, chemokines, lipid mediators, adhesion molecules, enzymes, free hemoglobin and heme, which create an intricate mechanism associated to clinical manifestations (6).

SCA individuals exhibit a wide range of clinical events, as they have been associated to VO, such as vaso-occlusive pain crisis, acute chest syndrome and osteonecrosis, while pulmonary hypertension, priapism, leg ulcers, stroke and splenomegaly have been associated to chronic hemolysis (7).

Notably, in SCA both extra and intravascular hemolysis are important events. Extravascular hemolysis consists in the removal of senescent RBC by macrophages from the mononuclear phagocytic system in the spleen and liver, while intravascular hemolysis is mainly associated to the rigid membrane of the irreversibly sickled red blood cells, which are lysed into the vessel, releasing pro oxidant products (2, 4, 8). Intravascular hemolysis releases arginase, which competes with endothelial nitric oxide synthase (eNOS) for the L-arginine pool, decreasing the nitric oxide (NO) production. Likewise, free heme and hemoglobin are capable of inactivate NO, which in turn leads to endothelial dysfunction and VO (2). Moreover, free heme and hemoglobin have been considered erythrocytic danger-associated molecular pattern (eDAMP) molecules, which can activate the innate immune system, and could also contribute to the chronic inflammatory response (2, 9).

Several inflammatory mechanisms have been described in SCA, including pro-inflammatory mediator production, chronic cell activation, hemolysis and hemoglobin products, ischemia-reperfusion injury and oxidative stress (10). Inflammatory cytokines

have also been described playing a pivotal role on the differentiation of sickle cell crisis and steady-state of the disease (6). Many pro-inflammatory cytokines are produced by leukocytes, vascular endothelial cells and platelets, such as IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-17 and transforming growth factor beta (TGF-β) during hemolysis, microvascular occlusion or infection, and all have been implicated in the development of clinical complications in SCA (11).

The interleukin-8 (IL-8) is a pro-inflammatory chemokine from the CXC family (Chemokine [C-X-C Motif] Ligand 8), produced by monocytes, macrophages, T lymphocytes, endothelial cells and other cell types (12). IL-8 exhibits a considerable importance in neutrophil activation with release of granule enzymes as well as the generation of reactive oxygen species (ROS), chemotaxis and migration due its ability to re-organize the cytoskeleton as result of a great Ca+2 influx (12, 13); moreover, it can also delay the apoptosis of this cell (14). In SCA, it has been shown that IL-8 is able to increase the adherence of sickle erythrocytes to endothelial cells via fibronectin (15), thus contributing to VO, which is validated by increased IL-8 serum levels in SCA patients during vaso-occlusive crisis (16). The IL-8 production is thought to be modulated by polymorphisms on the gene promoter (17, 18) and, importantly, even a CXCL8 gene haplotype with six single-nucleotide polymorphisms (SNP) has been identified in the context of infectious diseases (19). The rs4073 (-251A>T) SNP in CXCL8 gene was previously investigated among SCA patients, and the promising role of the T allele to protect from splenomegaly was suggested (20). Therefore, considering the multiple inflammatory mechanisms associated to SCA pathophysiology, we attempted to evaluate the rs4073 polymorphism in addition to IL-8 plasma levels in SCA patients associated to classical laboratory parameters, useful in the clinical practice, as well as the association with clinical manifestations. Our findings corroborate with previous studies on IL-8 and we suggest an association of decreased IL-8 levels with occurrence of splenomegaly.

2. METHODS

2.1. Subjects

Eligible subjects included patients with SCA with confirmed HbSS genotype in steady-state. This cross-sectional study included 43 SCA children who were regularly seen at Bahia Hemotherapy and Hematology Foundation (HEMOBA). Patients were aged 14.8 ± 3.2 years and 20 (46.5%) were female. Steady-state was defined as the absence of acute crisis in the past three months prior to blood collection procedures. Twenty-four patients were under hydroxyurea therapy; in addition, all the patients were taking folic acid supplementation. This study received approval from the Institutional Research Board (protocol number: 1400535) and was conducted in compliance with the ethical principles established by the revised Declaration of Helsinki. All patients had been informed about the purpose and procedures of this study and an informed written consent was obtained from each SCA patient’s guardian.

2.2. Biological analyses

Blood samples were collected at the time of enrollment. Hematological parameters were evaluated using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) and blood smears were stained with Wright's stain and examined by light optical microscopy. Reticulocytes were counted after staining supravitally with brilliant cresyl blue dye. Hemoglobin patterns were confirmed by high-performance liquid chromatography employing an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA).

Biochemical determinations, including lipid profile, total bilirubin and fractions and lactate dehydrogenase were determined in serum samples using an automated A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain). C-reactive protein (CRP) and alpha-1 antitrypsin (AAT) levels were measured using IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). Laboratory parameters were analyzed at the Clinical Analyses Laboratory of the College of Pharmaceutical Sciences (Universidade Federal da Bahia).

2.3. IL-8 measurement

IL-8 plasma concentrations were investigated through particle-based immunoassay using the BD™ Cytometric Bead Array (CBA) Human Inflammation Kit (BD Bioscience, San Jose, CA, USA), according to the manufacturer's protocol. The bead population was obtained in a BD FACSArray™ bioanalyzer (BD Bioscience, San Jose, CA, USA) and the Software FlowJo, LLC (BD Bioscience, San Jose, CA, USA) was used to analyze the data.

2.4. Characterization of rs4073 A/T polymorphism

Identification of genetic variant rs4073 -251A/T of the interleukin-8 promoter region was performed with PCR-RFLP technique as previously described (20, 21).

2.5. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism version 6.0 (Graphpad Software, San Diego, California, USA), which was also used to assemble the graphs, in addition to Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM, Armonk, New York, USA). Baseline values of selected variables are expressed as means with their respective standard variation. We tested each variable distribution employing the Shapiro-Wilk test. The Mann-Whitney U test and independent t-test were

used to compare the groups according to the normality of the distribution for each variable. Parametric ANOVA was used to analyze the means of quantitative or numerical variables with normal distributions, while the nonparametric Kruskal–Wallis test was used for data with non-normal distribution. Spearman correlation rank analysis was performed to test correlations between reticulocyte counts and IL-8 plasma levels. P values <0.05 were considered to be statistically significant. Association analysis between laboratory parameters and polymorphisms, using a genetic dominant model, and multivariate linear regression analyses were performed to evaluate the influence of gene polymorphisms on laboratory parameters.

3. RESULTS

3.1. IL-8 plasma levels are associated to different genotypes of rs4073 in SCA

Hemolysis markers, leukocytes and platelets counts as well as inflammatory determinations of SCA patients are described in Supplementary Table 1. We have found that 9 patients carry the wild-type genotype (AA), 20 patients were heterozygous and 14 were variant homozygous, frequencies were in Hardy-Weinberg equilibrium (Figure 1.A). Moreover, IL-8 plasma levels were also associated to different genotypes of rs4073 polymorphism (Figure 1.B). Addressing a dominant genetic model IL-8 plasma levels were also statistically different between the genotypes (Figure 1.C).

3.2. Association of IL-8 plasma levels and laboratory biomarkers

We attempted to investigate associations of IL-8 plasma levels and laboratory parameters in SCA, regardless of the polymorphism. IL-8 plasma levels were categorized according to the median value (3.6 pg/mL) and increased IL-8 levels were associated to increased lymphocyte counts (Figure 2. A), decreased reticulocyte counts (Figure 2. B) and increased AAT levels (Figure 2.C).

3.3. Splenomegaly in SCA associated to IL-8 plasma levels

Considering the pro-inflammatory nature of IL-8 we also evaluated the associations with clinical manifestations. We found that patients with previous history of splenomegaly (splenomegaly +) exhibited decreased IL-8 plasma levels when compared to those who had not (splenomegaly -) (Figure 3.A).

Nevertheless, our subset of patients with positive history splenomegaly was composed by 19 individuals; aged 15.4 ± 3.0 years, and mean age of splenomegaly occurrence was

6 years old. Conversely, patients without history of splenomegaly were aged 14.3 ± 3.3 years, and no statistical difference was found regarding age of the groups.

3.4. IL-8 plasma levels and reticulocytes are associated in splenomegaly

After investigating the IL-8 plasma levels according to previous history of splenomegaly we tested in correlation analysis which laboratory parameters would be associated in each group. Importantly, we have found that in patients with previous history of splenomegaly reticulocyte counts were negatively correlated to IL-8 plasma levels (Figure 3. B), while in the group without splenomegaly these determinations were not statistically significant (Figure 3. C). In addition, multivariate linear regression in SCA patients showed that IL-8 plasma levels were independently associated to previous history of splenomegaly (Table 1).

4. DISCUSSION

Chronic intravascular hemolysis in addition to vaso-occlusive events are known to promote inflammation in SCA, which creates a feedback loop by enhancing the adhesiveness of leukocytes, platelets, endothelial and RBCs, thus promoting VO.

We decided to investigate the role of polymorphism rs4073 CXCL8 -251A>T and plasma levels in SCA patients. Frequencies of rs4073 genotypes in our study are in agreement with a previous cross-sectional investigation of this polymorphism in SCA patients (20); in addition, similar frequencies were also identified in a different population of SCA patients (22). The influence of rs4073 polymorphism on IL-8 levels is thought to be at transcriptional level, once when the A allele is present, the transcriptional activity is increased and, therefore, IL-8 levels are also higher (19, 23). Our findings show statistical difference between the genotypes and also comparing the variant genotypes with wild-type, with higher levels exhibited by those carrying the AA genotype. SCA individuals carriers of the *IL-8* AA genotype also presented increased IL-8 serum levels in a different study (22).

Besides the polymorphism we also sought to investigate the association of IL-8 plasma levels with laboratory determinations. SCA individuals with IL-8 values higher than the median value also had increased lymphocyte counts. Previous report supports the role of IL-8 as an important chemotactic factor for T lymphocytes, as well as neutrophils granule-derived factors after IL-8 stimulation can induce in vitro T cell chemotaxis (24). Reticulocyte counts are a very useful and reliable hematological parameter to monitor hemolysis in SCA (25); in our study, SCA individuals with higher IL-8 levels also exhibited decreased reticulocyte counts. IL-8 levels were not associated to haptoglobin in SCA individuals (26), neither to coagulation factors in HbSC disease (27). Still, in

vitro evaluation of neutrophil exposure to heme lead to an increase in cell migration and ROS production, and it was suggested that the signaling pathway was similar to chemotactic receptors (28). Altogether, these results indicate that IL-8 may be an important chemokine associated to hemolysis and hemolysis by-products, although further studies are needed. Alpha1-antitrypsin (AAT) is an acute-phase protein mainly produced by the liver with potent anti-inflammatory properties (29). In SCA individuals AAT levels were shown to be increased in steady-state of the disease when compared to control group (30). Our results show that individuals with higher IL-8 levels also had increased AAT levels. The association of IL-8 and AAT was previously evaluated in an in vitro study in which neutrophil chemotaxis was dose-dependent of the IL-8:AAT ratio, in addition, the complex mounted by IL-8 and AAT was able to change neutrophil cytoskeleton, hence, modulating the chemotaxis (31). Collectively, these results corroborate the role of IL-8 as a chemokine involved in leukocyte chemotaxis and inflammatory process.

The severity of clinical complications in SCA is variable and several disease modifiers are implicated. Our evaluation of IL-8 plasma levels associated to clinical manifestations suggests that SCA patients with previous history of splenomegaly had decreased IL-8 levels. It was suggested before that the presence of the T allele of the rs4073 polymorphism is a protect factor for splenomegaly, although, no association was found regarding IL-8 levels (20). Splenomegaly in the context of hemolytic anemias is associated to two mechanisms. First, the overwhelming amount of extra and intravascular hemolysis leads to hypertrophy of the reticuloendothelial system with increased sequestration of erythrocytes with abnormal morphology from the circulation. The second mechanism is due to hypoxia which acts a homing factor for erythroblasts to migrate from the bone marrow to the spleen, causing obstruction with the proliferative cells (32, 33). Both mechanisms may be attributed to SCA. Splenic complications are a frequent cause of clinical events in SCA, such as increased susceptibility to encapsulated bacterial infections (34). Splenomegaly and splenic loss of function may be evidenced very early in infants with SCA, due to increased pitted red blood cell counts. It is estimated that the loss of splenic function begins before 12 months of age (35). We also found that IL-8 plasma levels were negatively correlated with reticulocyte counts in the group of patients with previous history of splenomegaly, which reinforces the notion that hemolysis may be associated to IL-8 levels. In a mouse model knock-out for the IL-8 murine receptor the animals developed splenomegaly due to increased expansion of the white pulp associated to the proliferation of myeloid cells, although the erythroid series remained unaffected (36). The role of hemolysis was also tested in a mouse model, which found that heme-challenged hemopexin-deficient mice accumulated more heme in the spleen when compared to wild-type controls, and that splenic macrophages exhibited increased expression of CD86, consistent with a pro-inflammatory phenotype (37). Nevertheless, the biological relevance of decreased IL-8 levels and occurrence of splenomegaly in SCA still needs further studies. We hypothesized that macrophages from the spleen may be an important source of IL-8 in SCA; however, it still lacks functional studies to confirm this idea.

5. CONCLUSION

Evaluation of polymorphism rs4073 in CXCL8 among SCA individuals demonstrated the influence of the A allele with higher IL-8 levels; moreover, IL-8 plasma levels were associated to inflammatory and hemolysis biomarkers as well as to previous history of splenomegaly. Taken together, our results suggest that the inflammatory response observed in SCA individuals could be modulated by IL-8.

6. ACKNOWLEDGEMENTS

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7. FUNDING

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8. AUTHORSHIP

CCG designed the project, performed all the experiments and wrote the manuscript. CFLF and JSSN performed all the IL-8 genotype characterization. LMF performed the IL-8 measurement. SCMAY, MMA, CVBF, RPS, performed the laboratory characterization and helped with the sample collection. SPC and RMO contributed to all the experiments, supervised the study and helped with the sample collection. VMLN and LCR assisted the patients and helped with the sample collection. MSG conceptualized, performed a critical evaluation of the data, supervised the study and critically revised the manuscript.

9. DISCLOSURE OF CONFLICTS OF INTEREST

We declare that we have no conflict of interest

Table 1. Multivariate linear regression model of history of splenomegaly and confounding variables in SCA patients.

Independent variables	**Dependent variable**	**B**	**p-value**	**R^2**	**p-value of the model**
IL-8 pg/mL	Splenomegaly	-0.421	**0.008**	0.166	0.024
Reticulocytes, %		-0.046	0.764		

R^2: coefficient of determination; β: coefficient of regression.

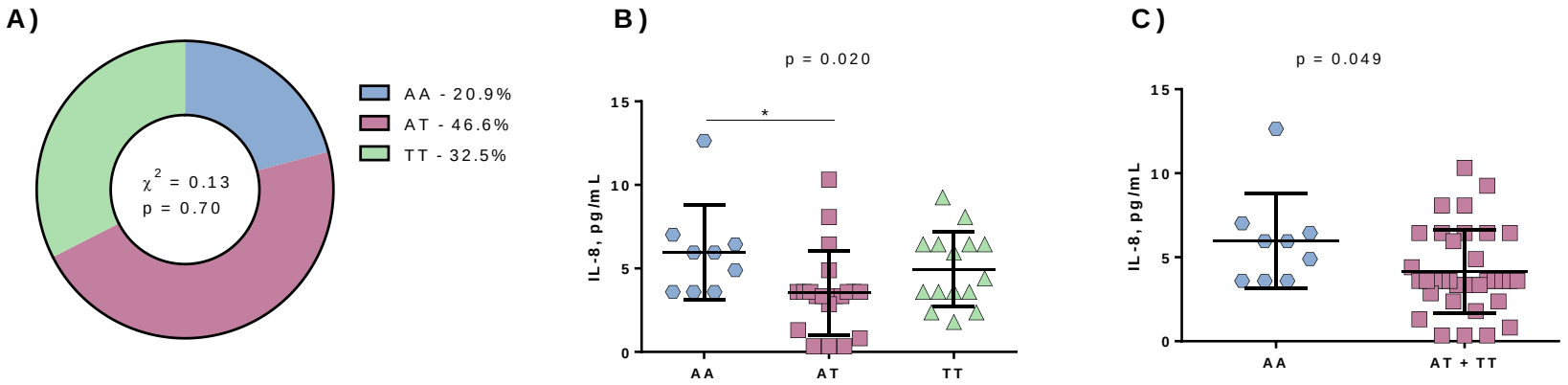

Figure 1. Frequency of the polymorphism rs4073 *IL8* and IL-8 plasma levels in SCA patients according to each genotype. A) Frequency of the polymorphism rs4073 in *IL8* gene were in Hardy-Weinberg equilibrium. B) Association of IL-8 plasma levels according to each genotype of polymorphism rs4073 in *IL8* gene. p value was obtained with Kruskal-Wallis. *p value < 0.05 obtained with Dunn's multiple comparisons test. C) Association of IL-8 plasma levels and polymorphism rs4073 in *IL8* gene according to dominant genetic model. p value was obtained with Mann-Whitney *U* test. χ^2: Chi-square.

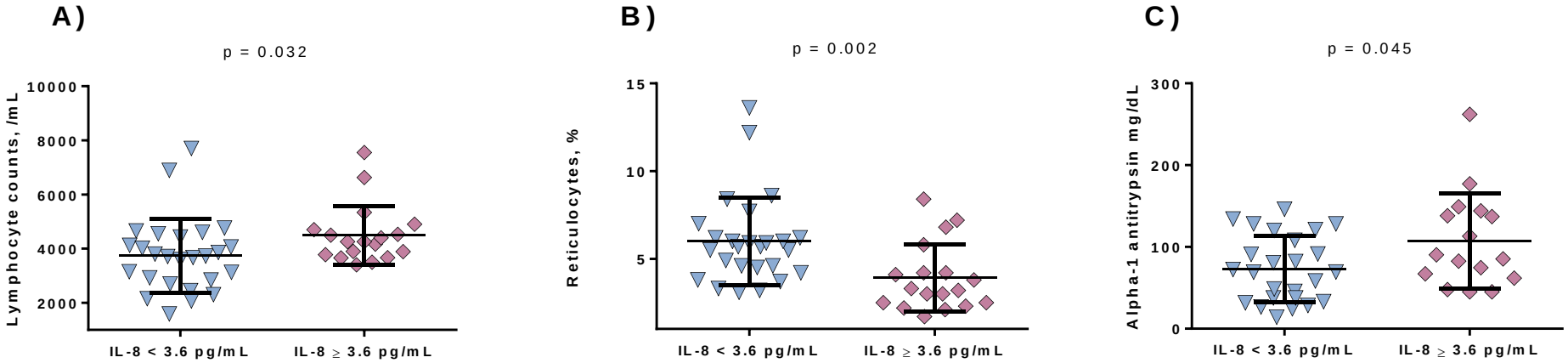

Figure 2. Association of IL-8 plasma levels according to the median value to laboratory parameters. A) Patients with IL-8 levels ≥ 3.6 pg/mL exhibited increased lymphocyte counts. B) Patients with IL-8 levels ≥ 3.6pg/mL exhibited decreased reticulocyte counts and C) Patients with IL-8 levels ≥ 3.6pg/mL exhibited increased alpha-1 antitrypsin levels. (IL-8 ≥ 3.6 pg/mL, n = 18 and IL-8 < 3.6 pg/mL, n = 25, for all comparisons). p values were obtained with Mann-Whitney *U* test.

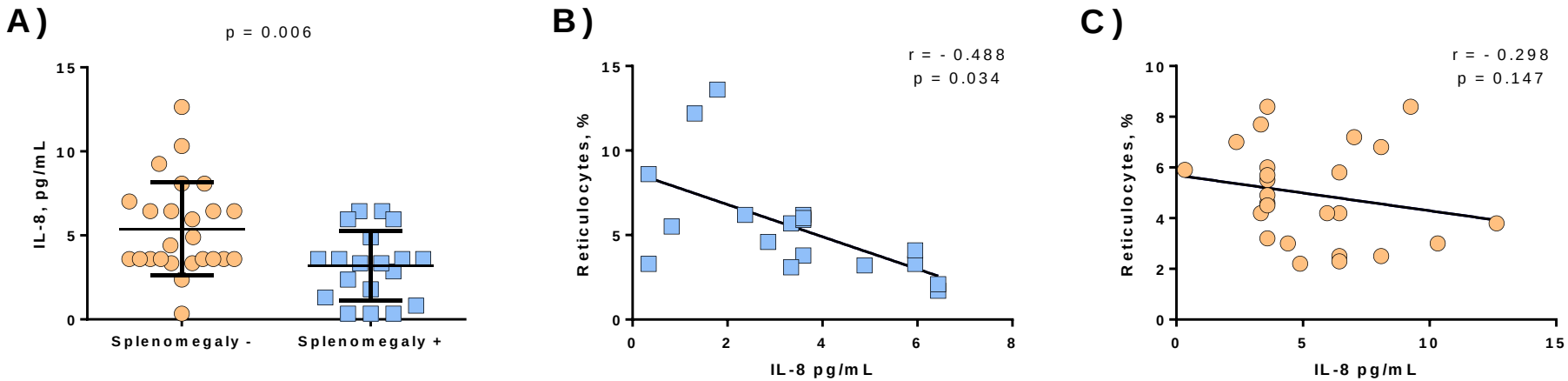

Figure 3. Association of IL-8 plasma levels in SCA with previous history of splenomegaly. A) Patients with previous history of splenomegaly (n = 19) presented decreased IL-8 levels. p value was obtained with Mann-Whitney *U* test. B) Reticulocyte counts and IL-8 plasma levels were negatively correlated in SCA patients with splenomegaly+. B) Reticulocyte counts and IL-8 plasma levels were not correlated in SCA patients with splenomegaly-. Data were compared using the Spearman correlation rank test.

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Supplementary Table 1. Baseline laboratory determinations of SCA patients.

Laboratory parameters	**HbSS (N = 43)**
	Mean ± SD
Sex, % of females	20 (46.5%)
Age, years	14.8 ± 3.2
Hemolysis markers	
RBC, 10^6/mL	2.66 ± 0.45
Hemoglobin, g/dL	8.37 ± 0.98
Hematocrit, %	24.87 ± 3.29
MCV, Fl	94.17 ± 11.78
MCH, ρg	31.84 ± 3.99
MCHC, g/dL	33.81 ± 1.08
Reticulocyte count, %	5.14 ± 2.49
RDW, %	22.81 ± 3.27
Total bilirubin, mg/dL	3.05 ± 1.65
Direct bilirubin, mg/dL	0.39 ± 0.15
Indirect bilirubin, mg/dL	2.66 ± 1.62
LDH, U/L	1158 ± 385.8
AST, U/L	47.19 ± 18.32
Hb pattern	
HbS, %	81.30 ± 11.09
HbF, %	8.69 ± 5.04
Leukocytes	
WBC /mL	11647 ± 2966
Neutrophils /mL	5764 ± 2538
Monocytes /mL	1108 ± 536.1
Eosinophils /mL	516.7 ± 602.0
Basophil /mL	119.3 ± 126.1
Lymphocytes /mL	4057 ± 1299
Platelets	
Platelet count, x10^3/mL	424.7 ± 144.6
Inflammatory markers	
CRP, mg/L	6.36 ± 9.85
AAT, mg/dL	86.51 ± 50.42
IL-8, pg/mL	4.44 ± 2.69

RBC: red blood cells; MCV: mean cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; LDH: lactate dehydrogenase; HbS: hemoglobin S; HbF: fetal hemoglobin; WBC: white blood cell; AST: aspartate amino-transferase; CRP: C-reactive protein; AAT: Alpha-1 antitrypsin; IL-8: interleukin-8.

6.4 MANUSCRITO 4

Título: Hydroxyurea alters circulating monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients

Autores: Caroline C. da Guarda, Paulo S. M. Silveira-Mattos, Sètondji C. M. A. Yahouédéhou, Rayra P. Santiago, Milena M. Aleluia, Camylla V. B. Figueiredo, Luciana M. Fiuza, Suellen P. Carvalho, Rodrigo M. Oliveira, Valma M. L. Nascimento, Nívea F. Luz, Valéria M. Borges, Bruno B. Andrade e Marilda S. Gonçalves.

Situação: Publicado no periódico *Scientific Reports*

Objetivos: Avaliar o perfil de monócitos no sangue periférico de pacientes com anemia falciforme, as citocinas produzidas por estas células e a influência do tratamento com hidroxiuréia em subtipos desse leucócito.

Principais resultados: pacientes em uso de HU tiveram contagem diminuída de monócitos em relação aos que não estavam em tratamento. Além disso, a HU reduziu a contagem de monócitos clássicos e aumentou a contagem de monócitos não clássicos. Entre as citocinas, observou-se redução na quantidade de células produtoras de TNF-α, IL-1β e IL-6; a HU não exerceu redução na produção de IL-8. A produção de fator tecidual (FT) também foi reduzida em função do tratamento com HU; no entanto, foi possível verificar a associação entre a porcentagem de monócitos que produziam FT e a ocorrência de vaso-oclusão. A polifuncionalidade dos monócitos também foi avaliada, e foi identificado que as células que eram positivas para FT também produziam a maior diversidade de citocinas. Tal polifuncionalidade das células também foi reduzida em consequência do tratamento com HU. Também foi verificado que os monócitos clássicos eram a fonte das citocinas e do FT expressos.

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Hydroxyurea alters circulating monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients

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Sickle cell anemia (SCA) is a hemolytic disease in which vaso-occlusion is an important pathophysiological mechanism. The treatment is based on hydroxyurea (HU), which decreases leukocyte counts and increases fetal hemoglobin synthesis. Different cell types are thought to contribute to vaso-occlusion. Nevertheless, the role of monocytes subsets remains unclear. We investigated frequencies of monocytes subsets in blood and their response to HU therapy, testing their ability to express pro-inflammatory molecules and tissue factor (TF). We identified major changes in monocyte subsets, with classical monocytes ($CD14^{++}CD16^{-}$) appearing highly frequent in who were not taking HU, whereas those with patrolling phenotype ($CD14^{dim}CD16^{+}$) were enriched in individuals undergoing therapy. Additionally, HU decreased the production of TNF-α, IL1-β, IL-6, IL-8 as well as TF by the LPS-activated monocytes. Likewise, frequency of TF-expressing monocytes is increased in patients with previous vaso-occlusion. Moreover, activated monocytes expressing TF produced several pro-inflammatory cytokines simultaneously. Such polyfunctional capacity was dramatically dampened by HU therapy. The frequency of classical monocytes subset was positively correlated with percentage cytokine producing cells upon LPS stimulation. These findings suggest that classical monocytes are the subset responsible for multiple pro-inflammatory cytokine production and possibly drive inflammation and vaso-occlusion in SCA which is damped by HU.

Sickle cell anemia (SCA) is a genetic disease associated with important alterations of morphology and function of red blood cells (RBC) which cause a wide range of clinical manifestations linked to vascular injury and coagulation abnormalities[1]. The SCA is characterized by homozygosity of the hemoglobin S (HbS), and patients with this disease exhibit the most severe clinical forms[1]. Of note, polymerization of HbS triggers biochemical and morphological changes in sickle erythrocytes, which interact with other erythrocytes, as well as with reticulocytes, leukocytes, platelets and endothelial cells leading to vaso-occlusive events (VOE)[1,2], which is the main pathophysiological mechanism underlying SCA. VOE is thought to be caused at least by three components: (i) activation of endothelial cells and leukocytes due to adherence of sickle erythrocytes; (ii) nitric oxide (NO) consumption by arginase and free hemoglobin as result of intravascular hemolysis; (iii) activation of coagulation cascades due to activation of endothelium and leukocytes, which drive blood flow obstruction and eventually

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VOE[1,3]. Understanding the mechanisms driving susceptibility to VOE is critical to develop optimization of clinical management and development of new therapeutic approaches of SCA patients.

Monocytes play an important role in innate immune responses. These cells originate from a myeloid progenitor from bone marrow, circulate in peripheral blood for approximately 2–3 days until they undergo apoptosis or migrate to the tissues where they become macrophages and maintain the innate immune surveillance[4,5]. They represent a very versatile leukocyte population which is responsible for a wide range of activities involved in immune defense against pathogens, maintenance of immune tolerance as well as of homeostasis[6]. Human monocyte subsets are characterized based on dichotomous expression of the surface markers CD14 and CD16 in classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical or patrolling ($CD14^{dim}CD16^{+}$) monocytes[7]. Such categorization is not stable and it has been shown that monocytes can turn from one subset to another depending on the microenvironment[8]. The diverse monocyte subsets exhibit distinct functions that can range from highly pro-inflammatory to immunosuppressant activities[9]. The involvement of monocytes subsets in the pathogenesis of several pathological scenarios has been evaluated, ranging from infectious diseases such as HIV infection[10] and tuberculosis[11] to inflammatory diseases such as atherosclerosis[12] and myocardial infarction[13].

In SCA, activated monocytes were shown to be associated with vascular dysfunction through different mechanisms. During vaso-occlusive crisis, monocytes activate endothelium by inducing nuclear factor-kappa B (NF-κB) translocation[14]. In addition, the direct contact with endothelial cells triggers upregulation of genes encoding adhesion molecules and cytokines[15], aside from production of lipid mediators, adhesion molecules, and coagulation factors[2,16], which may contribute to VOE. Importantly, increased levels of pro-inflammatory cytokines in SCA seem to be a critical factor contributing to onset of VOE. Elevated serum levels of TNF, IL-1β, IL-6 and IL-8 in SCA patients are correlated with endothelial cell activation, and increased cell expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), as well as of soluble forms of these molecules[17–19].

Moreover, monocytes are a main source of tissue factor (TF)[20], a critical molecule involved in activation of the extrinsic coagulation cascade leading to thrombin generation[20,21]. In a recent study, we have demonstrated that TF-expressing monocytes are in the epicenter of chronic inflammation and persistent activation of coagulation in patients living with HIV[10]. These cells produce multiple pro-inflammatory cytokines and are related to increased cardiovascular risk in HIV infection[10]. In SCA, expansion of monocytes producing TF has been reported during VOE[22]. The exact mechanisms by which TF-expressing monocytes may drive VOE and/or cardiovascular complications of SCA patients are not completely described.

Pharmacological treatment of SCA patients with severe clinical profile is based on hydroxyurea (HU) therapy, which has been associated to beneficial effects on the microvasculature and decreased occurrence of VOE and other clinical complications[23]. This drug exhibits cytostatic properties through the inhibition of ribonucleotide reductase, which stops cell division. Moreover, HU decreases neutrophil, monocyte and reticulocyte counts in peripheral blood, as well as the expression of adhesion molecules and cytokines; while increasing synthesis of fetal hemoglobin (HbF)[24]. Considering the intricate mechanisms related to SCA pathogenesis, we aimed to investigate in detail the effect of HU therapy on circulating monocytes subsets and on their ability to express TF as well as pro-inflammatory cytokines upon activation in SCA patients. Furthermore, we tested association between monocyte activation phenotypes and occurrence of VOE.

Our findings indicate that HU therapy induces substantial changes in frequency of monocyte subsets as well as in their capacity to promote inflammation and coagulation, which was associated to occurrence of VOE in SCA. Collectively, our data suggest that HU treatment modulate the inflammatory response driven by the monocytes.

Results

Impact of hydroxyurea therapy on laboratory parameters and clinical manifestations. The groups of participants were similar with regard to age and gender (Table S1). HU therapy was associated with improvement of most of biochemical and hematological parameters, including increases in hemoglobin levels and values of hematocrit, as well as reduction of LDH and AST concentrations. In addition, we observed a 2-fold increase in HbF levels and decrease of HbS levels (Table S1). HU use was also associated with decreased number of VOE, but no other change in clinical manifestations was noted in this specific study population (Table S2). The two patients undergoing HU therapy experienced one episode of VOE six months prior to blood drawn and referred HU use for the last 6 years.

Characterization of monocytes subsets of SCA patients under hydroxyurea therapy. Monocyte counts were decreased in SCA patients undergoing HU therapy (Fig. 1A). We next performed multicolor flow cytometry assays to better define the effects of HU treatment on monocyte subsets. The experiments revealed that HU decreased frequency of $CD14^{++}CD16^{-}$ monocytes, while $CD14^{dim}CD16^{+}$ were increased compared with that of patients not undergoing HU therapy (Fig. 1B,C). No statistical significance was found in frequency of monocytes subsets expressing both $CD14^{+}$ $CD16^{+}$ between individuals undertaking or not HU. Altogether these results suggest that HU induces substantial changes in monocytes subtypes in peripheral blood.

Modulation of cytokine production by monocytes driven by hydroxyurea. We tested the effect of HU on cytokine production by monocytes. In unstimulated conditions, frequencies of monocytes expressing TNF-α, IL-1β or IL-6 were similar between the groups of patients taking or not HU (Fig. 2). Nevertheless, monocytes producing IL-8 were significantly expanded in patients not undergoing HU therapy (Fig. 2). Upon LPS challenge *in vitro*, monocytes were able to increase the production of TNF-α, IL-1β, IL-6 and IL-8 independent of the clinical group (Fig. 2). Importantly, HU use was associated with decreased capacity to produce TNF-α, IL-1β or IL-6 relative to that in patients who were not under HU therapy (Fig. 2). Production of IL-8 was not affected

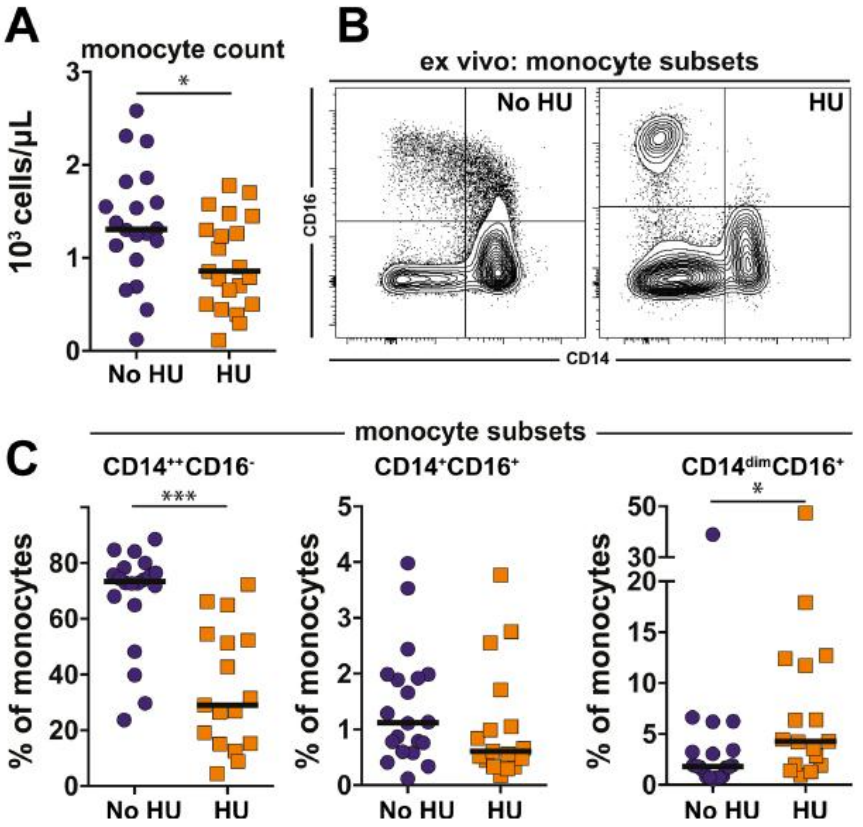

Figure 1. Hydroxyurea therapy induces major changes in peripheral blood monocyte subsets in patients with sickle cell anemia. (**A**) Total monocyte counts in blood examined by clinical cell counter in peripheral blood were compared between sickle cell anemia patients undertaking (n = 17) or not (n = 20) hydroxyurea using the Mann-Whitney *U* test. *p < 0.05. (**B**) Representative FACS plot of monocyte subsets examined by flow cytometry in PBMC. Overall gating strategy is shown in Supplementary Fig. 1. (**C**) Frequencies of indicated monocyte subsets PBMC between the study groups were compared using the Mann-Whitney *U* test. (HU group n = 17 and no HU group n = 20). *p < 0.05, ***p < 0.0001.

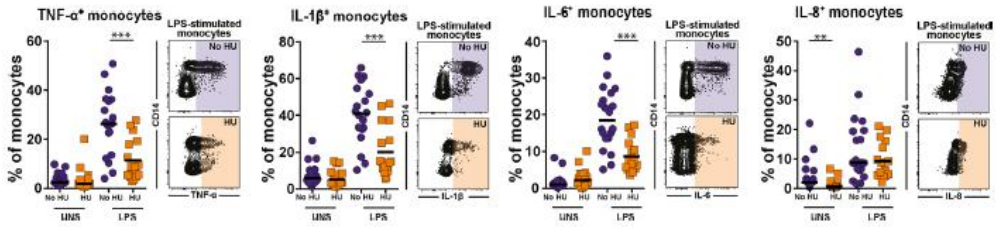

Figure 2. Hydroxyurea therapy negatively impacts production of pro-inflammatory cytokines of monocytes in response to LPS. PBMC from sickle cell anemia patients were incubated with 100 ng/mL LPS *in vitro* and intracellular cytokine staining assay was performed to test whether hydroxyurea treatment *in vivo* induces changes in the capacity of monocytes to respond to LPS by producing TNF-α, IL-1β, IL-6 and IL-8. Data represent frequency of monocytes. HU group n = 17 and no HU group n = 20. At each experimental condition, the study groups were compared using the Mann-Whitney *U* test. *p < 0.05, ***p < 0.0001.

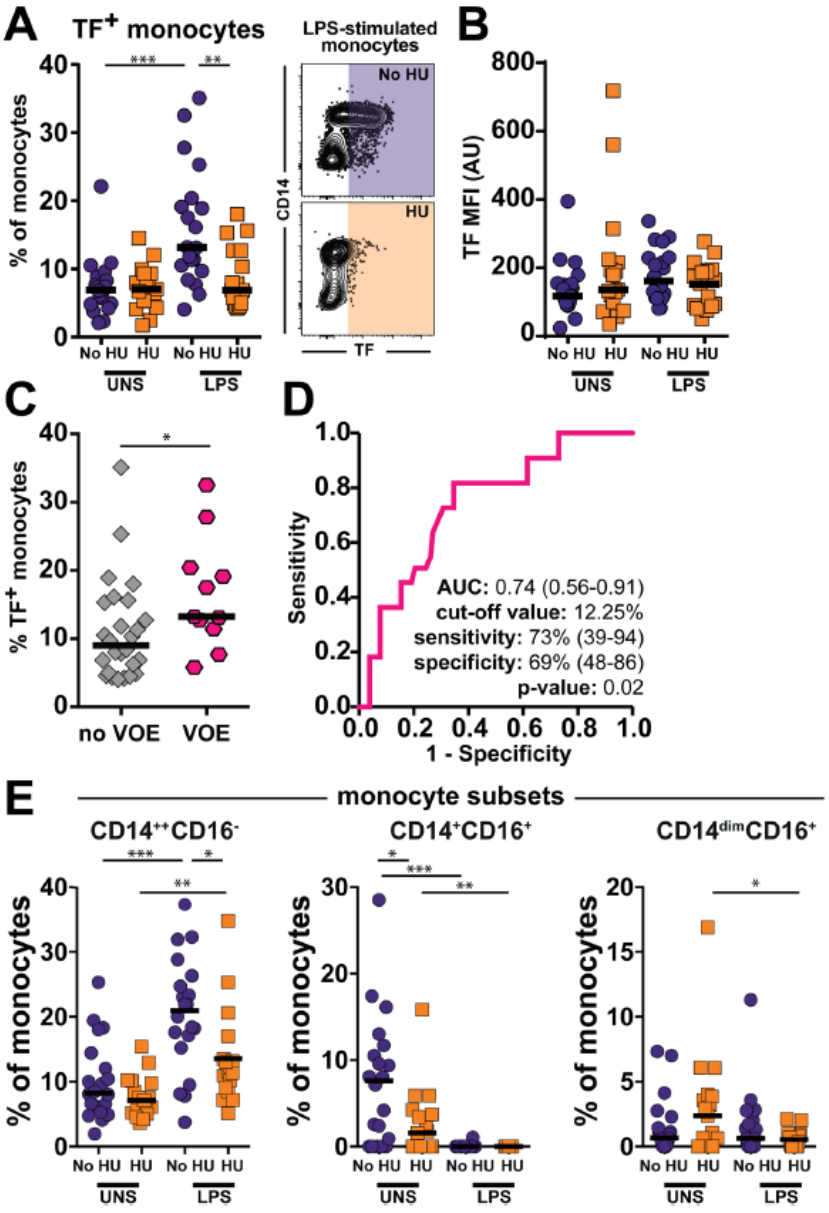

Figure 3. Sickle cell anemia-associated tissue factor production by monocytes in response to LPS is diminished by hydroxyurea treatment *in vivo*. (**A**) PBMC from sickle cell anemia patients were incubated with 100 ng/mL LPS *in vitro* and intracellular cytokine staining assay was performed to test whether hydroxyurea treatment *in vivo* induces changes in the capacity of monocytes to respond to LPS by producing tissue factor (TF). Data represent frequency of monocytes. HU group n = 17 and no HU group n = 20 At each experimental condition, the study groups were compared using the Mann-Whitney *U* test. $^{**}p < 0.01$, $^{***}p < 0.0001$. (**B**) Mean Fluorescence Intensity (MFI) of TF expression by monocytes at indicated experimental conditions is shown. No statistically significant differences were observed. HU group n = 17 and no HU group n = 20. (**C**) Frequency of TF-expressing monocytes upon LPS stimulation was compared between SCA patients presenting or not previous occurrence of vaso-occlusive events (VOE). VOE group n = 11 and no VOE n = 26. The study groups were compared using the Mann-Whitney *U* test. $^{*}p < 0.05$. (**D**) Receiver Operator Characteristics (ROC) curve analyses was employed to test whether frequency of TF-expressing monocytes after LPS stimulation could

discriminate patients with previous occurrence of VOE from those who had not, as a way to measure strength of association. AUC, area under the curve. (E) Frequencies of TF-expressing monocyte subsets was compared were compared between the indicated groups using the Mann-Whitney *U* test. HU group n = 17 and no HU group n = 20. *p < 0.05, ** p < 0.01, ***p < 0.0001.

by HU treatment. These effects of HU were not linked to differences in cell death before and after LPS stimulation (data not shown).

Effect of hydroxyurea treatment in tissue factor expression and vaso-occlusion events. Aside from producing pro-inflammatory cytokines upon stimulation, monocytes are also able to promote coagulation. Hence, we evaluated production of TF, a central molecule involved in activation of coagulation cascade, in our *in vitro* system. We found that unstimulated cells from both clinical groups displayed similar frequency of TF-expressing monocytes (Fig. 3A). Upon LPS-driven activation, percentage of TF-expressing monocytes was dramatically increased in patients not undergoing HU treatment but remained unchanged in those using HU (Fig. 3A). We did not find differences in mean fluorescence intensity values between the clinical groups and experimental conditions, which indicates that rather than interfering with magnitude protein production per cell basis, HU affected the expansion of cells expressing TF.

TF-expressing monocytes are associated to vaso-occlusive events. Additional analyses revealed that activated TF-expressing monocytes were associated with previous occurrence of VOE (Fig. 3C). ROC and C-statistics analyses were used to evaluate the association between VOE and TF-expressing monocytes. The greater the area under the ROC curve (AUC) the better the model is at discriminating between increased TF^+ monocytes frequency and patients who had VOE from those who had not. Patients who had previous history of VOE had increased frequency of TF-expressing monocytes (Fig. 3D). This finding indicated that frequency of TF-expressing monocytes may serve as a biomarker of VOE. We next, we evaluated the ability of the distinct monocyte subtypes to produce TF. Interestingly, in unstimulated cells, the HU therapy was associated with decreased frequency of TF-expressing $CD14^+CD16^+$ monocytes (Fig. 3E). However, LPS stimulation induced an increased in the frequency of TF-expressing $CD14^{++}CD16^-$, in patients not undergoing HU treatment compared with that using HU (Fig. 3E). These results uncover differential ability to induce TF expression among the distinct subsets of monocytes in SCA patients.

Capacity of monocyte to produce multiple inflammatory cytokines is affected by hydroxyurea. We next examined the capacity of monocytes to produce multiple pro-inflammatory cytokines simultaneously upon LPS-driven activation *in vitro*. Upon stimulation, TF^- monocytes from patients who were not taking HU predominantly produced IL-1β, TNF-α or both cytokines simultaneously (Fig. 4A). On the other hand, in the same clinical group, TF^+ monocytes exhibited the ability to produce more frequently IL-1β, IL-6, IL-8 and TNF-α simultaneously. Interestingly, HU therapy reduced the capacity of monocytes to produce multiple cytokines upon activation (Fig. 4A,B). Thus, the overall function profile in terms of cytokine production was different between TF^- and TF^+ and also between the two clinical groups stratified by HU therapy (Fig. 4C). The frequency of monocytes producing more than one cytokine after the LPS challenge was statistically different, and this polyfunctionality was shown to be dramatically reduced in the monocytes of patients who were taking HU (Fig. 4D).

Frequency of classical monocytes *ex vivo* and capacity to produce pro-inflammatory cytokines upon LPS stimulation *in vitro*. After assessing monocytes polyfunctionality, we sought to see whether frequency of classical monocytes in peripheral blood *ex vivo* was associated with capacity to produce pro-inflammatory cytokines upon LPS stimulation *in vitro*. Spearman correlation analyses revealed that frequency of monocytes expressing $CD14^{++}CD16^-$ in the entire study population exhibited strong positive association with percentage of monocytes expressing TNF-α^+, TF^+, IL-1β^+, IL-6^+ and IL-8^+ upon LPS stimulation (Fig. 5A). Noteworthy, in patients undergoing HU therapy reduction of $CD14^{++}CD16^-$ frequencies was proportional to reduction of cytokine production (Fig. 5A), implicating the classical monocyte subset was a potential source of such pro-inflammatory molecules. Of note, frequencies of the other monocyte subsets did not significantly correlated with the frequency of cells expressing these inflammatory mediators (Fig. 5B).

Discussion

Chronic inflammation and persistent activation of coagulation, with systemic involvement, are main features of SCA. This disease has a high prevalence and incidence worldwide and a very complex pathophysiology[1]. Although HU is considered the main therapeutic option for SCA, the specific mechanisms leading to improvement of clinical manifestations is not completely described. As previously described[25–27], HU therapy has been associated with improvement of hemolysis markers, increased HbF and decreased HbS levels as well as reduction of monocyte counts. Our results are in agreement with a previous study reporting that HU therapy reduced frequency of VOE and pain crisis[27].

The biological relevance of the role of monocytes in SCA has been previously demonstrated, such as involvement with VOE[14,15]. Nevertheless, details regarding monocytes subsets, activation pattern and cytokine production profile in SCA are not fully understood. Frequency of monocytes subsets identified herein are in agreement with previous characterization in healthy peripheral blood, where the majority has classical phenotype whereas around, 6.7% exhibits intermediate and 9.3% non-classic markers[7]. In contrast, a previous study has found that 75% of monocytes from patients with SCA exhibit a $CD14^+CD16^+$ pro-inflammatory phenotype[28]. Differences

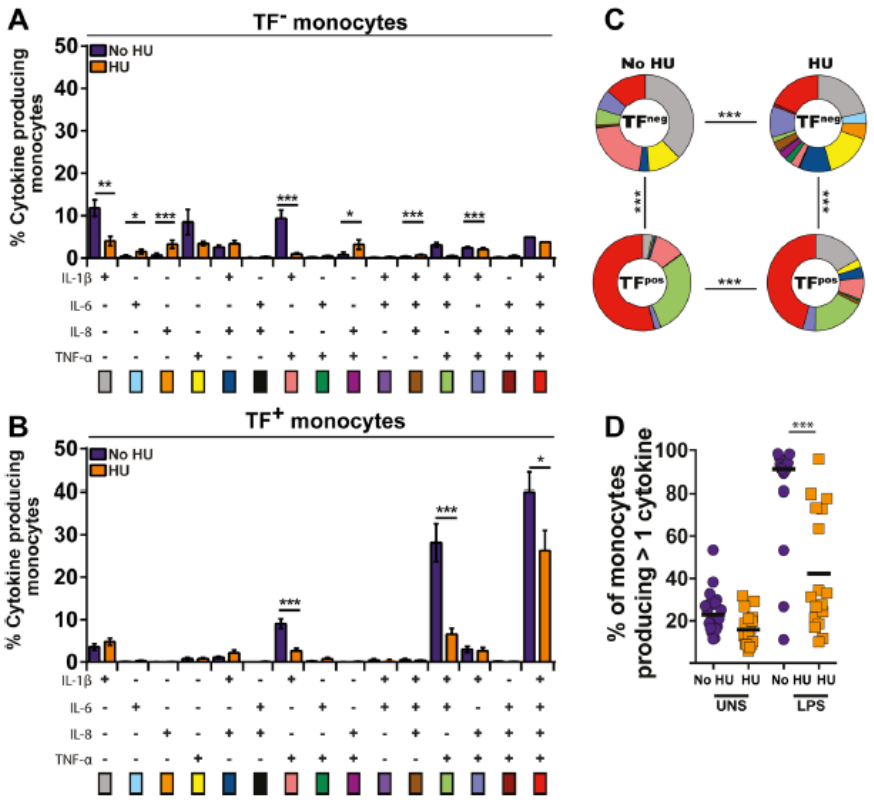

Figure 4. Hydroxyurea therapy reduces the capacity of activated monocytes to produce multiple pro-inflammatory cytokines. Polyfunctional analysis of TF^- (**A**) and TF^+ (**B**) monocytes upon LPS stimulation was performed in PBMC from SCA patients undertaking or not hydroxyurea. Data were compared using the Mann-Whitney *U* test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$. (**C**) The overall cytokine expression profiles of activated TF^- or TF^+ monocytes from SCA patients treated or not with hydroxyurea were compared using the chi-square test. $^{***}p < 0.0001$. (**D**) Frequencies of monocytes producing more than 1 cytokine *in vitro* were compared between SCA patients undertaking (n = 17) or not (n = 20) hydroxyurea using the Mann-Whitney *U* test. $^{***}p < 0.0001$.

in study populations and/or methodological in gating strategy during flow cytometry assays could at least in part explain these discrepancies. Our results demonstrated that HU therapy decreases frequency of classical monocytes ($CD14^{++}CD16^-$) while increasing percentage of non-classical monocytes ($CD14^{dim}CD16^+$). Previous studies have shown that HU increases frequency of non-classical monocytes[29] although the activation status of this subset has not been evaluated. We hypothesize that HU may directly induce differentiation of classical monocytes into non-classical/patrolling phenotype by increasing CD16 expression. Future studies are warranted to answer this question.

The specific pathways driving monocyte activation in SCA are not entirely elucidated. Previous studies have suggested participation of some agonists of toll-like receptor 4 (TLR4), such as free heme[30], high mobility group box 1 (HMGB1)[31] and heparan sulfate[32]. It is already known that monocyte activation through TLR4 leads to increased production of TNF-α[33] which can amplify TF and VCAM-1 expression in endothelial cells[33]. Our experiments demonstrated that upon LPS challenge, monocytes from SCA individuals who were not under HU therapy exhibit increased expression of TNF-α, IL-1β and IL-6 compared to that from those who were taking the drug. Furthermore, unstimulated monocytes from SCA individuals who were not under HU therapy already exhibited increased expression of IL-8. Therefore, the production of pro-inflammatory cytokines seems to be strongly modulated by HU[34]. Several studies include monocyte-activating molecules (such as LPS, TNF-α) in order to increase the responsiveness of the cells and to emphasize an activated phenotype[7,35]. Monocytes were obtained from patients in steady-state (in absence of inflammatory crisis), thus we decided to challenge the cells with LPS, in order to increase cytokine and TF production and to mimic an activation process. We found that monocytes from patients treated with HU produced less cytokine, which allows us to suggest that although LPS is able to activate the cells; their response is damped by the HU therapy. It has been shown that HU decreases levels

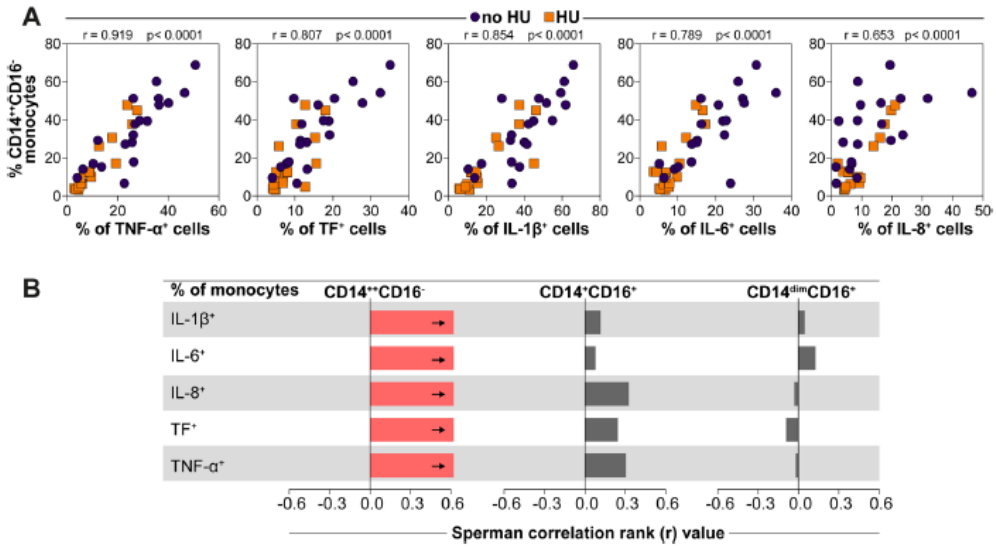

Figure 5. *Ex vivo* frequency of CD14^{++}CD16^{-} monocytes in peripheral blood directly correlates with the capacity of activated monocytes to produce pro-inflammatory cytokines. (**A**) Frequencies of classical monocytes in peripheral blood from SCA patients undertaking or not hydroxyurea were tested for correlations with frequencies of monocytes expressing indicated markers after LPS stimulation *in vitro*. Data were compared using the Spearman correlation rank test. (**B**) Spearman correlations between frequency of monocytes subsets and frequency of monocytes expressing IL-1 β, IL-6, IL-8, TF, or TNF-α in SCA patients. HU group n = 17 and no HU group n = 20. Bars represent the strength of correlation (r values). Red bar indicates statistically significant correlation ($p < 0.05$ after adjustment for multiple comparisons) while grey bars were nonsignificant.

of TNF-α, IL-8[19], IL-1β[36] and IL-6[37] in both plasma and serum of SCA individuals. More recently, it has been demonstrated that heme is able to increase IL-6 expression in SCA monocytes, since addition of iron chelator decreased its expression[38]. Considering that heme is released during hemolysis, these findings argue that intravascular hemolysis may play a pivotal role in monocyte activation in SCA. Collectively, these data indicate that HU affects not only monocyte subsets but also the ability of these cells to produce pro-inflammatory cytokines.

TF production at sites of vascular damage promotes the activation of VII factor, thrombin generation and fibrin deposition[39]. The mechanism underlying TF production and expression in both endothelial cells and monocytes has been extensively investigated in SCA[40]. A suggested that heme from intravascular hemolysis can activate endothelial cells and leading to NF-kB nuclear translocation[40]. These events promote transcription of adhesion molecules such as P-selectin and pro-inflammatory cytokines[40]. The participation of TF from monocytes and endothelial cells on VOE has also been related to microparticles production during steady state, and it is dramatically augmented during crisis[41], which can contribute to VOE. Here we found that HU therapy reduced TF expression by activated monocytes in patients undergoing treatment, corroborating with previous findings demonstrating decreased TF protein levels in plasma[42]. Our results further confirmed that TF^{+} monocytes are associated to occurrence of VOE in the study population. TF^{+} monocytes are described to be increased in SCA individuals (HbSS) compared to those with HbSC disease or controls[21]. In addition, frequency of TF^{+} monocytes has been shown to correlate with reticulocyte and leukocyte counts and soluble E-selectin levels[21]. Finally, other studies have shown that percentage of TF^{+} monocytes in peripheral blood increases during VOE[43].

Immune cells polyfunctionality, in terms of cytokine production, has been recently described in lymphocytes[44] and monocytes[10], in the context of infectious diseases. In sterile inflammatory conditions such as SCA, the polyfunctionality still remains to be evaluated. In the present study, we investigated the cytokine profile production of both TF^{+} and TF^{-} monocytes and also tested the effect of HU in production of multiple pro-inflammatory cytokines. Our data provide evidence that patients who were not under HU therapy have increased frequency of monocytes simultaneously producing TF, IL-1β, IL-6, IL-8 and TNF-α. Nonetheless, HU substantially dampened such production without affecting cell death. This result suggests that the inflammatory response promoted by activated monocytes relies on the production of multiple pro-inflammatory cytokines and is directly affected by HU therapy.

Lastly, our correlation analyses revealed that frequency of classical monocytes was positively correlated with percentage of cells producing TF as well as all the inflammatory cytokines examined in the entire study population. The role of classical monocytes on production of pro-inflammatory cytokines has been previously shown in healthy individuals[7]. In hematological diseases such as chronic myelomonocytic leukemia (CMML), it was shown that classical monocytes account for 94% of total monocytes and that this frequency could be useful to

distinguish between CMML and reactive monocytosis[45]. A model of lung ischemia-reperfusion injury has shown that classical monocytes were mobilized from the spleen and they also mediated neutrophil extravasation for the sites of injury[46]. During human immunodeficiency virus (HIV) infection, classical monocytes were shown to have increased capacity to promote activation of TF and to produce multiple pro-inflammatory cytokines suggesting their ability to crosstalk coagulation and inflammation[10]. Regarding sickle cell disease, previous evaluation of monocytes subsets has identified that non-classical or patrolling monocytes express low levels of TNF-α and IL-6 and they seem to be important protecting the microvasculature from VOE[35]. To our knowledge, this is the first study to determine *ex vivo* characterization of monocytes subsets and to identify their polyfunctionality in SCA. Of note, the association between TF-expressing monocytes and occurrence of VOE also highlights the importance of these cells in vascular complications linked to SCA.

In summary, our data corroborate with previous studies that show beneficial effects of HU therapy in SCA. We show that HU is associated with the improvement of laboratory parameters, to decreased frequency and activation of the classical inflammatory monocytes. Importantly, HU therapy directly dampened the polyfunctional capacity of monocytes, suggesting an overall anti-inflammatory property which the molecular mechanism still requires elucidation. Considerations regarding monocytes subsets, activation profile and cytokine production are useful to suggest novel therapeutic targets and may help to understand the inflammatory mechanism underlying SCA.

Material and Methods

Subjects. Thirty-seven pediatric SCA patients (HbSS genotype) were enrolled in the present study, eighteen (48.6%) of whom were female, all seen at the Bahia Hemotherapy and Hematology Foundation from August 2017 to December 2017. The patients had an average age of 14.16 ± 3.08 years and a median age of 14 years (interquartile range [IQR]: 12–17 years). All patients were in steady state of sickle cell anemia, characterized as the absence of acute crisis in the past three months prior to blood collection procedures. Three patients have had stroke and were under blood transfusion therapy; one patient had received transfusion 10 months before study enrollment and the other two had received 30 days before enrollment. Twenty patients were not under HU therapy while 17 patients were taking HU for at least 5 months. Prior to enrollment in the study informed consent was obtained from all individual participants. Legal guardians agreed to allow the biological sample collection procedures and signed terms of informed consent of all individuals under 18 years, while individuals older than 18 years have signed the assent form. This study received approval from the Institutional Research Board of São Rafael Hospital (protocol number: 1400535) and is in compliance with the ethical principles of the revised Declaration of Helsinki.

Clinical manifestations. At the time of enrollment, clinical data regarding the occurrence of previous clinical manifestations (e.g. VOE) were collected using a standardized questionnaire (self-reported or reported by the parents) and confirmed by the medical records. Patients or their legal guardians were asked whether they ever had or not, during their lifetime, any clinical manifestation related to SCA. Hospital admissions were defined as hospitalization for more than three days and VOE were defined as acute pain affecting any body part lasting several hours in association with swelling especially in the joints and soft tissues requiring medication. Patients with previous history of VOE presented at least one episode of VOE (ranging from 1 to 5 events) in the past six months.

Laboratory characterization. Hematological parameters were obtained using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) and hemoglobin patterns were confirmed by high-performance liquid chromatography employing an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA). Biochemical parameters, including lipid profile, total bilirubin and fractions, lactate dehydrogenase, iron, hepatic metabolism and renal profile were determined using an automated A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain). Ferritin levels were determined using Access 2 Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). C-reactive protein and alpha-1 antitrypsin levels were measured using IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). Laboratory parameters were analyzed at the Clinical Analyses Laboratory of the College of Pharmaceutical Sciences (Universidade Federal da Bahia).

***Ex vivo* monocyte phenotyping by flow cytometry.** Fresh peripheral blood mononuclear cells (PBMC) were obtained from SCA patients' blood samples collected with heparin, through gradient centrifugation on Ficoll Paque Plus (Gibco, GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) at room temperature. Isolated PBMC was cryopreserved in 90% of fetal bovine serum (FBS, Gibco, GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) and 10% of DMSO (Sigma, St. Louis, MO, USA) until flow cytometry assay. All the samples were processed within one hour after collection. PBMC were thawed and resuspended in RPMI 1640 supplemented with 10% FBS at 10^6 cells per well in 96-well plates. Cells were washed and resuspended in complete media with Brefeldin-A (Biolegend, San Diego, California, USA) and Monensin (Biolegend, San Diego, California, USA), two molecules capable to stop Golgi apparatus and vesicles secretion[47,48], in order to block cytokine secretion and stimulated with 100 ng/mL of LPS, a well-known TLR4 agonist in order to increase cytokine and TF expression (Sigma, St. Louis, MO, USA) for 6 hours at 37 °C in 5% CO2. Following stimulation, extracellular staining of phenotypic markers was performed. Monocyte immunophenotyping was carried out by detection of CD14 (Qdot 605), CD16 (PE-Cy7), HLA-DR (APC-Cy7) on cell surface. Several lineage markers including CD2, CD3, CD19, CD20, CD56 (Pacific Blue) were used to exclude other cells aside from monocytes of the analyses (see flow cytometry example plots in Supplementary Fig. 1). Dead cells and debris were also excluded by using Aqua fluorescent reactive Live/Dead dye (ThermoFisher Scientific, Waltham, MA, USA). Based on CD14 and CD16 surface expression, three monocyte subsets were examined: classical/inflammatory ($CD14^{++}CD16^{-}$),

intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) monocytes. To determine monocyte functionality, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set from eBioscience (ThermoFisher) and intracellular staining was performed detecting TNF-α (PerCP-Cy5.5), TF (APC), IL-8 (FITC), IL-1β (PE) and IL-6 (AF-700). Our results of flow cytometry assay are described as percentage of positive cells among $HLADR^{+}DUMP^{-}$ cells (which in the present investigation are denominated "monocytes", as described in overall gating strategy in the Fig. S1), per a total of 10^6 PBMC/well for each experiment. Description of antibody clones, conjugated fluorochromes, catalog numbers and dilutions used is shown in Table S3. Antibodies dilutions were carried out according to each manufacturer's instructions and validated in titration experiments. Acquisition of the stained cells was performed using a BD LSRFortessa™ cell analyzer (BD Bioscience, San Jose, CA, USA) and Software FlowJo, LLC (BD Bioscience, San Jose, CA, USA) was used to analyze the data.

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM, Armonk, New York, USA), JMP software v.12 (SAS Institute, Cary, North Carolina, USA) and GraphPad Prism version 6.0 (Graphpad Software, San Diego, California, USA), which was also used to assemble the graphs. Baseline values of selected variables are expressed as means with their respective standard variation. The Shapiro-Wilk test was used to determine variable distribution. The Mann-Whitney *U* test and independent t-test were used to compare the groups according to the normality of the distribution for each variable. Fisher's exact test was used to compare frequency of clinical manifestations as well as sex distribution between the patients groups. Spearman correlation rank analysis was performed to test correlations between frequency of monocyte subsets and cytokine production profiles. Results were adjusted for multiple comparisons using Bonferroni's method. Receiver Operator Characteristics (ROC) curve analysis was used to test the association between frequency of TF-expressing monocytes in blood and occurrence of VOE. Pearson's qui-square test was employed to compare the polyfunctionality profiles of monocytes[10]. All analyses were pre-specified. P values < 0.05, after correction for multiple measurements using the Holm-Bonferroni method were considered statistically significant.

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Author Contributions

C.C.G. and P.S.M.S.M. designed and performed all the experiments and co-wrote the manuscript. C.C.G., S.C.M.A.Y., R.P.S., M.M.A., C.V.B.F., L.M.F., S.P.C. and R.M.O. collected all the samples and performed the laboratory characterization. V.M.L.N. assisted the patients. N.F.L., V.M.B., B.B.A. and M.S.G. conceptualized and supervised the study and critically revised the manuscript.

Additional Information

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Supplementary Material

Hydroxyurea alters circulating monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients

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Table S1. Association of laboratory parameters in SCA patients taking or not HU

Characteristics	**No HU (N = 20)**	**HU (N = 17)**	***P* value**
	Mean ± SD	Mean ± SD	
Sex			
Females	11 (55.0%)	7 (41.2%)	-
Male	9 (45.0%)	10 (58.8%)	
Age, years	15 ± 3	13 ± 2	-
Hemolysis markers			
RBC, 10^6/mL	2.62 ± 0.29	2.56 ± 0.38	0.612
Hemoglobin, g/dL	8.15 ± 0.72	8.80 ± 1.08	**0.045**[#]
Hematocrit, %	23.85 ± 2.21	26.24 ± 3.34	**0.013**
MCV, fL	88.54 ± 7.88	101.19 ± 8.11	**0.000**
MCH, ρg	30.55 ± 2.61	34.57 ± 3.64	**0.000**
MCHC, g/dL	34.51 ± 0.83	33.54 ± 0.74	**0.001**
RDW, %	24.02 ± 0.97	19.71 ± 0.53	**0.001**
Reticulocyte count, %	6.93 ± 2.50	5.39 ± 2.83	0.089
Total bilirubin, mg/dL	3.25 ± 1.09	2.72 ± 1.51	0.231
Direct bilirubin, mg/dL	0.44 ± 0.19	0.43 ± 0.14	0.895
Indirect bilirubin, mg/dL	2.81 ± 1.09	2.29 ± 1.50	0.236
LDH, U/L	1304.05 ± 361.27	984.59 ± 296.83	**0.007**
Hb pattern			
HbS, %	87.42 ± 9.26	82.85 ± 5.27	**0.014**[#]
HbF, %	6.78 ± 5.13	12.86 ± 5.86	**0.003**
Leukocytes			
WBC /mL	12499.00 ± 2798.02	11371.76 ± 3716.55	0.300
Neutrophils /mL	6180.90 ± 2590.30	5849.18 ± 2916.16	0.537[#]
Monocytes /mL	1360.55 ± 622.39	966.29 ± 516.90	**0.046**
Eosinophils /mL	490.42 ± 245.36	343.65 ± 230.94	0.074
Basophil /mL	91.65 ± 77.35	92,06 ± 118.36	0.497[#]

Lymphocytes /mL	4087.25 ± 1133.09	3939.82 ± 988.26	0.679
Platelets			
Platelet count, x10^3/mL	440.25 ± 69.04	413.00 ± 134.63	0.458
MPV, fL	8.16 ± 0.84	7.94 ± 1.00	0.490
PCT, %	0.35 ± 0.06	0.30 ± 0.08	0.067
PDW, %	16.24 ± 0.37	15.98 ± 0.17	**0.026**
Lipid metabolism			
Total Cholesterol, mg/dL	125.37 ± 25.83	115.29 ± 23.67	0.233
HDL-C, mg/dL	34.95 ± 8.67	38.12 ± 8.14	0.268
LDL-C, mg/dL	70.35 ± 19.67	59.67 ± 19.20	0.081[#]
VLDL-C, mg/dL	20.06 ± 8.57	17.50 ± 5.22	0.283
Triglycerides, mg/dL	100.32 ± 42.89	87.53 ± 26.13	0.283
Iron metabolism			
Iron, mcg/dL	95.32 ± 31.24	132.18 ± 60.47	**0.038**[#]
Ferritin, ηg/mL	259.7 ± 490.8	673.4 ± 468.9	**0.001**[#]
Renal profile			
Urea, mg/dL	16.63 ± 4.94	20.21 ± 7.56	0.285[#]
Creatinine, mg/dL	0.41 ± 0.16	0.47 ± 0.11	0.212
Uric Acid, mg/dL	3.96 ± 1.17	3.66 ± 1.21	0.464
Hepatic profile			
AST, U/L	52.95 ± 17.22	37.53 ± 11.95	**0.004**
ALT, U/L	21.26 ± 12.06	15.59 ± 7.04	0.271[#]
GGT, U/L	21.53 ± 10.32	19.88 ± 9.08	0.683[#]
Alkaline phosphatase, U/L	145.00 ± 88.64	120.76 ± 43.26	0.573[#]
Inflammatory profile			
CRP, mg/L	4.95 ± 5.65	4.65 ± 3.55	0.845[#]
AAT, mg/dL	86.77 ± 42.79	68.43 ± 45.30	0.267[#]

RBC: red blood cells; MCV: mean cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; LDH: lactate dehydrogenase; HbS: hemoglobin S; HbF: fetal hemoglobin; WBC: white blood cell; MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet distribution width; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; AST: aspartate amino-transferase; ALT: alanine amino-transferase; GGT: gamma glutamyl-transferase; CRP: C-reactive protein; AAT: Alpha-1 antitrypsin. Bold values indicate significance at $p<0.05$; p-value obtained using t-test. [#]p-value obtained using Mann-Whitney *U* test.

Table S2. Frequency of clinical manifestations among SCA patients taking or not HU

Clinical manifestation	No HU (N = 20)	HU (N = 17)	*P* value
Previous hospital admissions	16 (80%)	17 (100%)	0.109
Pneumonia	12 (60%)	9 (52.9%)	0.746
Splenomegaly	7 (35%)	9 (52.9%)	0.331
Stroke	2 (10%)	1 (5.8%)	1.00
Vaso-occlusive events	9 (45%)	2 (11.7%)	**0.036**
Infections	15 (75%)	14 (82.3%)	0.701
Priapism	2 (10%)	0 (0%)	0.471
Leg ulcer	1 (5%)	1 (5.8%)	1.00
Acute chest syndrome	5 (25%)	4 (23.5%)	1.00
Cholelithiasis	5 (25%)	3 (17.6%)	0.701
Blood transfusion	3 (15%)	2 (11.7%)	1.00

Data were compared using the Fisher's exact test. Significant p values are shown in bold type font.

Table S3. List of antibodies used in the flow cytometry experiments.

Marker	Clone	Company	Catalog	Dilution
CD2	RPA-2.10	eBioscience	48-0029-42	1:200
CD3	UCHT1	eBioscience	48-0038-42	1:25
CD14	M5E2	Biolegend	301834	1:100
CD16	3G8	Biolegend	302015	1:100
CD19	HIB19	eBioscience	48-0199-42	1:200
CD20	2H7	eBioscience	48-0209-42	1:20
CD56	B159	BD Biosciences	560360	1:20
CD142 (TF)	HTF-1	eBioscience	17-1429-42	1:200
HLA-DR	L243	BD Biosciences	641393	1:200
IL-1β	JK1B-1	Biolegend	508206	1:25
IL-6	MQ2-13A5	eBioscience	56-7069-42	1:25
TNF-α	MAb11	Biolegend	502924	1:200
IL-8	AS14	BD Biosciences	340509	1:25

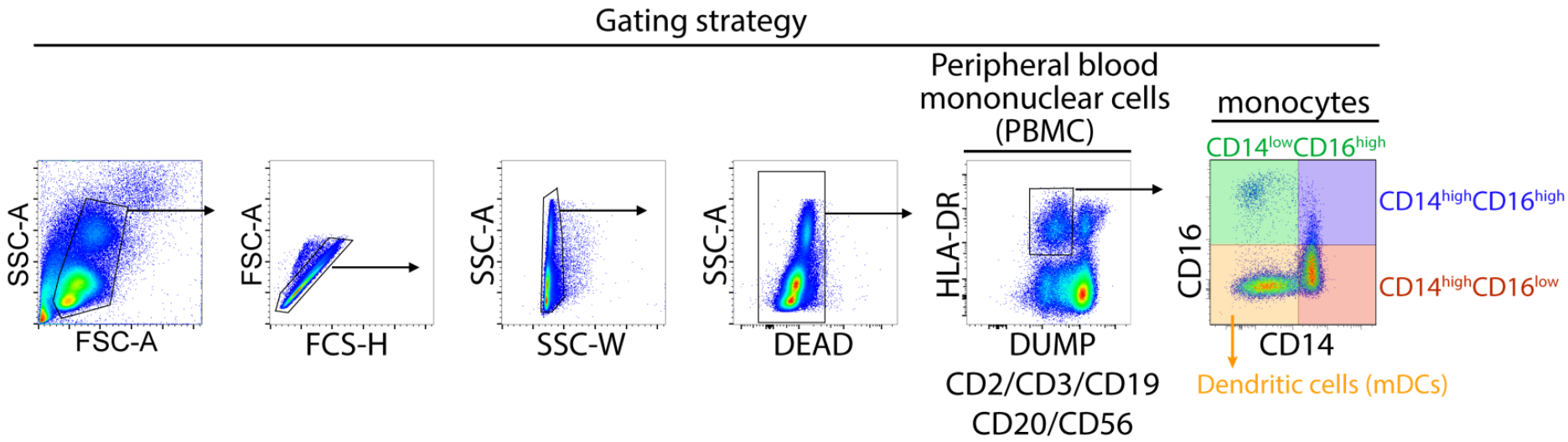

Figure S1. Overall gating strategy used for flow cytometry assays to evaluate monocytes in peripheral blood.

7 DISCUSSÃO

A DF é caracterizada por episódios vaso-oclusivos frequentes e por eventos de hemólise crônica, que dão origem ao quadro inflamatório crônico e sistêmico apresentado pelos pacientes (SUNDD *et al.*, 2018). Embora os mecanismos genéticos que envolvem a patogênese da doença já estejam esclarecidos, os pacientes desenvolvem complicações clínicas bastante heterogêneas (POWARS *et al.*, 2005; SUNDD *et al.*, 2018). O presente estudo investigou as diferenças laboratoriais entre indivíduos com AF e com HbSC, o perfil lipídico de pacientes com AF e a associação com manifestações clínicas; o papel da IL-8 na AF e, por fim, os subtipos de monócitos e as citocinas produzidas por essas células sob a influência da terapia pela HU.

O primeiro manuscrito buscou identificar as características laboratoriais e manifestações clínicas dos pacientes com AF e com HbSC. As características laboratoriais dos pacientes com AF são consistentes com outros estudos, demonstrando hemólise, leucocitose e inflamação sistêmica (WEST *et al.*, 1992; PLATT *et al.*, 1994; ALELUIA, *et al.*, 2017). A leucocitose com contagem total de leucócitos acima de 15 000 células por milímetro cúbico, bem como concentrações reduzidas de HbF, estão associadas ao risco elevado de morte precoce (PLATT *et al.*, 1994). A hemólise intravascular também está associada à gravidade dos prognósticos clínicos e também com a resposta inflamatória sistêmica apresentada pelos pacientes (NOURAIE *et al.*, 2013). Outros estudos já demonstraram que as proteínas de fase aguda AAT e PCR permanecem elevadas nos pacientes com DF mesmo no estado-estável da doença (BOURANTAS *et al.*, 1998; CARVALHO, *et al.*, 2017). A investigação laboratorial dos pacientes com HbSC revelou níveis aumentados de lipídios, creatinina e ácido úrico e níveis reduzidos de NO, o que está de acordo com outros estudos envolvendo pacientes HbSC (REES *et al.*, 2015; ALELUIA*, et al.*, 2017; SANTIAGO, *et al.*, 2017).

Os eventos clínicos na DF estão, principalmente, relacionados ao mecanismo fisiopatológico vaso-oclusivo. Todas as manifestações clínicas avaliadas foram mais prevalentes no grupo de pacientes com AF quando comparados aos indivíduos HbSC. Um estudo de coorte que acompanhou os pacientes por um

período de dez anos também identificou que o início das complicações ocorria em indivíduos mais jovens e com AF quando comparados aos HbSC, especialmente no que se refere a ocorrência de crises de dor e STA (GILL *et al.*, 1995). Na coorte de indivíduos investigados no presente estudo, verificou-se que a causa mais comum de hospitalização era as crises de dor, o que também é observado em outras populações, nas quais os episódios de dor aguda representam 94,6% dos casos de internamentos (BALLAS e LUSARDI, 2005). Biomarcadores de hemólise estiveram associados às crises de dor entre os pacientes com DF. Concentrações elevadas de RDW, contagem elevada de reticulócitos e contagem reduzida de plaquetas também estiveram associadas a diferentes fases das crises de dor na DF (BALLAS *et al.*, 2012). Considerando que a hemólise também desempenha papel importante na vaso-oclusão, a associação entre ambos os mecanismos fisiopatológicos a uma tríade de fatores (vaso-oclusão, inflamação e nocicepção) pode contribuir para o início da crise aguda de dor (BALLAS *et al.*, 2012).

O grupo de pacientes participantes do presente estudo e que possuía histórico de vaso-oclusão apresentou parâmetros laboratoriais relacionados, principalmente, à anemia e inflamação sistêmica. Concentrações elevadas de AAT também já foram associadas a infecções, colelitíase e transfusão sanguínea na DF (CARVALHO, *et al.*, 2017); adicionalmente, nos indivíduos com AF, as concentrações de PCR foram crescentes a medida que a gravidade da doença também aumentava (AKINLADE *et al.*, 2013). Além de contribuir para a ocorrência da vaso-oclusão, a hemólise contribui para a produção elevada de bilirrubina, que pode acumular no fígado formando cálculos de bilirrubinato de cálcio. A colelitíase é frequente na DF (MARTINS *et al.*, 2017).

A investigação laboratorial dos pacientes com DF também demonstrou que os parâmetros classicamente investigados para monitorar o curso clínico da doença são classificados em grupos (*clusters*) diferentes entre os diferentes genótipos da doença. Observamos a presença de dois *clusters* entre os pacientes com HbSC com biomarcadores de hemólise e disfunção endotelial, além de outros dois *clusters* com biomarcadores de acompanhamento hematológico e inflamação sistêmica. Entre os pacientes com AF observamos a classificação de um cluster contendo marcadores de hemólise, o segundo cluster com marcadores de inflamação e metabolismo dos lipídios e o terceiro

cluster com marcadores de anemia e ferro. O agrupamento entre os marcadores laboratoriais está de acordo com os fenótipos que são descritos na literatura para AF e para HbSC (GILL *et al.*, 1995; REES e GIBSON, 2012; NOURAIE *et al.*, 2013; ALELUIA, *et al.*, 2017; DU *et al.*, 2018). A classificação diferente dos mesmos parâmetros laboratoriais nos grupos HbSC e AF sugere que de fato, a mesma medida (ou o mesmo parâmetro laboratorial) obtida em um grupo de pacientes pode ter significado diferente quando os dois genótipos são comparados.

Os resultados do primeiro manuscrito corroboram os perfis clínicos e laboratoriais descritos para a AF e para a HbSC. A maior parte dos estudos com DF concentra-se apenas no grupo de pacientes com AF devido a sua maior gravidade clínica e a frequência maior dessa hemoglobinopatia. Portanto, para os estudos subsequentes, a casuística dos manuscritos foi composta por indivíduos com perfil da hemoglobinopatia HbSS. É importante observar que uma vez que os pacientes com HbSC tiveram níveis mais elevados de marcadores lipídicos quando comparados aos com AF, as determinações laboratoriais sugerem que os indivíduos com AF podem apresentar parâmetros dislipidêmicos (SEIXAS *et al.*, 2010; ZORCA *et al.*, 2010; FIGUEIREDO *et al.*, 2016; ALELUIA, *et al.*, 2017), o que foi melhor investigado no segundo manuscrito.

Conforme demonstrado por diversos estudos, os pacientes com AF apresentam alterações no perfil lipídico relacionados com parâmetros hematológicos, crises vaso-oclusivas, via do TGF-β, hipertensão pulmonar entre outras complicações (SEIXAS *et al.*, 2010; ZORCA *et al.*, 2010; AKINLADE *et al.*, 2014; FIGUEIREDO *et al.*, 2016; GUARDA *et al.*, 2017). No grupo de pacientes do presente estudo, verificou-se que os níveis de colesterol estiveram associados ao histórico de pneumonia, níveis de HbS, HbF e LDL. As complicações pulmonares na AF estão relacionadas com o comprometimento vascular e a vasoconstrição que culminam na vaso-oclusão. De forma semelhante, a frequência de pacientes com AF e histórico de pneumonia é diretamente relacionada com a frequência de STA, pois muitas vezes as apresentações clínicas são parecidas, e a STA geralmente está associada a embolia pulmonar gordurosa ou agentes infecciosos (VICHINSKY *et al.*, 2000). Em uma população diferente da deste estudo, já foi identificado

também que os níveis séricos de colesterol estiveram associados ao risco maior de hospitalização e óbito por doenças respiratórias (IRIBARREN *et al.*, 1997). Os resultados do presente estudo que descreveram a correlação positiva entre HbS e colesterol total e LDL nos pacientes com AF e histórico de pneumonia sugerem a associação dos lipídios com perfil mais grave da doença.

Foi possível verificar que os indivíduos com níveis elevados de HDL apresentaram melhores parâmetros hematológicos de hemólise, embora tenha sido observada associação com as crises de dor. Os resultados encontrados estão de acordo com achados prévios da literatura onde os níveis de HDL foram associados com contagens elevadas de hemácias e concentrações mais elevadas de hemoglobina e hematócrito (EMOKPAE e KULIYA-GWARZO, 2014). No estudo realizado por Zorca e colaboradores (2010) os autores avaliaram a associação de marcadores lipídicos e hipertensão pulmonar e também encontraram níveis de HDL negativamente correlacionados com níveis de LDH (ZORCA *et al.*, 2010); achados semelhantes ao que foi observado no grupo de pacientes que compuseram o presente estudo.

Estes resultados reforçam o possível papel do HDL na AF em associação com hemólise e disfunção endotelial. Concentrações mais elevadas de hematócrito têm sido associadas a reologia do sangue e a deformabilidade das hemácias, fatores que também contribuem para a vaso-oclusão e crises de dor (BARABINO *et al.*, 2010; SUNDD *et al.*, 2018). Correspondentemente, a taxa de dor aguda varia entre os pacientes; porém, tende a ser maior naqueles com concentrações maiores de hematócrito e menores de HbF (BALLAS *et al.*, 2012). Portanto, além das propriedades anti-inflamatórias e vasoprotetoras do HDL (NCEP., 2001), os resultados apresentados no presente estudo sugerem o papel importante para o HDL no ambiente vascular em associação com hemólise e anemia.

As concentrações de LDL entre os pacientes com AF investigados estiveram associadas ao histórico de úlceras de membros inferiores, pneumonia e parâmetros hematológicos (VCM e HCM). A anemia apresentada pelos indivíduos com AF está relacionada ao tempo menor de vida das hemácias (QUINN *et al.*, 2016) e hemólise intravascular (GUARDA *et al.*, 2017), que contribuem para o ambiente vascular pro-oxidante e pró-inflamatório que

culminam em disfunção endotelial (REITER *et al.*, 2002). No mesmo ambiente vascular, o LDL tem papel pró-inflamatório (GOLDSTEIN e BROWN, 2015), que também poderia contribuir com a vasculopatia da AF. Essa hipótese é sustentada pela evidência de que o LDL é suscetível a modificações oxidativas na AF, baseado na identificação de heme ligado a moléculas de LDL, o que poderia favorecer a produção de LDL oxidado (oxLDL) (VENDRAME *et al.*, 2018). Adicionalmente, vários mecanismos vasculares foram atribuídos a patogênese das úlceras de membros inferiores na AF, tais como obstrução física pelas hemácias irreversivelmente falcizadas, pouca recirculação venosa, infecções bacterianas, anemia, trombose in situ e redução na biodisponibilidade do NO (MOHAN *et al.*, 1997).

Embora neste estudo não tenham sido realizadas comparações entre concentrações elevadas de colesterol total, HDL e LDL com níveis reduzidos, a coorte de pacientes com AF investigados não apresentou hipercolesterolemia. Os achados do presente estudo estão de acordo com outros que também verificaram níveis menores de colesterol total, HDL e LDL entre indivíduos com AF quando comparados a indivíduos HbAA (SEIXAS *et al.*, 2010; ZORCA *et al.*, 2010; LALANNE-MISTRIH *et al.*, 2018). Acredita-se que a hipocolesterolemia encontrada na AF ocorre devido a maior utilização do colesterol durante a eritropoese como consequência da hemólise, apesar do mesmo resultado ser encontrado em anemias não-hemolíticas (WESTERMAN, 1975). É válido ressaltar também que o desenho do presente estudo é de corte-transversal; portanto, torna-se difícil estabelecer o papel de causa-efeito para as determinações lipídicas e as manifestações clínicas observadas; no entanto, diversas associações importantes foram encontradas e são úteis para estudos futuros.

A resposta inflamatória crônica é uma das principais características da AF influenciada por neutrófilos, plaquetas (ZHANG *et al.*, 2016), monócitos (SAFAYA *et al.*, 2012), hemólise intravascular e resposta imune inata (GUARDA *et al.*, 2017) e aumento dos mediadores pró-inflamatórios, tais como citocinas e mediadores lipídicos (CARVALHO *et al.*, 2018). Considerando a complexidade dos mecanismos envolvidos na inflamação da AF decidiu-se investigar o papel da quimiocina IL-8 nos parâmetros laboratoriais e manifestações clínicas em pacientes com AF.

Várias citocinas pró-inflamatórias são produzidas por leucócitos, células vasculares endoteliais e plaquetas, tais como IL-1β, TNF-α, IL-6, IL-8, IL-17 e TGF-β durante hemólise, oclusão microvascular ou infecção, e todas elas implicam no desenvolvimento de complicações clínicas na AF (KEIKHAEI *et al.*, 2013). A IL-8 é uma quimiocina com grande poder de quimiotaxia sobre neutrófilos e propriedades pró-inflamatórias; portanto, investigou-se os níveis plasmáticos e o polimorfismo rs4073. As frequências do polimorfismo encontradas no grupo de pacientes com AF estavam em equilíbrio de Hardy-Weinberg e de acordo com outros estudos também (HASSAN e ALZAHRANI, 2018). Os níveis plasmáticos de IL-8 foram maiores em indivíduos portadores do genótipo AA quando comparados aos genótipos TT + AT, também de acordo com a literatura (HASSAN e ALZAHRANI, 2018). Outro estudo também mostrou que pacientes com DF em crise vaso-oclusiva por motivos de infecção e desidratação tiveram níveis elevados de IL-8 quando comparados a indivíduos com DF fora da crise e indivíduos HbAA. Além disso, os níveis de IL-8 aumentaram antes da crise e reduziram cerca de 40 dias após a crise (DUITS *et al.*, 1998).

Foram encontradas também associações entre os níveis plasmáticos de IL-8 e contagens elevadas de linfócitos, níveis maiores de AAT e contagem diminuída de reticulócitos. Estudos anteriores sugerem que a IL-8 pode atuar também como fator quimiotático para linfócitos T (TAUB *et al.*, 1996). A contagem de reticulócitos é bastante útil para avaliar laboratorialmente a hemólise na AF (QUINN *et al.*, 2016), os resultados encontrados no presente estudo demonstraram que os indivíduos com níveis maiores de IL-8 apresentaram contagem menor de reticulócitos; por outro lado outros estudos não demonstraram associação da IL-8 com outros parâmetros de hemólise, tais como haptoglobina (PIERROT-GALLO *et al.*, 2015). Observou-se também que os indivíduos com níveis maiores de IL-8 também tinham níveis maiores de AAT. A associação entre IL-8 e AAT foi previamente avaliada em estudo *in vitro*, onde a quimiotaxia dos neutrófilos ocorreu de maneira dose-dependente da razão IL-8:AAT, bem como o complexo montado pela IL-8 e AAT é capaz de reorganizar o citoesqueleto do neutrófilo, modulando a quimiotaxia (BERGIN *et al.*, 2010).

O presente estudo investigou também as manifestações clínicas relacionadas aos níveis de IL-8. Os resultados mostraram que pacientes com história prévia de esplenomegalia apresentaram níveis menores de IL-8. Em estudo de corte-transversal foi observada a frequência de 9% de esplenomegalia em um total de 210 pacientes. O mesmo estudo mostrou que a presença do alelo T do polimorfismo rs4073 foi considerada fator protetor para a esplenomegalia. Por outro lado, os genótipos variantes deste polimorfismo não estiveram associados a diferentes concentrações séricas da IL-8 (CAJADO *et al.*, 2011). A esplenomegalia nas anemias hemolíticas está associada a dois mecanismos principais. Primeiro, a grande quantidade de hemólise extra e intravascular que leva a hipertrofia do sistema reticuloendotelial com maior sequestro dos eritrócitos de morfologia alterada na circulação. Segundo, a hipóxia atua como fator de "*homing*" para os eritroblastos migrarem da medula óssea para o baço, causando obstrução dos cordões esplênicos devido as células em proliferação (PAULSON *et al.*, 2011; MCKENZIE *et al.*, 2018). O presente estudo também descreveu, no grupo de pacientes com histórico prévio de esplenomegalia, a correlação negativa entre os níveis de IL-8 e a contagem de reticulócitos, o que reforça a ideia de que a hemólise está associada aos níveis de IL-8. Contudo, a relevância biológica dos níveis diminuídos de IL-8 e a ocorrência de esplenomegalia na AF ainda precisa ser elucidada.

Os indivíduos com AF apresentam contagem elevada de leucócitos, conforme identificado no manuscrito 1, o que constitui fator prognóstico importante para a gravidade clínica da doença. Entre os leucócitos, já foi associado aos monócitos o envolvimento no processo vaso-oclusivo (BELCHER *et al.*, 2000; SAFAYA *et al.*, 2012), embora os detalhes do perfil de ativação, quais citocinas são produzidas por essas células bem como a influência da HU ainda não tenham sido completamente esclarecidos.

No quarto manuscrito buscou-se caracterizar o perfil de monócitos do sangue periférico dos indivíduos com AF. Observou-se que a HU reduziu a contagem de monócitos, de acordo com o que a literatura traz (CHARACHE *et al.*, 1996; YAHOUEDEHOU *et al.*, 2018). Entre os subtipos de monócitos identificados, encontrou-se que a maior parte das células apresentavam fenótipo clássico, seguido por não-clássico e intermediário, na mesma proporção verificada em indivíduos saudáveis (BOYETTE *et al.*, 2017). Em relação ao uso de HU, os

pacientes que estavam utilizando o medicamento tiveram redução na frequência dos monócitos clássicos (representados por $CD14^{++}CD16^{-}$) e aumento na frequência dos monócitos não-clássicos (representados por $CD14^{dim}CD16^{+}$), o que também já foi demonstrado em outro estudo (FERTRIN *et al.*, 2012).

O perfil de ativação dos monócitos também constitui aspecto importante a ser avaliado. Observou-se que após o estímulo com lipopolissacarídeo (LPS), os monócitos dos indivíduos que não estavam em uso da HU aumentaram a expressão de TNF-α, IL-1β e IL-6 em comparação com aqueles que estavam sob o tratamento. Além disso, os monócitos não estimulados com LPS dos indivíduos que não tomavam HU demonstraram expressão maior de IL-8. Os resultados sugerem que a produção de citocinas pró-inflamatórias por estas células é fortemente modulada pelo uso de HU (PENKERT *et al.*, 2018). Diversos estudos já mostraram também a redução das citocinas TNF-α, IL-1β, IL-6 e IL-8 tanto no soro quanto no plasma de indivíduos com AF (LANARO *et al.*, 2009; KEIKHAEI *et al.*, 2013; BANDEIRA *et al.*, 2014).

Além da produção de citocinas, os monócitos produzem o FT, que atua como iniciador da cascata da coagulação através da via extrínseca. Na AF, a participação do FT de monócitos foi demonstrada através da análise das micropartículas liberadas por estas células, que contribuíam para a vaso-oclusão e crises de dor (SHET *et al.*, 2003). Os resultados do presente estudo mostraram que a HU também foi capaz de reduzir a expressão de FT pelos monócitos; adicionalmente, descreveu-se a associação entre os monócitos com produção de FT e o histórico de crises vaso-oclusivas apresentadas pelos pacientes. Outro estudo demonstrou que os pacientes com AF possuem frequência maior de monócitos que expressam FT quando comparados com HbSC e indivíduos controles, além de estarem correlacionados com a contagem de reticulócitos e leucócitos bem como com níveis de E-selectina solúveis (SETTY *et al.*, 2012). Já foi verificado também que o número de monócitos que expressam FT aumenta durante as crises vaso-oclusivas (SOLIMAN e RAGAB, 2015), o que reforça a associação encontrada com o histórico de vaso-oclusão.

Monócitos e linfócitos constituem tipos de leucócitos onde propriedades de polifuncionalidade já foram demonstrados no contexto de doenças infecciosas

(SCHECHTER *et al.*, 2017; HSU *et al.*, 2018). Em quadros de inflamação estéril, como a AF, essa propriedade ainda precisa ser mais bem esclarecida. Nossos resultados mostraram que os monócitos que produziam FT também produziam todas as outras citocinas pró-inflamatórias investigadas, revelando assim a polifuncionalidade destas células na AF. O tratamento com HU reduziu substancialmente a produção das citocinas pelos monócitos, sem induzir morte celular. Estes dados sugerem que a resposta inflamatória promovida pelos monócitos ativados é diretamente afetada pela terapia com HU.

Finalmente, buscou-se identificar se o perfil de monócitos polifuncionais correspondia aos monócitos clássicos. As análises mostraram que houve correlação positiva entre a frequência de monócitos clássicos e as citocinas inflamatórias expressas bem como o FT. O papel dos monócitos clássicos na produção de moléculas pró-inflamatórias foi demonstrado tanto em indivíduos saudáveis, quanto em indivíduos com doenças hematológicas, tais como leucemia mielomonocítica (BOYETTE *et al.*, 2017; SELIMOGLU-BUET *et al.*, 2017). Durante a infecção com o vírus da imunodeficiência humana (HIV), os monócitos clássicos eram capazes de aumentar a expressão de FT e também de produzir múltiplas citocinas pró-inflamatórias, sugerindo a habilidade destas células de intermediar coagulação e inflamação (SCHECHTER *et al.*, 2017). Na DF foi mostrado que os monócitos não-clássicos expressam níveis diminuídos de TNF-α e lL-6, além de serem capazes de proteger a microvasculatura da vaso-oclusão (LIU *et al.*, 2018). Acredita-se que este é o primeiro estudo a realizar a caracterização ex vivo dos subtipos de monócitos, identificar sua polifuncionalidade, bem como o efeito da HU na AF. Também é importante ressaltar a associação encontrada entre vaso-oclusão e monócitos que expressam FT, sugerindo a relevância destas células nas complicações vasculares da AF.

8 CONCLUSÕES

A DF é caracterizada pela tríade hemólise crônica, vaso-oclusão e inflamação sistêmica. A partir do primeiro manuscrito foram confirmados dados de literatura referentes a presença de alteração em marcadores laboratoriais associados a hemólise, leucocitose e inflamação nos indivíduos com AF, enquanto que nos indivíduos com HbSC as complicações laboratoriais e clínicas foram menos evidentes. No entanto, as crises de dor, como principal causa de hospitalização, estiveram presentes em ambos os genótipos da doença. No segundo manuscrito, pesquisou-se associações relevantes entre as determinações lipídicas e parâmetros laboratoriais, principalmente aqueles relacionados à hemólise e gravidade clínica da doença, tais como, as alterações nas concentrações da HbS e HbF. Da mesma forma, as manifestações clínicas identificadas no presente estudo estiveram associadas à hemólise por outros autores, reforçando o papel importante dos lipídios na patogênese da AF. Além disso, sabe-se que a resposta inflamatória presente na AF é mediada por diversos mecanismos, especialmente pelos leucócitos e pelas citocinas que estas células produzem. No terceiro manuscrito verificou-se associações entre os níveis de IL-8 e marcadores inflamatórios da doença e, principalmente, com a esplenomegalia. Estes resultados reforçam a ideia de que há um mecanismo inflamatório subjacente associado à hemólise e a anemia secundária na AF. Por fim, o quarto manuscrito trouxe informações novas relacionadas aos subtipos de monócitos na AF e a capacidade da HU em reduzir a produção de citocinas inflamatórias por estas células. Demonstrou-se também que os monócitos clássicos da AF são polifuncionais e produzem diversas citocinas pró-inflamatórias além de estarem associados a vaso-oclusão.

Coletivamente, os dados do presente estudo corroboram com dados anteriores sobre os aspectos clínicos e inflamatórios da DF, além de demonstrar associações novas entre os mecanismos fisiopatológicos da doença, marcadores laboratoriais e moléculas pró-inflamatórias. Dessa forma, acreditamos que os dados obtidos com este estudo podem auxiliar o manejo clínico e laboratorial dos pacientes com DF.

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9 APÊNDICE

A – MANUSCRITOS EM COLABORAÇÃO

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Leptin −2548 G > A gene polymorphism is associated with lipids metabolism and TGF-β alteration in sickle cell disease

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ABSTRACT

Background: Leptin is a protein with regulatory role in several body systems such as the immune system, and energy balance. Given that patients with sickle cell disease (SCD) have changes in cellular immunity and lipid metabolism, it is important to conduct research aimed understand the role of leptin in the pathophysiology of SCD. *Results:* We studied 103 patients with SCD from Northeast of Brazil in a case-control study. The investigation of the *leptin* −2548 G > A polymorphism in SCD individuals shows the frequency of 60.20% (62/103) for the wild genotype (GG); 34.95% (36/103) for the heterozygous genotype (AG) and 4.85% (5/103) for the variant homozygote genotype (AA). In the healthy volunteers group the polymorphism investigation indicated the frequency of 58.24% (53/91) for the wild genotype (GG); 37.36% (34/91) for the heterozygous genotype (AG) and 4.40% (4/91) for the variant homozygote genotype (AA). The AA genotype was associated with increased levels of very-low-density lipoprotein cholesterol (VLDL-C) and triglycerides among SCD patients. Furthermore, the presence of allele A was associated with the highest levels of transforming growth factor beta (TGF-β) in SCD patients. *Conclusion:* The results suggest that the presence of the variant allele may influence the disturbances in lipid metabolism and serum levels of TGF-β described in SCD patients.

1. Introduction

Sickle cell disease (SCD) designates a group of diseases that has in common the presence of the beta S allele (β^S) that can be found in homozygous state called sickle cell anemia (SCA) or in heterozygous paired with other alleles from variants hemoglobin. The hemoglobin S (HbS) is a variant hemoglobin resulting from GAG → GTG point mutation in the sixth codon of the beta globin gene (*HBB*) where valine replaces glutamic acid on the beta polypeptide chain (Silla, 1999; Steinberg, 2009). Clinical symptoms associated with the SCD are heterogeneous, with the presence of severe hemolytic anemia, pain crises, vaso-occlusive events, high susceptibility of infection, pulmonary hypertension, priapism, leg ulcers, and stroke among other clinical events (Ghosh et al., 2014).

Inflammation on SCD is also driven by several cytokines, such as transforming grow factors-beta (TGF-β), which are a pleiotropic cytokine family that can acts in both pro-inflammatory and anti-inflammatory pathways. Episodes of pain, occurrence of infection, stroke, leg ulcers, priapism, acute chest syndrome, pulmonary hypertension and renal failure are important clinical manifestations, being associated with higher levels of TGF-β (Nolan et al., 2006; Pereira et al., 2014). Lipids are also involved on the inflammatory milieu of SCD, and dyslipidemia has been described among SCD patients. Alterations in plasma cholesterol concentrations were reported in SCD, and studies demonstrated the association between decreased levels of high-density lipoprotein cholesterol (HDL-C) and increased levels of very-low-density lipoprotein cholesterol (VLDL-C) and triglycerides as biomarkers related to inflammation among this patient group (Seixas et al., 2010; Zorca et al., 2010).

Leptin is a peptide hormone secreted by adipocytes, formed by 167 amino acids, has a molecular weight of 16 kDa, transcribed from the *ob* gene in mice, and serves as an integral component in the physiological system, regulating the storage, balance and the use of energy by the body (Negrão and Licinio, 2000). Studies suggest that leptin also has the role of modulating the immune response, acting on inflammatory processes and immune-mediated pathologies.

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Endothelial Nitric Oxide Synthase (–786T>C) and Endothelin-1 (5665G>T) Gene Polymorphisms as Vascular Dysfunction Risk Factors in Sickle Cell Anemia

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ABSTRACT: Sickle cell anemia (SCA) patients have vascular complications, and polymorphisms in endothelin-1 (*ET-1*) and endothelial nitric oxide synthase (*eNOS*) genes were associated with ET-1 and nitric oxide disturbance. We investigate the association of *ET-1* 5665G>T and *eNOS* –786T>C polymorphisms with soluble adhesion molecules (sVCAM-1 and sICAM-1), biochemical markers, and medical history. We studied 101 SCA patients; carriers of *eNOS* minor allele (C) had the highest levels of sVCAM-1, and carriers of *ET-1* minor allele had more occurrence of acute chest syndrome (ACS). The multivariate analysis suggested the influence of the *ET-1* gene on ACS outcome and an association of the *eNOS* gene with upper respiratory tract infection. We suggest that *eNOS* and *ET-1* gene polymorphisms can influence SCA pathophysiology and that *eNOS* variant in SCA patients might be important to nitric oxide activity and vascular alteration. We found an association of the *ET-1* minor allele in ACS, showing the importance of genetic screening in SCA.

KEYWORDS: sickle cell anemia, eNOS, entothelin-1, gene polymorphisms

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Introduction

A single amino acid substitution in the hemoglobin (Hb) molecule is the molecular basis for sickle cell anemia (SCA). However, the disease clinical evolution is heterogeneous and involves multiple factors. The SCA is a vascular disease and is already known that genetic differences associated with endothelial function contribute to its phenotypic diversity.[1]

Endothelin-1 (ET-1) and nitric oxide (NO) are endothelium-derived mediators essential for maintaining vascular homeostasis. The correct balance between NO and ET-1 production seems to be essential in preventing vascular endothelial dysfunction.[2,3]

The endothelin is an endothelium-derived molecule and an important vasoconstrictor. Among the three isoforms of endothelin, ET-1 is the only isoform produced by endothelial cells. Various stimuli, such as thrombin, inflammatory mediators, and hypoxia, increase ET-1 levels that play a pivotal role in vascular function regulation and act through the smooth muscle producing vasoconstriction, cell growth, and cell adhesion.[2,3] Because of the role of ET-1 in vascular pathophysiology, polymorphic gene coding ET-1 increases vascular reactivity in several vascular disorders. A single nucleotide polymorphism in the *ET-1* gene involving a G-to-T replacement at nucleotide 5665 in exon 5 was correlated with an increased susceptibility of acute chest syndrome (ACS) in SCA individuals.[4]

The NO is synthesized by a family of NO synthase (NOS), and the dominant NOS isoform in the vasculature is the endothelial NOS (eNOS), an enzyme that can metabolize L-arginine and generate NO.[5,6] The NO is involved in the pathogenesis of several disease such as SCA and has vasodilatador and antithrombogenic properties that, if impaired, can contribute to the vasoconstriction that coupled with the adhesion of circulating cells may lead to the occlusion of microvessels.[5,7] The *eNOS* polymorphic variant –786T>C is associated with a decreased NO production because of the reduction

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Evaluation of Alpha-1 Antitrypsin Levels and *SERPINA1* Gene Polymorphisms in Sickle Cell Disease

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Alpha-1 antitrypsin (AAT) is an inhibitor of neutrophil elastase and a member of the serine proteinase inhibitor (serpin) superfamily, and little is known about its activity in sickle cell disease (SCD). We hypothesize that AAT may undergo changes in SCD because of the high oxidative stress and inflammation associated with the disease. We have found high AAT levels in SCD patients compared to controls, while mutant genotypes of *SERPINA1* gene had decreased AAT levels, in both groups. AAT showed negative correlation with red blood cells, hemoglobin (Hb), hematocrit, high-density lipoprotein cholesterol, urea, creatinine, and albumin and was positively correlated with mean corpuscular Hb concentration, white blood cells, neutrophils, Hb S, bilirubin, lactate dehydrogenase, ferritin, and C-reactive protein. Patients with higher levels of AAT had more infection episodes (OR = 1.71, CI: 1.05–2.65, $p = 0.02$), gallstones (OR = 1.75, CI: 1.03–2.97, $p = 0.02$), and had more blood transfusions (OR = 2.35, CI: 1.51–3.65, $p = 0.0001$). Our data on AAT association with laboratory indices of hemolysis and inflammation suggest that it may be positively associated with SCD severity; the negative correlations with renal parameters suggest a cytoprotective mechanism in SCD patients. In summary, AAT may need to be included in studies related to SCD and in the discussion of further therapeutic strategies.

Keywords: sickle cell disease, alpha-1 antitrypsin, *SERPINA1*, biomarkers, inflammation

INTRODUCTION

Clinical symptoms associated with sickle cell disease (SCD) are heterogeneous, with the presence of hemolytic anemia, vaso-occlusive events, infections, acute chest syndrome (ACS), pulmonary hypertension, stroke, and glomerulopathy, among others (1–5). SCD has several sub-phenotypes and the search for biomarkers related to the disease severity is very useful to patients' follow-up (6, 7).

Alpha-1 antitrypsin (AAT) is a glycoprotein of 52 kDa with 394 amino acids that is secreted and synthesized primarily not only in hepatocytes, but also on phagocytic cells, such as neutrophils, monocytes, and macrophages; lung epithelial cells; and intestinal cells. It is considered an acute phase protein, but it is also known as a hepatic stress protein, since its plasma levels increase during

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Evaluation of Cardiometabolic Parameters among Obese Women Using Oral Contraceptives

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Background: Combined oral contraceptive (COC) use has been associated with an unfavorable impact on carbohydrate and lipid metabolism in diverse populations of normal weight and obese women. The present study aimed to evaluate the cardiometabolic and inflammatory profiles of women in northeastern Brazil with respect to COC use and obesity.

Methods: We performed a cross-sectional study to verify cardiovascular parameters, including blood pressure (BP), fasting serum glucose, lipid, and inflammatory profile, in a population of women aged 15–45 years, considering obesity and COC use. Our sample consisted of 591 women, 481 women who were COC users, and 110 age-matched women who were COC non-users, classified as obese and non-obese according to BMI.

Results: COC use and obesity were associated with increased systolic ($p \leq 0.001$) and diastolic BP ($p = 0.001$), blood glucose ($p \leq 0.001$), total cholesterol ($p = 0.008$), low-density lipoprotein cholesterol ($p \leq 0.001$), very low-density lipoprotein cholesterol ($p \leq 0.001$), triglycerides ($p \leq 0.001$), ferritin ($p = 0.006$), C-reactive protein (CRP) ($p \leq 0.001$), and nitric oxide metabolites ($p \leq 0.001$), as well as decreased high-density lipoprotein cholesterol (HDL-c) ($p \leq 0.001$) in comparison to controls. CRP and HDL-c levels in obese COC users were determined to be outside reference range values. The odds of having lower levels of HDL-c and elevated CRP increased among obese COC users. COC use was independently associated with low levels of HDL-c, especially second-generation progestins ($p < 0.001$; OR = 8.976; 95% CI 2.786–28.914).

Conclusion: Obesity and COC use were associated with alterations in lipid and inflammatory cardiometabolic parameters, particularly increased CRP levels and decreased HDL-c, which are considered markers of cardiovascular disease (CVD) risk. Given the need to prevent unintended pregnancy among obese women, together with weight loss counseling, it is important to evaluate the most effective and safest contraceptive methods to avoid the potential risk of developing CVD.

Keywords: combined oral contraceptives, obesity, cardiometabolic parameters, women, C-reactive protein, high-density lipoprotein cholesterol, cardiovascular risk

Abbreviations: BMI, body mass index; COC, combined oral contraceptives; CRP, C-reactive protein; HDL-c, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein cholesterol; EE, ethinylestradiol; LEVO, levonorgestrel; CVD, cardiovascular disease, OB, obese; NOB, non-obese. SBP, systolic blood pressure; DBP, diastolic blood pressure; NO, nitric oxide; NOm, nitric oxide metabolites.

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RESEARCH ARTICLE Open Access

Comparative study of sickle cell anemia and hemoglobin SC disease: clinical characterization, laboratory biomarkers and genetic profiles

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Abstract

Background: In this study, we evaluate the association of different clinical profiles, laboratory and genetic biomarkers in patients with sickle cell anemia (SCA) and hemoglobin SC disease (HbSC) in attempt to characterize the sickle cell disease (SCD) genotypes.

Methods: We conducted a cross-sectional study from 2013 to 2014 in 200 SCD individuals (141 with SCA; 59 with HbSC) and analyzed demographic data to characterize the study population. In addition, we determined the association of hematological, biochemical and genetic markers including the β^S-globin gene haplotypes and the 3.7 Kb deletion of α-thalassemia ($-\alpha^{3.7Kb}$-thal), as well as the occurrence of clinical events in both SCD genotypes.

Results: Laboratory parameters showed a hemolytic profile associated with endothelial dysfunction in SCA individuals; however, the HbSC genotype was more associated with increased blood viscosity and inflammatory conditions. The BEN haplotype was the most frequently observed and was associated with elevated fetal hemoglobin (HbF) and low S hemoglobin (HbS). The $-\alpha^{3.7Kb}$-thal prevalence was 0.09 (9%), and it was associated with elevated hemoglobin and hematocrit concentrations. Clinical events were more frequent in SCA patients.

Conclusions: Our data emphasize the differences between SCA and HbSC patients based on laboratory parameters and the clinical and genetic profile of both genotypes.

Keywords: Sickle cell anemia, Hemoglobin SC disease, Biomarkers, Genetic profile

Background

Sickle cell disease (SCD) is a group of inherited diseases that includes sickle cell anemia (SCA), which is the homozygous state of the beta S (β^S) allele and the most severe SCD genotype. Likewise, the heterozygous state of the β^S allele is characterized by the presence of hemoglobin S (HbS) associated with changes in the structure or synthesis of the other globin chain and consists of a group of less severe SCD, including hemoglobin SC disease (HbSC). SCD has important implications for public health, as both worldwide incidence and prevalence are high, which reinforces it as a significant social problem in many countries [1, 2]. The clinical diversity of SCD includes hemolytic and vaso-occlusive episodes (VOE), infections, stroke, acute chest syndrome (ACS), pulmonary hypertension, multiple organ dysfunctions and other complications [3]. Several factors have been shown to modulate the clinical manifestations of SCD including hematological, biochemical, inflammatory and genetic

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Inflammatory mediators in sickle cell anaemia highlight the difference between steady state and crisis in paediatric patients

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Sickle cell anaemia (SCA) is a chronic inflammatory disease with a complex mechanism of pathogenesis. The rheological phenomenon of SCA has been directly associated with the activation of sickle red blood cells, reticulocytes, leucocytes, platelets and endothelial cells, with the expression of several molecules secondarily expressed in this inflammatory environment and on the surface of these cells (Ware *et al*, 2017).

Although inflammatory mediators have been studied among SCA patients, the immunological and inflammatory mechanisms associated with the disease pathogenesis, endothelial activation and dysfunction, and repair mechanisms, as well as their roles as biomarkers of the crisis and steady states, remain unclear.

Considering the complex network of mechanisms involved in SCA pathogenesis, we investigated systemic levels of cytokines, including tumour necrosis factor-alpha (TNF-α); interleukin (IL) 1β, IL6, IL8, IL10, and IL12; and transforming growth factor beta (TGF-β). We also investigated inflammatory mediators, such as prostaglandin E2 (PGE_2), leukotriene B4 (LTB_4) and the vascular remodelling modulators matrix metalloproteinase-9 (MMP9) and its inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP1), and free

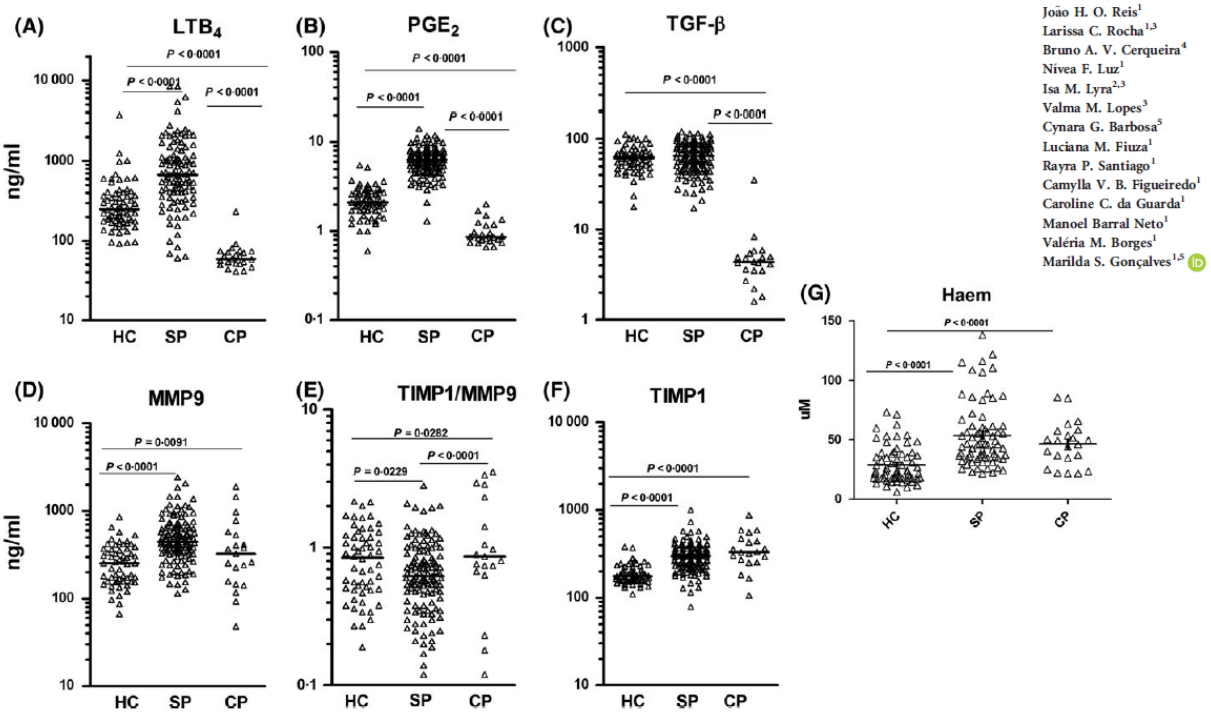

Fig 1. Inflammatory mediator and tissue remodelling marker concentrations in sickle cell anaemia patients and controls. Evaluation of LTB_4 (A), PGE_2 (B), TGF-β (C), MMP9 (D), TIMP1/MMP9 ratio (E), TIMP1 (F) and haem (G) levels among sickle cell anaemia patients in steady state and crisis, as well as healthy controls. LTB_4, PGE_2, TGF-β, MMP9 and TIMP1 were measured using an enzyme-linked immunosorbent assay and haem was measured using a commercially available QuantiChrom Heme Assay (BioAssay Systems, Hayward, CA, USA). The Mann–Whitney test and independent *t*-test were used to compare the variables according to each distribution. SP, stable (steady state) patients; CP, crisis patients; HC, healthy controls.

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Research Article

Laboratory and Genetic Biomarkers Associated with Cerebral Blood Flow Velocity in Hemoglobin SC Disease

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Reference values for cerebral blood flow velocity (CBFV) in hemoglobin SC disease (HbSC) have not been established. We aimed to investigate associations between laboratory and genetic biomarkers associated with CBFV in HbSC children. Sixty-eight HbSC children were included; CBFV was analyzed by transcranial Doppler, and the time-averaged maximum mean velocity (TAMMV) was estimated. Hematological, biochemical, immunological, and genetic analyses were performed. TAMMV was negatively correlated with red blood cell count (RBC) count, hemoglobin, hematocrit, and direct bilirubin (DB), yet positively correlated with monocytes and ferritin. We found that children with TAMMV ≥ 128 cm/s had decreased red blood cell distribution width (RDW) and nitric oxide metabolite (NOx) concentration. Children with TAMMV ≥ 143.50 cm/s had decreased hemoglobin and hematocrit, as well as increased ferritin levels. Decreased hemoglobin, hematocrit, RDW, and NOx and increased ferritin were detected in children with TAMMV ≥ 125.75 cm/s. The CAR haplotype was associated with higher TAMMV. In association analyses, RBC, hemoglobin, hematocrit, RDW, monocyte, DB, NOx, and ferritin, as well as the CAR haplotype, were found to be associated with higher TAMMV in HbSC children. Multivariate analysis suggested that high TAMMV was independently associated with hematocrit, RDW, and NOx. Additional studies are warranted to validate the establishment of a cutoff value of 125.75 cm/s associated with elevated TAMMV in HbSC children.

1. Introduction

Sickle cell disease (SCD) is characterized by the presence of hemoglobin S (HbS). The HbSS genotype, in which the beta allele S (β^S) is homozygous, is known as sickle cell anemia (SCA), the most severe type of SCD. In HbS-β^0 thalassemia, another severe form of SCD, the beta allele S is present in association with the absence of synthesis of the β gene on the second chromosome. In hemoglobin SC disease (HbSC), there is an association of HbS with another hemoglobin variant, HbC (β^C), that results in a typically milder form of SCD [1].

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ORIGINAL ARTICLE

Hemoglobin Variant Profiles among Brazilian Quilombola Communities

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ABSTRACT

Brazilian Quilombolas are communities composed of African-derived populations that have their territories guaranteed by the Brazilian Constitution. The present study investigated the hemoglobin (Hb) variants among these population groups. This study was conducted in a total of 2843 individuals of Brazilian Quilombola communities of the Bahia, Pará, and Piauí states. All the participants had their Hb profiles evaluated. The Hb S (*HBB*: c.20A>T) variant was described in all the studied localities. However, the individuals in Bahia State had the highest frequency of the Hb C (*HBB*: c.19G>A) variant; individuals from Piauí State had a higher frequency of the Hb D-Punjab (*HBB*: c.364G>C) variant compared to the other states, and individuals from Pará State only carried the Hb S variant. The present study revealed a specific distribution of Hb variants that could represent different waves of African influence in these Brazilian populations.

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KEYWORDS
Africans; hemoglobinopathy; hemoglobin (Hb) variant; Quilombola

Introduction

Historically, Brazil was colonized by Portugal for over three centuries, and its economy was based on the exploitation of cheap slave labor brought from Africa; these slaves had no political or social rights. The Portuguese colonization in Brazil was maintained by the Africans and their descendants and was the basis of the sociocultural and economic growth of the Brazilian population. During this period, the African people in Brazil had no assistance or health security and worked in poor conditions. Africans and their descendants organized and took refuge in places called Quilombos, where they lived according to their culture and custom. The term Quilombo (Kilombo) is of African origin from the Bantu language and means 'village.' The presence of Quilombos in Brazil was associated with the Bantu people who were brought from Africa and enslaved during the Brazilian colonization [1].

The Brazilian Constitution guarantees the human rights of African origin communities in its territory, provides public policies for Quilombola regularization, and ensures that the land belongs to the remnants of Quilombo communities. According to the Brazilian Ministry of Culture, there are 3524 identified Quilombola communities in Brazil, and of these, 1342 are legally recognized. The federal government seeks to improve the quality of life in these communities through public policies aimed at the Quilombola population [2].

Since 2004, the Brazilian Quilombola Program has coordinated governmental actions inside these communities, which have important socioeconomic deficiencies, as shown by the results of the Brazilian population census of 2010 [2,3]. Some of these actions are directed to assist the most prevalent pathologies, including hemoglobinopathies, a group of genetic diseases that have a high incidence and prevalence in African countries and, most likely, in their descendant groups such as the Quilombola communities [4].

Hemoglobinopathies are classified by structural or synthesis changes in genes associated with the synthesis of globins, the protein portion of the hemoglobin (Hb) molecule. These diseases include structural Hb variations and thalassemias. Sickle cell disease is a group of diseases associated with the presence of Hb S (*HBB*: c.20A>T); the most severe form is called sickle cell anemia, which has the Hb SS (β^S/β^S) homozygous phenotype. However, Hb S can be found in association with other Hb variants or with thalassemia, such as in Hb SC disease (*HBB*: c.19G>A), and Hb S-β-thalassemia (Hb S-β-thal). Notably, patients with sickle cell disease have a high morbidity and mortality and a heterogeneous clinical picture, requiring frequent hospitalization and specific vaccines, therapies, and medical follow-up; a high cost of public resources is spent on monitoring these patients [5].

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REVIEW

Heme-mediated cell activation: the inflammatory puzzle of sickle cell anemia

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ABSTRACT

Introduction: Hemolysis triggers the onset of several clinical manifestations of sickle cell anemia (SCA). During hemolysis, heme, which is derived from hemoglobin (Hb), accumulates due to the inability of detoxification systems to scavenge sufficiently. Heme exerts multiple harmful effects, including leukocyte activation and migration, enhanced adhesion molecule expression by endothelial cells and the production of pro-oxidant molecules.

Area covered: In this review, we describe the effects of heme on leukocytes and endothelial cells, as well as the features of vascular endothelial cells related to vaso-occlusion in SCA.

Expert commentary: Free Hb, heme and iron, potent cytotoxic intravascular molecules released during hemolysis, can exacerbate, modulate and maintain the inflammatory response, a main feature of SCA. Endothelial cells in the vascular environment, as well as leukocytes, can become activated via the molecular signaling effects of heme. Due to the hemolytic nature of SCA, hemolysis represents an interesting therapeutic target for heme-scavenging purposes.

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KEYWORDS
Inflammation; heme; hemolysis; sickle cell anemia; sickle cell disease

1. Sickle cell anemia

Sickle cell anemia (SCA) was the first genetic disease in which an altered protein capable of causing clinical symptoms was verified. The underlying abnormality consists of a single nucleotide substitution (GAG → GTG), which replaces one amino acid (Glu → Val) in the sixth position of the β globin ($\beta^{6Glu\text{-}Val}$) amino terminal region. This produces the Hb variant S (HbS; $\alpha_2\beta_2^S$), forming long polymers when exposed to low oxygen concentrations [1–3] which consequently provokes the deformation of erythrocytes that participate in the pathophysiological mechanism of SCA [4].

Sickling, the process in which polymers are assembled and erythrocytes suffer morphological changes, has been associated with three conditions: deoxygenation and alterations in both intracellular HbS and fetal Hb (HbF) concentrations. Changes in physicochemical properties, in addition to deformities and tensing of the erythrocyte membrane, are also responsible for the pathologic event of vaso-occlusion (VOC), since erythrocytes become predisposed to adhere to the vascular endothelium [3,5]. Repeated sickling cycles can cause severe injury to the erythrocyte membrane and generate reactive oxygen species (ROS). This process can also lead to an abnormal cation homeostasis, resulting in dehydrated, irreversibly sickled red blood cells, whose morphology tends to exacerbate the underlying hemolytic anemia and vascular obstructions seen in SCA [6].

Irreversibly sickled erythrocytes are usually removed from the bloodstream by the mononuclear phagocyte system, a phenomenon that shortens the erythrocyte lifespan from 120 days to nearly 31 days, thus contributing to SCA severity [7]. The removal of senescent red blood cells by macrophages in the spleen and liver (the mononuclear phagocyte system) is known as extravascular hemolysis, which is mainly regulated by the proteins responsible for heme degradation, for example, heme oxygenase (HO-1), and those involved in iron metabolism, such as iron importer transferrin receptor 1 (TfR1), divalent metal transport 1 (DMT1), iron exporter ferroportin 1 (FPN1), and the iron regulatory hormone hepcidin [8]. This process does not usually elicit an inflammatory response, although it is believed that increased bacterial infection may result from functional asplenia, possibly due to recurrent ischemic accidents in the red pulp [9]. SCA patients present elevated vulnerability to chronic hemolysis and higher susceptibility to infection, in addition to VOC resulting in chronic ischemic injury to many organs, as well as endothelial dysfunction and early mortality [10].

SCA patients exhibit ongoing hemolysis even in the absence of an acute clinical hemolytic event. The pathologic mechanism of hemolysis leads to several complications, including renal, pulmonary, and gastrointestinal manifestations, as well as priapism and leg ulcers [11]. It is accepted that hemolysis occurring in vascular spaces results in toxicity due to the release of free hemoglobin (Hb), heme and

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Lipids in Health and Disease

RESEARCH Open Access

Association of classical markers and establishment of the dyslipidemic sub-phenotype of sickle cell anemia

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Abstract

Background: Sickle cell anemia (SCA) patients exhibit sub-phenotypes associated to hemolysis and vaso-occlusion. The disease has a chronic inflammatory nature that has been also associated to alterations in the lipid profile. This study aims to analyze hematological and biochemical parameters to provide knowledge about the SCA sub-phenotypes previously described and suggest a dyslipidemic sub-phenotype.

Methods: A cross-sectional study was conducted from 2013 to 2014, and 99 SCA patients in steady state were enrolled. We assessed correlations and associations with hematological and biochemical data and investigated the co-inheritance of -$\alpha^{3.7Kb}$-thalassemia (-$\alpha^{3.7Kb}$-thal). Correlation analyses were performed using Spearman and Pearson coefficient. The median of quantitative variables between two groups was compared using *t*-test and Mann-Whitney. *P*-values <0.05 were considered statistically significant.

Results: We found significant association of high lactate dehydrogenase levels with decreased red blood cell count and hematocrit as well as high levels of total and indirect bilirubin. SCA patients with low nitric oxide metabolites had high total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol and reduced very low-density cholesterol, triglycerides, direct bilirubin level and reticulocyte counts. In SCA patients with high-density lipoprotein cholesterol greater than 40 mg/dL, we observed increased red blood cell count, hemoglobin, hematocrit, and fetal hemoglobin and decreased nitric oxide metabolites levels. The presence of -$\alpha^{3.7Kb}$-thal was associated with high red blood cell count and low mean corpuscular volume, mean corpuscular hemoglobin, platelet count and total and indirect bilirubin levels.

Conclusions: Our results provide additional information about the association between biomarkers and co-inheritance of -$\alpha^{3.7Kb}$-thal in SCA, and suggest the role of dyslipidemia and nitric oxide metabolites in the characterization of this sub-phenotype.

Keywords: Sub-phenotype, Sickle cell anemia, Dyslipidemia, α-thalassemia

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frontiers in Microbiology

ORIGINAL RESEARCH
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Nasopharyngeal and Oropharyngeal Colonization by *Staphylococcus aureus* and *Streptococcus pneumoniae* and Prognostic Markers in Children with Sickle Cell Disease from the Northeast of Brazil

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We investigated the nasopharynx and oropharynx microbiota in sickle cell disease (SCD) to identify the microorganisms, antibiotic sensitivity, prevalent serotypes, and association of with laboratorial markers. Oropharynx/nasopharynx secretions were investigated in 143 SCD children aging 6 months to 17 years. Pathogens were isolated using standard procedures, and laboratorial markers were performed by automated methods. *Staphylococcus aureus* (*S. aureus*) was isolated from nasopharynx and oropharynx of 64 and of 17 SCD children respectively. *Streptococcus pneumoniae* (*S. pneumoniae*) was isolated from the nasopharynx and oropharynx of eight SCD patients. Serotypes of *S. pneumoniae* were 19F, 23F, and 14. All isolates were susceptible to penicillin, and patients whose nasopharynx and oropharynx were colonized by *S. pneumoniae* had high concentrations of aspartate transaminase, alanine transaminase, and ferritin. *S. pneumoniae* isolated were not penicillin-resistant serotypes suggesting that the use of penicillin for prophylaxis and/or treatment of infections is safe. Our finding of colonization and laboratory evaluation in SCD patients suggests that microorganisms are involved in the modulation of chronic inflammatory. The association of colonized microorganisms and laboratorial markers suggest a new approach to these patients follow-up, and additional studies of microorganism colonization and their association with SCD patients' clinical outcome will improve control and prevention strategies.

Keywords: nasopharyngeal, oropharyngeal, serotype, *Staphylococcus aureus*, *Streptococcus pneumoniae*

INTRODUCTION

Infections are the major cause of death in children with sickle cell anemia (SCA) (Williams et al., 2009). Likewise, bacterial infection is the primary cause of death during childhood; infants and children younger than 3 years of age are at risk of mortality and morbidity from sepsis (Iughetti et al., 2016). *Streptococcus pneumoniae* (*S. pneumoniae*) is a genetically variable organism

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Genetic modulation of fetal hemoglobin in hydroxyurea-treated sickle cell anemia

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To the Editor:

HbF levels are associated with haplotypes of the *HBB* gene cluster although the mechanisms accounting for this are largely unknown. Genome-wide association studies (GWAS) have revealed three quantitative trait loci (QTL), *HBG2* on chromosome 11p15, *HBS1L-MYB* (*HMIP*) intergenic region on chromosome 6q23 and *BCL11A* on chromosome 2p16, which account for 20%-50% of HbF variation in sickle cell anemia (SCA). The olfactory receptors genes might have a regulatory role in γ-globin gene expression.[1,2]

Hydroxyurea (HU) induces the production of HbF in SCA, providing a pharmacological therapeutic approach for ameliorating clinical complications.[3] Accordingly, we analyzed *HBB* haplotypes along with SNPs in HbF associated QTL to evaluate their role in regulating HbF in SCA treated with HU.

The study was conducted from 2013 to 2014 in 141 SCA patients, 42 on and 99 not on HU, who attended Sickle Cell Disease Reference Center in Itabuna, Bahia, Brazil. Mean age was 15.2 ± 11.1 years (median, 13 years) with 71 females. Laboratory variables were measured in patients without clinical manifestations of vaso-occlusive crisis and no transfusions in the preceding three months. The study was approved by the Research Board of the CPqGM-FIOCRUZ-Bahia-Brazil.

Hematological analyses were done on a Sysmex Count KX 21 N (Sysmex Corporation, Tokyo, Japan) and blood chemistries on a Cobas (Roche Diagnostics, Salt Lake City, Utah, USA). Hemoglobin fractions were quantified by high-performance liquid chromatography (HPLC) (BioRad, Hercules, CA, USA) at the Laboratory of Research in Anemia (LPA/UFBA) at the Universidade Federal da Bahia and Laboratory of Hematology, Genetic and Computational Biology (LHGB) at CPqGM-FIOCRUZ-Bahia-Brazil.

β^S-globin gene cluster haplotypes were ascertained by polymerase chain reaction (PCR) and restriction fragments length polymorphisms (RFLP). SNPs of *BCL11A* (rs6732518, C > T; rs766432, A > C), *HBS1L-MYB* interval (rs11759553, A > C; rs35959442, C > G), and *OR51B5/6* genes (rs4910755, A > C; rs7483122, C > T), corresponding to QTL on chromosomes 2, 6, and 11 respectively, were analyzed by Real-Time PCR (Applied Biosystems, Foster City, California, USA).

Variables for analysis were evaluated in means, medians and percentile. Quantitative variables were compared using the *t*-test for normal data, and Mann-Whitney for non-normal data. Differences in laboratory data associated with SNP genotypes and dose of HU (mg/kg/day) were determined by the Kruskal-Wallis test. Multivariate linear regression analyses were performed to estimate the likelihood of having HbF levels as outcome and a possible interaction with age, sex, HU use, CAR haplotype, and polymorphisms in genes related to HbF

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Research Article

Sickle Cell Anemia Patients in Use of Hydroxyurea: Association between Polymorphisms in Genes Encoding Metabolizing Drug Enzymes and Laboratory Parameters

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This study investigated associations between SNPs in genes encoding metabolizing drug enzymes and laboratory parameters in sickle cell anemia patients under hydroxyurea (SCA-HU$^+$). We evaluated hematologic and biochemical parameters by electronic methods and SNPs by PCR-RFLP and multiplex PCR in 35 SCA-HU$^+$ patients and 67 SCA-HU$^-$ patients. The HbS, total cholesterol, lactate dehydrogenase, aspartate aminotransferase, total bilirubin and fractions levels, and leukocyte, eosinophil, monocyte, and erythroblast counts were reduced in SCA-HU$^+$ patients ($p < 0.05$). Moreover, they presented higher HbF, C-reactive protein, and ferritin levels and elevated MCH and MCV values ($p < 0.05$). Genotype frequencies of variants GA + AA of *MPO* −463G>A and c1c2 + c2c2 of *CYP2E1* −1293G>C/−1053C>T were higher in SCA-HU$^+$ patients ($p < 0.05$). Independent associations were found between the variant A allele and lower total cholesterol, between c2 allele and low alpha-1 antitrypsin and between the null GSTT1 variant and high indirect and total bilirubin in SCA-HU$^+$ patients. In SCA-HU$^-$ patients, independent associations were found between the variant A allele and high uric acid and between c2 allele and high urea. Our results suggest that SNPs *MPO* −463G>A, *CYP2E1* −1293G>C/−1053C>T, and *GSTT1* can be associated with alterations in lipid, inflammatory, renal, hemolytic, and hepatic profiles. However, further studies are needed to elucidate these associations.

1. Introduction

Sickle cell anemia (SCA) is a monogenic disease, characterized by clinical heterogeneity [1]. The clinical diversity of SCA patients has been attributed to several factors, such as sociodemographic, socioeconomic, environmental, and genetic factors [2, 3]. Fetal hemoglobin (HbF: $\alpha_2\gamma_2$) is a classic genetic modulator associated with a less-severe SCA outcome, and high concentration of HbF inhibits the polymerization of the hemoglobin variant S (HbS) by formation of asymmetric hybrids with gamma (γ) chain and β^S chain ($\alpha_2\gamma\beta^S$) that present high affinity for oxygen [4, 5]. Hydroxyurea (HU) is the most used drug to treat SCA patients with severe profile and increased HbF [6, 7]. Several studies have demonstrated that HU use in SCA can improve the clinical profile by reducing painful crises, hospital stay, blood transfusion, and acute chest syndrome episodes [1, 8]. Despite the HU beneficial effects, there is an interindividual

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REVIEW ARTICLE

Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism

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Abstract

Hydroxyurea (HU) was approved to be used in the treatment of sickle cell disease (SCD) because of its anti-sickling potential. However, there is variability in HU response among SCD patients and this can be due to physiological, socioeconomic, environmental, metabolic and/or genetic factors. The present review focuses on the latter two. Three quantitative trait loci, *HBG2*, *BCL11A* and *HMIP*, have been suggested as important markers for HU response. Other genes (*ASS1, KLF10, HAO2, MAP3K5, PDE7B, TOX, NOS1, NOS2A*, *FLT1, ARG1, ARG2, UGT1A1, OR51B5/6*, *SIN3A*, *SALL2*, *SAR1A*, *UTB*, *OCTN1*, *CYP2C9*, *AQP9*, *MPO*, *CYP2E1*, and *GSTT1*) have also been considered. Studies implicate catalase, urease, horseradish peroxidase and enzymes of CYP450 family in HU metabolism. However, little is known about these enzymes. Therefore, further studies are needed to elucidate the metabolic pathway of HU, which will facilitate pharmacogenomic studies and help in identification of candidate genes for predicting HU response.

Introduction

Hydroxyurea (HU), or hydroxycarbamide, is a hydroxylated analogue of urea (Fig. 1; CAS Registry Number, 127-07-1) [1, 2], first synthesized in 1869 by Dresler and Stein and later tested in an experimental model in 1928 by Rosenthal, who suggested its myelosuppressive potential [3, 4]. HU has been used to treat myeloproliferative syndromes, particularly chronic myeloid leukemia, polycythemia vera and psoriasis [5, 6], as well as AIDS, since it inhibits DNA synthesis in human immunodeficiency virus type I (HIV-I) by reducing intracellular dNTP levels in activated lymphocytes [7, 8].

HU, due to its anti-sickling potential, was approved in 1999 by the U.S. Food and Drug Administration for the treatment of sickle cell disease (SCD) in patients with severe clinical profiles [9–11]. The benefits of HU in SCD patients have been attributed to increasing fetal hemoglobin (HbF) levels, which inhibits the polymerization of the variant hemoglobin S, leading to a reduction in the incidence of painful crises, as well as decreased rates of hospitalization, acute chest syndrome, blood transfusion and mortality among SCD patients [6, 9]. HU is also associated with increasing hemoglobin and mean cell volume of red cells; reducing white cell, platelet and reticulocyte counts; in addition to reducing expression of adhesion molecules and release of nitric oxide (NO) [12, 13]. However, increase in HbF levels and the clinical response induced by HU have been variable among different patients, necessitating elevated dosages and increasing toxicity [9, 14]. Differences in responses can be attributed to various factors, including physiological, socioeconomic and environmental factors. However, genetic factors have been considered as some of the most important determinants of variations in drug therapy response and tolerance [15]. Recently, studies in SCD patients showed that in addition to genomic variations within the β-globin gene (*HBB*), variants in modifier genes outside *HBB* are also significantly associated with increase in HbF levels, and, consequently, HU treatment response [15].

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Research Article

Genetic Polymorphisms Associated with Environmental Exposure to Polycyclic Derivatives in African Children

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Background. The nonracial leukopenia may be a result of exposure to polycyclic derivatives (benzene-toluene-xylene (BTX)) and may arise from a possible change in the bone marrow microenvironment. The present study sought to evaluate the association of genetic polymorphisms in xenobiotic-metabolizing enzymes with hematological and biochemical profiles. *Methods.* We evaluated 89 African descendant children, exposed indirectly to benzene derivatives. Laboratory parameters were investigated by automated methods and genetic polymorphisms by PCR-RFLP and PCR multiplex. *Results.* Children with leukopenia had significantly decreased white blood cells (WBCs) and platelet counts, which is not consistent with benign leukopenia. In the same group, we have found that carriers of the *CYP2E1* variant allele had decreased WBC and lymphocytes. Those with *NQO1* variant allele had decreased WBC, neutrophil, eosinophil, monocyte, and lymphocyte counts. Carriers of the *MPO* variant allele had decreased WBC, neutrophil, eosinophil, basophil, monocyte, lymphocyte, and platelet counts and an elevated free iron level. Children with *GSTT* and *GSTM* null exhibited decreased WBC, neutrophil, basophil, and lymphocyte counts. Our multivariate analysis model reveals that females were independently associated with leukopenia. *Conclusion.* Our results suggest that the polymorphisms investigated were associated with hematological changes in the studied population. These alterations could be heightened by exposure to benzene derivatives.

1. Introduction

Xenobiotic compounds are classified as any foreign chemical substance inside the biological system. Most xenobiotics that the humans are exposed come from environmental pollution, food additives, cosmetics, agricultural products, toxic agents, and drugs. Usually xenobiotics are lipophilic, and if they do not undergo regular metabolism, they can be potentially harmful to exposed humans. Under physiological conditions, humans exhibit mechanisms responsible for enzymatic metabolism or biotransformation of xenobiotics. This involves the biotransformation based on phase I and phase II reactions. During the first phase, the oxidation and reduction of hydrophobic chemicals occur, while in the second phase the conjugation reactions (acetylation, methylation, and glucuronidation) take place in order to remove the byproducts from the human organism as urine or sweat [1, 2].

Human exposure to refining and petroleum refinery process derivatives can happen indirectly in the environment.

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Letter to the Editor

Serum haptoglobin and hemopexin levels are depleted in pediatric sickle cell disease patients

ARTICLE INFO

Editor: Mohandas Narla

To the Editor:

Anemia, hemolysis and vaso-occlusion are the hallmarks of sickle cell disease (SCD). The release of hemoglobin (Hb) and heme into the intravascular milieu can promote inflammatory responses including vasculopathy, leukocyte, platelet and endothelial cell activation, thrombosis, and even renal injury [1]. Nature defends the vasculature from hemoglobin/heme by plasma haptoglobin and hemopexin, which tightly bind free hemoglobin and heme, respectively. Haptoglobin-hemoglobin and hemopexin-heme complexes bind to CD163 and CD91 receptors found primarily on macrophages and hepatocytes respectively, and are taken up by receptor-mediated endocytosis [2]. While it is generally accepted that haptoglobin and hemopexin are depleted in SCD patients [3], we found a limited number of publications that have reported human plasma haptoglobin levels in SCD patients [4–11] and only two papers from 1968 and 1971 that have reported human plasma hemopexin levels in SCD patients, albeit in limited numbers [5, 7]. We found no papers that compared hemopexin levels in SS, SC and AA children. In this letter, we examined serum haptoglobin and hemopexin levels and biomarkers of hemolysis in SS, SC and AA children in Brazil.

Table 1
Association of laboratory parameters in SS and SC patients and AA individuals.

	SS patients $N = 179$	SC patients $N = 93$	AA individuals $N = 28$	p value	Dunn's multiple comparisons test		
	Mean ± SE	Mean ± SE	Mean ± SE		SS vs SC	SS vs AA	SC vs AA
Gender, % female	39.88	54.63	51.72				
Age, years	9.77 ± 0.52	10.70 ± 0.93	8.82 ± 0.66				
Hemolysis markers							
RBC, 10^6/mL	2.73 ± 0.03	4.34 ± 0.05	4.71 ± 0.34	< 0.001	< 0.001	< 0.001	0.290
Hemoglobin, g/dL	8.46 ± 0.09	11.45 ± 0.10	12.81 ± 0.17	< 0.001	< 0.001	< 0.001	0.029
Reticulocytes, %	7.28 ± 0.17	3.95 ± 0.18	0.84 ± 0.04	< 0.001	< 0.001	< 0.001	< 0.001
Reticulocytes, 10^4/mL	19.94 ± 0.56	17.44 ± 0.89	3.97 ± 0.22	< 0.001	0.0122	< 0.001	< 0.001
Total bilirubin, mg/dL	2.30 ± 0.08	1.01 ± 0.05	0.49 ± 0.03	< 0.001	< 0.001	< 0.001	0.006
Indirect bilirubin, mg/dL	1.75 ± 0.07	0.70 ± 0.04	0.25 ± 0.02	< 0.001	< 0.001	< 0.001	0.003
LDH, U/L	1231.00 ± 36.70	587.00 ± 20.13	426.30 ± 16.40	< 0.001	< 0.001	< 0.001	0.075
Hemopexin, μg/mL	251.70 ± 17.36	815.70 ± 42.02	2077.00 ± 124.20	< 0.001	< 0.001	< 0.001	0.002
Haptoglobin, μg/mL	49.15 ± 3.47	60.14 ± 6.30	493.70 ± 63.30	< 0.001	0.898	< 0.001	< 0.001
Total heme, μM	80.67 ± 4.96	38.06 ± 1.91	46.45 ± 4.11	< 0.001	< 0.001	0.002	0.472
Leukocytes							
WBC, 10^9/mL	13.00 ± 0.32	8.58 ± 0.28	7.43 ± 0.51	< 0.001	< 0.001	< 0.001	0.436
Platelets							
Platelets, 10^3/mL	418.6 ± 10.86	274.80 ± 11.42	314.70 ± 13.13	< 0.001	< 0.001	0.002	0.344
Lipid metabolism							
Total cholesterol, mg/dL	123.60 ± 1.78	136.20 ± 2.92	163.70 ± 7.21	< 0.001	< 0.001	< 0.001	0.002
HDL-C, mg/dL	32.25 ± 0.65	40.18 ± 0.97	49.96 ± 2.58	< 0.001	< 0.001	< 0.001	0.016
LDL-C, mg/dL	72.93 ± 1.41	79.10 ± 2.26	95.96 ± 6.72	< 0.001	0.070	< 0.001	0.070
VLDL-C, mg/dL	17.26 ± 0.52	15.29 ± 0.63	16.71 ± 1.34	0.048	0.043	0.999	0.821
Inflammation							
CRP, mg/L	5.47 ± 0.33	3.51 ± 0.32	1.28 ± 0.12	< 0.001	< 0.001	< 0.001	< 0.001

SE: Standard error; RBC: Red blood cells; LDH: Lactate dehydrogenase; WBC: White blood cells; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very low-density lipoprotein cholesterol; CRP: C-reactive protein. p value obtained using the Kruskal-Wallis test. Comparisons between groups were obtained using Dunn's multiple comparisons test.

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ORIGINAL ARTICLE

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Differential gene expression analysis of sickle cell anemia in steady and crisis state

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Abstract

Sickle cell anemia is one of the most prevalent genetic diseases worldwide, showing great clinical heterogeneity. This study compared the gene expression patterns between sickle cell anemia pediatric patients in steady state and in crisis state, as compared to age-paired, healthy individuals. RNA sequencing was performed from these groups of patients/controls using Illumina HiSeq 2500 equipment. The resulting differentially expressed genes were loaded into QIAGEN's ingenuity pathway analysis. The results showed that EIF2 pathway and NRF2-mediated oxidative stress-response pathways were more highly activated both in steady state and in crisis patients, as compared to healthy individuals. In addition, we found increased activation of eIF4 and p70S6K signaling pathways in crisis state compared to healthy individuals. The transcription factor *GATA-1* was found exclusively in steady state while *SPI* was found exclusively in crisis state. *IL6* and *VEGFA* were found only in crisis state, while *IL-1B* was found exclusively in steady state. The regulator effects analysis revealed IgG1 as an upstream regulator in steady state compared to healthy individuals, resulting in invasion of prostate cancer cell lines as the disease/function outcome. For crisis-state patients versus healthy individuals, two networks of regulator effects revealed STAT1, CD40LG, TGM2, IRF7, IRF4, and IRF1 acting as upstream regulators, resulting in disease/function outcomes, including engulfment of cells and aggregation of blood cells and inflammation of joints. Our results indicated genes and pathways that can provide clues on the molecular events involved in the severity of sickle cell disease.

KEYWORDS

gene expression, sickle cell anemia, signaling pathways, vaso-occlusive crisis

1 | INTRODUCTION

Sickle cell anemia (SCA) is one of the most severe and prevalent genetic diseases worldwide. This disease presents as progressive organ damage with the occurance of acute episodes. The severity of SCA varies substantially. Some individuals exhibit very mild clinical course; others have a progressive clinical course and do not live past early childhood. The clinical manifestations of SCA affect every organ system, and the hallmark of SCA is the vaso-occlusive crisis (VOC). The factors that determine the variability of SCA severity are poorly understood, although certain markers are well accepted, such as the elevated levels of fetal hemoglobin (HbF), which is associated with decreased morbidity. On

B – TERMOS DE CONSENTIMENTO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PARA MENORES DE 18 ANOS

Você está sendo convidado a consentir com a participação do menor _____no estudo chamado: "Alfa-1-Antitripsina e Anemia Falciforme: Avaliação Genética, Proteômica e de Mecanismos Associados a Inflamação e a Homeostase Sanguínea ", uma vez que oficialmente é o seu representante legal**.**

A participação do menor é totalmente voluntária e a sua permissão para a sua participação no estudo pode ser retirada a qualquer momento, não resultando em punições.

A anemia falciforme é uma doença genética muito comum na população de Salvador, sendo que o indivíduo doente apresenta crise de dor decorrente da oclusão das veias pelas células vermelhas que possuem o formato de foice, podendo também possuir infecção e outros tipos de alterações clínicas tais como alteração nos olhos, rins, coração, pulmão e cérebro.

Nessa pesquisa serão investigados pacientes com anemia falciforme, que possuem a hemoglobina S, alteração que muda a forma das células vermelhas que ficam rígidas, facilitando a obstrução de veias e juntamente com as células brancas participam das crises de dor e podem contribuir para a ocorrência de derrame, problemas no coração, nos olhos, nervos e pulmões. O sangue retirado será destinado ao estudo do DNA, RNA, células brancas e de algumas substâncias que ajudam na ligação das células às veias, além do estudo de fatores que contribuem para os fenômenos de vaso-oclusão.

Tendo em vista os motivos apresentados, convidamos o menor_____ a participar desta pesquisa.

Os registros da participação do menor no estudo serão mantidos confidencialmente, sendo do conhecimento apenas da equipe participante do projeto e do médico que o acompanha. Os dados individuais dos exames e informações do prontuário serão do conhecimento somente dos pesquisadores envolvidos na pesquisa. Desta forma, a sua identidade será mantida em segredo e nenhum outro grupo terá acesso às informações

coletadas, tais como seguradoras, empregadores ou superiores, de acordo com a resolução Res. CNS 340/2004, item V.1.e.

A permissão para que o menor _____ participe deste estudo implicará na retirada **de 20 ml de sangue**, quantidade igual a três colheres de sopa cheia, para que possamos ser realizados o estudo das células do sangue, do DNA e RNA. Também queremos que você concorde que as amostras colhidas sejam armazenadas e possam ser utilizadas em estudos futuros, desde que estes estudos adicionais sejam analisados por um Comitê de Ética em Pesquisa em Seres Humanos e sigam os aspectos éticos determinados nas resoluções 466/12 e 347/05 do Conselho Nacional de Saúde, além de contribuir para a obtenção de conhecimentos novos relacionados à doença.

Comunicamos que o sangue será colhido do braço, podendo acarretar em riscos e desconfortos, como formação de hematomas, sangramento e dor. Entretanto, a coleta de sangue será realizada por pessoal habilitado e especializado, visando diminuir estes riscos. A realização de coletas adicionais dependerá do médico e estará relacionada, simplesmente, ao acompanhamento clínico e avaliação periódica do menor.

A participação do menor no estudo não trará benefícios diretos, mas possibilitará a realização de exames que não são realizados na rotina, podendo trazer informações importantes referentes à anemia falciforme, proporcionando a obtenção de dados que poderão ser utilizados futuramente no acompanhamento dos pacientes, na busca de novos medicamentos e na implantação de políticas de saúde.

Você teve todas as explicações sobre o projeto e receberá uma cópia deste termo de consentimento livre e esclarecido.

Por favor, entre em contato com a pesquisadora responsável pelo desenvolvimento do projeto, caso você necessite de maiores esclarecimentos:

Se tiver qualquer dúvida, você pode procurar a Dra. Marilda de Souza Gonçalves na FIOCRUZ (telefone: 3176-2226), ou a aluna Caroline Conceição da Guarda (telefone: 3176-2256).

C – QUESTIONÁRIO

Projeto: Alfa 1-antitripsina e Anemia Falciforme: Avaliação molecular e proteômica de mecanismos associados à inflamação e a homeostase sanguínea

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. Qual a sua cor? {cor} ( ) Branca[0] ( ) Negra[1] ( ) Parda[2] ( ) Amarela[3] ( ) Indígena[4]
02. Você estuda? {EST} ( ) NÃO [0] ( ) SIM [1]
03. Nível de escolaridade: {NESC} ( ) Alfabetiz.[0] ( ) Até 4 FM[1] ( ) Até 8 FM[2] ( ) Até 3 MD[3]
04. Número de irmãos: {NIRM} ( ) 0 [0] ( ) 1 [1] ( ) 2 [2] ( ) 3 [3] ( ) 4 ou + [4]
05. Familiares com DF? {FDFALC} ( ) Nenhum[0] ( ) Pai [1] ( ) Mãe [2] ( ) Irmão [3]
06. Idade primeira menstruação: {IPM} ( ) Não menst.[0] ( ) 09-11[1] ( ) 12-14 [2] ( ) 15-17 [3]
07. Já engravidou? {ENGRA} ( ) NÃO [0] ( ) SIM [1]
08. Está grávida? {GRA} ( ) NÃO [0] ( ) SIM [1]
09. Usa anticoncepcional? {ANTICO} ( ) NÃO [0] ( ) SIM [1]
10. Menstruação é regular? {MREG} ( ) NÃO [0] ( ) SIM [1]
11. Idade do 1° diagnóstico de Doença Falciforme: {ID} ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2] ( ) 10 - 14anos [3] ( ) 15 - 17anos [4]
12. Eletroforese de Hb {EHB} ( ) AA[0] ( ) SS[1] ( ) SC[2] ( ) SB+[3] ( ) SB_0[4] ( ) SD[5]
13. Haplótipo {HAPL} ( ) Sen[0] ( ) Car[1] ( ) Ben[2] ( ) Cam[3] ( ) Sau-Ara [4] ( ) Atip[5] ( ) I[6] ( ) II[7] ( ) III[8]
14. 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]

15. 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 [
( ) Outras [9]

16. 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} _____

17. 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} _____

18. 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]

19. Já teve AVC? {AVC} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas vezes? {QAVC} ( ) 1 [0] ( ) 2 [1] ( ) 3 [2] ( ) 4 ou + [3]
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]

20. Esplectomizado? {ESPLECTO} ( ) NÃO [0] ( ) SIM [1]
Esplenectomia: {TIPOESPLECTO} ( ) Total [0] ( ) Parcial [1]

21. 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+
Faz uso regular de nebulização? {NEBU} ( ) [0] NÃO ( ) SIM [1]

22. 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+
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? {HIDROXI} ( ) NÃO [0] ( ) SIM [1]

23. Faz uso de alguma medicação? {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]

24. Vaso-Ocusão: {VO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QVO} _____
Fez uso de alguma medicação? {MVO} ( ) NÃO [0] ( ) SIM [1]

25. Retinopatia: {RETIN} ( ) NÃO [1] ( ) SIM [2]
Se SIM, fez uso de alguma medicação? {MRETIN} ( ) NÃO [0] ( ) SIM [1]
Faz consultas periódicas com oftalmo? {CONSOFTAL} ( ) NÃO [0] ( ) SIM [1]

26. Infecções: {INFEC} ( ) NÃO[0] ( ) SIM [1]
Quais? {DESCINFEC} ( ) Rinite [0] ( ) Sinusite [1] ( ) Otite [2]
( ) Faringite [3] ( ) Amigdalite [4] ( ) Outros [5]
Fez uso de alguma medicação? {MINFEC} ( ) SIM [0] ( ) NÃO [1]

27. Priapismo: {PRIAP} ( ) NÃO [0] ( ) SIM [1]
N° de vezes: {QPRIAP} ( ) Até 4 [0] ( ) 05-09 [1] ( ) 10 ou +[2]
Fez uso de alguma medicação? {MPRIAP} ( ) NÃO [0] ( ) SIM [1]

28. Úlcera maleolar: {ULCMALEO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QULCMALEO} _____
Idade da primeira úlcera: {IDULC} ( ) Até 4 anos [0] ( ) 5-9 [1] ( ) 10 ou + [2]
Tratou a úlcera? {TRATULC} ( ) NÃO [0] ( ) SIM [1]
Qual tratamento? {QUALTRAT} _____

29. Síndrome toráxica aguda: {SDTOR} ( ) NÃO [0] ( ) SIM [1]
Quantas vezes? {QSDTOR} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

30. Alterações ósseas: {ALTOSSEA} ( ) NÃO [0] ( ) SIM [1]
Quais? {DESCALTOSSEA} _____

31. Insuficiência Renal Aguda: {INSRENAG} ( ) NÃO [0] ( ) SIM [1]
Quantas vezes? {QINSRENAG} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

32. 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]

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

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

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

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

38. Cirurgia: {CIRURG} ( ) NÃO [0] ( ) SIM [1]
Quais? {QUALCIRURG} _____

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

40. 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]

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

Se SIM, quantas vezes ao ano? {QHEMODER} _____

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

Quais? {DESCPATOLOG} ( ) Hipertensão [0] ( ) Diabetes [1] ( ) Obesidade [2] ( ) Outras [3]

43. Você trabalha? {TRAB} ( ) NÃO [0] ( ) SIM [1]

Tipo de profissão: {QTRAB} _____

Se SIM, manipula alguma substância química? {SUBQUIM} ( ) NÃO [0] ( ) SIM [1]

Qual? {QSUBQUIM} _____ Freqüência ? {FREQSUBQUI} _____

Manipula diretamente esta subst? {MANIDIRE} ( ) NÃO [0] ( ) SIM [1]

44. Pratica esportes? {ESPOR} ( ) NÃO [0] ( ) SIM [1]

45. Faz uso de bebida alcoólica? {BEBE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que freqüência? {FREQBEBE} _____

46. Você fuma? {FUMA} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que freqüência? {FREQFUMA} _____

47. Faz uso de alguma droga? {DROGA} ( ) NÃO [0] ( ) SIM [1]

Em caso de SIM, que freqüência? {FREQDROGA} _____

48. Além dos seus pais quantos membros da família ou parentes são apegados a vc? {APEG}

( ) 01[0] ( ) 02 – 03 [1] ( ) 04 – 06[2] ( ) 07 – 10[3] ( ) nenhum[4]

49. Quantos amigos vc têm aproximadamente? {AMIGO}

( ) 01[0] ( ) 02 – 03 [1] ( ) 04 – 06[2] ( ) 07 – 10[3] ( ) nenhum[4]

50. Com que freqüência vc se reúne com seus parentes, amigos ou vizinhos? {REUNI}

( ) Diariamente ou quase todos os dias [0] ( ) Várias vezes na semana [1]

( ) Várias vezes no mês [2] ( ) Várias vezes por ano [3] ( ) Quase nunca [4]

Data da próxima consulta no HEMOBA: ____/____/____

D – APROVAÇÃO DO COMITÊ DE ÉTICA

HOSPITAL SÃO
RAFAEL/MONTE TABOR-BA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ALFA-1-ANTITRIPSINA E ANEMIA FALCIFORME: AVALIAÇÃO GENÉTICA, PROTEÔMICA E DE MECANISMOS ASSOCIADOS À INFLAMAÇÃO E A HOMEOSTASE SANGUÍNEA

Pesquisador: Marilda Gonçalves

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 52280015.1.0000.0048

Instituição Proponente: Hospital São Rafael/Monte Tabor-BA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.400.535

Apresentação do Projeto:

A doença falciforme (DF) é caracterizada pela presença de hemoglobina S (HbS) em homozigose ou heterozigose duplo com outras variantes de
hemoglobina e hemoglobinopatias de síntese. A HbS é devido à mutação GAG GTG no sexto codon do gene da beta-globina (gene HBB) onde a
valina substituir o ácido glutâmico na posição seis da cadeia beta polipeptídio. O quadro clínico da anemia hemolítica varia de DF para crises
dolorosas, com a ocorrência de oclusão dos vasos sanguíneos, inflamação e tecidual. Indivíduos com anemia falciforme apresentam mortalidade
elevada, sendo que a busca de novos biomarcadores de prognóstico é de grande interesse, uma vez que pode contribuir para modificar a história
natural da doença. O estado da Bahia tem a maior incidência brasileira de SCD, dados confirmados pela triagem neonatal e da diversidade étnica da
população, que tem uma predominância de ascendência africana, população historicamente mais afetada pela doença. A alfa-1-antitripsina (A1AT) é

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HOSPITAL SÃO
RAFAEL/MONTE TABOR-BA

Continuação do Parecer: 1.400.535

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_626122.pdf	13/11/2015 00:02:35		Aceito
Folha de Rosto	FRCarol.pdf	13/11/2015 00:01:34	Marilda Gonçalves	Aceito
Orçamento	carol2.pdf	12/11/2015 23:50:22	Marilda Gonçalves	Aceito
Orçamento	carol1.pdf	12/11/2015 23:49:46	Marilda Gonçalves	Aceito
Orçamento	Carol.pdf	12/11/2015 23:49:31	Marilda Gonçalves	Aceito
Outros	Carta_de_Anuencia_Alfa.png	12/11/2015 23:32:22	Marilda Gonçalves	Aceito
Outros	QUESTIONARIO_AAT.pdf	12/11/2015 22:45:18	Marilda Gonçalves	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_AAT.pdf	12/11/2015 22:28:13	Marilda Gonçalves	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termos.pdf	12/11/2015 22:27:45	Marilda Gonçalves	Aceito
Outros	TermodeCompromissoparaUtiliacaodeDadoseProntuarios_AAT.pdf	12/11/2015 22:25:43	Marilda Gonçalves	Aceito
Outros	Termo_de_Compromisso_coberturadoscustosdapesquisa_AAT.pdf	12/11/2015 22:23:53	Marilda Gonçalves	Aceito
Outros	Curriculum_VITAE_Marilda_AAT.pdf	12/11/2015 22:23:02	Marilda Gonçalves	Aceito
Outros	Curriculum_VITAE_AAT.pdf	12/11/2015 22:22:09	Marilda Gonçalves	Aceito
Outros	Declaracao_do_orientador_AAT.pdf	12/11/2015 22:20:45	Marilda Gonçalves	Aceito
Declaração de Pesquisadores	Declaracao_do_Pesquisador_Participante_AAT.pdf	12/11/2015 22:19:23	Marilda Gonçalves	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SALVADOR, 02 de Fevereiro de 2016

Assinado por:
Regina Maria Pereira Oliveira
(Coordenador)