UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
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CENTRO DE PESQUISAS GONÇALO MONIZ

UFBA FIOCRUZ

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

DOENÇA FALCIFORME: AVALIAÇÃO DE BIOMARCADORES RELACIONADOS AO PERFIL LIPÍDICO E DISFUNÇÃO ENDOTELIAL

MAGDA OLIVEIRA SEIXAS CARVALHO

Salvador – Bahia – Brasil

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Orientadora: Drª Marilda De Souza Gonçalves

Tese apresentada ao Colegiado do Curso de Pós-graduação em Patologia Humana, como requisito obrigatório para obtenção do grau de Doutor.

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"DOENÇA FALCIFORME: AVALIAÇÃO DE BIOMARCADORES RELACIONADOS AO PERFIL LIPIDICO E DISFUNÇÃO ENDOTELIAL".

MAGDA OLIVEIRA SEIXAS CARVALHO

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De tudo ficaram três coisas:
A certeza de que estamos começando
A certeza de que é preciso continuar
A certeza de que podemos ser interrompidos antes de terminar.

Façamos da interrupção um caminho novo
Da queda, um passo de dança
Do medo, uma escada
Do sonho, uma ponte.

Fernando Sabino

Dedico este trabalho a minha família por iluminar a minha vida.

A Maurício, meu marido, companheiro, melhor amigo e amor.

A minha amada e querida mãe que sempre está ao meu lado.

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RESUMO

A patogênese da doença falciforme (DF) envolve mecanismos diversos, sendo a inflamação crônica, a hemólise e a disfunção vascular fenômenos importantes nesta patologia. A busca por biomarcadores de prognóstico é um desafio e um ponto crucial nas pesquisas referentes a doença. O objetivo desta tese foi avaliar possíveis marcadores de prognóstico, associados à hemólise, inflamação e a disfunção endotelial, e suas possíveis relações com a gravidade clínica na DF. Para tanto, investigamos, prospectivamente, marcadores bioquímicos e inflamatórios em pacientes com DF em estado estável e em crise comparando-os a indivíduos saudáveis. Dessa forma, além das determinações laboratoriais rotineiras (perfis lipídico, hematológico, renal, hepático, lactato desidrogenase (LDH) e proteína C reativa CRP)), também foram avaliados os níveis séricos de marcadores de remodelamento vascular (inibidor tecidual da metaloproteinase-1 (TIMP-1), metaloproteinase de matriz-9 (MMP-9)), de inflamação (leucotrieno B4 (LTB_4), prostaglandina E2 (PGE_2), citocinas anti-inflamatórias e inflamatórias (Fator beta transformador do crescimento (TGF-β), interleucina (IL)-1β, IL-6, IL-8, IL-12, fator de necrose tumoral alfa (TNF-α) e IL-10), de hemólise (heme livre), e níveis séricos de alfa-1 antitripsina (ATT) e os polimorfismos do gene *SERPINA1*. Os dados clínicos dos pacientes foram coletados de prontuários médicos. A comparação entre pacientes e controles revelou diferenças significativas para a maioria dos marcadores estudados com destaque para o colesterol HDL (HDL-C) que mostrou associação inversa com marcadores de hemólise, de inflamação e, cujos níveis reduzidos foram associados a alterações cardíacas, pneumonia e aumento da utilização de hemoderivados. Os pacientes com AF, em estado estável, apresentaram concentrações elevadas de LTB4, PGE2, TGF-β, IL-8 e IL-12 em comparação aqueles em crise. No entanto, os indivíduos com AF em crise apresentaram maiores concentrações de IL-1β, IL-6, IL-10 e TNF-α em comparação aos pacientes estáveis. Os níveis séricos de heme livre, MMP-9 e TIMP-1 estavam aumentados em ambos os grupos de pacientes e foram associados a marcadores de inflamação, hemólise e estresse oxidativo. A AAT, por sua vez apresentou correlação negativa com marcadores hematológicos, HDL-C, ureia, creatinina e albumina e correlação positiva com leucócitos, bilirrubinas, LDH, HbS, CRP e ferritina. Pacientes com níveis mais elevados de AAT apresentaram mais infecção, litíase biliar e receberam mais transfusões sanguíneas. A presença de um alelo mutante no gene da *SERPINA1* foi relacionada à redução dos níveis séricos de ATT. Os resultados deste trabalho sugerem que alguns pacientes com DF apresentam sub-fenótipo dislipidêmico específico, caracterizado por níveis reduzidos de HDL-C, hipertrigliceridemia e elevação de VLDL-C. A análise da curva ROC mostrou que o LTB4, PGE2 e TGF-β são marcadores relacionados ao estado estável e que a IL-1β, IL-6 e TNF-α são marcadores de crise na DF. Além disso, a manutenção dos níveis séricos elevados de heme livre, provavelmente, está associada à hemólise crônica. Enquanto que, a MMP-9 e o TIMP-1 parecem estar associados a inflamação crônica nos pacientes com AF em crise e estado estável, contribuindo para um microambiente que resulta no aumento da gravidade clínica da doença. A AAT, por conseguinte, pode ser considerada como um marcador de prognóstico na DF, devido as suas associações com marcadores laboratoriais e clínicos relevantes para a doença. Os resultados dos genótipos da *SERPINA1* enfatizam o papel do alelo mutante na redução da produção de AAT, o que pode representar um fator de risco para desfechos graves nos pacientes estudados. Consideramos que esses resultados representam um passo importante em direção a um

prognóstico clínico mais completo; entretanto, estudos adicionais são necessários para testar as hipóteses sugeridas e os mecanismos envolvidos nesta rede complexa de marcadores, bem como o seu papel na patogênese da DF.

Palavras-chave: Doença falciforme; Inflamação; Hemólise; Disfunção endotelial; Biomarcadores de prognóstico.

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ABSTRACT

The pathogenesis of sickle cell disease (SCD) involves several mechanisms, and chronic inflammation, hemolysis and vascular dysfunction, which are important phenomena in this pathology. The search for biomarkers of prognosis is a challenge and a crucial point in researches related to DF. The aim of this thesis was to evaluate possible prognostic markers, associated with hemolysis, inflammation and endothelial dysfunction and their possible relationships with clinical severity in SCD. To do so, we investigated prospectively, biochemical and inflammatory markers in patients with SCD in steady state and crisis in comparing them to healthy subjects. Thus, in addition to the routine laboratory tests (lipid, hematological, renal and hepatic profiles, lactate dehydrogenase (LDH) and C-reactive protein (CRP), we investigated serum markers of vascular remodeling, such as tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-9 (MMP-9); inflammation, such as leukotriene B4 (LTB_4), prostaglandin E2 (PGE_2). Inflammatory and anti-inflammatory cytokines were investigates, including beta transforming growth factor (TGF-β), interleukin (IL) -1β, IL-6, IL-8, IL-12, tumor necrosis factor alpha (TNF-α) and IL-10). We evaluated hemolysis marker, the free heme, serum levels of alpha-1 antitrypsin (ATT) and gene polymorphisms of *SERPINA1*. We also collected clinical data from medical patients' records, and made comparison between patients and controls, which showed significant differences for most of the markers studied, especially the high-density lipoprotein cholesterol (HDL-C) that showed inverse association with markers of hemolysis, inflammation and whose low levels have been associated with cardiac abnormalities, pneumonia and increased use of blood transfusion. Patients with SCA in steady state showed high concentrations of LTB_4, PGE_2, TGF-β, IL-8 and IL-12 when compared to those in crisis. However, individuals in crisis with SCA had higher concentrations of IL-1β, IL-6, IL-10 and TNF-α compared to stable patients. Serum levels of free heme, MMP-9 and TIMP-1 levels were elevated in both groups of patients and was associated with markers of inflammation, oxidative stress and hemolysis. AAT, in turn, presents negatively correlated with hematological markers, HDL-C, urea, creatinine and albumin and positive correlation with leukocytes, bilirubin, LDH, Hb, CRP and ferritin. Patients with higher levels of AAT showed more infection, gallstones and received more blood transfusions. We found an association of the *SERPINA1* mutant allele and decreased serum levels of ATT. These results suggest that some patients with SCD have a specific dyslipidemic sub-phenotype, characterized by reduced levels of HDL-C, hypertriglyceridemia and elevated VLDL-C. The ROC curve analysis showed that LTB_4, PGE_2 and TGF-β are markers related to the steady state and that IL-1β, IL-6 and TNF-α are markers of crisis in SCD. Furthermore, the maintenance of high serum levels of free heme is probably associated with chronic hyper-hemolysis. Whereas MMP9 and TIMP-1 are probably associated with chronic inflammation in patients with SCA and stable state and in crisis, contributing to a microenvironment those results in increased clinical severity. We found an association of AAT as a prognostic marker in SCD due to their relevant clinical and laboratory function in the disease. The results of the genotypes *SERPINA1* emphasize the role of the mutant allele in reducing production of AAT, which may represent a risk factor for severe outcomes in these patients. We believe these results represent an important step toward of a more complete clinical follow-up; however, additional studies will be need to test the

hypotheses and mechanisms involved in this complex network of markers, and its role in the pathogenesis of SCD.

Keywords: Sickle Cell Disease; Inflammation; Hemolysis; Endothelial Dysfunction; Prognostic biomarkers.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

β^A	Alelo beta A
β^S	Alelo beta S
AA	Ácido araquidônico
AAT	Alfa-1 Antitripsina
AF	Anemia falciforme
APAE	Associação de Pais e Amigos dos Excepcionais
AVC	Acidente vascular cerebral
Ca^{++}	Íon cálcio
CD163	Cluster de diferenciação 163 (do inglês, *Cluster of differentiation 163*)
Cl^-	Íon cloreto
CO	Monóxido de carbono
COX-1	Ciclooxigenase 1
COX-2	Ciclooxigenase 2
CRP	Proteína C reativa (do inglês, *C reactive Protein*)
CSSCD	Estudo Cooperativo da Doença Falciforme (do inglês, *Cooperative Study of Sickle Cell Disease)*
DM	Diabetes Melito
DTC	*Doppler* transcraniano
DF	Doença falciforme (do inglês, SCD – *Sickle cell disease*)
ET-1	Endotelina-1
Fe	Ferro
HbA	Hemoglobina do adulto
HbAA	Homozigoto para a hemoglobina A, normal do adulto
HbAS	Heterozigoto para a hemoglobina S
HbF	Hemoglobina Fetal
HBB	Gene da globina beta
HBG	Gene da globina gama
HbS	Hemoglobina S
HO-1	Hemeoxigenase – 1 (do inglês, *Heme oxynase – 1*)
HP	Hipertensão pulmonar
HU	Hidroxiuréia
ICAM	Molécula de adesão intracelular (do inglês, *Intracellular adhesion molecule*)
IL	Interleucinas (do inglês, *Interleukins*)
INF-γ	Interferon gama
iNOs	Óxido nítrico sintase induzível (do inglês, *Inducible nitric oxide synthase*)
K^+	Íon potássio
LDH	Desidrogenase lática
LTBs	Leucotrienos B

LTB4	Leucotrieno B4
LPS	Lipopolissacarideo
MCP-1	Proteína quimiotática de monócitos -1 (do inglês, *Monocyte chemoattractant protein-1*)
MMPs	Metaloproteinases de matriz (do inglês, *Matrix metalloproteinases*)
MMP-9	Metaloproteinase de matriz – 9 (do inglês, *Matrix metalloproteinase- 9*)
MPO	Mieloperoxidase
NF-κB	Fator nuclear kappa B (do inglês, *Factor nuclear kappa B*)
NO	Óxido nítrico
NOS	Sintases de óxido nítrico (do inglês, *Nitric oxide synthase*)
PAI-1	Inibidor do ativador do plasminogênio (do inglês, *Plasminogen activator inhibitor-1*)
PAMPs	Padrões moleculares associados a patógenos (do inglês*, Pathogen-associated molecular patterns*)
PGs	Prostaglandinas
PGH_2	Prostaglandina H_2
PGE_2	Prostaglandina E_2
VER	Fosfatidilserina (do inglês, P*hosphatidylserine*)
ROS	Radicais livres de oxigênio (do inglês, *Reactive oxygen species*)
SE	Sequestro Esplênico
SM	Síndrome Metabólica
SNPs	Polimorfismos de um único nucleotídeo (do inglês, *single nucleotide polymorphism*)
SS	Homozigoto para a hemoglobina S
STA	Síndrome Torácica Aguda
TGF-β	Fator beta transformador do crescimento (do inglês, *transforming growth factor beta*)
Th1	Linfócito T auxiliar 1 (do inglês, *T helper 1)*
Th2	Linfócito T auxiliar 2 (do inglês, *T helper 2)*
TIMPs	Inibidores teciduais das metaloproteinases (do inglês, *Tissue inhibitors of metalloproteinases*)
TIMP-1	Inibidor tecidual da metaloproteinase – 1 (do inglês, *Tissue inhibitor of metalloproteinase – 1*)
TLRs	Receptores semelhantes ao Toll (do inglês, *Toll like receptors*)
TNF	Fator de necrose tumoral (do inglês, *Tumor necrosis factor*)
VCAM	Molécula de adesão à célula vascular (do inglês, *Vascular cell adhesion molecule*)
VLDL-c	Lipoproteína de muito baixa densidade ligada ao colesterol (do inglês, *Very low density lipoprotein*)
VOE	Eventos vaso-oclusivos (do inglês, *Vaso occlusive events*)
VO	Vaso oclusão
vWF	Fator de Von Willebrand (do inglês, *Von Willebrand factor*)

SUMÁRIO

1. INTRODUÇÃO

1.1. A MOLÉCULA DE HEMOGLOBINA

A hemoglobina (Hb) é uma proteína globular encontrada nos eritrócitos, tendo como principal função o transporte de oxigênio para todos os tecidos com trocas gasosas com o dióxido de carbono nos mesmos. A Hb é constituída pela globina, porção proteíca, e por um grupamento prostético formado por um complexo Protoporfirina IX - Ferro 2^+ (Fe 2^+) denominado grupo heme. A Hb é composta por quatro cadeias polipeptídicas agrupadas duas a duas, sendo um par de cadeias do tipo alfa (α – alfa e ξ - zeta) e outro de cadeias tipo não alfa (β - beta, δ - delta, γ - gama e ε - epsilon), cujas associações formam os diferentes tipos de Hb que são correspondentes a cada estagio de desenvolvimento ontogênico do indivíduo (BUNN e FORGET, 1986; WEATHERALL e PROVAN, 2000). O heme é formado por quatro núcleos pirrólicos condensados, que possuem estrutura plana e apresentam um átomo Fe 2^+ na região central. O heme e a globina são produzidos separadamente, sendo associados pós-traducionalmente para a formação da estrutura terciária e queternária da molécula de Hb; assim, quando o heme se dissocia da globina a mesma fica mais susceptível a desnaturação (HUNTSMAN e LEHMAN, 1974).

A hemólise é caracterizada pela lise dos eritrócitos com liberação de seu conteúdo celular. Os produtos do catabolismo eritrocitário, como a Hb, o heme e o ferro, são liberados pela hemólise e podem causar danos aos vasos e tecidos expostos, visto que, nessa condição, estão desprovidos dos antioxidantes normalmente produzidos e decorrentes das vias metabólicas do próprio eritrócito (BENZ *et al*, 1995). Para neutralizar esses compostos, o organismo tem captadores plasmáticos especializados na retirada e transporte de moléculas oxidantes além de outras moléculas e substâncias redutoras que contribuem para a proteção do organismo contra agentes oxidantes. Entretanto, esses sistemas de compensação e de desintoxicação são deficientes na doença falciforme (DF), em indivíduos que usam hemocomponentes, na malária ou na sepse, tendo em vista a exacerbação da hemólise intravascular, bem como o aumento de produtos oxidantes, que contribuem para a geração de espécies reativas de oxigênio (ROS) e para o estabelecimento de um ambiente altamente inflamatório (HUNTSMAN e LEHMAN, 1974; SCHAER *et al*., 2014).

Alterações genéticas na molécula da Hb são denominadas hemoglobinopatias e apresentam prevalência mundial elevada (NAGEL e STEINBERG, 2001), sendo classificadas como estruturais e de síntese. As hemoglobinopatias estruturais são decorrentes de mutações nos genes da globina, resultando em alterações na estrutura das cadeias polipeptídicas, com a

formação de hemoglobinas variantes, sendo exemplos mais comuns o das hemoglobinas S, C e E. As hemoglobinopatias de síntese ou as talassemias resultam de mutações nos genes da globina que levam a diminuição ou ausência na produção de uma ou mais cadeias polipeptídicas (MODELL e DARLISON, 2008). Nesse segundo grupo estão incluídos os indivíduos com persistência hereditária de hemoglobina fetal (PHHF) (LEE *et al.*, 1992; BUNN, 1994; BUNN, 1997; WEATHERALL e PROVAN, 2000).

1.2. A HEMOGLOBINA S E A DOENÇA FALCIFORME

A HbS é resultante da mutação de ponto GAG > GTG, que ocorre no sexto códon do gene da globina beta (*HBB*), resultando na substituição de um ácido glutâmico por valina na posição 6 da extremidade N-terminal da cadeia beta (β) globínica (STEINBERG *et al*.,1995). A HbS apresenta as mesmas funções da Hb normal, exceto quando a Hb está no estado desoxigenado; nesta condição a mutação que caracteriza a HbS leva à exposição de uma superfície hidrofóbica em torno da valina que produz interações entre as cadeias cadeias β1 e β2 de moléculas da HbS desoxigenadas, resultando em um núcleo polimérico e, em seguida, um feixe de polímero longo (GLADWIN E SACHDEV, 2012). O polímero de HbS preenche o eritrócito, alterando sua estrutura e flexibilidade, promovendo a desidratação e o aumento do estresse físico e oxidativo (STEINBERG, 2009). A taxa e a extensão da polimerização da HbS são proporcionais à extensão e a duração da desoxigenação da Hb, a concentração de HbS intracelular, e a concentração da hemoglobina fetal (HbF) nos eritrócitos, o que reduz de forma eficaz a concentração de HbS.

A DF é caracterizada pela presença da hemoglobina S (HbS) associada a outras hemoglobinas variantes (C e D, por exemplo), como na doença SC (HbSC), ou a hemoglobinopatias de síntese, como a talassemia beta. A DF possui prevalência mundial elevada o que leva produz impactos sociais e econômicos importantes, influenciando no aumento das taxas de abandono escolar, dos custos com internações e da incapacidade laboral dos pacientes (WHO, 2012). A anemia falciforme (AF) é a forma mais grave da doença, na qual o alelo beta S (β^S) encontra-se em homozigose, caracterizando o genótipo HbSS. A presença simutanea do alelo normal beta A (β^A) da Hb do adulto (HbA) e do alelo β^S caracteriza os indivíduos heterozigotos (HbAS), que são portadores assintomáticos da HbS (BUNN *et al.*, 1986; STEINBERG, 2001). A gravidade da doença está associada a taxa e a propagação da polimerização da HbS, que se relacionam a presença concomitante de fatores genéticos que modulam os níveis intracelulares de HbS ou a concentração de HbF, tais como

os efeitos protetores da alfa (α)-talassemia ou associação com a persistência hereditária de HbF (REES *et al*.; 2010). Assim, ocorre alteração da forma e diminuição da flexibilidade da hemácia, com consequente comprometimento da fluidez e das propriedades reológicas do sangue (KAUL e HEBBEL, 2000).

1.3. EPIDEMIOLOGIA DA DOENÇA FALCIFORME

A HbS é distribuída mundialmente, com frequência elevada na África, principalmente nas regiões Centro-Ocidental, Atlântico-Ocidental e Sul. O alelo β^S apresenta frequência entre 0,12 a 0,14 no Congo e Zaire e 0,08 a 0,10 no Senegal. A HbS também é encontrada em países do Mediterrâneo, incluindo a Itália e Grécia, bem como na Arábia Saudita, Kuait e Índia. Nos Estados Unidos da América e América Latina, aproximadamente 8% da população negra é portadora da HbS. A DF afeta aproximadamente 1/500 afro-americanos e 1/1200 hispano-americanos e estima-se que 72.000 americanos têm DF (WANG e LUKENS, 1998; REES, 2010) (Figura 1).

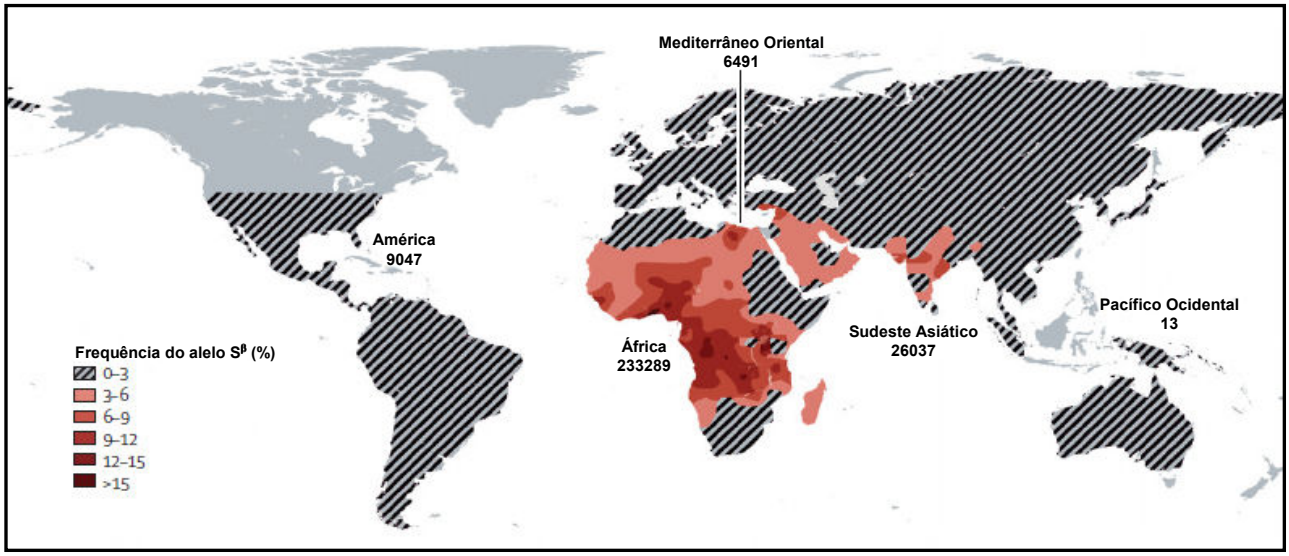

Figura 1. Distribuição mundial do alelo β^S. Os números indicam as estimativas para o número anual total de indivíduos HbSS, HbSC e HbS/β-talassemia por região, segundo dados da OMS (adaptado de REES *et al.,* 2010).

O Brasil apresenta distribuição heterogênea do alelo β^S entre os diferentes estados, com variação da prevalência de acordo com o grupo populacional estudado (Figura 2). O estado da Bahia apresenta a frequência brasileira mais elevada para a HbS, tendo sido encontrados 9,8% para os indivíduos HbAS e a prevalência de 0,9% para os heterozigotos duplos SC (HbSC) e 0,2% para a AF, em recém-nascidos de Salvador-Bahia (ADORNO *et al*., 2005). A Associação de Pais e Amigos dos Excepcionais (APAE) descreveu as frequências de 9,5 e 11,4% para indivíduos HbAS em estudo realizado na região do

Recôncavo Baiano (SILVA *et al*., 2006). Dados da triagem neonatal entre os anos de 2007 e 2009 relatam a razão de uma criança com DF para cada 565 nascidos vivos no estado da Bahia (AMORIM *et al*., 2010) (Figura 2).

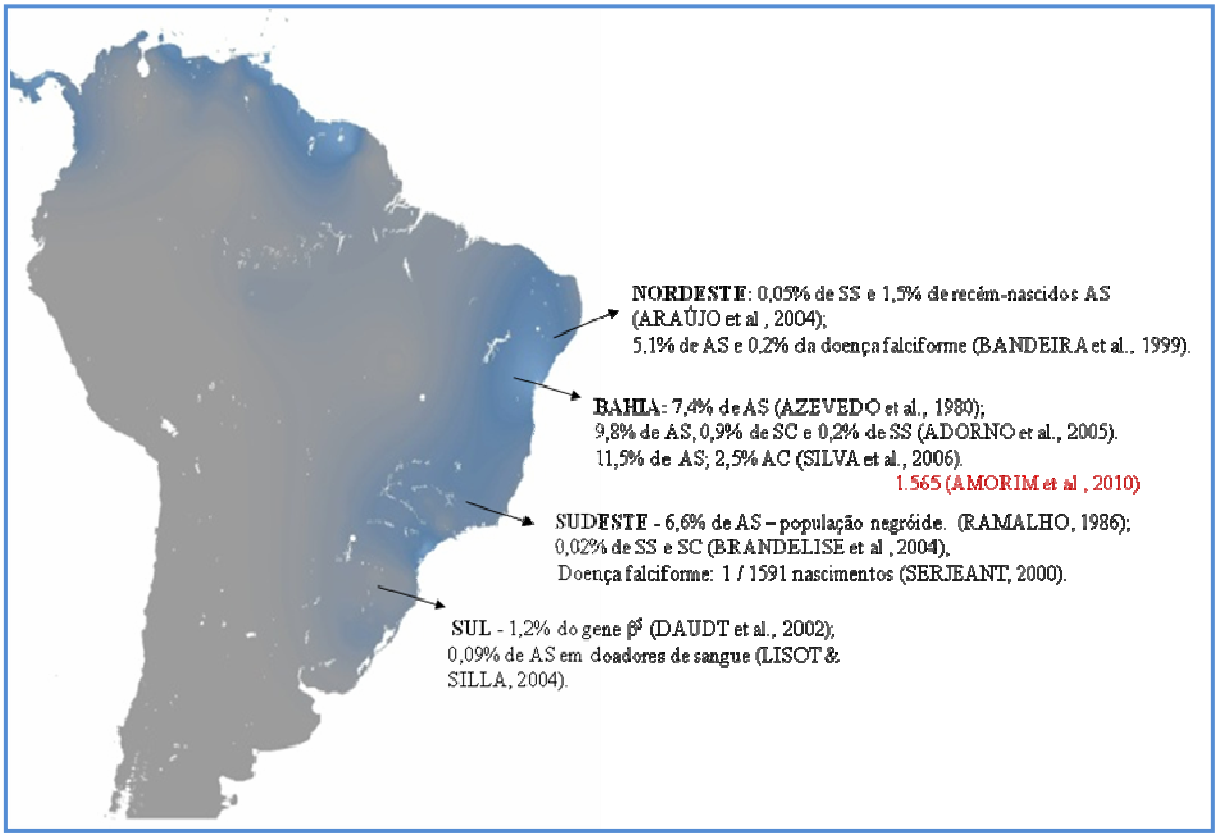

Figura 2. Distribuição da HbS no Brasil. Distribuição do alelo β^S em algumas regiões brasileiras conforme dados de estudos realizados no país entre 1980 e 2010 (adaptado de AZEDEDO *et al*, 1980; ADORNO *et al*, 2005; SILVA *et al,* 2006 e AMORIM *et al*, 2010)

1.4. MANIFESTAÇÕES CLÍNICAS DA DOENÇA FALCIFORME

Os indivíduos com DF apresentam quadro clínico heterogêneo, com grande variabilidade na gravidade das manifestações clínicas, sendo estas, influenciadas pela idade, gênero, características genéticas, fatores ambientais e socioeconômicos (LYRA *et al*., 2005; BRASIL, 2008). Entre as manifestações clínicas mais comuns na DF podemos destacar os eventos de vaso-oclusivos (VOE) e dolorosos, o acidente vascular cerebral (AVC), a síndrome torácica aguda (STA), o priaprismo, a hipertensão pulmonar (HP), a retinopatia, a anemia hemolítica, o sequestro esplênico (SE), a osteonecrose, a litíase biliar, as infecções e as úlceras maleolares, dentre outras (KATO *et al.*, 2007; SONATI, 2008, STEINBERG, 2009; SARAF *et al.*, 2014). As crianças com DF podem apresentar eventos graves desde os primeiros anos de vida, sendo que as manifestações que levam ao número maior de internações até os 10 anos são os VOE e dolorosos, as infecções, SE, STA e AVC (POWARS *et al.,* 2005).

Os fenômenos vaso-oclusivos e dolorosos constituem os principais eventos clínicos da DF, sendo decorrentes da oclusão microvascular, secundária a fatores múltiplos associados à participação de diversos tipos celulares, como reticulócitos, leucócitos, plaquetas e hemácias falcizadas. A taxa de polimerização da HbS tem sido destacada como fator principal associado a adesão e desidratação dos eritrócitos e, consequentemente, ao desenvolvimento de VOE (KAUL *et al*., 1989; KAUL e HEBBEL*,* 2000; TURHAN *et al.*, 2002; Looney e Matthay, 2009).

A oclusão microvascular pode levar a necrose tecidual e a ação de mediadores inflamatórios desencadeando uma resposta dolorosa. Os VOE são frequentemente associados a episódios febris e podem estar ou não relacionados aos processos infecciosos (PLATT *et al*.,1994; BALLAS, 2002; HOPPE, 2014). As infecções são as principais causas de óbito entre os pacientes com DF e podem ocorrer de forma recorrente e acredita-se que estão associadas a alterações no sistema imunológico e à asplenia funcional. Dentre as infecções mais comuns estão àquelas causadas por microorganismos encapsulados, como o *Streptococcus pneumoniae* (METHA *et al.,* 2006).

A oclusão microvascular relaciona-se a episódios dolorosos e a alterações na microcirculação cerebral. O AVC observado nos indivíduos com DF e, frequentemente, em crianças com AF, muitas vezes acontece de forma silenciosa e resulta de um comprometimento vascular das grandes artérias do polígono de Willis levando a infartos e ao comprometimento da perfusão vascular ou, eventualmente, embolia arterial (MESCHIA E PANKRATZ, 2005; ADAMS, 2007). O AVC é uma das complicações mais graves da DF, sendo o isquêmico mais comum que o hemorrágico; este último equivale a cerca de 5% dos casos e é mais frequente em adultos, apresentando morbimortalidade elevada, como consequência de ruptura de pequenos vasos, a partir de neoformações vasculares ou de aneurismas (STROUSE *et al.,* 2011). O Estudo Cooperativo da DF (CSSCD), o maior estudo longitudinal e observacional dos Estados Unidos da América sobre complicações da DF, relatou a prevalência global de AVC de 3,75% em indivíduos com DF, sendo que em pacientes com AF na faixa etária menor que 20 anos de idade, a prevalência de AVC foi de 11%. A incidência maior de AVC (1,02 por 100 pessoas/ano) foi encontrada em crianças com AF entre 2 e 5 anos de idade (OHENE-FREMPONG *et al.,* 1998).

As sequelas do AVC comprometem as atividades socio-econômicas dos indivíduos com DF e têm reflexo na estrutura familiar (BRASIL, 2008). Atualmente, métodos de diagnóstico precoce das alterações vasculares cerebrais têm sido usados para monitoramento de indivíduos com DF. O *doppler* transcraniano (DTC), por exemplo, permite o

monitoramento dinâmico da velocidade do fluxo sanguíneo cerebral e pulsatilidade vascular com uma resolução temporal elevada (ADAMS, 2005). O DTC vem se tornando cada vez mais acessível, reprodutível e a sua portabilidade oferece conveniência maior em relação a outros métodos de imagem. As limitações do DTC estão relacionadas à experiência do operador e ao fato de 10 a 20% dos pacientes terem janelas acústicas transtemporais inadequadas (ADAMS, 2005; NAQVI *et al.*, 2013).

A STA é uma complicação frequente na DF, associada usualmente à febre, dor torácica e/ou tosse, que ocorre em pacientes com as formas mais graves da doença; tem relação com a oclusão vascular ou infecção, embora outras etiologias estejam envolvidas (STEINBERG *et al*., 2001). A infecção predomina em pacientes pediátricos e a obstrução vascular nos adultos, embora possam ocorrer de forma simultânea e concorrente (GASTON *et al.*, 1986; VICHINSKY *et al*., 2000; STEINBERG, 2009).

A HP é uma complicação reconhecida na DF (GLADWIN, *et al*., 2004), sendo uma condição grave, com taxa de mortalidade elevada (CASTRO *et al.,* 2003). A patogênese da HP na DF tem relação com a hemólise recorrente e redução sistêmica do óxido nítrico (NO) pela ação da Hb plasmática livre e disfunção endotelial, resultando em hiperplasia da íntima vascular pulmonar, hipoxemia crônica, ativação plaquetária excessiva e asplenia (WUN *et al.,* 1998; VILLAGRA *et al*., 2007). Vários fatores vêm sendo associados à presença de HP em pacientes com DF, entre eles destacam-se a endotelina-1 (ET-1), um aminoácido endógeno, produzido pelas células endoteliais pulmonares e sistêmicas, que apresenta ação vasoconstrictora potente e de longa duração (HICKEY *et al.*, 1985; YANAGISAWA *et al.,* 1998). A ET-1 participa de vários processos fisiológicos relacionados aos sistemas nervoso, renal, cardiovascular, respiratório, gastrointestinal e endócrino (MASAKI, 1998; ARCHER, 2000; KEDZIERSKI E YANAGISAWA, 2001). Além disso, a ET-1 tem sido relacionada a diversas complicações clínicas da DF, incluindo alterações cardíacas e hipertensão pulmonar (MINNITI *et al*., 2009; GLADWIN E SACHDEV, 2012).

A redução da capacidade física em indivíduos adultos e pediátricos com DF está associada à gravidade da anemia. Neste caso, os exames cardíacos estão geralmente alterados, sendo que pode se observar alterações como hiperatividade, sopro sistólico, bem como o encontro frequente de contração prematura (COVITZ *et al.,* 1995). O eletrocardiograma de indivíduos com DF, em geral não apresenta anormalidades específicas e mostra sinais de hipertrofia ventricular (STEINBERG, 2001). As alterações cardíacas na DF estão associadas à concentração diminuída de Hb e a alterações das funções sistólica e diastólica (COVITZ *et*

al., 1995; GLADWIN E SACHDEV, 2012), não sendo comum a ocorrência de colapso cardiovascular devido à distensibilidade hepática limitada (TAYLOR *et al*., 2004).

O SE se caracteriza pelo aumento súbito do baço, diminuição da Hb, reticulocitose, palidez e plaquetopenia (variável), podendo ocorrer insuficiência cardíaca e choque hipovolêmico (STEINBERG *et al*., 2001). O SE está relacionado ao aumento da mortalidade em crianças com DF nos primeiros cinco anos de vida; entretanto, pode atingir indivíduos em qualquer faixa etária, com genótipo HbSC e Hb S/β-talassemia (HbS/β-tal), devido à persistência da esplenomegalia (TAYLOR *et al*., 2004). O SE deve ser reconhecido e tratado rapidamente, sendo responsável por 13% dos óbitos ocorridos entre os pacientes com DF menores de 20 anos (STEINBERG *et al*., 2001; POWARS *et al.,* 2005).

Outra manifestação clínica importante na DF é o priapismo, caracterizado pela diminuição acentuada do fluxo sanguíneo e o empilhamento de hemácias falcizadas no corpo cavernoso, sendo uma manifestação clínica que pode ocorrer de forma rápida ou prolongada nesses pacientes (STEINBERG *et al*., 2001). Nos casos de eventos prolongados, além da hidratação e da analgesia, pode ser necessária a intervenção cirúrgica (STUART e NAGEL, 2004). Alguns estudos sugerem que a origem desse fenômeno pode estar relacionada a mecanismos moleculares complexos que envolvem, por exemplo, a participação da guanilato ciclase e de uma fosfodiesterase do tipo 5A (BURNETT, 2003; CHAMPION *et al.*, 2005).

Os pacientes com DF podem também desenvolver colelitíase, que é uma consequência do metabolismo acelerado de bilirrubina, tipicamente observada nas anemias hemolíticas. A litíase biliar pode ser observada na primeira década de vida, sendo que a maioria dos adultos é afetada e o método diagnóstico preferencial é a ultrassonografia (STEINBERG, 2001).

Os indivíduos com AF podem desenvolver úlceras de pernas, que são decorrentes de lesões traumáticas, que levam um período longo para cicatrização (SERJEANT *et al*., 2005). As condições clínicas, como úlcera de perna e retinopatia têm sido associadas ao risco elevado de lesões a órgãos e óbito precoce nesses pacientes (POWARS *et al.*, 2005).

1.5. FISIOPATOLOGIA DA DOENÇA FALCIFORME

Em grande parte, a morbimortalidade na DF decorre de complicações associadas aos VOEs, a isquemia, lesão por reperfusão e infarto em diversos orgãos e tecidos (STEINBERG, 2006; CHARNESKI E CONGDON, 2010). Dessa forma, os eventos básicos relacionados às manifestações clínicas da DF são a VO, a hemólise e a inflamação, que se manisfestam de forma aguda e crônica; esses eventos não estão totalmente elucidados, mas acredita-se que sejam resultado da interação celular complexa e dinâmica com participação de reticulócitos,

hemácias falcizadas, hemácias normais, células endoteliais, leucócitos, plaquetas, além da produção exacerbada de radicais livres e citocinas (LEE *et al*., 2006).

1.5.1. Hemólise

A lise dos eritrócitos no compartimento vascular libera Hb para o plasma, que forma dímeros que se ligam rapidamente a haptoglobina (Hp). O complexo haptoglobina-hemoglobina (Hp-Hb) expõe um epítopo que é reconhecido com afinidade elevada pelo CD163, presente na superfície de monócitos/macrófagos, levando a endocitose do complexo Hp-Hb e a sua degradação (NAGEL E GIBSON, 1971; KRISTIANSEN *et al*., 2001). Uma vez que a haptoglobina não é reciclada, a formação de quantidades grandes do complexo Hp-Hb conduz à depleção rápida da referida proteína; assim, nas doenças hemolíticas graves, como DF, a Hp plasmática geralmente é indetectável (TABBARA, 1992).

O metabolismo do heme, por sua vez, consiste na oxidação do heme ferroso ($Fe\ 2^{+}$), o componente de ligação da Hb ao oxigênio, ao heme férrico ($Fe\ 3^{+}$), que se liga, com afinidade elevada a uma glicoproteína plasmática, a hemopexina (Hpx) (RYTER *et al.*, 2002). O heme ligado a Hpx é degradado em uma série de passos enzimáticos no fígado. A hemeoxigenase 1 (HO-1), posteriormente, degrada o heme livre, que apresenta características pró-oxidantes e pró-inflamatórias, produzindo o monóxido de carbono (CO), biliverdina e ferro (Fe) (RYTER *et al.*, 2002; JISON *et al.*, 2004). O CO tem propriedades antioxidantes, vasodilatadora, antiproliferativa, anti-inflamatórias, enquanto que a biliverdina é um antioxidante que é convertido pela biliverdina redutase em bilirrubina (BARANANO *et al.*, 2002). As substâncias oxidantes derivadas do ferro hêmico são diretamente capturadas e inativadas pela ferritina (SEARS, 1970; ROLTHER *et al*., 2005). Além disso, a ligação do CD163 ao complexo Hp-Hb tem ação anti-inflamatória pela indução da produção de IL-10 e HO-1 pelos monócitos circulantes (PHILIPPIDIS *et al*., 2004). Assim, as ações antioxidantes, anticoagulantes, antiproliferativas e os efeitos vasodilatadores do sistema CD163/HO-1/biliverdina redutase são mecanismos compensatórios a redução dos níveis de NO e aos efeitos vasoconstritores, proliferativos, inflamatórios e pró-oxidantes promovidos pelo aumento plasmático de Hb, heme e ferro hêmico (SEDLAK E SNYDER, 2004; ROLTHER *et al*., 2005).

A hemólise, assim como a VO, é um dos eventos primários mais importantes na patogênese da DF (ODIÈVRE *et al.*, 2011; REES E GIBSON, 2011). As alterações na permeabilidade a cátions e na organização de proteínas da membrana eritrocitária, levam a

redução da flexibilidade e maleabilidade dos eritrócitos, além da desidratação e a baixa tensão de oxigênio desencadeando a polimerização da HbS e a hemólise (STEINBERG, 2001).

O aumento da desidratação é uma das consequências mais relevante de lesão da membrana dos eritrócitos ricos em HbS. As contribuições mais importantes para esse processo são dos cotransportadores de potássio (K^+)/cloreto (Cl^-) e cálcio (Ca^{++})-ativado e o efluxo de K^+ (Lew *et al*., 1991). As taxas de cotransporte de K^+/Cl^- são muito mais elevadas nos eritrócitos dos indivíduos com AF quando comparadas aqueles normais (HbAA). O cotransporte de K^+/Cl^- é induzido pela presença de edema celular e também por acidificação (BRUGNARA *et al*., 1985; CANESSA *et al*., 1986; LEW *et al*., 1991). Dessa forma, o cotransporte desses íons parece ter papel importante na desidratação elevada não só dos eritrócitos SS, mas também daqueles SC, resultando em uma característica morfológica comum na DF, que é a presença de hemácias em forma de alvo (BRUGNARA *et al*., 1985; CANESSA *et al*., 1986). Os eritrócitos SS têm quantidades aumentadas de cálcio, compartimentada em vesículas intracelulares, mesmo com concentrações normais de Ca^{++} no citosol. Quando a membrana celular é alterada pela falcização, no entanto, há o aumento transitório na concentração citosólica de Ca^{++}, sendo este aumento suficiente para provocar alteração no canal de Ca^{++} -dependente de K^+, contribuindo com uma segunda via para a perda de K^+ e de água induzidas pela falcização, além da desidratação eritrocitária (Lew *et al*., 1985; BUNN, 1997).

A lesão endotelial e a hemólise, por sua vez, contribuem para manutenção do dano vascular crônico decorrente do aumento nos níveis de citocinas inflamatórias, da mobilização de células mononucleares e dos níveis patológicos da adesão de monócitos ao endotélio vascular (SWITZER *et al.,* 2006; CONRAN *et al.,* 2007). Em geral, estas condições inflamatórias exacerbadas, bem como as interações entre células mononucleares e endoteliais, podem contribuir para o espessamento da camada íntima e estreitamento do lúmen vascular. Essas são condições observadas na HP e no AVC em crianças com DF, que representam de 20-30% das mortes relacionadas à DF em pacientes adultos (VERDUZCO *et al.,* 2009; MAÎTRE *et al.,* 2011) (Figura 3).

A lise das hemácias falcizadas ocasiona uma série de alterações bioquímicas que são percebidas, laboratorialmente, pelo aumento das bilirrubinas (principalmente a indireta), diminuição da Hp e aumento dos níveis da lactato desidrogenase lática (LDH), enzima que participa do processo metabólico de produção de energia e que é encontrada em grandes quantidades nos eritrócitos (REES *et al*., 2010; REES E GIBSON, 2011).

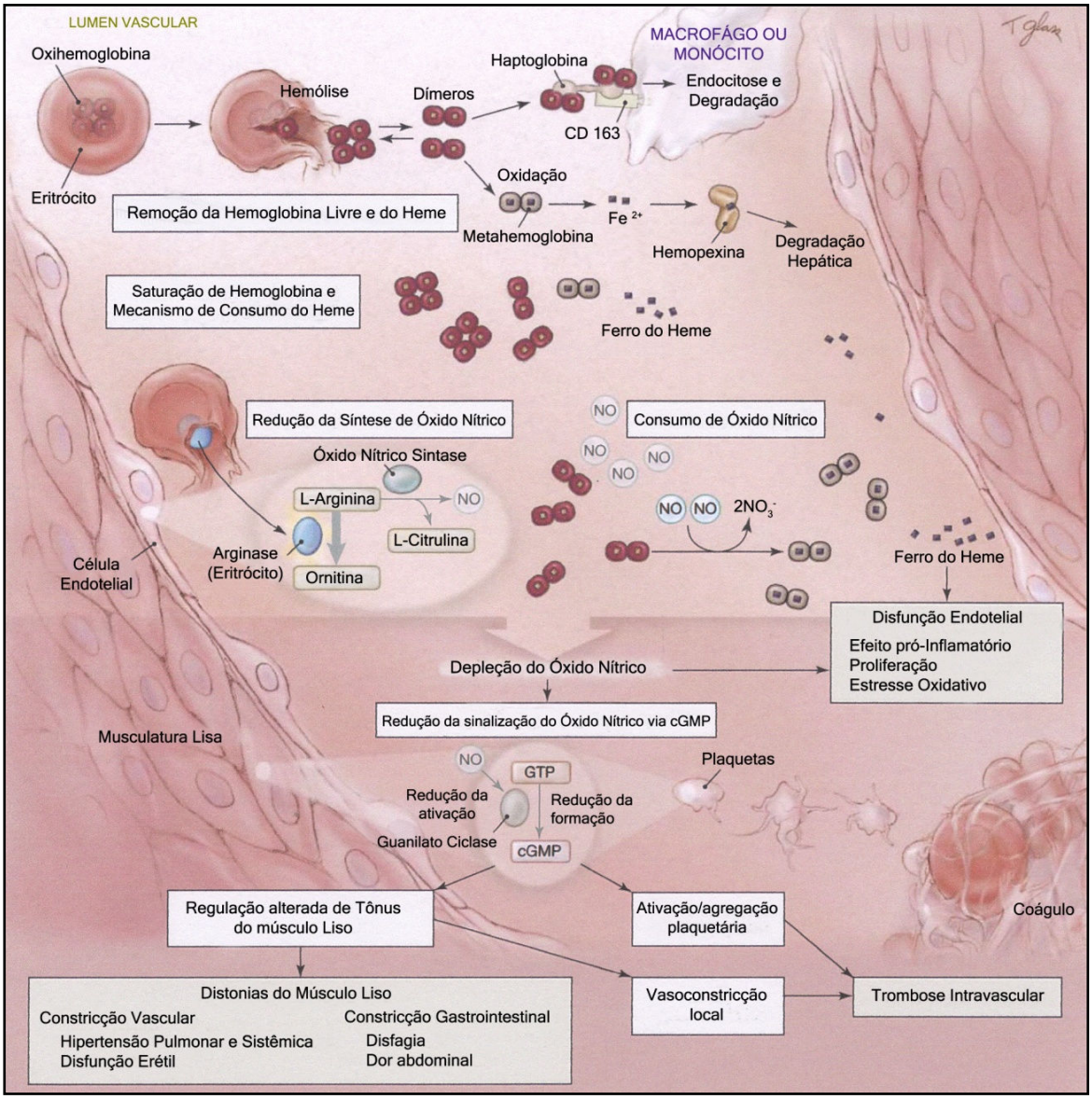

Figura 3. Hemólise intravascular. Durante a hemólise intravascular, a hemoglobina é liberada no plasma, de onde é normalmente retirada pela haptoglobina, CD163 e hemopexina. Os complexos de haptoglobina - hemoglobina ligam-se a CD163, na superfície de macrófagos/monócitos, iniciando a endocitose e a degradação dos mesmos. Durante o processo de oxidação da hemoglobina há também a liberação do heme férrico, que se liga a hemopexina e é degradado pelos hepatócitos. A hemólise excessiva satura e esgota esses sistemas de remoção de hemoglobina e leva a um acúmulo da hemoglobina e do heme no plasma. A hemoglobina plasmática e o heme medeiam os efeitos pró-inflamatório, proliferativo e pró-oxidantes nas células do endotélio vascular. O óxido nítrico (NO) é normalmente produzido a partir da L-arginina nas células endoteliais do vaso pela ação da enzima óxido nítrico sintase (NOS). O NO mantém o relaxamento da musculatura lisa e inibe a ativação e agregação plaquetárias, regulando assim o tônus vascular e promovendo a homeostase sistêmica. Durante a hemólise intravascular, a disponibilidade do NO pode ser severamente limitada pela reação com oxihemoglobina e pela degradação da L-arginina, pela arginase eritrocitária, apesar dos níveis elevados de NOS (diminuição da síntese de NO). A depleção do NO resulta na diminuição da ativação da guanilato ciclase, uma enzima necessária para a geração de guanina monofosfato cíclico (cGMP). A redução nos níveis de cGMP desrregula o tônus da musculatura lisa resultando em distonias, incluindo hipertensão arterial sistêmica e pulmonar, disfunção erétil, disfagia e dor abdominal. A diminuição dos níveis de cGMP através da depleção de NO também pode conduzir à ativação e agregação das plaquetas, promovendo a formação de coágulos. GTP indica trifosfato de guanosina. Adapatado de ROLTHER *et al*., 2005.

1.5.2. Inflamação

A DF é caracterizada pela presença de inflamação aguda e crônica associadas à ocorrência de infecções recorrentes, à leucocitose, à ativação leucocitária e endotelial, sendo estes eventos relacionados à VO (JANEWAY, 2001; HOPPE, 2014). Os aspectos imunológicos da DF têm sido cada vez mais estudados, sendo que alguns autores descrevem níveis elevados de citocinas Th2, como as interleucinas (IL) 4, 6 e 10 (IL-4, IL-6 e IL-10) e de citocinas pró-inflamatórias, como a IL-8 e o Fator de Necrose Tumoral alfa (TNF-α) no plasma de indivíduos com AF em estado estável, alterações que estão associadas ao aumento na expressão ou que promovem a ativação de moléculas de adesão em leucócitos, plaquetas e no endotélio vascular desses indivíduos (MALAVE, *et al.*, 1993; TAYLOR *et al.*, 1997).

Nas fases iniciais de uma infecção ocorre a diferenciação das células T em duas principais classes efetoras CD4+, as células Th1 (quando estimuladas por IL-12 e interferon gama (INF-γ)) ou Th2 (estimuladas pela produção de IL-4 e de IL-6). Nesse contexto, há uma indução à citotoxicidade e a resposta inflamatória via produção de citocinas Th1, como a IL-12, IL-2, o IFN-γ e o TNF-α; a resposta Th2, por sua vez, conta com a participação da IL-4, IL-5, IL-6 e IL-10, que são importantes na formação de anticorpos. As células Th1 e Th2 desempenham papéis distintos em várias condições fisiológicas ou patológicas. Essas respostas são também antagônicas, uma vez que o IFN-γ modula negativamente a resposta Th2, e as IL-4 e IL-10 modulam negativamente a resposta Th1, o que permite a homeostasia do sistema imune e a resposta imunológica balanceada (JANEWAY, 2001; FRENETTE, 2004; MACHADO, *et al.* 2004).

As células fagocíticas desempenham papel importante na imunidade inata e adquirida, pela capacidade de reconhecimento, fagocitose e promoção da resposta imune pela produção de mediadores inflamatórios e liberação de ROS (MEDZHITOV e JANEWAY, 2000). No que se refere à AF, alguns autores descrevem níveis plasmáticos elevados de citocinas Th2 e de citocinas pró-inflamatórias, como a IL-8 e TNF-α, em estado estável, sendo que, possivelmente, essas alterações estão associadas ao aumento na expressão ou promovem a ativação de moléculas de adesão em leucócitos, plaquetas e no endotélio vascular desses indivíduos (MALAVE *et al.,* 1993; TAYLOR *et al.*, 1997; CAJADO *et al*., 2011).

O processo inflamatório é modulado por diversos tipos de moléculas, tais como aminas vasoativas, eicosanóides derivados de lipídios, citocinas e quimiocinas. Mediadores lipidicos endógenos têm sido descritos como importantes nesse processo por apresentarem efeito imunomodulador. Nesse grupo se destacam os leucotrienos B (LTBs), que são moléculas resultantes da ação das lipoxigenases; o LTB_4, por exemplo, foi associado ao

aumento da produção de citocinas pró-inflamatórias e de NO (PETERS-GOLDEN *et al.,* 2005). Outro grupo de mediadores lipídicos em destaque são as prostaglandinas (PGs), cuja produção se inicia com a liberação de ácido araquidônico (AA) a partir de fosfolípidos da membrana celular sob a ação da fosfolipase A2, em resposta a estímulos inflamatórios. O AA é convertido em prostaglandina (PG) H_2 pela ação das ciclooxigenases 1 e 2 (COX-1 e COX-2). A prostaglandina E_2 (PGE_2), por sua vez, apresenta ações em diversos tipos celulares, de acordo com ligações a receptores específicos nessas células; dessa forma, a PGE_2 desempenha papel importante, como a inibição da produção de citocinas pró-inflamatórias, como IFN-γ, TNF-α, IL-12, e IL-1β e a produção de citocinas de perfil Th2, como IL-10 e IL-4 (HARRIS et al., 2002). Estudos prévios têm associado a interação anormal de hemácias falcizadas com o endotélio hiperestimulado ao aumento nos níveis dos eicosanóides, sugerindo um possível aumento na síntese dos mesmos (IBE *et al*., 1997; HARRIS et al., 2002).

O fator beta transformador do crescimento (TGF-β) faz parte de uma família de proteínas com propriedades diveras e alguns de seus receptores são amplamente distribuídos pelos tecidos do organismo vivo. As ações do TGF-β estão relacionadas ao desenvolvimento e diferenciação tecidual, além da regulação de células do sistem imune (MILLS *et al.*, 2000). A deficiência de TGF-β, em modelos animais, relaciona-se a um processo inflamatório grave que atinge diversos orgãos, sendo associada à ação de citocinas e de linfócitos T (GORELIK E FLAVELL, 2002). Keikhaei e cols. (2013) relataram que pacientes com DF em VO apresentaram níveis médios aumentados de todas as citocinas quando comparados a indivíduos em estado estável, mas com significancia estatística apenas para a IL-8. Por outro lado, os níveis de TGF-β e IL-17 foram mais elevados nos pacientes em estado estável que nos controles normais. Vilas-Boas e cols. (2010), em estudo realizado com indivíduos com AF, relataram a associação direta entre os níveis de TGF-β e arginase, sugerindo que o TGF-β pode ter papel importante na ativação vascular (Figura 4).

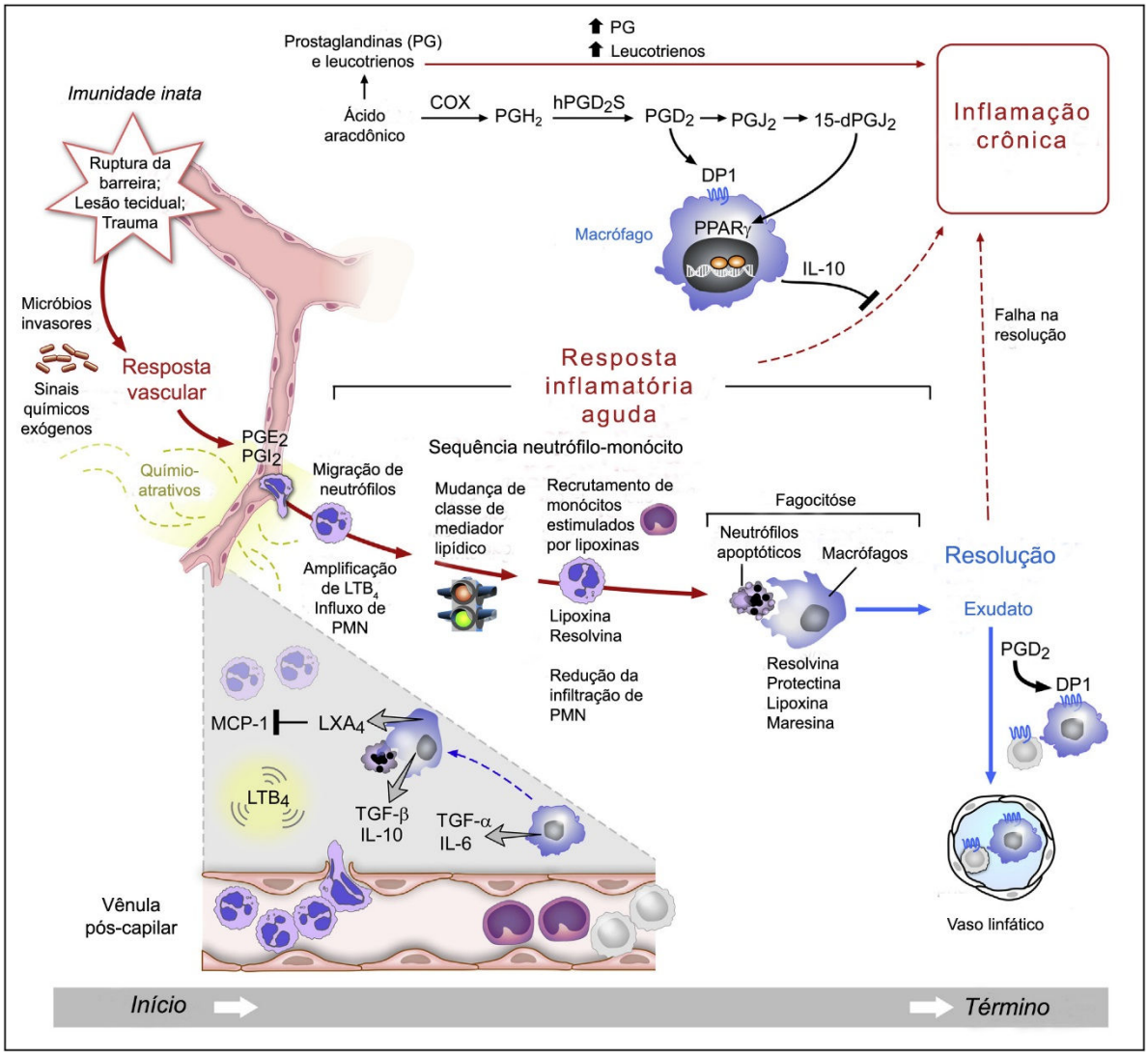

Figura 4. A resposta inflamatória aguda e o papel dos mediadores lipídicos na sua resolução ou falha. A resposta aguda tem início com alterações no fluxo sanguíneo, estimuladas por PGE_2 e PGI_2, e LTB_4, os quais são produzidos a partir do ácido araquidônico e estimulam o recrutamento de polimorfonucleares (PMN). O excesso de prostaglandinas e leucotrienos contribuem para a inflamação crônica. A ciclooxigenase (COX) desencadeia a produção de PGD_2, via sintase PGD_2 humana ($hPGD_2s$), ativa seu receptor DP1 que estimula a IL-10, citocina anti-inflamatória, que bloqueia a instalação da inflamação crônica. PGD_2 pode ser convertida em PGJ_2 e 15-$dPGJ_2$ e produtos que ativam PPAR-g que leva a resolução da inflamação. A mudança de classe do mediador lipídico é a chave temporal, em exsudatos inflamatórios, que ativa a produção de lipoxina (LXA). A LXA_4 regula a MCP-1, recrutamento de monócitos e interrompe o influxo de PMN estimulado por LTB_4. Lipoxinas e resolvinas limitam ainda mais influxo PMN para o local e estimulam a fagocitose e a depuração de restos celulares pelos macrófagos. Resolvinas, protetinas, lipoxinas e maresinas (SPM) estimulam e melhoraram a fagocitose, promovendo a resolução do processo. A perda de qualquer um dos receptores celulares ou de mediadores químicos pode, em teoria, conduzir à falha de resolução que e ocasionar uma inflamação persistente, crônica, associada a diversas doenças (Adaptado de Buckley *et al.*, 2014).

1.5.3. Lesão Endotelial

O endotélio vascular dos indivíduos com DF apresenta-se frequentemente estimulado pela ação de citocinas pró-inflamatórias, pela sobrecarga de ferro, pelo aumento das ROS e da adesividade celular através da expressão de moléculas de adesão como a molécula de adesão

intercelular-1 (ICAM-1) e a molécula de adesão celular vascular-1 (VCAM-1). Além disso, a ativação plaquetária e de fatores da cascata de coagulação contribuem para a ocorrência do fenômeno vaso-oclusivo na DF (OKPALA, 2004; MORRIS, 2008).

A homeostase vascular é regulada por diversas substâncias dentre as quais se destaca o óxido nítrico (NO), um potente vasodilatado produzido no endotélio a partir da L-arginina, substrato obrigatório, que é convertida em citrulina por uma família de enzimas, as sintases do NO (NOS) (MORRIS *et al.,* 2005). O NO tem propriedades que podem afetar todos os aspectos da DF, desde a redução da ativação plaquetária e expressão de receptores de adesão ao endotélio vascular até a diminuição da proliferação de músculo liso vascular, o que limita a lesão de isquemia-reperfusão, modula a proliferação endotelial e regula a inflamação (ROTHER *et al*., 2005; DURANTE *et al*., 2007). A redução dos níveis de NO tem um papel crucial na disfunção endotelial, e, por conseguinte, o desequilíbrio no metabolismo do NO é um processo comum entre os diversos mecanismos da vasculopatia falciforme (MORRIS *et al*., 2000). A DF é caracterizada por um estado de resistência, inativação e biodisponibilidade baixa do NO, embora a expressão e a atividade da óxido nítrico sintase (NOs) esteja aumentada na referida patologia. Indivíduos adultos com DF em estado estável da doença apresentam deficiência de arginina; já as crianças com DF têm níveis plasmáticos comparáveis aos controles saudáveis (ENWONWU, 1989; MORRIS *et al*., 2000). Logo, acredita-se que a deficiência de arginina está relacionada à idade do indivíduo e é influenciada por eventos agudos. As concentrações séricas de arginina diminuem, em adultos e crianças, durante os VOE e a STA, e estão associadas à redução dos níveis de metabólitos do NO. Em última análise, a biodisponibilidade baixa da arginina está associada à mortalidade precoce em adultos com DF, principalmente em condições de hemólise elevada, inflamação e/ou estresse oxidativo (MORRIS *et al*., 2005).

Conhecimentos novos à cerca da disfunção endotelial na DF vêm sendo atribuídos à hemólise e a interferência desse fenômeno em diversas etapas da via arginina-NO (GLADWIN *el al*., 2004; ROTHER *et al*., 2005). Em condições normais, a Hb é encontrada no interior dos eritrócitos; entretanto, durante a hemólise, a mesma é descompartimentalizada e liberada no plasma, onde reage e destrói, de forma rápida, o NO. Esse processo resulta em consumo anormalmente elevado de NO e a formação de ROS e, em última análise, a inibição da vasodilatação (MORRIS *et al.*, 2007; TAYLOR *et al.,* 2008). A redução do NO pela ação da Hb livre relaciona-se a uma hiperestimulação do endotélio vascular, através da ativação da transcrição de moléculas de adesão, incluindo a VCAM-1 e a E-selectina, e vasoconstritores potentes, tais como a endotelina. A liberação simultânea da arginase eritrocitária durante a

hemólise vai limitar a disponibilidade de arginina para a NOs, contribuindo para uma deficiência de NO (DURANTE *et al*., 2007). A arginase também redireciona o metabolismo de L-arginina a L-ornitina e há a formação de poliaminas e de L-prolina, que são essenciais para o crescimento de células do músculo liso e para a síntese de colágeno. Dessa maneira, a indução da arginase também pode promover a remodelação aberrante do endotélio vascular e a formação de neoíntima (FIELD *et al*., 2008). As complicações pulmonares na DF comprometem a oxigenação e contribuem para a manutenção da falcização dos eritrócitos (MORRIS *et al.,* 2005). Através da criação de uma mudança para o metabolismo ornitina, a arginase desencadeia o processo que contribui para a vasculopatia proliferativa comumente encontrada em desordens hemolíticas (ENWONWU, 1989; FIELD *et al.,* 2008; GLADWIN e VICHINSKY, 2008) (Figura 5).

O estresse oxidativo constitui outro mecanismo importante de vasculopatia associada à hemólise, uma vez que nessas patologias, o eritrócito pode ser um dos principais determinantes do ambiente redox. Os eritrócitos de pacientes com DF ou talassemia apresentam aumento das concentrações de ROS em comparação com os eritrócitos normais (HEBBEL *et al*., 1982; ASLAN e FREEMAN, 2004). A superprodução de ROS, como superóxido, promove o estresse oxidativo intravascular que também pode interferir na homeostase do NO e produzir o peroxinitrito, um agente com potencial oxidante elevado. Na DF, as alterações dos lipídios da membrana e a exposição anormal de fosfatidilserina (VER) pelos eritrócitos são consequência, em parte, do estresse oxidativo, que contribui para a morte precoce das hemácias. A exposição da VER também induz a ligação dos eritrócitos às células endoteliais, levando ao sequestro de células que expõem VER nos vasos sanguíneos periféricos (ASLAN e FREEMAN, 2004; MORRIS, 2008). Este processo pode contribuir para a disfunção vascular, hemólise e um estado pró-trombótico (PRITCHARD *et al*., 2004; NEIDLINGER *et al*., 2006). Em adição a este fato, as alterações no sistema da glutationa, comum a essas afecções, podem tornar eritrócitos incapazes de suportae o aumento da carga oxidante, que se tornam sensíveis a hemólise (REID *et al*., 2006). Dados recentes sugerem que a depleção da concentração de glutamina eritrocitária e aberrações no metabolismo da glutationa dos eritrócitos estão ligadas à gravidade da HP na DF e a biomarcadores de hemólise (HSU *et al*., 2007). Dessa forma, a disfunção vascular na DF é o resultado final da interação entre os referidos fenômenos complexos e multifatoriais que se manifestam de forma variada como fenótipos clínicos da doença (MORRIS, 2008).

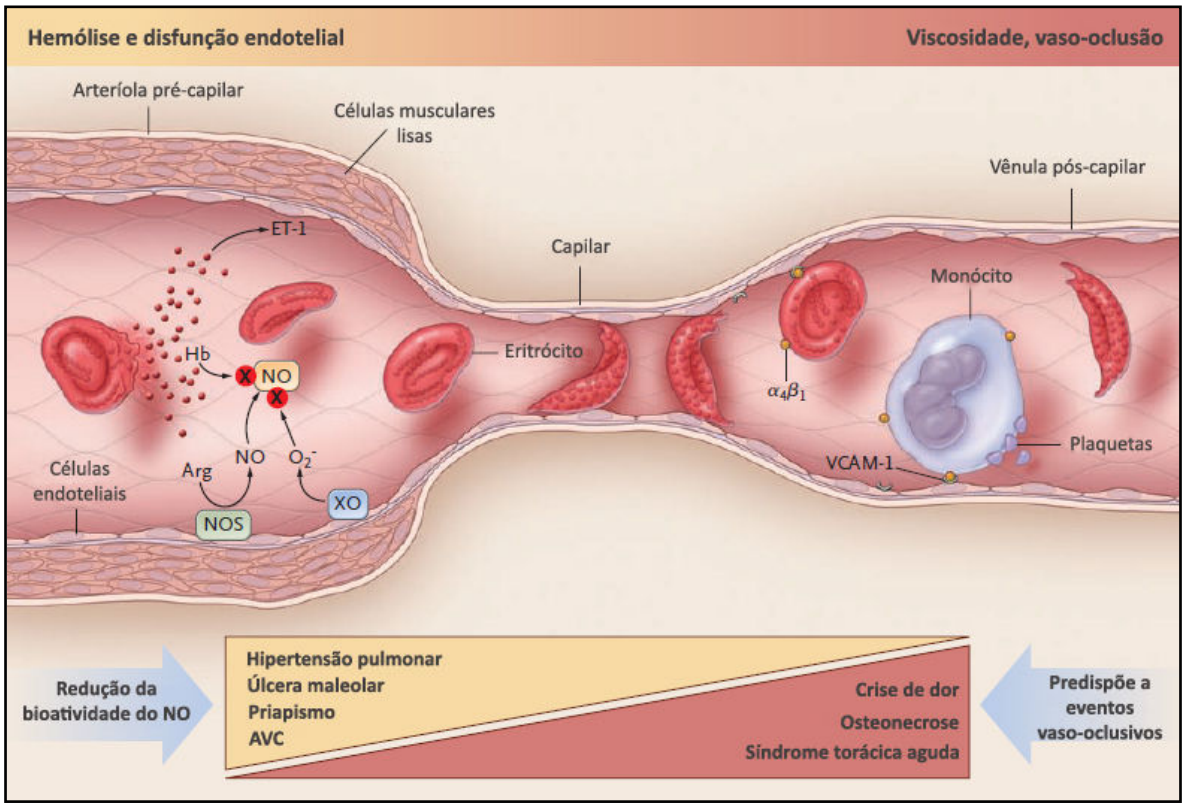

Figura 5. Mecanismos hipotéticos de subfenótipos clínicos da doença falciforme. Supõe-se que muitas das complicações clínicas presentes na DF podem ser divididas em dois subtipos que se superpõem, mas que são, cada um, movidos por mecanismos distintos. As úlceras maleolares, priapismo, hipertensão pulmonar, morte súbita e acidente vascular encefálico estão associados, em indivíduos em estado estável da doença, a níveis reduzidos de hemoglobina (Hb) e ao aumento da taxa de hemólise intravascular (lado esquerdo da figura). Estas complicações vasculares, provavelmente, resultam da disfunção endotelial, mediada tanto pela inativação de óxido nítrico (NO), devido ao aumento da Hb livre no plasma e de espécies reativas de oxigênio, assim como o catabolismo da arginina (Arg) pela arginase plasmática. Este processo de disfunção endotelial associada à hemólise também pode causar a ativação hemostática e proliferação da íntima e da musculatura lisa. Tais complicações clínicas como as crises vaso-oclusivas e dolorosas, a síndrome torácica aguda, a necrose avascular óssea, e retinopatia estão associadas à contagem elevada de leucócitos no estado estável e concentrações elevadas de Hb. Estas complicações resultam da obstrução dos capilares e das vênulas pós-capilares por eritrócitos contendo polímeros de HbS e por leucócitos (monócito, por exemplo), como mostrado no lado direito da figura. ET-1: endotelina-1, NOS: óxido nítrico sintetase, $O2^-$: superóxido, VCAM: molécula de adesão à célula vascular -1 e XO: xantina oxidase. Adapatado de GLADWIN e VICHINSKY, 2008.

O endotélio vascular dos pacientes com DF está susceptível a ação de produtos provenientes da exposição a microrganismos, o aumento da expressão de moléculas de superfície e seus receptores e a ativação de fatores transcricionais, como o fator nuclear kappa B (NF-κB) (BLASIUS e BEUTLER, 2010; LEE E DING, 2013).

O NF-κB, por sua vez, regula o crescimento e a diferenciação celular, além de ativar os genes envolvidos no controle da inflamação, do estresse oxidativo e da apoptose. De fato, o aumento da ativação do NF-κB pode ser mediado tanto pela produção elevada de ROS, como

ocorre na DF, como pelo o aumento da transcrição de genes de citocinas pró-inflamatórias, entre elas o TNF-α, IL-1, IL-2, IL-6 e IL-12 (WANG *et al.*, 2002; KIM *et al.*, 2010). A estimulação dos monócitos pelo lipopolissacarídeo **(**LPS), por exemplo, ativa muitos RNAs mensageiros e vias de transdução de sinal, que por sua vez ativam fatores de transcrição, incluindo aqueles da família do NF-kB, responsáveis pela indução de genes que codificam mediadores inflamatórios (O'CONNELL *et al*., 1998; KIM *et al*., 2010).

A agregação, a ativação e adesão plaquetárias e a trombose podem ser induzidas pela ativação da coagulação e, normalmente, refreadas pela ação do NO (JIN *et al.*, 2005; SCHAFER *et al.*, 2004). A ativação plaquetária elevada tem sido destacada em pacientes com DF (WESTWICK, *et al*., 1983; WUN *et al.,* 1998), sendo relacionada à ação da Hb livre sobre as plaquetas (ex vivo). Nos pacientes com DF que apresentam hemólise intensa e HP observa-se a redução do NO e o aumento estatisticamente significativo da ativação plaquetária (VILLAGRA *et al*., 2007), o que sugere a relação direta entre a redução da biodisponibilidade do NO e ativação das plaquetas em indivíduos com a referida patologia. Villagra e cols. (2007) utilizaram um modelo com sildenafil, um medicamento que amplifica a sinalização da via do NO, e observaram a redução da ativação plaquetária, in vivo, em pacientes com DF com HP (RUF, *et al*., 1997). Dessa forma, é concebível que a ativação plaquetária desempenhe papel importante na progressão da HP, podendo estar ligada a liberação de fatores contidos nos grânulos plaquetários que podem contribuir para a ativação e a disfunção endotelial (RUF, *et al*., 1997). Outro fator importante é a desregulação da protease do fator de Von Willebrand (vWF) em indivíduos com DF, que associada ao aumento da liberação da Hb livre, durante a hemólise, pode gerar multímeros de vWF de alto peso molecular, contribuindo para as complicações vasculares (SCHNOG *et al*., 2006; ZHOU *et al.,* 2009). A ativação das plaquetas e de fatores de coagulação também contribuem para a manutenção dos diversos fenômenos envolvidos na patogênese da DF (STEINBERG E RODGERS, 2001; EMBURY, 2004; OKPALA, 2004).

Kato e cols. (2009) associaram níveis elevados da enzima lactato desidrogenase (LDH) a hemólise subclínica e a um estado generalizado de ativação do endotélio vascular como reflexo da elevação de moléculas de adesão solúveis, em especial a VCAM-1; logo, o aumento da LDH sérica apresenta relação direta com complicações graves, como a HP que aumenta em mais de dez vezes o risco de mortalidade precoce em indivíduos com DF. A inibição da ativação do endotélio vascular em camundongos transgênicos foi associada a ações dos glicocorticóides e da sulfasalazina; em parte, esse efeito se deve a eventos múltiplos, a saber: alteração na transcrição do NF-kB, redução da ativação de células

endoteliais; redução da expressão de E-selectina e de ICAM-1, além de ligantes como o CD162 (AZIZ E WAKEFIELD, 1996; KAUL *et al*., 2004; BELCHER *et al*., 2005).

1.6. METALOPROTEINASES E SEUS INIBIDORES

As metaloproteinases de matriz (MMPs) são endopeptidases dependentes do zinco, responsáveis pelo remodelamento tecidual em condições fisiológicas e patológicas, com atuação sobre os elementos da matriz extracelular (KHOKHA *el al.*, 2013; SEIZER E MAY, 2013). As MMPs são reguladas pelos inibidores teciduais das metaloproteinases (TIMP) e estão associadas com a degradação da matriz extracelular; no entanto, as MMPs também atuam sobre as citocinas e quimiocinas e servem como reguladores de inflamação e imunidade (REYNOLDS, 1996; PARKS *et al*., 2004; BOURBOULIA E STETLER-STEVENSON, 2010).

A regulação da ação das MMPs é realizada por um grande número de inibidores endógenos que evitam a proteólise descontrolada. Dentre estes, os mais específicos são os TIMPs, compostos por proteínas secretadas que se ligam as MMPs em níveis diferentes de especificidades; o TIMP-1, por exemplo, possui afinidade elevada pela MMP-9, enquanto que o TIMP-2 é um inibidor mais eficaz da MMP-2 (KHOKHA *et al.*, 2013). O TIMP-1 é um inibidor da atividade da MMP-9 e tem a capacidade de estimular o crescimento de vários tipos celulares. No entanto, o TIMP-1 apresenta atividade diversa, dependente ou independente, da sua capacidade inibitória da MMP-9 (BERTAUX E HORNEBECK, 1993; GOMEZ *et al*., 1997; LAMBERT *et al*., 2009; OULD-YAHOUI *et al*., 2009).

A presença das MMPs, incluindo a MMP-9, também conhecida como gelatinase B, varia de acordo com o tipo celuluar, sendo que a sua expressão, secreção e ativação nas células do sistema imunológico, são reguladas por citocinas e quimiocinas inflamatórias. A MMP-9 é encontrada nos grânulos terciários dos neutrófilos, grânulos de gelatinase, sendo liberada, imediatamente, após a estimulação pela IL-8, TNF ou um agente quimioatratante (MA *et al*., 2014).

1.7. O METABOLISMO LIPÍDICO NA DOENÇA FALCIFORME

A produção de energia é fundamental para a manutenção da homeostase orgânica e nesse âmbito se destacam moléculas de diversas vias metabólicas, como a insulina, a glicose, os lipídios e as lipoproteínas (DUONG *et al.,* 2006). Cumpre ressaltar que existe uma relação estreita entre carboidratos, lipídios e a insulina, sendo a última um hormônio peptídico necessário para a homeostase da glicose, com papel importante na regulação do metabolismo

lipídico (Diretrizes da Sociedade Brasileira de Diabetes, 2009). O estudo de Ahmed E Goldstein (2008) descreveu a redução aguda dos níveis séricos de diferentes moléculas após a administração, por via intravenosa, de insulina. Entre as moléculas, cujos níveis séricos foram alterados, encontram-se a ICAM-1, proteína quimiotática de monócitos-1 (MCP-1), fatores de transcrição pro-inflamatórios, como o inibidor do ativador do plasminogênio-1 (PAI-1), MMP-9, proteína C reativa (CRP), expressão da oxido nítrico sintase induzível (iNOs) e dos níveis plasmáticos de nitrito e nitrato. Em adição às funções citadas, a ação da insulina nas plaquetas, macrófagos e fatores da coagulação resulta na manutenção da integridade vascular, em indivíduos saudáveis (AHMED E GOLDSTEIN, 2008).

O desequilíbrio na produção, secreção ou ação insulínica está associado ao conjunto de distúrbios metabólicos, como o Diabetes Melito (DM) e a Síndrome Metabólica (SM). Essas patologias têm como características a hiperglicemia, o aumento do colesterol total e a redução da lipoproteína de alta densidade ligada ao colesterol (HDL-c). Estudos relatam que o aumento nos níveis séricos de mediadores inflamatórios e de proteínas de fase aguda, tais como fibrinogênio, CRP, PAI-1, IL-6 e da contagem de leucócitos, pode estar associado à incidência de DM tipo 2 (SCHMIDT *et al.,* 1999; BARZILAY *et al*., 2001; DUNCAN *et al.*, 2003).

A HDL é uma lipoproteína aceptora no processo de transporte reverso do colesterol dos tecidos periféricos ao fígado, que é eliminado pela via biliar (DUONG *et al.,* 2006). Outra função importante da HDL é a atividade anti-aterogênica marcante, além de atuar inibindo a quimiotaxia de monócitos, a adesão leucocitária ao endotélio e a ativação do complemento, apresentando ainda funções antiinflamatórias, antioxidante, anticoagulante e pró-fibrinolítica. A HDL exerce papel importante também no endotélio vascular, interferindo na interação entre monócitos e moléculas de adesão expressas pelo endotélio, como a VCAM-1, ICAM-1 e Selectina-E (NOFER *et al.,* 2002).

As alterações no metabolismo de lipídios e carboidratos estão relacionadas as manifestações clínicas graves descritas em diversas patologias, incluindo a DF. A hiperlipidemia e a hipertensão, por exemplo, têm sido associadas ao aumento do estresse oxidativo e ao dano à membrana do eritrócito nos indivíduos com DF (KUYPERS, 2007; KUMAR *et al.*, 2008). Taylor e cols. (1979) relacionaram a presença da DF com níveis diminuídos de colesterol tanto em homens quanto em mulheres. Os níveis elevados de lipídios são associados ao aumento do estresse oxidativo e este aumento é ainda maior nos indivíduos com hemoglobinopatias; desta forma, sugere-se que nesses indivíduos ocorra o aumento do dano à membrana do eritrócito (KUYPERS, 2007). Ainda tendo como base o estudo de

lipidios na DF, Zorca e cols. (2010) correlacionaram a hipertrigliceridemia em indivíduos com DF e marcadores de hemólise intravascular, disfunção vascular e hipertensão pulmonar.

Os processos referidos anteriormente fazem parte de um fenômeno sistêmico, complexo, dinâmico e evolutivo associado a ativação endotelial e a adesão de hemácias falcizadas e leucócitos ao endotélio vascular; a expressão de moléculas de adesão (VCAM-1, ICAM-1); a alterações na concentração de Hb total e hemoglobina fetal (HbF); ao aumento do número de leucócitos; a ativação de monócitos e a expressão de proteínas de fase aguda, citocinas e quimiocinas, contribuindo para o estado inflamatório crônico e pró-oxidante que pode ser agravado por alterações no metabolismo de lipídico (HEBBEL e VERCELLOTI, 1997; WANG e LUKENS, 1998; OHENE-FREMPONG e STEINBERG, 2001; CANALLI *et al.,* 2004; CONRAN *et al*., 2004; STUART e NAGEL, 2004; KUTLAR, 2005; REDDING-LALLINGER e KNOLL, 2006).

1.8. TRATAMENTO

As condutas clínicas e os tratamentos farmacológicos utilizados pelos indivíduos com DF objetivam a redução dos eventos graves associados à doença e a melhoria da qualidade de vida dos pacientes. A busca por um tratamento seguro e eficaz para a DF passou pela avaliação de moléculas com ação promissora, incluindo agentes demetilantes, como a 5-azacitidina, e ácidos graxos de cadeia curta, como o butirato; entretanto, as limitações para o uso dessas substâncias são inúmeras, desde a forma de administração à segurança e eficácia dos referidos agentes farmacológicos (LEY *et al.*, 1983; ATWEH *et al.*, 1999; Kaul *et al.*, 2004). Nesse contexto, a hidroxiuréia (HU), fármaco citotóxico e antimetabólico associado ao aumento de HbF, surgiu como agente terapêutico potencial (PLATT *et al.*, 1984). A HU associa a facilidade de administração por via oral, com a toxicidade aceitável, sendo que a eficácia do tratamento pode ser avaliada pela dosagem da HbF e a contagem de leucócitos; além disso, o uso de HU tem sido associado à redução de eventos clínicos graves, como os VOE (DAVIES e GILMORE, 2003), apesar dos mecanismos pelos quais a HU age, em especial os relacionados ao aumento da concentração de HbF, não serem totalmente conhecidos. Os efeitos citotóxicos e mecanismos mais complexos têm sido também associados ao tratamento com a HU, entre eles pode-se citar a indução da expressão do gene gama da globina (*HBG*), a produção de NO, da guanilato ciclase solúvel e a ativação da via da proteína cinase dependente-GMPc (IKUTA *et al.*, 2001; COKIC *et al.*, 2003; MABAERA *et al*., (2008).

O uso de hemocomponentes na DF tem a função de restaurar os níveis de Hb quando estes estão muito baixos, reduzir os níveis de HbS e, consequentemente, prevenir complicações vaso-oclusivas graves, como o AVC (STEINBERG, 2001). A avaliação neonatal adequada, por sua vez, possibilita o diagnóstico precoce da DF e permite a utilização de medidas profiláticas, como a antibióticoterapia e esquemas de vacinação (METHA *et al.,* 2006).

1.9. TALASSEMIA ALFA

As síndromes talassêmicas compreendem um grupo de alterações hereditárias que resultam na síntese reduzida ou ausente da Hb normal, em decorrência de alterações nos genes da globina (STEINBERG, 2001; MARTIN e THOMPSON, 2013). A concomitância da talassemia alfa (TA) com a HbS está associada à diminuição da concentração relativa da Hb variante e, na DF, a presença da TA está associada a diminuição da ocorrência de úlceras maleolares e ao aumento da sobrevida dos pacientes (SONATI, 1990; TAKEKOSHI *et al.*, 1995). Por outro lado, a redução da hemólise e o aumento do hematócrito, resultantes dessa associação, leva ao aumento da viscosidade sanguínea e a predisposição elevada a eventos vaso-oclusivos, dolorosos e retinopatias (SADELAIN *et al.*, 1995; ADORNO *et al.*, 2004; LYRA *et al.*, 2005).

1.10. ALFA 1 ANTITRIPSINA

A alfa 1 antitripsina (AAT) é uma glicoproteína responsável pela inibição de proteases, preservando os tecidos contra a ação de elastases neutrofílos e de monócitos (CARRELL, 1996). Estudos têm demonstrado a participação da AAT na inibição da toxicidade de linfócitos e quimiotaxia de neutrófilos e monócitos (BREIT *et al.,* 1985, CRYSTAL *et al.*, 1991). O gene *SERPINA1*, que codifica a proteína AAT, está localizado no braço longo do cromossomo 14 (14q31-32) e é altamente polimórfico, sendo que mais de 100 variantes alélicas já foram identificadas (KÖHNLEIN; WELTE, 2008). Entre os alelos associados à deficiência da AAT, os mais frequentes são o S e o Z (BRANTLY *et al.*, 1988). A variante Z é resultante de mutação no éxon V do gene *SERPINA1*, onde ocorre a substituição de guanina por adenina na posição 9985. A presença em homozigose do alelo Z está associada à redução dos níveis séricos da AAT a valores equivalentes a 10-15% do nível normal, com tendência para o desenvolvimento de enfisema pulmonar. O alelo S, o segundo mais frequente, é resultante da substituição de adenina por timina no éxon III do gene *SERPINA1*, sendo sua presença associada à degradação de AAT recém-sintetizada. Os indivíduos homozigotos para

o alelo S apresentam produção intermediária de AAT, suficiente para proteger o pulmão da ação proteolítica da elastase neutrófilica; por outro lado, os indivíduos heterozigotos SZ são susceptíveis ao desenvolvimento de doença pulmonar (CRYSTAL, 1991).

2. JUSTIFICATIVA

A DF possui variabilidade clínica, com ocorrência de episódios agudos e crônicos e propensão à lesão progressiva em órgãos (STEINBERG, 2009). Apesar da gravidade da DF, o manejo clínico é composto por medidas para minimizar os sintomas, uma vez que não existem terapias que atuem como um todo no processo patológio da doença (REES *et al*., 2010), sendo que os mecanismos fisiopatológicos da DF ainda não foram totalmente elucidados. Nesse ponto, a hemólise, aumento do estresse oxidativo e lesão endotelial, têm destaque entre os mecanismos estudados na DF. A VO também representa papel importante na patogênese da DF, com ênfase para a polimerização da HbS, participação de citocinas pró-inflamátorias, eicosanóides, metaloproteinases e seus inibidores fisiológicos (REES *et al.*, 2011).

Diante do exposto, o presente estudo avaliou a participação de moléculas relacionadas à hemólise (heme livre, haptoglobina, LDH), mediadores do processo inflamatório (citocinas, PGE_2, LTB_4 e TGF-β) e moléculas relacionadas à lesão endotelial (MMP-9 e TIMP-1), investigando suas possíveis associações com marcadores laboratoriais de rotina, genéticos (polimorfismos no gene da *SERPINA1*) e dados do histórico clínico apresentados pelos pacientes com DF. Indivíduos com DF em estado estável e em crise fizeram parte da casuística do presente estudo, visando contribuir a compreenção da relação entre os fenômenos citados, as manifestações e gravidade clínica da doença.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar marcadores potenciais de prognóstico na DF, em especial aqueles associados à hemólise, inflamação e disfunção endotelial, sugerindo os mecanismos pelos quais os mesmos se relacionam a gravidade da doença e investigando a sua possível utilização no acompanhamento clínico dos pacientes.

3.2. OBJETIVOS ESPECÍFICOS

- ✓ Avaliar o perfil laboratorial de pacientes DF;
- ✓ Avaliar os lipídios como possíveis marcadores de prognóstico na DF e a influência dos mesmos na quimiotaxia de neutrófilos;
- ✓ Avaliar o heme livre em pacientes com DF (em estado estável e em crise);
- ✓ Determinar os níveis séricos das citocinas (IL-1β, IL-4, IL-6, IL-8, IFN-γ, TNF-α, IL-12, IL-13, IL-10 e TGF-β), em pacientes com DF (em estado estável e em crise);
- ✓ Investigar a participação das citocinas investigadas nos fenômenos vaso-oclusivos e inflamatórios presentes na DF;
- ✓ Avaliar os níveis de LTB_4, PGE_2, MMP-9 e TIMP-1 em pacientes estáveis e em crise;
- ✓ Avaliar os níveis de AAT e suas possíveis associações com dados clínicos, bioquímicos, hematológicos e mutações no gene da *SERPINA1*.

4. MANUSCRITOS

4.1. MANUSCRITO I

Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease

Magda O Seixas, Larissa C Rocha, Mauricio B Carvalho, Joelma F Menezes, Isa M Lyra,Valma ML Nascimento, Ricardo D Couto, Ájax M Atta, Mitermayer G Reis, Marilda S Goncalves. **Lipids Health Dis, 27; 9:91, 2010.**

Este trabalho avalia a associação entre as liporoteínas ligadas ao colesterol, em especial a HDL-c e triglicerídeos, bem como suas possíveis associações com marcadores bioquímicos e clínicos na DF.

Resumo: A busca por biomarcadores de prognóstico na DF é um desafio. A identificação desses marcadores pode auxiliar na compreensão das complicações clínicas graves, no acompanhamento dos pacientes e na busca de alvos terapêuticos novos. Neste estudo investigamos, prospectivamente, biomarcadores bioquímicos, inflamatórios e hematológicos em 152 crianças com DF em estado estável e em 132 indivíduos saudáveis. Os dados clínicos foram coletados de prontuários médicos. Nas crianças com DF observamos associação positiva entre os níveis de HDL-c com a hemoglobina (p <0,001), hematócrito (p <0,001) e colesterol total (p <0,001) e associação significativa negativa com reticulócitos (p = 0,046), leucócitos (p = 0,015), monócitos (p = 0,004) e plaquetas (p = 0,005), bilirrubina total (p <0,001), direta (p <0,001) e indireta (p <0,001); ferro (p <0,001), transaminase AST (p = 0,004) e ALT (p = 0,035); LDH (p <0,001), ureia (p = 0,030), alfa 1-antitripsina (p <0,001), VLDL-c (p = 0,003), triglicerídeos (p = 0,005) e hemoglobina S (p = 0,002). As concentrações diminuídas de HDL-c apresentaram relação direta com a presença de alterações cardíacas (p = 0,025), pneumonia (P = 0,033) e uso de hemoderivados (p = 0,025). Lipídios e marcadores inflamatórios foram associados a presença de colelitíase. Nossa hipótese é que alguns pacientes com DF apresentam subfenótipo dislipidêmico específico, caracterizado por níveis reduzidos de HDL-c, com hipertrigliceridemia e níveis elevados de VLDL-c. Consideramos que esses resultados representam um passo importante em direção a mais um tipo de prognóstico clínico na doença. Estudos adicionais são necessários para testar essa hipótese e os mecanismos envolvidos nesta rede complexa de marcadores e seu papel na patogênese da DF.

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LIPIDS IN HEALTH AND DISEASE

RESEARCH Open Access

Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease

Magda O Seixas[1,2], Larissa C Rocha[1,3], Mauricio B Carvalho[2], Joelma F Menezes[1,2], Isa M Lyra[3,4], Valma ML Nascimento[3], Ricardo D Couto[2], Ájax M Atta[2], Mitermayer G Reis[1], Marilda S Goncalves[1,2*]

Abstract

Background: The search for sickle cell disease (SCD) prognosis biomarkers is a challenge. These markers identification can help to establish further therapy, later severe clinical complications and with patients follow-up. We attempted to study a possible involvement of levels of high-density lipoprotein cholesterol (HDL-C) in steady-state children with SCD, once that this lipid marker has been correlated with anti-inflammatory, anti-oxidative, anti-aggregation, anti-coagulant and pro-fibrinolytic activities, important aspects to be considered in sickle cell disease pathogenesis.

Methods: We prospectively analyzed biochemical, inflammatory and hematological biomarkers of 152 steady-state infants with SCD and 132 healthy subjects using immunochemistry, immunoassay and electronic cell counter respectively. Clinical data were collected from patient medical records.

Results: Of the 152 infants investigated had a significant positive association of high-density lipoprotein cholesterol with hemoglobin ($P < 0.001$), hematocrit ($P < 0.001$) and total cholesterol ($P < 0.001$) and a negative significant association with reticulocytes ($P = 0.046$), leukocytes ($P = 0.015$), monocytes ($P = 0.004$) and platelets ($P = 0.005$), bilirubins [total bilirubin ($P < 0.001$), direct bilirubin ($P < 0.001$) and indirect bilirubin ($P < 0.001$], iron ($P < 0.001$), aminotransferases [aspartate aminotransferase ($P = 0.004$), alanine aminotransferase ($P = 0.035$)], lactate dehydrogenase ($P < 0.001$), urea ($P = 0.030$), alpha 1-antitrypsin ($P < 0.001$), very low-density lipoprotein cholesterol ($P = 0.003$), triglycerides ($P = 0.005$) and hemoglobin S ($P = 0.002$). Low high-density lipoprotein cholesterol concentration was associated with the history of cardiac abnormalities ($P = 0.025$), pneumonia ($P = 0.033$) and blood transfusion use ($P = 0.025$). Lipids and inflammatory markers were associated with the presence of cholelithiasis.

Conclusions: We hypothesize that some SCD patients can have a specific dyslipidemic subphenotype characterized by low HDL-C with hypertriglyceridemia and high VLDL-C in association with other biomarkers, including those related to inflammation. This represents an important step toward a more reliable clinical prognosis. Additional studies are warranted to test this hypothesis and the probably mechanisms involved in this complex network of markers and their role in SCD pathogenesis.

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Background

Sickle cell disease (SCD) clinical outcomes vary widely from mild to severe and the disease has been associated with multi-organ damage and risk of early mortality [1,2]. Acute and chronic clinical manifestations of SCD include vaso-occlusive pain episodes (VOE), impaired blood flow as a result of intravascular sickling in capillaries and small vessels, inflammation processes and high susceptibility to infection. Researchers have found a complex network of associations among laboratory analyses and clinical events predicting a probably risk of death [1,3,4].

The sickle cell disease vaso-occlusive phenomenon has been described as a complex event with the participation of stressed reticulocytes, sickled erythrocytes, leukocytes, platelets and endothelium activation [2,5-8]. Reactive oxygen species (ROS), scavenger molecules and nitric oxide (NO) play important roles as regulators of vascular homeostasis in SCD pathogenesis [9].

Several biomarkers have been associated with SCD clinical prognosis; some, such as fetal hemoglobin (HbF) concentration, leukocytes count and reticulocyte count are considered to be classic [2,5]. Recently, serum lactate dehydrogenase (LDH), a well-known marker of intravascular hemolysis, was described as a biomarker of prognosis in SCD [10]. It has been associated with nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in SCD patients [11].

We conducted a prospective study to investigate high-density lipoprotein cholesterol (HDL-C) levels, including also determination of total cholesterol, low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C) and triglycerides to test the hypothesis that they can be used as a marker of prognosis among steady-state sickle cell disease children. This potential biomarker and their association with others laboratory determination and medical history were investigated in order to identify sub-phenotypes associated with the disease.

Subjects and Methods

Subjects and Controls

Of 152 steady-state SCD children from Salvador city, state of Bahia, in Brazil were prospectively analyzed for laboratory (biochemical and hematological) markers. Brazil is the largest country in South America, with one of the most heterogeneous populations due to several waves of immigration that have resulted in cultural, socioeconomic, and ethnic diversity in different geographic regions. Salvador is the largest city in Bahia, a Northeastern Brazilian state. Among the local population, 86% is of African origin, and Salvador has the highest incidence of SCD in Brazil [12].

The study was conducted from March 2007 to November 2008 and included patients from the Fundação de Hematologia e Hemoterapia do estado da Bahia (HEMOBA), a reference center attending to sickle cell disease patients who are seen for routine visits at the outpatient clinic. The study also included 132 healthy children randomly selected from the Clinical Laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (UFBA); these were matched to cases by age, gender and African ethnic origin as a control group. The study was approved by the Fundação de Pesquisa Oswaldo Cruz human subject research board, and all officials responsible provided written informed consent, in accordance with the Declaration of Helsinki of 1975, as revised in 2000.

Laboratory Methods

Clinical laboratory analyses were performed in the Clinical Analyses Laboratory of the PHAR-UFBA and the Pathology and Molecular Biology Laboratory of the Centro de Pesquisas Goncalo Moniz da Fundação de Pesquisa Oswaldo Cruz. Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain). Serum ferritin was measured by immunoassay using an Access® 2 Immunoassay system X2 (Beckman Coulter, Fullerton, CA). C-reactive protein (CRP), alpha 1-antitripsin (A1AT) and antistreptolysin-O (ASO) were measured by immunochemistry (Immage® 800 system, Beckman Coulter, Fullerton, CA). Hematological analyses were carried out using an electronic cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC/VARIANT I; BIO-RAD, CA, USA).

Definition of Clinical Events

Clinical data were collected from patient medical records. Demographic data were provided by interviews with patients and parents or guardians. Eligibility criteria included only SCD patients of pediatric age. All patients were in the steady-state of the disease when samples were collected; steady-state was characterized as a period without any acute events and no blood transfusion for 120 days prior to blood sampling. Exclusion criteria included infection or inflammatory episodes and previous blood transfusion (within four months prior to the study). To identify possible associations between HDL-C levels and clinical characteristics in SCD we assessed medical history from patients' records, including prevalence of stroke, number of hospitalizations, painful episodes, VOE, infection, pneumonia, priapism, splenomegaly, splenic sequestration, leg ulcers, cardiac

abnormalities, respiratory insufficiency and cholelithiasis. Pneumonia was defined as an acute infection of the lung by virus, bacteria or atypical organisms with a clinical outcome that did not meet the criteria for ACS [8].

Statistical analysis

Baseline characteristics were summarized as means and proportions of selected variables. Distribution of quantitative variables was determined using the Kolmogorov-Smirnov test. Mean values of quantitative variables between groups were compared using the unpaired t-test for normal data distribution and Mann-Whitney for non-normal data. Bivariate correlation analyses were carried out to determine correlations between pairs of variables using Pearson's and Spearman's rank correlation (R). The nonparametric Kruskal-Wallis test was used to compare means among two or more groups as measured by interval variables. The level of 40 mg/dl was considered as a reference range and interactions between low HDL-C (less than 40 mg/dl) and high HDL-C (at least 40 mg/dl) and baseline characteristics were evaluated using independent t-test and Mann-Whitney tests. The interactions between low HDL-C (less than 40 mg/dl) and high HDL-C (at least 40 mg/dl) and specific categorical clinical variables were tested for significance using a χ^2 test or Fisher's exact test, taking into account the expected frequency in the cell tables. All tests were considered significant if p values were less than .05. Data analyses were performed using Prism 5.01 (Graphpad Software, San Diego, CA), EPIinfo 6.04 (CDC, Atlanta, Georgia) and STATA SE 10 software (StataCorp, Texas, USA).

Results

First of all we compared the analyses of markers of intravascular hemolysis, hemolysis and hepatic involvement, leukocyte and platelet counts, renal involvement, lipid metabolism, inflammation and Hb profile in order to establish how much are the difference between those markers between control and patients groups (Table 1).

HDL-C association with markers of hemolysis, inflammation and vascular dysfunction

The high-density lipoprotein cholesterol was positively correlated with red blood cells (RBC), Hb, hematocrit and total cholesterol and urea concentrations and negatively correlated with hematimetric indexes of mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC); reticulocytes, hemoglobin S (HbS), hemolysis and hepatic markers, total leukocytes, monocytes and platelets, alanine aminotransferase (ALT), iron and A1AT. However, it was not correlated with LDL-C. Steady state triglycerides were negatively correlated with RBC, Hb, hematocrit and HDL-C, and positively correlated with HbS, LDH, AST, total bilirubin, platelet, total protein, total cholesterol, and VLDL-C (Table 2).

We next determined whether the levels of HDL-C in SCD group (HDL-C less than 40 mg/dl vs. 40 mg/dl or more) showed difference among the laboratorial markers. In the first group, there were 80 *HBSS* and 23 *HBSC* patients, and in the second group, there were 23 HBSS and 25 HBSC patients. Sickle cell patients with low HDL-C presented lower RBC counts as well as Hb and hematocrit concentrations than patients from the group with normal HDL-C levels. The low concentration HDL-C group had higher erythroblast, leukocyte, platelet, neutrophil, monocyte and reticulocyte counts and higher iron, AST, total bilirubin, direct bilirubin, indirect bilirubin, LDH and A1AT concentrations. There was no difference in LDL-C concentration between the two HDL-C subgroups, but the VLDL-C and triglycerides concentrations were higher in the low HDL-C group (Table 3).

Association of HDL-C with sickle cell disease clinical history

We assessed possible associations between HDL-C levels and a series of clinical characteristics in SCD medical history, including prevalence of stroke, number of hospitalizations, painful episodes, VOE, infection, pneumonia, priapism, splenomegaly, splenic sequestration, leg ulcers, cardiac abnormalities, respiratory insufficiency and cholelithiasis. To compare these categorical variables with HDL-C concentration, we divided patients into two groups. The low HDL-C group (less than 40 mg/dl) comprised 103 sickle cell disease patients (80 HBSS and 23 HBSC), with an HDL-C range of 16-39 mg/dl and mean of 28.95 mg/dl. The high HDL-C group (at least 40 mg/dl) comprised 48 SCD patients (23 HBSS and 25 HBSC), with an HDL-C range of 41-85 mg/dl and mean of 51.2 mg/dl.

The prevalence of pneumonia (OR = 2.42, 95%CI: 1.06-5.53; P = 0.033) and the prevalence of cardiac abnormalities (OR = 2.88, 95%CI: 1.12-7.59, P = 0.025) were significantly different between the HDL-C groups. Forty-one children in the low HDL-C group had cardiac abnormalities typical of hemolytic anemia on auscultation. However, among these, 24 had electrocardiograph arrhythmia, and 3 had tricuspid regurgitant jet velocity of at least 2.6 m/sec, indicating a possible presence of pulmonary hypertension. These results were obtained from previously performed echocardiograms that were not preformed at the same time of the present study. The low HDL-C concentration group underwent more blood transfusions (OR = 2.52, 95%CI: 1.11-5.77, P = 0.025).

Table 1 Patient and control group characteristics

	Patients		Controls		
Characteristics	**N**	**Mean ± SD**	**N**	**Mean ± SD**	***p***
Age (Years)	152	9.2 ± 4.0	132	8.7 ± 3.2	
Gender					
Male	82	53.9*	68	51.5*	
Female	70	46.1*	64	48.5*	
Hemoglobins					
AA	–	—	132	100.0	
SS	103	67.8	–	—	
SC	48	31.5	–	—	
SD	01	0.7	–	—	
Hemoglobin					
Fetal **(%)**	142	7.51 ± 6.20	130	0.47 ± 0.46	**<0.001**
Hemolysis					
RBC (× 10^6/cu mm)	152	3.24 ± 0.97	131	4.74 ± 0.39	**<0.001**
Hemoglobin (g/dL)	152	8.93 ± 2.01	131	12.83 ± 1.03	**<0.001**
Hematocrit (%)	152	27.65 ± 6.20	131	38.47 ± 2.78	**<0.001**
Mean Cell Volume (fL)	152	87.44 ± 10.85	131	81.37 ± 5.16	**<0.001**
Mean Cell Hemoglobin (pg)	152	28.29 ± 3.73	131	27.14 ± 1.95	**0.007**
Reticulocyte Count (%)	140	7.61 ± 4.88	122	0.846 ± 0.256	**<0.001**
Leukocytes					
Leukocyte Count (× 10^9/L)	152	13.1 ± 5.8	131	7.0 ± 2.2	**<0.001**
Neutrophil Count (× 10^9/L)	152	6161.72 ± 3779.49	131	3240.32 ± 1686.15	**<0.001**
Monocyte Count (× 10^9/L)	152	817.15 ± 481.83	131	488.67 ± 204.90	**<0.001**
Platelets					
Platelet Count (× 10^9/L)	152	403.93 ± 158.66	131	308.21 ± 67.35	**<0.001**
Lipid metabolism					
Total Cholesterol (mg/dL)	151	121.12 ± 26.16	124	164.08 ± 34.55	**<0.001**
HDL Cholesterol (mg/dL)	151	35.65 ± 12.34	123	48.90 ± 13.67	**<0.001**
LDL Cholesterol (mg/dL)	151	64.95 ±22.19	123	97.41 ± 33.54	**<0.001**
VLDL Cholesterol (mg/dL)	151	20.44 ± 9.38	123	17.75 ± 10.37	**<0.001**
Triglycerides (mg/dL)	150	102.07 ± 46.86	123	88.31 ± 51.73	**0.002**
Hemolysis plus Hepatic					
Aspartate aminotransferase (U/L)	152	48.05 ± 24.92	122	30.28 ± 11.13	**<0.001**
Total bilirubin (mg/dL)	151	2.73 ± 1.76	118	0.49 ± 0.21	**<0.001**
Direct bilirubin (mg/dL)	151	0.66 ± 0.46	118	0.250 ± 0.082	**<0.001**
Indirect bilirubin (mg/dL)	151	2.08 ± 1.59	118	0.244 ± 0.182	**<0.001**
Iron serum (mcg/dL)	126	123.40 ± 119.94	119	71.14 ± 40.31	**<0.001**
Lactate dehydrogenase(U/L)	151	858.22 ± 503.81	119	406.27 ± 132.03	**<0.001**
Hepatic					
Alanine aminotransferase (U/L)	152	28.25 ± 21.34	121	17.36 ± 7.10	**<0.001**
Total protein (g/dL)	151	7.33 ± 0.848	119	7.31 ± 0.62	0.695
Albumin (g/dL)	151	4.07 ± 0.675	119	4.24 ± 0.49	0.249
Globulin (g/dL)	151	3.26 ± 0.781	119	3.06 ± 0.63	0.109
Albumin/Globulin ratio	151	1.35 ± .54	112	1.44 ± .42	0.289
Renal					
Urea nitrogen (mg/dL)	150	17.73 ± 6.41	120	21.65 ± 5.92	**<0.001**
Creatinine (mg/dL)	151	0.51 ± 0.50	120	0.523 ± 0.185	0.708
Inflammation					
C-reactive protein (mg/L)	148	7.08 ± 11.97	102	2.01 ± 2.29	**<0.001**
Alpha 1-antitrypsin (mg/dL)	151	152.50 ± 46.18	129	137.48 ± 43.36	**0.013**
Ferritin (ng/mL)	152	313.32 ± 361.44	117	37.29 ± 28.28	**<0.001**
Antistreptolysin-O(UI/mL)	148	192.70 ± 285.42	101	132.75 ± 131.19	0.181

* percentage Mann-Whitney test

Table 2 Laboratory value associations with HDL-C and Triglycerides in sickle cell disease

	HDL Cholesterol (mg/dL)		Triglycerides (mg/dL)	
	r	*p*	*r*	*p*
Hemoglobin				
S hemoglobin (%)	-0.311	**0.002**	0.286	**0.005**
Fetal hemoglobin (%)	-0.048	0.644	-0.685	0.685
Hemolysis				
RBC (x 10^6/cu mm)	0.328	**<0.001**	-0.190	**0.019**
Hemoglobin (g/dL)	0.292♣	**<0.001**	-0.202	**0.013**
Hematocrit (%)	0.309	**<0.001**	-0.189	**0.020**
Mean Cell Volume (fL)	-0.273♣	**0.006**	0.126	0.125
Mean Cell Hemoglobin (pg)	-0.284♣	**0.002**	0.111	0.175
Reticulocyte Count (%)	-0.170♣	**0.046**	0.082	0.339
Leukocyte				
Leukocyte Count (x 10^9/L)	-0.198♣	**0.015**	0.081	0.325
Neutrophil Count (x 10^9/L)	0.017♣	0.838	-0.154	0.061
Monocyte Count (x 10^9/L)	-0.234	**0.004**	0.139	0.089
Platelets				
Platelet Count (x 10^9/L)	-0.228♣	**0.005**	0.233	**0.004**
Hemolysis plus Hepatic				
Aspartate aminotransferase (U/L)	-0.235♣	**0.004**	0.207	**0.011**
Total bilirubin (mg/dL)	-0.298♣	**<0.001**	0.165	**0.044**
Direct bilirubin (mg/dL)	-0.471	**<0.001**	0.035	0.669
Indirect bilirubin (mg/dL)	-0.287	**<0.001**	0.140	0.088
Iron Serum (mcg/dL)	-0.186	**0.038**	0.159	0.076
Lactate dehydrogenase (U/L)	-0.375	**<0.001**	0.167	**0.041**
Hepatic				
Alanine aminotransferase (U/L)	-0.172	**0.035**	0.075	0.364
Total protein (g/dL)	-0.021♣	0.793	0.274	**0.001**
Albumin (g/dL)	0.102	0.213	0.142	0.083
Globulin (g/dL)	-0.124♣	0.129	0.133	0.104
Albumin/Globulin ratio	0.033	0.689	-0.033	0.684
Renal				
Urea nitrogen (mg/dL)	0.178	**0.030**	0.020	0.806
Creatinine (mg/dL)	0.118	0.152	0.105	0.201
Lipid metabolism				
Total Cholesterol (mg/dL)	0.299♣	**<0.001**	0.268	**0.001**
HDL Cholesterol (mg/dL)	——	——	-0.228	**0.005**
LDL Cholesterol (mg/dL)	-0.083♣	0.312	0.068	0.409
VLDL Cholesterol (mg/dL)	-0.242	**0.003**	0.998	**<0.001**
Triglycerides (mg/dL)	-0.228	**0.005**	——	——
Inflammation				
C-reactive protein (mg/L)	0.048	0.563	-0.031	0.714
Alpha 1 antitrypsin (mg/dL)	-0.327♣	**<0.001**	-0.074	0.378
Ferritin (ng/mL)	-0.032	0.699	0.102	0.220
Antistreptolysin O (UI/mL)	-0.079	0.339	0.157	0.058

Spearman or Pearson correlation coefficients (r) and p values (*p*) ♣ r = Pearson correlation coefficient

High serum levels of LDL-C, VLDL-C and total cholesterol lipoproteins, TRIG, ferritin and A1AT but not HDL-C were associated with the occurrence of cholelithiasis (Figure 1).

Discussion

The present study analyzed levels of HDL-C in steady-state children with SCD. Children with SCD, even in steady-state, have differences in several biomarkers as compared to healthy age-matched children [13]. Those differences are related to numerous mechanisms associated with infection, inflammation and VOE in the disease [1,2]. Several biomarkers associated with hemolysis, inflammation, renal metabolism, hepatic metabolism, and lipid metabolism in children with SCD and healthy subjects were studied, and the findings of normal concentrations of protein and globulin as well as the albumin/globulin ratio among the SCD patients suggest an absence of early severe liver cell damage in the studied group [13]. Normal levels of creatinine in the patient group confirm previous observations that an increased rate of creatinine secretion by dysfunctional renal tubules may lead to a falsely normal plasma creatinine and creatinine clearance. A more accurate evaluation of different aspects of SCD nephropathy, emphasizing proteinuria and hyperfiltration, needs to be developed in children in order to detect early renal alteration [14-16].

Hypocholesterolemia has been described in SCD patients with significantly decreased LDL-C and HDL-C [17-22] and has been also described for our group as a potential biomarker for SCD clinical severity [23]. A negative association was found for HDL-C and VLDL-C, which was directly associated with triglycerides. Triglyceride-rich VLDL-C particles availability may play an important role in lipid oxidization in SCD patients. It has been suggested that VLDL-C is an important factor for atherosclerosis development. VLDL-C particles assemble by a complex process that includes an apolipoprotein B (apoB)-containing VLDL precursor and a VLDL-sized lipid droplet lacking apoB. Both particles fuse to produce a mature VLDL particle [24]. The increase of triglycerides probably contributes to an increase in the hepatic production of VLDL-C, increasing the number of receptors for LDL-C that is extensively metabolized, decreasing its serum levels. However, the role of cholesterol and triglycerides and the regulation of assembly and production of VLDL-C are poorly understood.

A negative association was observed between LDH and HDL-C, showing that HDL-C, as measured by its

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Table 3 Laboratory values for sickle cell disease patients with different steady-state levels of HDL-C

	**HDL less than 40 mg/dL		***HDL at least 40 mg/dL		
	N	Mean ± SD	N	Mean ± SD	*p
Hemolysis					
RBC (× 10^6/cu mm L)	103	3.01 ± 0.85	48	3.75 ± 1.0	**<0.001**
Hemoglobin (g/dL)	103	8.57 ± 2.02	48	9.76 ± 1.75	**0.001**
Hematocrit (%)	103	26.42 ± 6.17	48	30.43 ± 5.37	**<0.001**
Mean Cell Volume (fL)	103	89.17 ± 10.34	48	83.52 ± 11.01	**0.003**
Mean Cell Hemoglobin (pg)	103	28.94 ± 3.52	48	26.82 ±3.79	**0.001**
Mean Cell Hemoglobin Concentration (%)	103	32.44 ± 0.96	48	32.07 ± 0.86	**0.025**
Erythroblast (%)	103	1.90 ± 2.31	48	1.02 ± 2.48	**0.034**
Reticulocyte count (%)	97	8.34 ± 4.55	42	5.90 ± 5.25	**0.006**
Hemoglobins					
S hemoglobin (%)	97	79.22 ± 16.16	44	60.75 ± 18.22	**<0.001**
Fetal hemoglobin (%)	97	7.55 ± 5.99	44	7.41 ± 6.78	0.899
Leukocyte					
Leukocyte Count (× 10^9/L)	103	14105.83 ± 6085.37	48	10868.75 ± 4416.13	**0.001**
Neutrophil Count (× 10^9/L)	103	6723.36 ± 4167.75	48	4971.83 ± 2459.10	**0.002**
Monocyte Count (× 10^9/L)	103	910.35 ± 499.84	48	604.69 ± 361.83	**0.004**
Platelets					
Platelet Count (× 10^9/L)	103	424.90 ± 160.26	48	357.69 ± 148.02	**0.015**
Hemolysis plus Hepatic					
Aspartate aminotransferase (U/L)	103	51.45 ± 26.29	48	40.65 ± 20.32	**0.013**
Total bilirubin (mg/dL)	103	3.13 ± 1.82	48	1.88 ± 1.25	**<0.001**
Direct bilirubin (mg/dL)	103	0.79 ± 0.47	48	0.38 ± 0.25	**<0.001**
Indirect bilirubin (mg/dL)	103	2.34 ± 1.68	48	1.50 ± 1.20	**0.002**
Iron serum (mcg/dL)	95	136.65 ± 133.77	31	82.77 ± 40.17	**0.029**
Lactate dehydrogenase (U/L)	103	977.19 ± 524.50	48	602.92 ± 339.59	**<0.001**
Lipid metabolism					
Total Cholesterol (mg/dL)	103	116.49 ± 25.17	48	131.06 ± 25.73	**0.001**
LDL Cholesterol (mg/dL)	103	65.78 ± 21.47	48	63.19 ± 23.81	0.506
VLDL Cholesterol (mg/dL)	103	21.62 ± 10.31	48	17.90 ± 6.38	**0.023**
Triglycerides (mg/dL)	102	107.74 ± 51.64	48	90.02 ± 31.86	**0.030**
Hepatic					
Alanine aminotransferase (U/L)	103	29.90 ± 22.10	48	24.58 ± 19.56	0.156
Total protein (g/dL)	103	7.40 ± .89	48	7.46 ± .82	0.684
Albumin (g/dL)	103	4.01 ± .75	48	4.23 ± .59	0.054
Globulin (g/dL)	103	3.36 ± .85	48	3.23 ± .72	0.333
Albumin/Globulin ratio	103	1.34 ± .55	48	1.38 ± .53	0.709
Renal					
Urea nitrogen (mg/dL)	102	17.25 ± 6.70	47	18.77 ± 5.73	0.181
Creatinine (mg/dL)	103	0.49 ±0.50	47	0.57 ± 0.50	0.315
Inflammation					
C-reactive protein (mg/L)	101	7.79 ± 14.02	46	5.39 ± 5.18	0.133
Alpha 1 antitrypsin (mg/dL)	102	163.13 ± 44.06	48	128.92 ± 42.13	**<0.001**
Ferritin (ng/mL)	103	300.76 ± 399.59	46	323.46 ± 348.87	0.740
Antistreptolysin O (UI/mL)	101	198.62 ± 288.98	46	183 ± 282.56	0.759

* unpaired t-test ** 80 HBSS and 23 HBSC ***23 HBSS and 25 HBSC

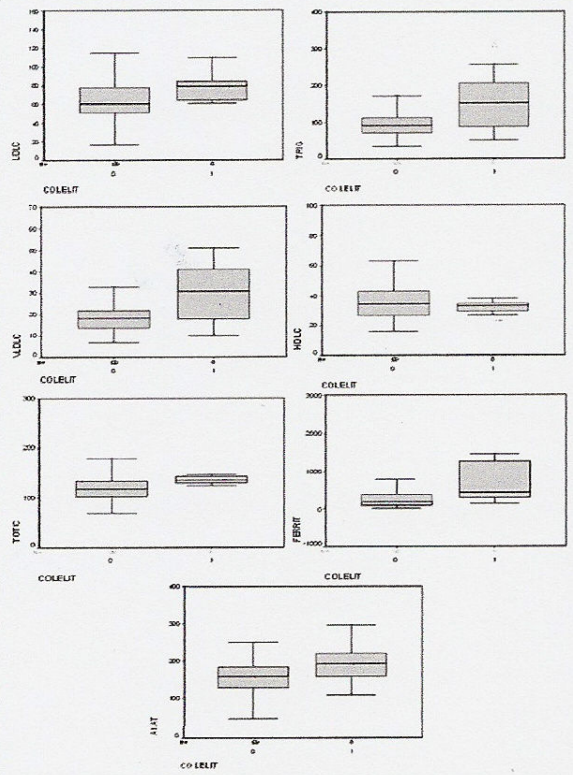

Figure 1 Box-plots of cholelithiasis and biochemical markers among sickle cell disease steady-state children. Number 0 represents absence and 1 presence of cholelithiasis (COLELIT) analyzed accord to different serum concentrations of triglycerides (TRIG) (P = 0.047), very-low density lipoprotein cholesterol (VLDLC) (P = 0.044), low-density lipoprotein cholesterol (LDLC) (P = 0.033), total cholesterol (P = 0.007), high-density lipoprotein cholesterol (HDLC) (P = 0.349), alpha 1-antitrypsin (A1AT) (P = 0.040) and ferritin (FERRIT) (P = 0.008). The p values were estimated by the Kruskal-Wallis test.

concentration, may function as a prognostic marker of intravascular hemolysis and endothelial dysfunction given its anti-inflammatory, anti-oxidative, anti-aggregation, anti-coagulant and pro-fibrinolytic activities [25,26].

Sickle cell disease patients with higher HDL-C levels presented a low risk of hemolysis and endothelial dysfunction, including lower reticulocyte and erythroblast counts as well as a lower HbS concentration and it may be related to the high consumption of cholesterol due to acceleration of blood marrow cell production during hemolysis crisis. Sickle cell disease patients with higher HDL-C levels had lower leukocyte, monocyte and platelet counts as well as a lower concentration of hepatic and hemolytic markers and significantly lower VLDL-C, triglycerides and A1AT concentrations; this may reflect the action of the anti-inflammatory and anti-oxidative properties of this biomarker [25,26]. The high-density lipoprotein cholesterol removes excess cholesterol from peripheral tissues and transports it to the liver for excretion via bile by reverse cholesterol transport. The high-density lipoprotein is made up of several particles with different composition and function [24,27,28].

Further confirmation of these associations came from comparing HDL-C concentrations and patients' clinical records, which revealed a higher occurrence of pneumonia and cardiac abnormalities among those with lower HDL-C levels. The results related to pneumonia risk can be explained by the production of auto-antibodies specific to oxidized phospholipids; these auto-antibodies have been shown to inhibit macrophage uptake of oxidized LDL and to provide protection against virulent pneumococcal infection [29]. Low levels of HDL-C are an important cardiovascular risk factor, and HDL-C and apoA-I have been shown to decrease lesions and improve vascular reactivity in animal models of atherosclerosis and in humans; these changes may be due to the reduction of oxidized lipids and the enhancement of reverse cholesterol transport [30]. The presence of pulmonary hypertension was shown to be associated with several laboratory test alterations [31]. Recent study has also demonstrated the important role of the apolipoprotein pathway and its association with endothelial dysfunction in SCD patients with pulmonary hypertension [31].

Patients with lower HDL-C levels were also likely to have had more blood transfusions; this can be linked with a more severe clinical course of disease, once that it is a therapeutic strategy used to prevent several clinical symptoms, such as stroke [1].

It is well known that gallstones in patients with hemolytic anemia are said to be calcium bilirubinate stones. In view our results of correlation of cholesterol and triglycerides with hemolysis, we propose that the stones in SCD patients could be related directly to hemolysis and bilirubin generation, and indirectly to cholesterol and lipids and it could be a novel observation and needs to be confirmed by further studies. The association of acute-phase proteins and cholelithiasis may be explained by the response to stress due to traumatic injury or infection-related mechanisms including hypermetabolism and protein catabolism associated with a cytokine-driven inflammatory response.

Conclusions

In conclusion, we hypothesize that some SCD patients can have a specific dyslipidemic subphenotype characterized by low HDL-C with hypertriglyceridemia and high VLDL-C in association with other biomarkers, including those related to inflammation. This represents an important step toward a more reliable clinical

prognosis. Additional studies are warranted to test this hypothesis and the probably mechanisms involved in this complex network of markers and their role in SCD pathogenesis.

Abbreviations

SCD: Sickle cell disease; VOE: vaso-occlusive pain episodes; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; LDH: lactate dehydrogenase; CRP: C-reactive protein; A1AT: alpha 1-antitripsin; ASO: antistreptolysin-O; HB: hemoglobin; HBF: hemoglobin fetal; HPLC: high performance liquid chromatography; RBC: red blood cells; MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; HBS: hemoglobin S; COLELIT: cholelithiasis.

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Authors' contributions

MOS performed experiments and analyzed the results; LR, MBC, JM, RDC, and AMA performed experiments; IML, VMLN and LR performed clinical evaluation of patients; MGR analyzed the results; and MSG was the principal investigator and takes primary responsibility for the paper, designed the research, analyzed the results and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Ohene-Frempong K, Steinberg MH: **Clinical Aspects of sickle cell anemia in adults and children.** In *Disorders of Hemoglobin: Genetics, In Patophysiology, and Clinical Management.* Edited by: Steinberg MH, Forget BG, Higgs DR, Nagel RL. New York: Cambridge University Press; 2001:611-70.
2. Stuart MJ, Nagel RL: **Sickle cell disease.** *Lancet* 2004, **364**:1343-60.
3. Nagel RL, Platt OS: **General pathophysiology of sickle cell anemia.** In *Disorders of Hemoglobin: Genetics, In Pathophysiology, and Clinical Management.* Edited by: Steinberg MH, Forget BG, Higgs DR, Nagel RL. New York: Cambridge University Press; 2001:494-526.
4. Sebastiani P, Nolan VG, Baldwin CT, Abad-Grau MM, Wang L, Adewoye AH, McMahon LC, Farrer LA, Taylor JG, Kato GJ, Gladwin MT, Steinberg MH: **A network model to predict the risk of death in sickle cell disease.** *Blood* 2007, **110**:2727-2763.
5. Okpala I: **The intriguing contribution of white blood cells to sickle cell disease- a red cell disorder.** *Blood Reviews* 2004, **18**:65-73.
6. Telen MJ: **Role of adhesion molecules and vascular endothelium in the pathogenesis of sickle cell disease.** *Hematology Am Soc of Hematol Educ Program* 2007, **2007**:84-90.
7. Villagra J, Shiva S, Hunter LA, Machado RF, Gladwin MT, Kato GJ: **Platelet activation in patients with sickle cell disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin.** *Blood* 2007, **110**:2166-2172.
8. Johnson C, Telen MJ: **Adhesion molecule and hydroxyurea in the pathophysiology of sickle cell disease.** *Hematologica* 2008, **93**:481-486.
9. Gladwin MT, Crawford JH, Patel RP: **Serial review: Biomedial implications for hemoglobin interactions with nitric oxide.** *Free Radical Biology & Medicine* 2003, **36**:707-717.
10. Kato GJ, McGowan V, Machado RF, Little JA, Taylor J, Morris CR, Nichols JS, Wang X, Poljakovic M, Morris SM Jr, Gladwin MT: **Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease.** *Blood* 2006, **107**:2279-2285.
11. O'Driscoll S, Height SE, Dick MC, Rees DC: **Serum lactate dehydrogenase activity as a biomarker in children with sickle cell disease.** *Br J Haematol* 2007, **140**:206-209.
12. Azevêdo ES: **Subgroup studies of black admixture within a mixed population of Bahia, Brazil.** *Ann Hum Genet* 1980, **44**:55-60.
13. Isichei UP: **Liver function and the diagnosis significance of biochemical changes in the blood of African children with sickle cell disease.** *J Clin Pathol* 1980, **33**:626-630.
14. Skalar AH, Campbell H, Caruana RJ, Lightfoot BO, Gaier JG, Milner P: **A population study of renal function in sickle cell anemia.** *Int J Artif Organs* 1990, **13**:231-236.
15. Skalar AH, Perez JC, Harp RJ, Caruana LRJ: **Acute renal failure in sickle cell anemia.** *Int J Artif Organs* 1990, **13**:347-351.
16. Marouf R, Mojiminiyi O, Abdella N, Kortom M, Al Wazzan H: **Comparison of renal function markers in Kuwaiti patients with sickle cell disease.** *J Clin Pathol* 2006, **59**:345-351.
17. Sasaki J, Waterman MR, Buchanan GR, Cottam GL: **Plasma and erythrocyte lipids in sickle cell anaemia.** *Clin Lab Haematol* 1983, **5**:35-44.
18. VanderJagt DJ, Huang YS, Chuang LT: **Phase angle and n-3 polyunsaturated fatty acids in sickle cell disease.** *Arch Dis Child* 2002, **87**:252-254.
19. VanderJagt DJ, Shores J, Okorodudu A, Okolo SN, Glew RH: **Hypocholesterolemia in Nigerian children with sickle cell disease.** *J Trop Pediatr* 2002, **48**:156-161.
20. Shores J, Peter son J, Vanderjagt D, Gless RH: **Reduced cholesterol levels in African-American adults with sickle cell disease.** *J Natl Med Assoc* 2003, **95**:813-817.
21. Djoumessi S, Zekeng L, Lando G, Zeukeng D: **Serum lipids and atherogenic risk in sickle- cell trait carriers.** *Ann Biol Clin* 1994, **52**:663-665.
22. Zorca S, Freeman L, Hildesheim M, Allen D, Remaley AT, Taylor JG, Kato GJ: **Lipid levels in sickle-cell disease associated with haemolytic severity, vascular dysfunction and pulmonary hypertension.** *Br J Haematol* 2010, **149**:436-45.
23. Magda Oliveira Seixas, Larissa Rocha, Mauricio Carvalho, Joelma Menezes, Isa Lyra, Valma Nascimento, Ricardo Couto, Ajax Atta, Mitermayer Galvão Reis, Marilda Souza Goncalves: **Lipoprotein Cholesterol and Triglycerides in Children with steady-state sickle cell anemia [Abstract].** *Blood* 2009, **114**:s1547.
24. Prinsen BHCMT, Romiju JA, Bisschop PH: **Endogenous cholesterol synthesis is associated with VLDL-2 apo-B100 production in healthy humans.** *J Lipid Res* 2003, **44**:1341-1348.
25. Nofer JR, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A: **HDL arteriosclerosis: beyond reverse cholesterol transport.** *Atherosclerosis* 2002, **161**:1-16.
26. Fredenrich A, Bayer P: **Reverse cholesterol transport, high density lipoproteins and HDL cholesterol: recent data.** *Diabetes Metab* 2003, **29**:201-205.
27. Miyazaki O, Fukamavhi I, Mori A: **Formation of preβ1-HDL during lipolysis of triglyceride-rich lipoprotein.** *Biochem Biophys Res Commun* 2009, **379**:55-59.
28. Pearson T, Wattis JA, O'Malley B: **Mathematical modeling of competitive LDL/VLDL binding and update by hepatocytes.** *J Math Biol* 2009, **58**:845-880.
29. Navab M, Berliner JA, Subbanagounder G, Hama S, Lusis AJ, Castellani LW, Reddy S, Shih D, Shi W, Watson AD, Van Lenten BJ, Vora D, Fogelman AM: **HDL and inflammatory response induced by LDL-derived oxidized phospholipids.** *Arterioscler Thromb Vasc Biol* 2001, **21**:481-488.

30. Navab M, Ananthramaiah GM, Reddy ST: **The oxidation hypothesis hypothesis of atherogenesis: the role of oxidized phospholipids and HDL.** *J Lipid Res* 2004, **45**:9932-1007.
31. Minniti CP, Sable C, Campbell A, Rana S, Ensing G, Dham N, Onyekwere O, Nouraie M, Kato GJ, Gladwin MT, Castro OL, Gordeuk VR: **Elevated tricuspid regurgitant jet velocity in children and adolescents with sickle cell disease: association with hemolysis and hemoglobin oxygen desaturation.** *Haematologica* 2009, **94**:340-347.

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4.2. MANUSCRITO II

Evaluation of Lipids, Prostaglandin-E_2 pattern, and neutrophils chemotaxis in sickle cell disease

Magda O S Carvalho[1,2]; Larissa C Rocha[1,3]; Valma M L Nascimento[3]; Mauricio B Carvalho[3]; Thassila Pitanga[1]; Sânzio Silva Santana[1]; Théo Araújo-Santos[4]; Deboraci Brito Prates[1,2]; Valéria M. Borges[1]; and Marilda S Gonçalves[1,2]

Este trabalho avalia as possíveis associações entre os lipídios, a prostaglandina E2, a quimiotaxia de neutrófilos e as manifestações clínicas nos pacientes com doença falciforme.

Resumo: A inflamação crônica e a heterogeneidade clínicas são características importantes na doença falciforme (DF). Os produtos do metabolismo do ácido araquidônico e outras moléculas lipídicas já foram associados a doenças inflamatórias, metabólicas e neoplásicas. Neste trabalho descrevemos associação de prostaglandina E2 (PGE_2) e marcadores de gravidade clínica na DF, bem como o perfil lipídico e associação com dados clínicos e quimiotaxia de neutrófilos. A casuística do presente estudo foi composta por 356 pacientes com DF, estáveis, e 167 indivíduos saudáveis. Foram investigados os níveis séricos de PGE_2 e suas possíveis associações com marcadores bioquímicos, hematológicos, inflamatórios e dados clínicos. As informações sobre as histórias clínicas do paciente foram coletadas dos registros médicos dos pacientes. Com base nessas análises observou-se que os níveis de PGE_2 foram estatisticamente associados a dados bioquímicos e hematológicos. O colesterol ligado a lipoproteína de alta densidade (HDL-c), o triglicérides e o colesterol total foram associados aos marcadores estudados e a diversas características clínicas encontradas nos pacientes com DF, tais como vaso-oclusão, úlcera maleolar, internação, pneumonia, esplenomegalia, acidente vascular cerebral, crises dolorosas, anormalidades cardíacas e colelitíase. O ensaio de quimiotaxia revelou a redução no recrutamento de neutrófilos sob a ação da sinvastatina, e o aumento do mesmo sob o estímulo de eritrócitos do próprio paciente. Acreditamos que a PGE_2 pode ser considerada como um marcador de gravidade na DF, bem como os níveis séricos de lipídios. Os resultados obtidos a partir da avaliação da quimiotaxia de neutrófilos sugerem que alterações lipídicas nos eritrócitos falciformes, podem estimular a migração de neutrófilos, além de reforçar o papel da sinvastatina sobre níveis séricos de lipídios oxidados presentes em pacientes com DF.

Evaluation of Lipids, Prostaglandin-E_2 pattern, and neutrophils chemotaxis in sickle cell disease

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ABSTRACT

The sickle cell disease (SCD) has chronic inflammatory properties and clinical heterogeneity being hallmarks of the disease. We study prostaglandin E2 (PGE_2) association with markers of SCD severity, clinical outcomes and its role in neutrophil chemotaxis. A total of 351 SCD patients were studied, and we investigate PGE_2 serum levels and its association with markers of the disease severity. PGE_2 levels were statistically associated to biochemical and hematological markers. The lipid pattern was associated with other laboratorial parameters, and with SCD patients' clinical characteristics. Simvastatin, an important lipid-lowering agent, decreased the red blood cells-induced neutrophil recruitment. Our results show PGE_2 and systemic lipids molecules as markers of SCD severity, and suggest an action of lipid membrane disturbance in sickle red erythrocytes as a stimulator of neutrophil migration, and a role of simvastatin in systemic oxidized lipids in SCD patients.

Key Words: Sickle cell disease, prostaglandin E_2, lipids, leukocytes chemotaxis, erythrocytes

INTRODUCTION

The sickle cell disease (SCD) is the denomination of a group of genetic disease characterized by the hemoglobin S (HbS) presence, which has a point mutation in the beta globin gene (*HBB*) where adenine is replaced by thymine (G<u>A</u>G → G<u>T</u>G), and valine replaces glutamic acid at sixth position of the N-terminus of the beta globin chain (β). The more severe type of SCD is the homozygous state the beta S allele (β^S) (HbSS), commonly referred as sickle cell anemia (SCA). Though, some others SCD types have also a severe clinical picture as described in the hemoglobin SC disease (HbSC), characterized by the β^S allele in heterozygosis with β^C allele, related to the hemoglobin C (HbC) presence (Steinberg, 2001; Ohene-Frempong e Steinberg, 2001; Rees *et al.*, 2010).

The SCD has a heterogeneous clinical outcome, with multi-organ damage, systemic metabolic alteration, and early mortality risk (Steinberg, 2001; Rees *et al.*, 2010). SCD patients has a diversified clinical picture and exhibit hemolytic anemia, infection susceptibility, vaso-occlusive events, pulmonary complication, such as pneumonia, acute chest syndrome (ACS), and pulmonary hypertension (PH). SCD patients' children commonly experience stroke episodes, and in general, may present nephropathy and retinopathy amongst others complications (Steinberg, 2001; Kato *et al.*, 2007; Galdwin, 2011; Ataga *et al.*, 2014).

All clinical alteration described in SCD has been associated with a strong inflammatory component, ischemia-reperfusion injury-associated, endothelial dysfunction, and metabolic changes, including lipids and correlate metabolites (Kato *et al.*, 2009; Hebbel, 2014; Oztas *et al.*, 2011). Previous reports, including study developed in our research group, have been related the contribution of lipids in metabolic alterations in SCD patients (Zorca *et al.*, 2010; Seixas *et al.*, 2010; Frenette e Atweh, 2007; Rahimy *et al.*, 2006**)**. All of these processes involve the activation of several molecules and cells, which are related to hemolysis, inflammation, vasculopathy, generation of oxidative process and reactive oxygen species (ROS), and exacerbation of several pathways related to the immune system (Okpala *et al.*, 2002; Haynes *et al.*, 2008; Kaul *et al.*, 2009; Krishnan *et al.*, 2010).

Changes in circulating lipids have been described in SCD patients, which present lipid oxidation as well as decrease of molecules related to lipid metabolism, such as total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) (el-Hazmi *et al.*, 1995; Njoku *et al.*, 1996; Belcher *et al.*, 1999; Vanderjagt *et al.*, 2002; Shores *et al.*, 2003; Rahimy *et al.*, 2006). On the other hand, the changes occurring in the red blood cells (RBC) membrane in SCD bring phospholipids alterations and repercussions in several metabolic pathways, generating ion and coagulation cascade disturbance (Kavecansky *et al.*, 1995; Marzouki e Khoja, 2003; Ren *et al.*, 2005; Frenette e Atweh, 2007**)**.

Additionally, eicosanoids and diacylglycerol are released by endothelial cells secondary to sickle red cell stimulation, increasing arachidonic acid products, such as prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4), whose synthesis has been implicated in the control of the inflammatory response (Kuehl *et al.*, 1980), and the increase of eicosanoids synthesis was described in rat lungs perfused with HbSS erythrocytes, suggesting an aberrant erythrocyte-endothelium interactions in SCD (Bo e*t al*., 1997). The increase of PGE2 concentration has been described in plasma of SCD patients in steady state and crisis (Graido-Gonzalez *et al*., 1998; Aslan *et al*., 2014), and in urinary and plasma concentrations together with thromboxane B_2 levels among SCA subjects, and related to platelet and endothelial cell activation (Kurantsin-Mills *et al*., 1994). However, the increase of PGE_2 has been associated to the induction of discocyte to echinocyte transformation, but with any effect on the viscosity or osmotic fragility of normal or sickle cell erythrocytes (Gruber e Gilbertson, 1978).

Based on these fact, several biomarkers have been associated with different sub-phenotypes exhibited by SCD patients (Kato *et al.*, 2007; Rees e Gibson, 2012), including also those related to disturbances of lipids and their action on mechanisms that contribute to the maintenance of sub-clinical disease, even in SCD patients in steady state. In this report, we propose evaluate levels of PGE_2, LTB_4, and molecules associated to lipids systemic pattern and their association with laboratorial parameters, analyzing also the influence of RBC in the chemotaxis of neutrophils and the effects of simvastatin on this recruitment.

SUBJECTS AND METHODS

Subjects and Controls

Of 351 unrelated steady state SCD patients, 235 with sickle cell anemia (SCA) and 116 with hemoglobin SC disease (HbSC) Brazilian children were prospectively enrolled at the present study. Patients were followed at the Hematology outpatient clinics of the Fundação de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA). The HEMOBA is a reference center to attend hematological disease and realize the follow-up of more than 2,000 SCD patients in Salvador, state of Bahia, in the Northeast of Brazil. All patients were in steady state, characterized by an absence of blood transfusion in a period of four months prior to blood sampling. Inclusion criteria for steady state SCD patients were a period of three months without any acute events, absence of general symptoms, hospitalization, or infections. None of patients had taken antibiotics or corticosteroids ten days prior to blood sampling, and hydroxyurea, but were on folic acid therapy. Blood samples were taken during a regular clinical visit, and the medical history of the SCD patients was recorded from patients' files.

To identify possible associations between lipids molecules, such as total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), and triglycerides levels and clinical pattern of SCD, we assessed medical history from patients' records, including prevalence of vaso-occlusive events (VOE), presence of leg ulcer, number of hospitalizations, events of pneumonia, splenic sequestration, splenomegaly, stroke, painful episodes, infection, priapism, cardiac abnormalities, respiratory insufficiency, gallstones, and use of blood therapy. Cardiac abnormalities typical of hemolytic anemia on auscultation were not considered. However, patients with cardiac abnormalities had electrocardiograph arrhythmia, and history of tricuspid regurgitation, but these evaluations were not realized at the same time of the present study.

PGE_2, LTB_4, heme, tissue inhibitor of metalloproteinase 1 (TIMP-1), and transforming growth factor beta (TGF-β) dosages were evaluated among 146 SCD patients (99 with SCA, and 47 with HbSC), which were parts of the 351 SCD patients evaluated at the present study.

The study included 167 healthy children without clinical and biochemical evidence of SCD, randomly selected from the Clinical Laboratory of the "Faculdade de Farmácia da

Universidade Federal da Bahia (FAR-UFBA)"; control individuals were matched with the SCD patients by age and gender. The study was approved by the "Centro de Pesquisa Gonçalo Moniz- Fundação Oswaldo Cruz (CPqGM-FIOCRUZ)" human subject research board, and all officials responsible provided written informed consent, in accordance with the Declaration of Helsinki of 1975, as revised in 2000.

Biochemical and Hematological evaluation

Biochemical laboratorial markers were performed by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain). The serum ferritin was measured by immunoassay using an Access® 2 Immunoassay system X2 (Beckman Coulter, Fullerton, CA). C-reactive protein (CRP), alpha 1-antitripsin (AAT), and antistreptolysin-O (ASO) were measured by immunochemistry (Immage® 800system, Beckman Coulter, Fullerton, CA).

Hematological analyses were carried out using an electronic cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC / VARIANT I; BIO-RAD, CA, USA).

All these evaluations were developed in the Clinical Analyses Laboratory of the FAR-UFBA and the Laboratory of Hematology, Genetic, and Computational Biology (LHGB) at the CPqGM-FIOCRUZ.

PGE_2, TIMP-1, LTB4, TGF-β, and free heme plasma measurement

Plasma levels of PGE_2 were investigated by the enzyme-linked immunoassay (ELISA) technique following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). TIMP-1, LTB4, and TGF-β plasma concentrations were estimated using the ELISA technique in accordance with the manufacturer's instructions (ReD Systems, Minneapolis, MN, USA). Plasmatic concentrations of total free heme were measured with a QuantiChrom Heme Assay Kit (Bioassay Systems, Hayward, CA, USA) following the manufacturer's protocol.

Chemotaxis assays

The chemotaxis assay evaluated neutrophil migration from patients with SCD and healthy controls to different stimuli as described. We added to the bottom of a 96-well chemotaxis plate ChemoTx system (Neuro Probe, Gaithersburg, MD, USA) fresh serum from HbSS, HbSC or HbAA; 10 mg/mL simvastatin suspension in each serum; 5% suspension of RBC from each group in physiological saline; and medium (RPMI) and lipopolysaccharides (LPS).We developed the assay with stimuli under the same condition. Neutrophils were obtained from SCD patients and healthy controls by separation on Ficoll (Histopaque 1077 Sigma) from peripheral blood freshly collected in EDTA and were diluted in RPMI medium before being added to the top wells (10^5 cells/well) of the same chemotaxis plate. We incubated the plate at 37 ° C under 5% CO2 for 1h. Following incubation, we counted neutrophils that migrated to the bottom wells in a hemocytometer. We used as a negative control neutrophil migration toward pure RPMI (random chemotaxis) and migration against lipopolysaccharides (LPS) as positive control. We calculated the chemotactic index as the ratio between the number of neutrophils that migrated under stimuli and neutrophils that migrated with RPMI alone.

Statistical analysis

We summarized baseline characteristics as means and proportions of selected variables. We determined the distribution of quantitative variables using the Kolmogorov-Smirnov test. We compared mean values of quantitative variables between two groups using the unpaired t-test for normal data distribution and Mann-Whitney for non-normal data. We analyzed bivariate correlation between pairs of variables using Pearson and Spearman's rank correlation (R). We used the nonparametric Kruskal-Wallis test to compare means of three or more variables values. We analyzed levels of PGE_2 as $\geq$ or < of 50^{th} (6.0 ng/mL) and of LTB_4 as $\geq$ or < of 50^{th} (249.0 ng/mL). We assessed correlation analyses between lipids molecules and chemistry and hematological variables using Pearson and Spearman's rank correlation (R). We assessed statistical analyses of clinical characteristics and HDL-C, LDL-C, VLDL-C, and triglycerides with independent t-test and Mann-Whitney tests. We compared means values obtained from the neutrophils chemotaxis assay by the nonparametric Kruskal-Wallis test. We considered all tests as significant if p values were less than .05. Data analyses were performed using Prism 5.01 (Graphpad Software, San Diego, CA), EPIinfo 6.04 (CDC, Atlanta, Georgia), and STATA SE 10 software (StataCorp, Texas, USA).

RESULTS

Patients and control groups laboratorial characteristics

SCD patients had mean ± standard deviation age of 13.96 ± 9.91 years, with median of 12.00, and 25th percentile of 8.00 and 75th percentile of 16.00, and 47.51% (162/341) were females.

Association of levels of Prostaglandin E2 and Leukotriene B4 with hematological and biochemical markers

Higher levels of PGE_2 were associated with altered levels of biochemistry and hematological markers and to SCD severity. However, higher levels of LTB_4 were associated to high concentration of urea (Figure 1).

Correlation of the high-density lipoprotein cholesterol to biochemical and hematological markers

The HDL-C was correlated to biochemical markers of renal function, hemolysis and hepatic dysfunction, and inflammation (Figure 2), indicating its correlation with metabolic changes in SCD patients. The HDL-C was also correlated to hematological markers of anemia, hemolysis, circulating leukocytes activation, and hemoglobin pattern, showing a participation of this molecules on possible mechanisms related to hemolysis (Figure 3), a common clinical feature in SCD, and other reactions resulting from the cascade of events that follow the hemolysis, such as the generation of ROS (Ren *et al.*, 2008).

Correlation of triglycerides to hematological and chemistry markers

Triglycerides were correlated to hematological markers of anemia, hemolysis, and hemoglobin pattern, showing a participation of this molecule on possible mechanisms related to the disease severity (Figure 4). Triglycerides were correlated to biochemical markers of renal function, hemolysis and hepatic dysfunction, and oxidative stress (Figure 4), indicating its correlation with metabolic changes in SCD patients.

Correlation of total cholesterol to hematological and chemistry markers

The total cholesterol was correlated to hematological markers of anemia, and platelet activation, showing a participation of this molecule on possible mechanisms related to the anemia, hypoxia and ischemia, and reperfusion injury (Eltzschig e Eckle, 2011; Hebbel, 2014) (Figure 5). The total cholesterol was correlated to biochemical markers of renal function, and

hemolysis and hepatic dysfunction and, consequently, to the oxidative stress (Figure 5), indicating its correlation with metabolic changes in SCD patients.

Association of lipid pattern to clinical characteristics exhibited by SCD patients

The lipids altered pattern has been associated to vaso-occlusive events, leg ulcer presence (Figure 6); hospitalization, pneumonia, splenomegaly, and stroke events (Figure 7); painful crises and cardiac abnormalities (Figure 8); cholelithiasis, and blood therapy (Figure 9).

Chemotaxis assay of neutrophils

We finally investigated whether SCD patient sera (with or no simvastatin) and its RBC suspension are able to induce neutrophil recruitment in vitro. Chemotaxis assay revealed that the patient's serum alone induced autologous neutrophil recruitment in vitro (Figure 10). The addition of simvastatin (10 mg / ml for 1 h) reduced the rate of neutrophil chemotaxis in patients with SCD, independent of genotype (SCA and HbSC), but not in healthy controls (HbAA) (Figure 10). The response of neutrophil chemotaxis front of a 5 % suspension of washed RBC from the individual himself revealed the highest levels of neutrophil chemotaxis in each experiment, suggesting a role of sickle erythrocytes in neutrophil migration in SCD (Figure 10).

DISCUSSION

The present study analyzed the association of levels of PGE_2, LTB_4, and molecules associated to lipids pattern among SCD children in steady state. SCD patients usually have changes in hematological markers, with presence of anemia, altered hemolysis markers, and also metabolic changes when compared to healthy children, even when patients are in steady state (Steinberg, 1996; Kato *et al.*, 2007; Seixas *et al.*, 2010; Milton *et al.*, 2012). Those alterations have been related to the inflammatory, vaso-occlusive, painful episodes and with numerous clinical events present in the disease, with exacerbation of several signaling pathways, with activation of leukocytes, RBC, platelets and endothelial cells (Okpala *et al.*, 2002; Okpala, 2004; Okpala, 2006). Also the presence of hypoxia and the lipids changes in RBC membrane have been described to contribute to the inflammatory environment and other metabolic disorders maintenance in the disease (Connor *et al.*, 1997; Setty *et al.*, 1996; de Jong *et al.*, 1997).

The PGE_2 is a lipid component, cyclooxygenase (COX)-derived from the arachidonic acid metabolism, which mediates several physiological mechanisms, and exerts effect by binding prostaglandin receptors (EP), characterized as G protein-coupled receptors (Reader *et al.*, 2011). The endothelium has the property to control the releasing of relaxing and contracting factors to regulate local vascular tone. Increased levels of PGE_2 and COX-2 have been associated to several disorders, including inflammatory diseases, and also with endothelial dysfunction (Wong *et al.*, 2010). Our study described the association of high systemic levels of PGE_2 with changes in markers related to hepatic dysfunction and hemolysis (Aspartate transferase, bilirubin and fractions, lactate dehydrogenase, and free heme); also with TIMP-1, and with markers of anemia (Hb, Hct), and leukocytes and platelets activation. The LTB_4 was only related to urea alterations. The inflammatory property of PGE_2 was described in SCD patients (Graido-Gonzalez *et al*., 1998), also the increase of PGE_2 levels was associated to increase of inflammatory cytokine and with leukocyte activation (North et al., 2007; Konya *et al*., 2011; Barrie *et al*., 2011; Hegyi *et al*., 2012), aspects very well known in SCD, emphasizing a possible role as a biomarker of the disease severity. Larsson *et al.* (2015) described that beside the lipid mediators have inflammatory property, and in this group they include the PGE_2 and its role on proinflammatory states and also contribution on neoplastic disease, in the growth and malignancy of the tumor. Related to the association of increased LTB_4 and the increase of urea, we did not find any description in literature and it may be involved in kidney alterations described among these patients.

Ours findings about HDL-C, total cholesterol, and triglycerides correlation to biochemical and hematological markers in SCD were already described, but studies emphasize lipids alteration have increased and are showing the association of SCD with these metabolic changes. The decrease of HDL-C has been described not quantitatively, but also qualitatively, mainly related to the change of HDL-C to inflammatory action and its use as potential biomarker for SCD clinical severity (Sasaki et al., 1983; Djoumessi et al., 1994; VanderJagt DJ et al., 2002a; VanderJagt DJ et al., 2002b; Shores et al., 2003; Seixas et al., 2010; Zorca et al., 2010). Results related to clinical alteration and have been show and may be associated to the production of oxidized phospholipids, increasing metabolic alteration and systemic changes, contributing to several organ damage and dysfunction among our SCD patients (Navab et al., 2001).

Changes in the lipid membrane of RBC in the SCD have been previously described (Kucuk *et al*., 1992; Connor *et al.*, 1997; Setty *et al.*, 1996; de Jong *et al.*, 1997). Our results of increase of neutrophil migration may be explained looking for the lipid changes in the erythrocyte membrane and the exposition of these metabolic products, contributing to the inflammatory milieu present in SCD. Indeed, we can confirm by the finding of a decrease of neutrophil migration after exposition of patient serum to simvastatin, probably by the action in the oxidized cholesterol. An important finding in our study was the characteristic of simvastatin did not have a strong action on the reduction of the neutrophil migration among HbAA individual, what can be related to different metabolites synthetized in the absence of oxidized cholesterol.

Conclusions

In conclusion, we emphasize the importance of the dyslipidemic sub-phenotype in SCD as an important mechanism to be involved in target therapeutic strategies. Besides, PGE_2 is associated with markers of the SCD severity and also with lipids patterns, and an important role of simvastatin therapy action in the oxidized cholesterol production and inflammatory milieu in this disease.

REFERENCES

1. Steinberg, M.H., Forget, B.G., Higgs, D.R., Nagel R.L. (2001) Disorders of hemoglobin genetics, pathophysiology and clinical management, Cambridge University Press.

2. Ohene-Frempong K, Steinberg MH (2001): Clinical Aspects of sickle cell anemia in adults and children. In: Disorders of Hemoglobin: Genetics, In Phatophysiology, and Clinical Management. Edited by Steinberg MH, Forget BG, Higgs DR, Nagel RL. New York: Cambridge University Press, 611-70.

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

4. Kato GJ, Gladwin MT, Steinberg MH (2007) Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev 21:37-47.

5. Gladwin MT. (2011) Prevalence, risk factors and mortality of pulmonary hypertension defined by right heart catheterization in patients with sickle cell disease. Expert Rev Hematol.;4(6):593-6.

6. Ataga KI, Derebail VK, Archer DR. (2014) The glomerulopathy of sickle cell disease. Am J Hematol.;89(9):907-14.

7. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT. (2009) Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol;84(9):618-25.

8. Hebbel RP. (2014) Ischemia-reperfusion injury in sickle cell anemia: relationship to acute chest syndrome, endothelial dysfunction, arterial vasculopathy, and inflammatory pain. Hematol Oncol Clin North Am.:28(2):181-98.

9. Oztas YE, Sabuncuoglu S, Unal S, Ozgunes H, Ozgunes N. (2011) Hypocholesterolemia is associated negatively with hemolysate lipid peroxidation in sickle cell anemia patients. Clin Exp Med.;11(3):195-8. Erratum in: Clin Exp Med. 2012 Jun;12(2):133. Yesim, Oztas Er [corrected to Oztas, Y E]; Suna, Sabuncuoglu [corrected to Sabuncuoglu, S]; Selma, Unal [corrected to Unal, S]; Hilal, Ozgunes [corrected to Ozgunes, H]; Nuriman, Ozgunes [corrected to Ozgunes, N].

10. Zorca S, Freeman L, Hildesheim M, Allen D, Remaley AT, Taylor JG 6th, Kato GJ. (2010) Lipid levels in sickle-cell disease associated with haemolytic severity, vascular dysfunction and pulmonary hypertension. Br J Haematol,149:436-45.

11. Seixas MO, Rocha LC, Carvalho MB, Menezes JF, Lyra IM, Nascimento VM, Couto RD, Atta ÁM, Reis MG, Goncalves MS. (2010) Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease. Lipids Health Dis.; 9:91.

12. Frenette PS, Atweh GF. (2007) Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest.; 117(4):850-8.

13. Rahimi Z, Merat A, Haghshenass M, Madani H, Rezaei M, Nagel RL. (2006) Plasma lipids in Iranians with sickle cell disease: hypocholesterolemia in sickle cell anemia and increase of HDL-cholesterol in sickle cell trait. Clin Chim Acta.;365(1-2):217-20.

14. Okpala I, Daniel Y, Haynes R, Odoemene D, Goldman J (2002) Relationship between the clinical manifestations of sickle cell disease and the expression of adhesion molecules on white blood cells. Eur J Haematol 69:135-144.

15. Haynes J Jr, Obiako B, Hester RB, Baliga BS, Stevens T. (2008) Hydroxyurea attenuates activated neutrophil-mediated sickle erythrocyte membrane phosphatidylserine exposure and adhesion to pulmonary vascular endothelium. Am J Physiol Heart Circ Physiol.;294(1):H379-85.

16. Kaul DK, Finnegan E, Barabino GA. (2009) Sickle red cell-endothelium interactions. Microcirculation.;16(1):97-111.

17. Krishnan S, Setty Y, Betal SG, Vijender V, Rao K, Dampier C, Stuart M. (2010) Increased levels of the inflammatory biomarker C-reactive protein at baseline are associated with childhood sickle cell vasocclusive crises. Br J Haematol.;148(5):797-804.

18. el-Hazmi MA, Warsy AS, al-Swailem A, al-Swailem A, Bahakim H. (1995) Red cell genetic disorders and plasma lipids. J Trop Pediatr,;41(4):202-5.

19. Njoku OU, Ononogbu IC, Alumunan EO, Nwanjoh J. (1996) Serum lipids, ABO blood group and sickle cell trait. Indian J Physiol Pharmacol.;40(2):171-4.

20. Belcher JD, Marker PH, Geiger P, Girotti AW, Steinberg MH, Hebbel RP, Vercellotti GM. (1999) Low-density lipoprotein susceptibility to oxidation and cytotoxicity to endothelium in sickle cell anemia. J Lab Clin Med.;133(6):605-12.

21. Vanderjagt DJ, Huang Y-S, Chuang L-T. (2002) Phase angle and n-3 polyunsaturated fatty acids in sickle cell disease. Arch Dis Child, 87:252-254.

22. Shores J, Peter son J, Vanderjagt D, Gless RH. (2003) Reduced cholesterol levels in African-American adults with sickle cell disease. J Natl Med Assoc.; 95: 813-817.

23. Kavecansky J, Schroeder F, Joiner CH. (1995) Deoxygenation-induced alterations in sickle cell membrane cholesterol exchange. Am J Physiol.;269(5 Pt 1):C1105

24. Marzouki ZM, Khoja SM. (2003) Plasma and red blood cells membrane lipid concentration of sickle cell disease patients. Saudi Med J.;24(4):376-9.

25. Ren H, Obike I, Okpala I, Ghebremeskel K, Ugochukwu C, Crawford M. (2005) Steady-state haemoglobin level in sickle cell anaemia increases with an increase in erythrocyte membrane n-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids.; 72(6):415-21.

26. Kuehl FA Jr, Egan RW (1980) Prostaglandins, arachidonic acid, and inflammation. Science; 210:978-984**.**

27. Rees DC, Gibson JS. (2012) Biomarkers in sickle cell disease. Br J Haematol.; 156(4):433-45.

28. Ren H, Ghebremeskel K, Okpala I, Lee A, Ibegbulam O, Crawford M. (2008) Patients with sickle cell disease have reduced blood antioxidant protection. Int J Vitam Nutr Res.; 78(3):139-47.

29. Eltzschig HK, Eckle T. (2011) Ischemia and reperfusion--from mechanism to translation. Nat Med.; 17(11):1391-401.

30. Steinberg MH. (1996) Modulation of the phenotypic diversity of sickle cell anemia. Hemoglobin.;20(1):1-19.

31. Milton JN, Sebastiani P, Solovieff N, Hartley SW, Bhatnagar P, Arking DE, Dworkis DA, Casella JF, Barron-Casella E, Bean CJ, Hooper WC, DeBaun MR, Garrett ME, Soldano K, Telen MJ, Ashley-Koch A, Gladwin MT, Baldwin CT, Steinberg MH, Klings ES. (2012) A genome-wide association study of total bilirubin and cholelithiasis risk in sickle cell anemia. PLoS One.;7(4):e34741.

32. Okpala I (2004) The intriguing contribution of white blood cells to sickle cell disease - a red cell disorder. Blood Rev 18:65-73.

33. Okpala I (2006) Leukocyte adhesion and the pathophysiology of sickle cell disease. Curr Opin Hematol; 13:40-44.

34. Connor WE, Lin DS, Thomas G, Ey F, DeLoughery T, Zhu N. (1997) Abnormal phospholipid molecular species of erythrocytes in sickle cell anemia. J Lipid Res.; 38(12):2516-28.

35. Setty BN, Chen D, Stuart MJ. (1996) Sickle red blood cells stimulate endothelial cell production of eicosanoids and diacylglycerol. J Lab Clin Med.;128(3):313-21.

36. de Jong K, Geldwerth D, Kuypers FA. (1997) Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes. Biochemistry.;36(22):6768-76.

37. Reader J, Holt D, Fulton A. (2011) Prostaglandin E2 EP receptors as therapeutic targets in breast cancer. Cancer Metastasis Rev.;30(3-4):449-63.

38. Wong SL, Wong WT, Tian XY, Lau CW, Huang Y. (2010) Prostaglandins in action indispensable roles of cyclooxygenase-1 and -2 in endothelium-dependent contractions. Adv Pharmacol. 2010;60:61-83.

39. Graido-Gonzalez E, Doherty JC, Bergreen EW, Organ G, Telfer M, McMillen MA. (1998) Plasma endothelin-1, cytokine, and prostaglandin E2 levels in sickle cell disease and acute vaso-occlusive sickle crisis. Blood.;92(7):2551-5.

40. North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, Fitzgerald GA, Daley GQ, Orkin SH, Zon LI. (2007) Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature. Jun 21;447(7147):1007-11.

41. Konya V, Philipose S, Bálint Z, Olschewski A, Marsche G, Sturm EM, Schicho R, Peskar BA, Schuligoi R, Heinemann A. (2011) Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. Eur J Immunol.;41(8):2379-89.

42. Barrie A, Khare A, Henkel M, Zhang Y, Barmada MM, Duerr R, Ray A. (2011) Prostaglandin E2 and IL-23 plus IL-1β differentially regulate the Th1/Th17 immune response of human CD161(+) CD4(+) memory T cells. Clin Transl Sci. Aug;4(4):268-73.

43. Hegyi B, Kudlik G, Monostori E, Uher F. (2012) Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. Biochem Biophys Res Commun. 9;419(2):215-20.

44. Larsson K, Kock A, Idborg H, Arsenian Henriksson M, Martinsson T, Johnsen JI, Korotkova M, Kogner P, Jakobsson PJ. (2015) COX/mPGES-1/PGE2 pathway depicts

an inflammatory-dependent high-risk neuroblastoma subset. Proc Natl Acad Sci U S A. pii: 201424355. [Epub ahead of print].

45. Montrose DC, Nakanishi M, Murphy RC, Zarini S, McAleer JP, Vella AT, Rosenberg DW. (2015) The role of PGE2 in intestinal inflammation and tumorigenesis. Prostaglandins Other Lipid Mediat; 116-117:26-36.

46. Sasaki J, Waterman MR, Buchanan GR, Cottam GL. (1983) Plasma and erythrocyte lipids in sickle cell anaemia. Clin Lab Haematol, 5: 35-44.

47. VanderJagt DJ, Huang Y-S, Chuang L-T. (2002a) Phase angle and n-3 polyunsaturated fatty acids in sickle cell disease. Arch Dis Child, 87:252-254.

48. VanderJagt DJ, Shores J, Okorodudu A, Okolo SN, Glew RH. (2002b) Hypocholesterolemia in Nigerian children with sickle cell disease. J. Trop Pediatr, 48: 156-161.

49. Djoumessi S, Zekeng L, Lando G, Zeukeng D: Serum lipids and atherogenic risk in sickle- cell trait carriers. Ann Biol Clin 1994, 52:663-665.

50. Navab M, Berliner JA, Subbanagounder G, Hama S, Lusis AJ, Castellani LW, Reddy S, Shih D, Shi W, Watson AD, Van Lenten BJ, Vora D, Fogelman AM. (2001) HDL and inflammatory response induced by LDL-derived oxidized phospholipids. Arterioscler Thromb Vasc Biol, 21:481-488.

51. Kucuk O, Lis LJ, Dey T, Mata R, Westerman MP, Yachnin S, Szostek R, Tracy D, Kauffman JW, Gage DA, et al. (1992) The effects of cholesterol oxidation products in sickle and normal red blood cell membranes. Biochim Biophys Acta.;1103(2):296-302.

LEGENDS

Figure 1. Association of biochemical and hematological markers in steady state sickle cell disease (SCD) patients with Prostaglandin E 2 (PGE_2) concentrations higher and lower than the 50^{th} percentile (6.42 ng/mL), and with Leukotriene B4 (LTB_4) higher and lower than the 50th percentile (249.0 ng/mL) .

Figure 2. Correlation of high-density lipoprotein cholesterol (HDL-C) with other biochemical markers in steady state sickle cell disease patients.

Figure 3. Correlation of high-density lipoprotein cholesterol (HDL-C) with hematological markers in steady state sickle cell disease patients.

Figure 4. Correlation of triglycerides with hematological and chemistry markers in steady state sickle cell disease patients.

Figure 5. Correlation of total cholesterol with hematological and chemistry markers in steady state sickle cell disease patients.

Figure 6. Association of lipids and clinical events of vaso-occlusive events and legs ulcers in sickle cell disease patients in steady state.

Figure 7. Association of lipids with hospitalization and clinical events of pneumonia, splenomegaly, and stroke in sickle cell disease patients in steady state.

Figure 8. Association of lipids and clinical events of painful crises, and cardiac abnormalities in sickle cell disease patients in steady state.

Figure 9. Association of lipids with blood therapy and clinical events of cholelithiasis in sickle cell disease patients in steady state.

Figure 10. Neutrophil recruitment toward sickle cell disease patient serum. Neutrophils migration toward serum from sickle cell anemia (SCA), SC hemoglobin disease (HbSC) patients, and health controls (HbAA); all plus simvastatin or not, and red blood cells from each group of donors. Following incubation, the migrated neutrophils were counted, and the chemotactic index was calculated. Medium is the negative control and lipopolysaccharide (LPS) is the positive control. Crtl, Negative control of random chemotaxis. Data are representative of three independent experiments performed in triplicate for each sample.

FIGURE 1

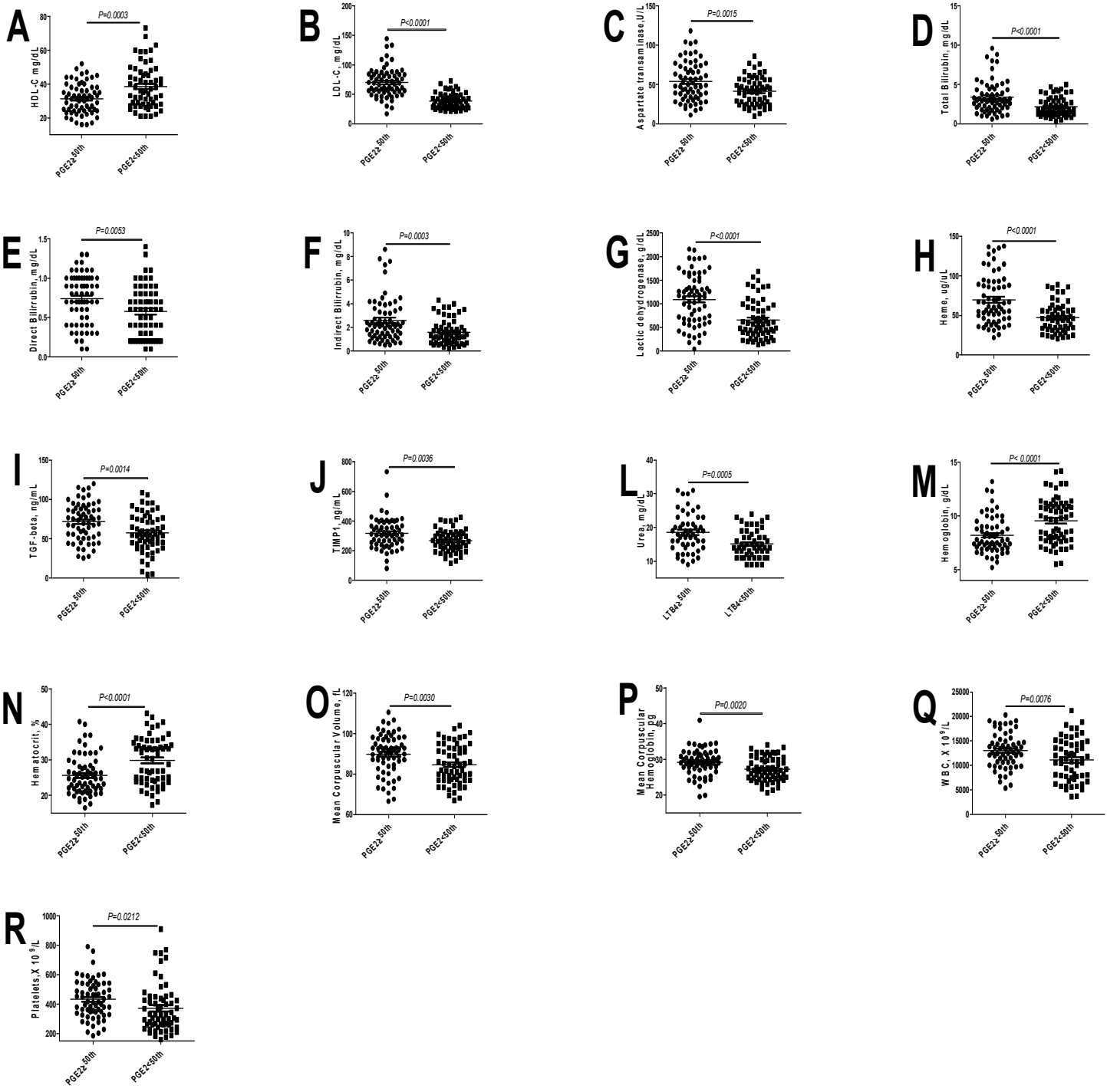
A
HDL-C mg/dL
P=0.0003
PGE2≥50th
PGE2<50th
B
LDL-C, mg/dL
P<0.0001
C
Aspartate transaminase,U/L
P=0.0015
D
Total Bilirubin, mg/dL
P<0.0001
E
Direct Bilirrubin, mg/dL
P=0.0053
F
Indirect Bilirrubin, mg/dL
P=0.0003
G
Lactic dehydrogenase, g/dL
P<0.0001
H
Heme, ug/uL
P<0.0001
I
TGF-beta, ng/mL
P=0.0014
J
TIMP1, ng/mL
P=0.0036
L
Urea, mg/dL
P=0.0005
LTB4≥50th
LTB4<50th
M
Hemoglobin, g/dL
P< 0.0001
N
Hematocrit, %
P<0.0001
O
Mean Corpuscular Volume, fL
P=0.0030
P
Mean Corpuscular Hemoglobin, pg
P=0.0020
Q
WBC, X 10 9 /L
P=0.0076
R
Platelets,X 10 9 /L
P=0.0212

FIGURE 2

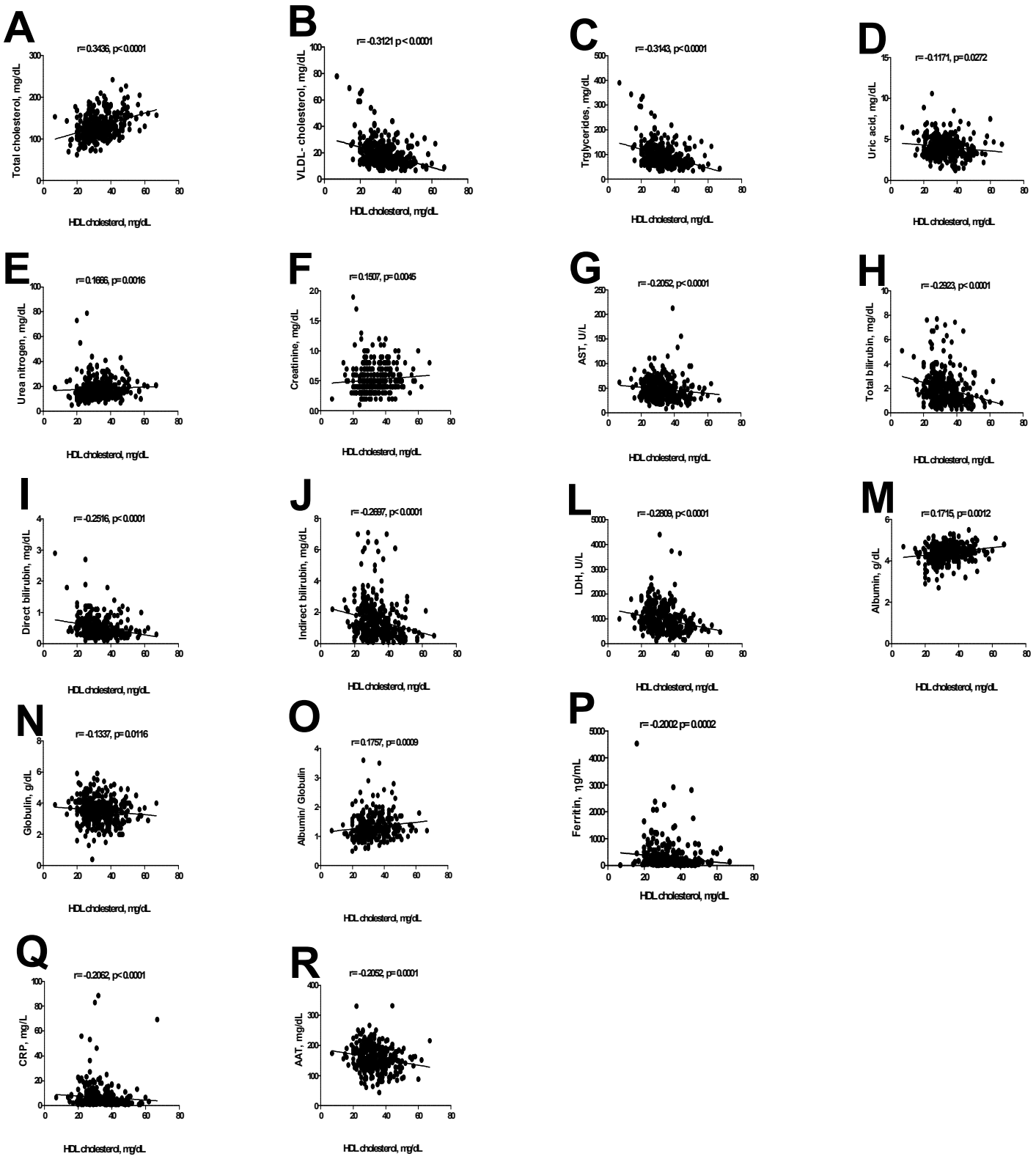
A
r= 0.3436, p< 0.0001
Total cholesterol, mg/dL
HDL cholesterol, mg/dL
B
r= -0.3121 p < 0.0001
VLDL- cholesterol, mg/dL
HDL cholesterol, mg/dL
C
r= -0.3143, p< 0.0001
Trglycerides, mg/dL
HDL cholesterol, mg/dL
D
r= -0.1171, p= 0.0272
Uric acid, mg/dL
HDL cholesterol, mg/dL
E
r= 0.1666, p= 0.0016
Urea nitrogen, mg/dL
HDL cholesterol, mg/dL
F
r= 0.1507, p= 0.0045
Creatinine, mg/dL
HDL cholesterol, mg/dL
G
r= -0.2052, p< 0.0001
AST, U/L
HDL cholesterol, mg/dL
H
r= -0.2923, p< 0.0001
Total bilirubin, mg/dL
HDL cholesterol, mg/dL
I
r= -0.2516, p< 0.0001
Direct bilirubin, mg/dL
HDL cholesterol, mg/dL
J
r= -0.2697, p< 0.0001
Indirect bilirubin, mg/dL
HDL cholesterol, mg/dL
L
r= -0.2809, p< 0.0001
LDH, U/L
HDL cholesterol, mg/dL
M
r= 0.1715, p= 0.0012
Albumin, g/dL
HDL cholesterol, mg/dL
N
r= -0.1337, p= 0.0116
Globulin, g/dL
HDL cholesterol, mg/dL
O
r= 0.1757, p= 0.0009
Albumin/ Globulin
HDL cholesterol, mg/dL
P
r= -0.2002 p= 0.0002
Ferritin, ηg/mL
HDL cholesterol, mg/dL
Q
r= -0.2062, p< 0.0001
CRP, mg/L
HDL cholesterol, mg/dL
R
r= -0.2052, p= 0.0001
AAT, mg/dL
HDL cholesterol, mg/dL

FIGURE 3

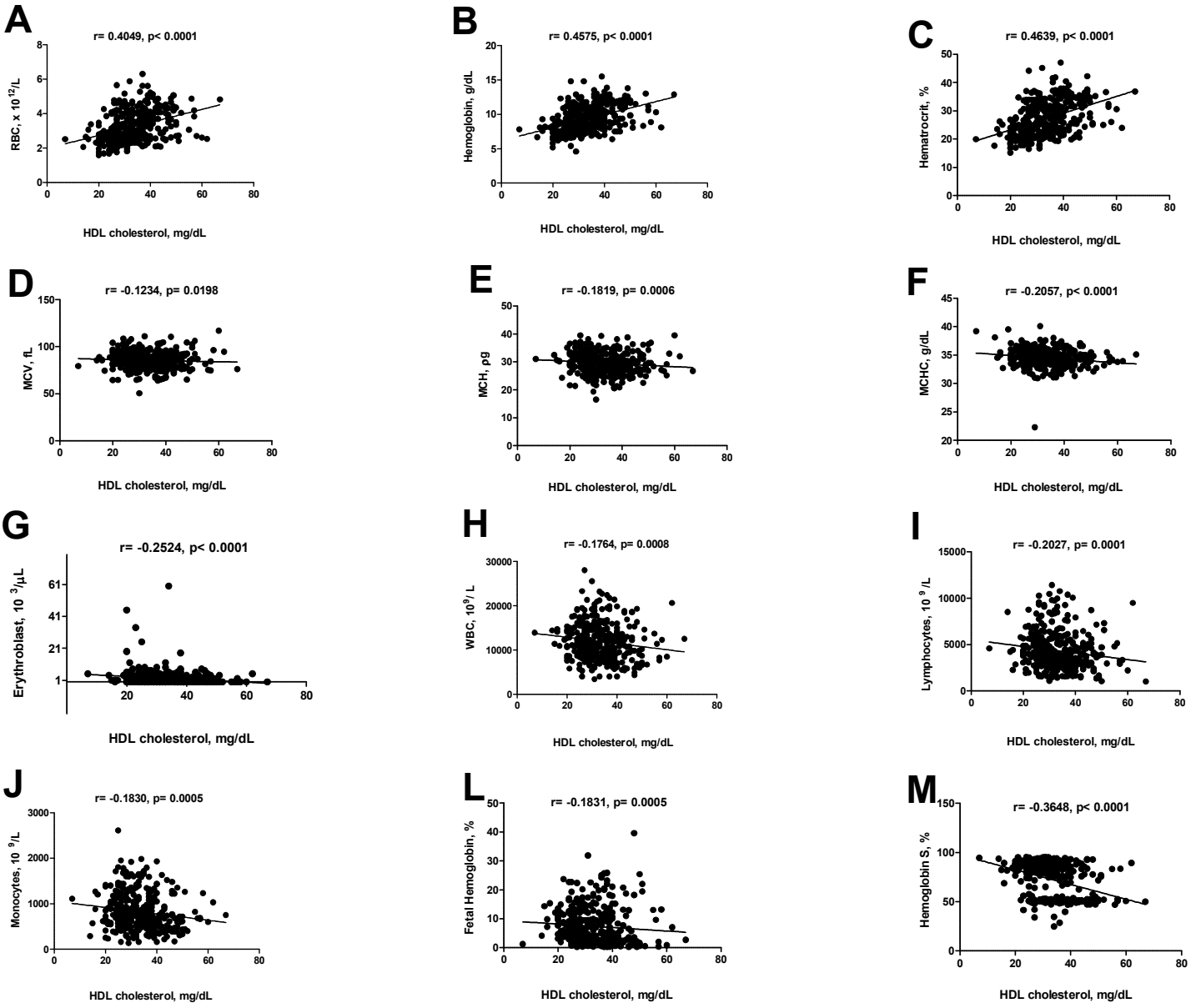
A
r= 0.4049, p< 0.0001
RBC, x 10 12/L
HDL cholesterol, mg/dL
B
r= 0.4575, p< 0.0001
Hemoglobin, g/dL
HDL cholesterol, mg/dL
C
r= 0.4639, p< 0.0001
Hematrocrit, %
HDL cholesterol, mg/dL
D
r= -0.1234, p= 0.0198
MCV, fL
HDL cholesterol, mg/dL
E
r= -0.1819, p= 0.0006
MCH, pg
HDL cholesterol, mg/dL
F
r= -0.2057, p< 0.0001
MCHC, g/dL
HDL cholesterol, mg/dL
G
r= -0.2524, p< 0.0001
Erythroblast, 10 3/µL
HDL cholesterol, mg/dL
H
r= -0.1764, p= 0.0008
WBC, 10 9/ L
HDL cholesterol, mg/dL
I
r= -0.2027, p= 0.0001
Lymphocytes, 10 9 /L
HDL cholesterol, mg/dL
J
r= -0.1830, p= 0.0005
Monocytes, 10 9/L
HDL cholesterol, mg/dL
L
r= -0.1831, p= 0.0005
Fetal Hemoglobin, %
HDL cholesterol, mg/dL
M
r= -0.3648, p< 0.0001
Hemoglobin S, %
HDL cholesterol, mg/dL

FIGURE 4

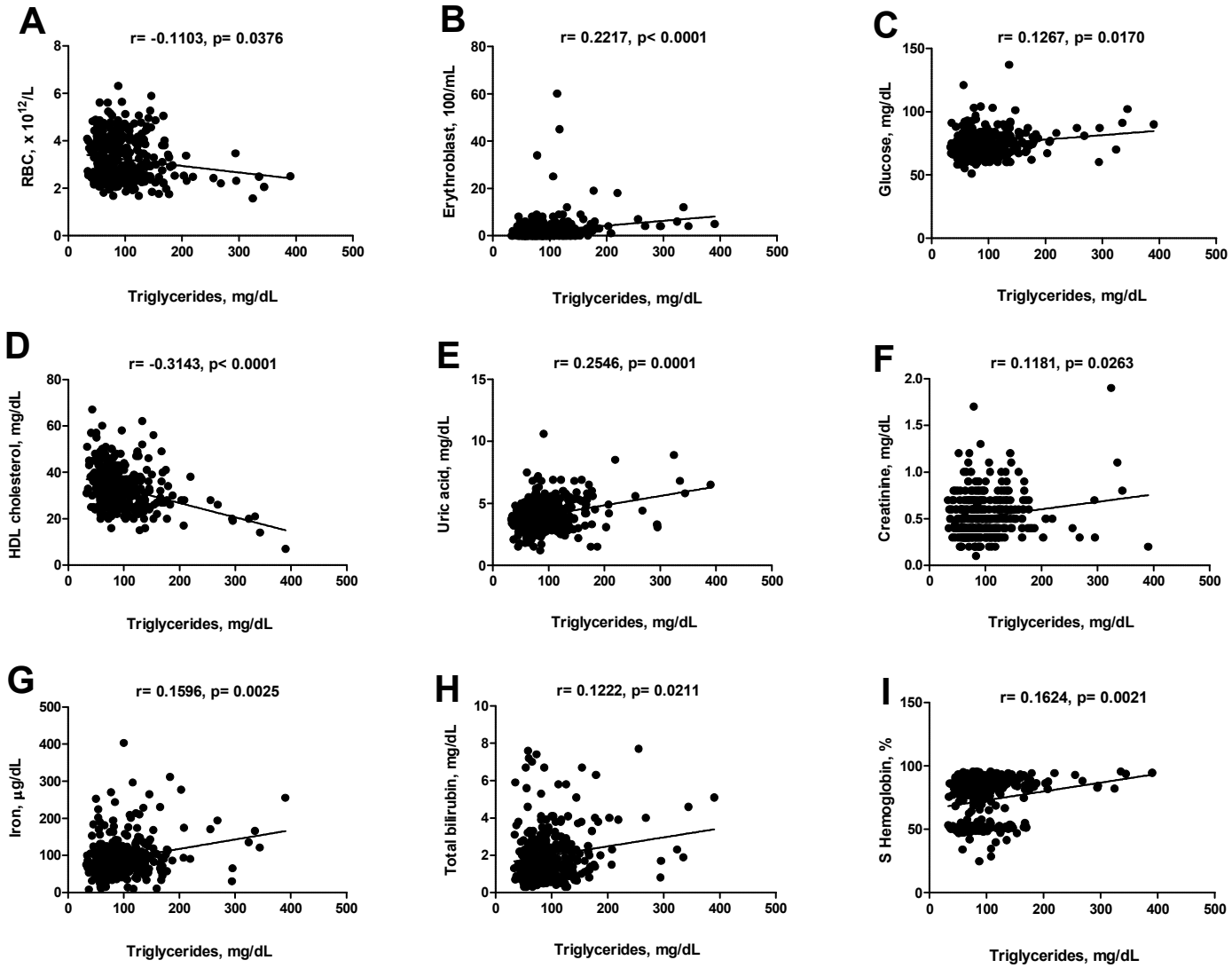
A
r= -0.1103, p= 0.0376
RBC, x 10^12/L
Triglycerides, mg/dL
B
r= 0.2217, p< 0.0001
Erythroblast, 100/mL
Triglycerides, mg/dL
C
r= 0.1267, p= 0.0170
Glucose, mg/dL
Triglycerides, mg/dL
D
r= -0.3143, p< 0.0001
HDL cholesterol, mg/dL
Triglycerides, mg/dL
E
r= 0.2546, p= 0.0001
Uric acid, mg/dL
Triglycerides, mg/dL
F
r= 0.1181, p= 0.0263
Creatinine, mg/dL
Triglycerides, mg/dL
G
r= 0.1596, p= 0.0025
Iron, µg/dL
Triglycerides, mg/dL
H
r= 0.1222, p= 0.0211
Total bilirubin, mg/dL
Triglycerides, mg/dL
I
r= 0.1624, p= 0.0021
S Hemoglobin, %
Triglycerides, mg/dL

FIGURE 5

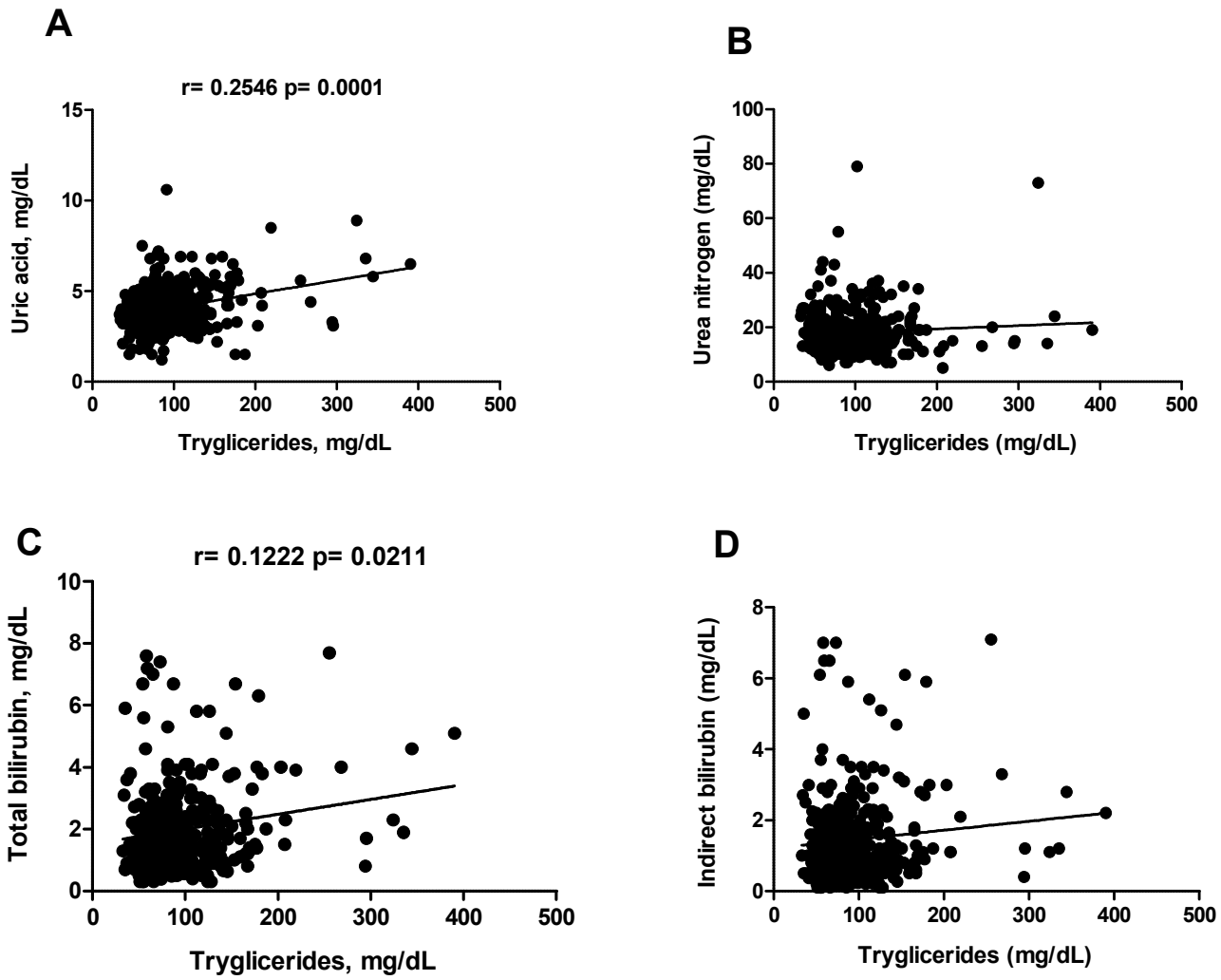
A
r= 0.2546 p= 0.0001
Uric acid, mg/dL
Tryglicerides, mg/dL
B
Urea nitrogen (mg/dL)
Tryglicerides (mg/dL)
C
r= 0.1222 p= 0.0211
Total bilirubin, mg/dL
Tryglicerides, mg/dL
D
Indirect bilirubin (mg/dL)
Tryglicerides (mg/dL)

FIGURE 6

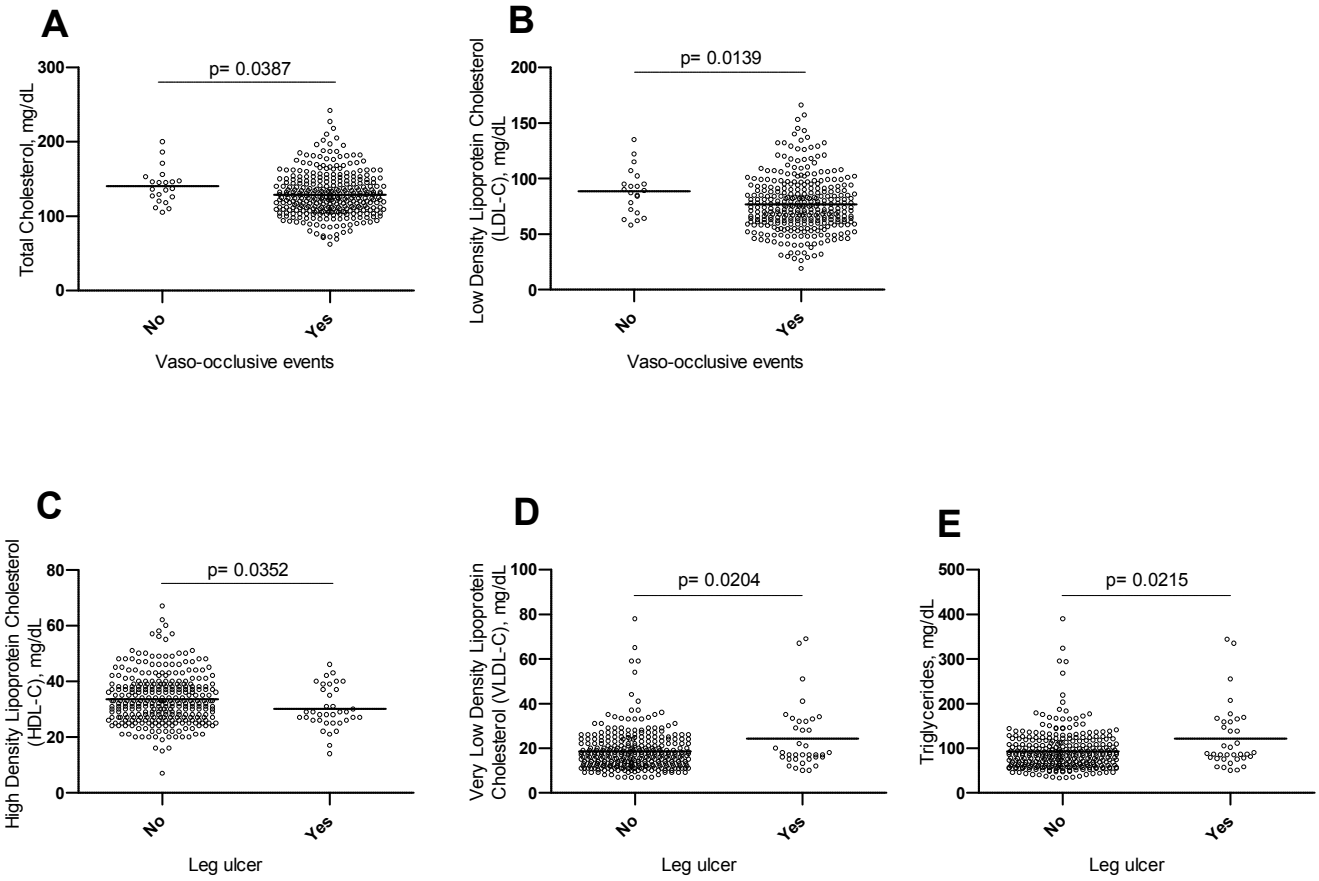
A
p= 0.0387
Total Cholesterol, mg/dL
300
200
100
0
No
Yes
Vaso-occlusive events
B
p= 0.0139
Low Density Lipoprotein Cholesterol (LDL-C), mg/dL
200
150
100
50
0
No
Yes
Vaso-occlusive events
C
p= 0.0352
High Density Lipoprotein Cholesterol (HDL-C), mg/dL
80
60
40
20
0
No
Yes
Leg ulcer
D
p= 0.0204
Very Low Density Lipoprotein Cholesterol (VLDL-C), mg/dL
100
80
60
40
20
0
No
Yes
Leg ulcer
E
p= 0.0215
Triglycerides, mg/dL
500
400
300
200
100
0
No
Yes
Leg ulcer

FIGURE 7

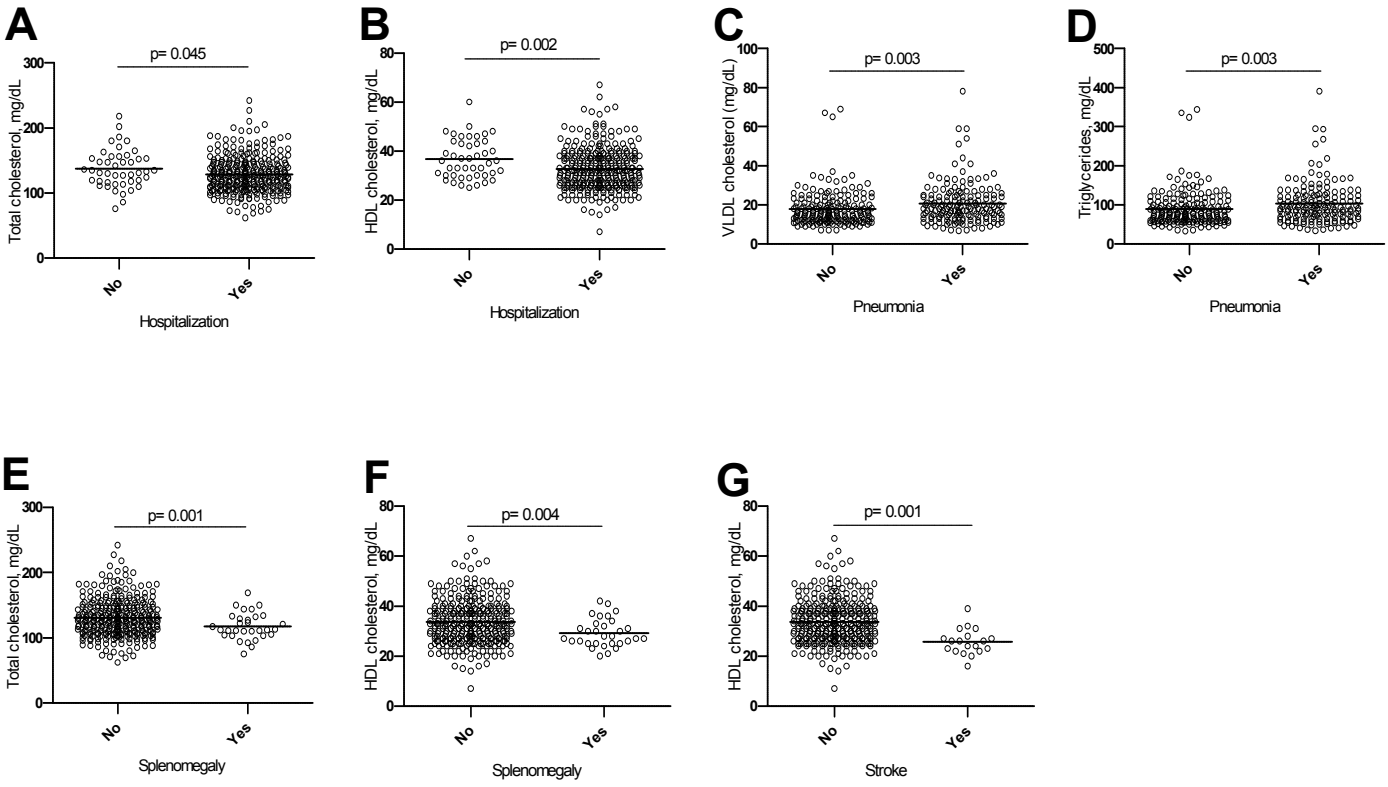

FIGURE 8

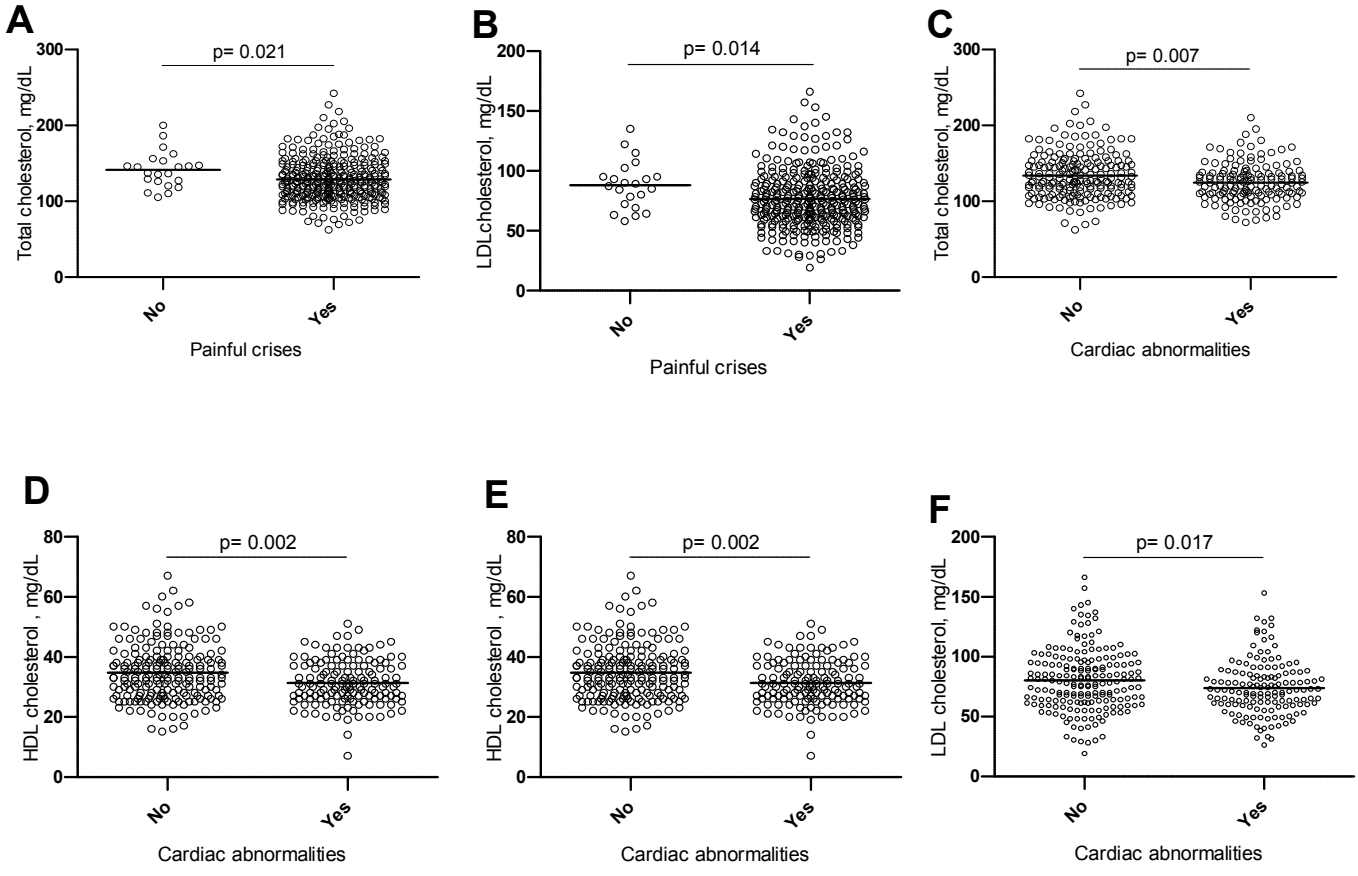

FIGURE 9

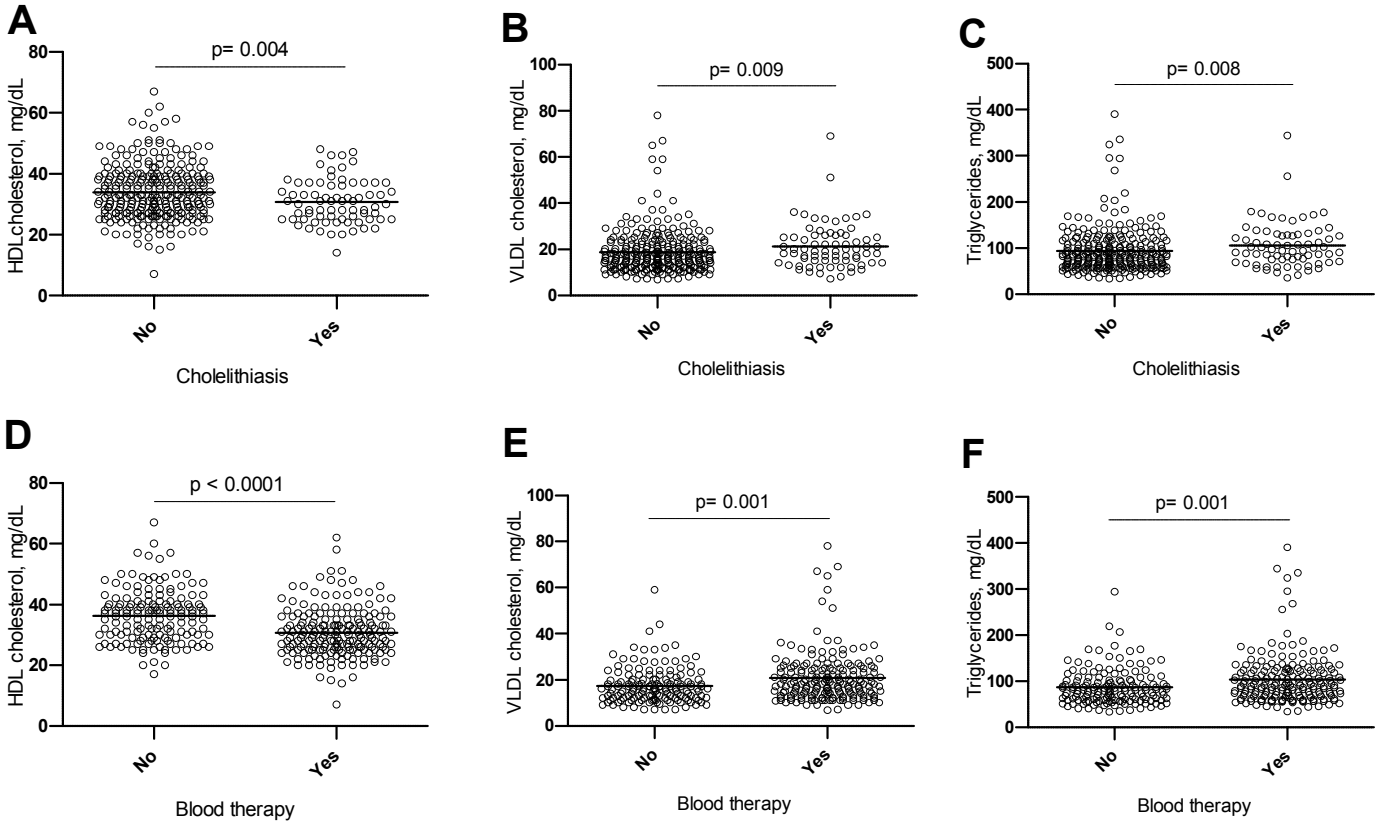

FIGURE 10

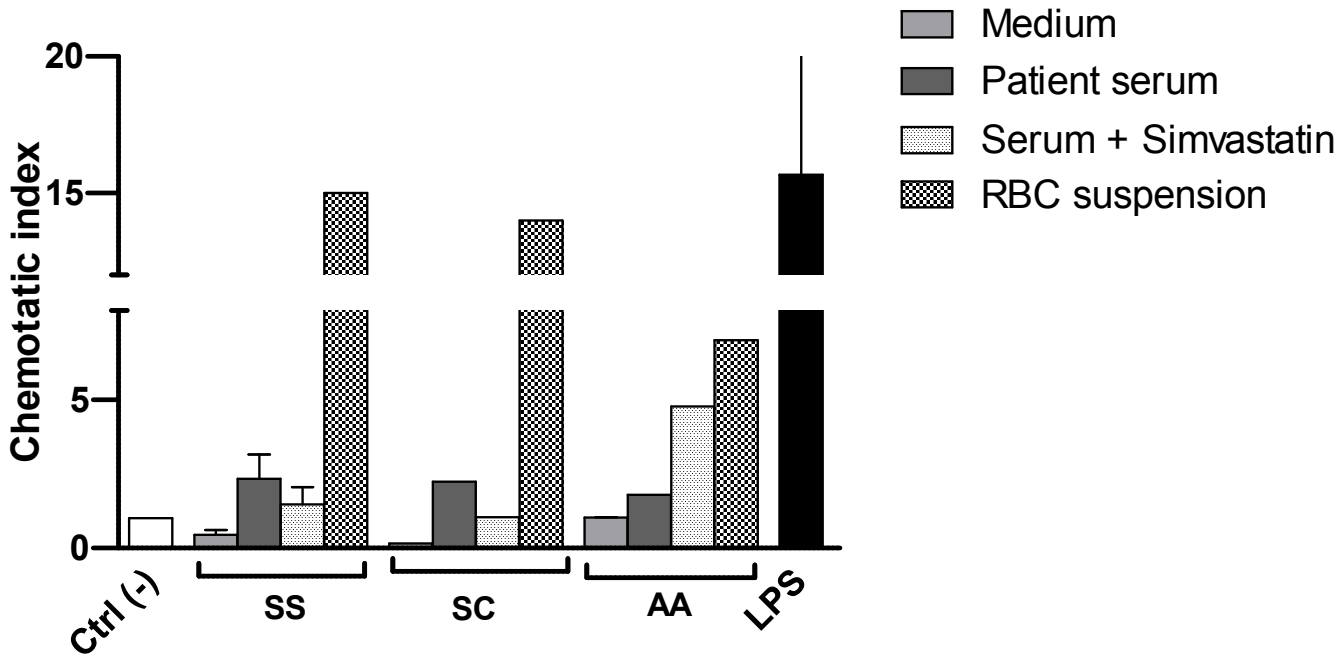

Table 1 – Laboratory characteristics of sickle cell disease patients and control group

Laboratory value	Patients (Mean ± SD) (N=351)	Controls (Mean ± SD) (N=167)	
RBC, $x10^{12}$/L	3.25 ± 0.94	4.57 ± 0.40	**<0.0001**
Hemoglobin, g/dL	9.35 ± 1.95	13.07 ± 1.20	**<0.0001**
Hematocrit, %	27.18 ± 6.00	39.06 ± 3.40	**<0.0001**
MCV, fL	85.62 ± 9.21	85.69 ± 5.48	0.937
MCH, ρg	29.62 ± 3.78	28.70 ± 2.21	**0.001**
MCHC, g/dL	34.56 ± 1.63	33.41 ± 1.26	**<0.0001**
Reticulocyte Count, %	6.07 ± 2.58	1.23 ± 0.69	**<0.0001**
WBC, x 10^9/L	11955.31 ± 4180.38	7406.25 ± 2383.61	**<0.0001**
Neutrophil count, x 10^9/L	5891.08 ± 2789.25	3805.62 ± 1848.54	**<0.0001**
Eosinophil count , x 10^9/L	768.17 ± 712.07	356.38 ± 407.76	**<0.0001**
Basophil count , x 10^9/L	85.64 ± 86.77	94.69 ± 52.82	0.214
Lymphocyte count, x 10^9/L	4327.80 ± 2027.55	2693.90 ± 1060.62	**<0.0001**
Monocyte count , x 10^9/L	826.21 ± 402.89	488.53 ± 202.41	**<0.0001**
Platelet count, $x10^3$/mL	407.21 ± 158.74	275.65 ± 71.19	**<0.0001**
Glucose, mg/dL	75.14 ± 19.72	86.01 ± 9.07	**<0.0001**
Total Cholesterol, mg/dL	130.25 ± 28.39	171.77 ± 41.86	**<0.0001**
HDL-C, mg/dL	33.21 ± 8.73	47.84 ± 10.85	**<0.0001**
LDL-C, mg/dL	77.88 ± 24.35	104.31 ± 35.15	**<0.0001**
VLDL-C, mg/dL	19.23 ± 9.73	19.62 ± 10.03	0.679
Triglycerides, mg/dL	96.18 ± 48.47	98.10 ± 50.16	0.680
ALT, U/L	22.99 ± 14.72	27.63 ± 10.55	**<0.0001**
AST, U/L	47.68 ± 22.13	33.18 ± 8.46	**<0.0001**
Iron serum, mcg/dL	91.67 ± 51.01	70.23 ± 26.89	**<0.0001**
Ferritin, ηg,/mL	305.68 ± 458.89	77.88 ± 78.32	**<0.0001**
Total bilirubin, mg/dL	1.99 ± 1.35	0.70 ± 0.27	**<0.0001**
Direct bilirubin, mg/dL	0.51 ± 0.47	0.25 ± 0.08	**<0.0001**
Indirect bilirubin, mg/dL	1.49 ± 1.23	0.45 ± 0.23	**<0.0001**
Total protein, g/dL	7.96 ± 0.80	7.95 ± 0.45	0.802
Albumin, g/dL	4.45 ± 0.54	4.35 ± 0.25	**0.031**
Globulin, g/dL	3.54 ± 0.82	3.60 ± 0.41	0.361
Uric acid, mg/dL	4.12 ± 1.27	3.20 ± 1.03	**<0.0001**
Urea nitrogen, mg/dL	18.17 ± 8.07	23.97 ± 7.19	**<0.0001**
Creatinine, mg/dL	0.54 ± 0.51	0.84 ± 0.22	**<0.0001**
CRP, mg/L	6.74 ± 9.57	4.09 ± 4.00	0.116
AAT, mg/dL	158.67 ± 41.42	97.42 ± 26.65	**<0.0001**
ASLO, UI/mL	177.68 ± 339.37	90.91 ± 66.44	0.112
Haptoglobin, mg/dL	8.82 ± 15.75	104.20 ± 55.28	**<0.0001**
LDH, U/L	964.05 ± 531.42	415.89 ± 111.25	**<0.0001**

RBC: Red blood cells; MCV: Mean Cell Volume; MCH: Mean Cell Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very Low-density lipoprotein cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CRP: C-reactive protein; AAT: Alpha 1-antitrypsin; ASLO: Antistreptolysin-O; LDH: Lactate d

4.3. MANUSCRITO III

Biomarkers of steady and crisis states in sickle cell disease patients: the roles of heme, MMP-9 and TIMP-1

Magda O S Carvalho[1,4,5], Théo Araujo-Santos[1], João H O Reis[1], Larissa C Rocha[1,3], Bruno A V Cerqueira[1,5], Nívea F Luz[1], Isa M Lyra[3,4], Valma M Lopes[3], Cynara G Barbosa[2,3], Manoel Barral-Netto[1], Valéria M Borges[1], Marilda S Goncalves[1,2,5]

Este trabalho avalia os níveis de marcadores de remodelamento vascular, inflamação e hemólise, buscando identificar a relação entre estes e o estado estável ou de crise em indivíduos com DF.

Resumo: A doença falciforme (DF) é caracterizada por hemólise, inflamação, disfunção vascular, sendo que os mecanismos que contribuem para a gravidade da doença são ainda controversos. Neste trabalho buscamos investigar marcadores que possam estar associados ao estado estável ou de crise na DF. Foram avaliados os níveis séricos de marcadores de remodelamento vascular (Inibidor tecidual da metaloproteinase-1 (TIMP-1), Metaloproteinase de matriz-9 (MMP-9)), de inflamação (leucotrieno B4 (LTB_4), prostaglandina E2 (PGE_2), citocinas anti-inflamatórias e inflamatórias (Fator beta transformador do crescimento (TGF-β), interleucina (IL)-1β, IL-6, IL-8, IL-12, fator de necrose tumoral alfa (TNF-α) e IL-10) e de hemólise (heme livre) em um total de 148 indivíduos com DF em estado estável. Também foram avaliados 23 paciente com anemia falciforme (AF) em crise e 148 indivíduos saudáveis pareados por idade e sexo. Os pacientes com AF, em estado estável, tiveram concentrações elevadas de LTB_4, PGE_2, TGF-β, IL-8 e IL-12 em comparação com os pacientes em crise. No entanto, os indivíduos com AF em crise apresentaram níveis mais elevados de interleucina (IL)-1β, IL-6, IL-10 e TNF-α em comparação com o grupo de pacientes estáveis. Os níveis séricos de heme livre, MMP-9 e TIMP-1 estavam aumentados em ambos os grupos de pacientes com DF e foram associados a marcadores de inflamação, hemólise e estresse oxidativo. A curva ROC mostrou que o LTB_4, PGE_2 e TGF-β são marcadores relacionados com os pacientes com AF em estado estável e que a IL-1β, IL-6 e TNF-α são marcadores de crise. Além disso, a manutenção de níveis séricos elevados de heme livre, provavelmente está associada a hiper-hemólise crônica. Por sua vez, MMP-9 e TIMP-1 provavelmente estão associados ao processo inflamatório crônico dos pacientes com AF em crise e estado estável, contribuindo para um microambiente que resulta no aumento da gravidade clínica da doença.

Estudos adicionais são necessários para desvendar os mecanismos envolvidos e a possibilidade de criação de estratégias terapêuticas novas na DF.

Biomarkers of steady and crisis states in sickle cell disease patients: the roles of heme, MMP-9 and TIMP-1

Magda O S Carvalho[1,4,5], Théo Araujo-Santos[1], João H O Reis[1], Larissa C Rocha[1,3], Bruno A V Cerqueira[1,5], Nívea F Luz[1], Isa M Lyra[3,4], Valma M Lopes[3], Cynara G Barbosa[2,3], Manoel Barral-Netto[1], Valéria M Borges[1], Marilda S Goncalves[1,2,5]

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Abstract

Background: Sickle cell disease (SCD) is characterized by hemolysis, inflammation and vascular dysfunction, and the mechanisms determining disease severity remain controversial. This study aimed to identify biomarkers related to SCD crisis and steady states.

Methods: Systemic levels of vascular remodeling (tissue inhibitor of metalloproteinase (TIMP-1) and matrix metalloproteinase 9 (MMP-9)), inflammation (leukotriene B4 (LTB_4), prostaglandin E2 (PGE_2)), anti-inflammatory and inflammatory cytokines (transforming growth factor beta (TGF-β), interleukin (IL)-1β, IL-6, IL-8, IL-12, IL-10, tumor necrosis factor alpha (TNF-α)) and hemolytic (free heme) markers were monitored in 148 steady-state SCD patients, 23 crisis-state sickle cell anemia patients (SCA) and 148 age- and sex-matched healthy individuals.

Results: The steady-state SCA patients had higher LTB_4, PGE_2, TGF-β, IL-8 and IL-12 levels than the crisis patients. However, the crisis-state SCA patients had higher levels of interleukin (IL)-1β, IL-6, IL-10 and TNF-α than the steady-state patients. The systemic levels of free heme, MMP-9 and TIMP-1 were increased in both SCA patient groups and were associated with inflammation, hemolysis and oxidation markers in SCD patients. Receiver operating characteristic (ROC) curves demonstrated that LTB_4, PGE_2 and TGF-β are markers of steady-state SCA and that IL-1β, IL-6 and TNF-α are markers of crisis-state SCA.

Conclusions: LTB_4, PGE_2 and TGF-β are considered predictors of steady-state SCA, and IL-1β, IL-6 and TNF-α are considered predictors of crisis-state SCA. Additionally, the continuous synthesis of systemic free heme is most likely associated with chronic hyper-hemolysis, and the expression of MMP-9 and TIMP-1 is most likely associated with the chronic inflammatory state observed in crisis- and steady-state SCA patients, contributing to a hazardous microenvironment that results in increased clinical severity. Further studies related to these molecules are required to elucidate the underlying mechanisms and the possibility of designing further therapeutic strategies for SCD.

Introduction

Sickle cell disease (SCD) patients have heterogeneous clinical features. The disease is characterized by overall organ failure; premature risk of death; hemolysis; vaso-occlusive episodes (VOE); neurological alterations, such as vascular encephalic accident (VEA); pain; infections; acute chest syndrome (ACS); pulmonary hypertension syndrome; and other systemic symptoms. Sickle cell anemia (SCA) is the homozygous state of the beta S (β^S) allele (HbSS) and is the SCD genotype with the greatest clinical severity [1-3].

SCD is a chronic inflammatory disease with a complex mechanism of pathogenesis. The rheological phenomenon of SCD has been directly associated with the activation of red sickle blood cells (RBCs), reticulocytes, white blood cells (WBCs), platelets, and endothelial cells, and several molecules are secondarily expressed in this inflammatory environment and on the surface of these cells. Additionally, adhesion molecules and their ligands are overexpressed, stimulating the endothelium and the immune and inflammatory responses [4-6]. Based on the complex biological characteristics exhibited by SCD patients, there are several classical markers related to disease morbidity and mortality, including a decrease in fetal hemoglobin (HbF) concentration, an increase in WBC and reticulocyte counts, and the presence of dactylitis during childhood [7].

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

Considering the complex network of mechanisms involved in SCD pathogenesis, we investigated the systemic levels of inflammatory and anti-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α); interleukin (IL)-1 beta (β), IL-6, IL-8, IL-10, and IL-12; and transforming growth factor beta (TGFβ). We also investigated inflammatory mediators, such as prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4), and the vascular remodelling modulator matrix metalloproteinase-9 (MMP-9) and its inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1). To test the hypothesis that the systemic levels of these molecules can be associated with steady and crisis states in SCD patients, we investigated the hematological and chemical markers associated with oxidative stress, hemolysis, and inflammation to identify biomarkers of prognosis and establish risk factors for each phase of the disease.

Our results suggest that high systemic levels of PGE_2, LTB_4 and TGF-β expression during the steady-state period may be related to the maintenance of the chronic inflammatory state commonly described in SCA. The alteration of proinflammatory cytokines in SCD patients is well established; however, in this study, increased levels of TNF-α, IL-1β and IL-10 were found in crisis-state SCA patients and could be related specifically to this disease stage and to the inflammatory marker cascade commonly expressed in this disease. Our results suggest that heme, MMP-9 and TIMP-1 may be considered key molecules in SCD pathogenesis. These molecules were associated with the maintenance of the chronic hyper-hemolysis state and with chronic inflammation because their systemic concentrations were heightened in both the crisis- and steady-state SCA groups. Finally, to test this statement, the relationships between the systemic levels of heme, MMP-9 and TIMP-1 and the markers of hemolysis, oxidative stress, and inflammation were tested in a group of steady-state SCD patients.

Materials and Methods

Patient and control group characteristics

This case-control study was performed between March 2010 and November 2012. Venous blood was prospectively and randomly collected from 148 SCD children in a steady state of SCD; 101 patients with SCA comprised the group of stable patients (SP), while the remaining 47 patients had SC disease (HbSC). However, there were differences in the number of patients analysed for some variables, and these specific casuistic are described in supplementary table S1.

In total, 23 SCA patients in vaso-occlusive crisis were included in the study and comprised the crisis patient (CP) group (Table S1); 146 healthy control (HC) individuals comprised the casuistic of the study. However, there were differences in the number of healthy individuals analysed for certain variables, and these specific casuistic are described in supplementary table S1. The patients and controls were age- and sex-matched and selected from within the state of Bahia, Brazil, which is a country with a high degree of racial admixture [8]. The crisis-state SCA patients were not receiving hydroxyurea (HU) therapy, and blood was collected immediately after hospital admission, without any therapeutic intervention.

The eligibility criteria for this study were as follows: homozygous HbSS and double heterozygous HbSC, older than 5 years, and not receiving HU therapy. The steady-state SCD patients did not present acute clinical events, infection or inflammatory episodes and did not undergo any blood transfusion procedures before sampling. The crisis-state patients were hospitalized at the time of sample collection. The SCD patients in the steady state were selected from Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), which is a reference center that provides routine visits at an outpatient clinic. Patients in vaso-occlusive crisis were admitted to the Children's Hospital of Obras Sociais Irmã Dulce (HCOSID). The individuals in the healthy control group were selected randomly from the chemistry laboratory of the Faculdade de Farmácia da Universidade Federal da Bahia (PHAR-UFBA). The human subject research board of the Centro de Pesquisa Gonçalo Moniz- Fundação Oswaldo Cruz (CPqGM-FIOCRUZ) and the Children's Hospital of Obras Sociais Irmã Dulce (HCOSID) HCOSID approved this study (protocol number 0016.0.225.000-09). Each child's guardian signed the consent form, and the study followed the Brazilian standards for the development of research on humans. This work was in accordance with the Helsinki Declaration of 1975 and its revision.

Chemical, immunological and hematological marker analyses

Chemical and immunological markers, including analyses of antistreptolysin O (ASO), ferritin, alpha 1-antitrypsin (A1AT), and C-reactive protein (CRP), were evaluated using immunochemistry and immunoassay techniques (A25 system, BIOSYSTEMS SA, Barcelona, Spain; Access® 2 Immunoassay system X2, Beckman Coulter, Fullerton, CA, USA; Immage® 800 system, Beckman Coulter, Fullerton, CA, USA). An electronic cell counter (Coulter Corporation, Miami, FL, USA) was used to quantify hematological parameters. The hemoglobin (Hb) pattern and its concentration were estimated using high-performance liquid chromatography (HPLC; Bio-Rad, Hercules, CA, USA).

Cytometric bead array

Plasma levels of IL-1β, IL-6, IL-8, IL-12, TNF-α and IL-10 were measured using a cytometric bead array (CBA, BD Biosciences Pharmingen, USA) following the manufacturer's protocol. Standard curves were generated using quality control reagents to confirm the established result.

Inflammatory mediator plasma measurement

The PGE_2 levels in the plasma samples were estimated using an enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The plasma concentrations of TIMP-1, MMP-9, LTB_4 and TGF-β were measured using the ELISA technique according to the manufacturer's instructions (ReD Systems, Minneapolis, MN, USA).

Total free heme measurement

The total systemic free heme in the plasma samples was measured using a QuantiChrom Heme Assay Kit (Bioassay Systems, Hayward, CA, USA) following the manufacturer's protocol.

Statistical analysis

The baseline characteristics of the variables were investigated by analysing their means and medians. The quantitative variable distribution was evaluated using the Kolmogorov-Smirnov test. The Mann-Whitney test and unpaired Student's t-test analyses were used to estimate differences in cytokine levels and inflammatory mediators between the steady- and crisis-state SCA patients. Additionally, receiver operator characteristics (ROC) curve and C-statistics analyses were performed to estimate the predicted disease severity for acute and chronic events using data from each candidate SCA biomarker. The Mann-Whitney test and unpaired Student's t-test analyses were also used to estimate the associations between median free heme, MMP-9, and TIMP-1 concentrations and hematological and chemical markers in the steady-state SCD patient group. Differences with p values less than 0.05 were considered significant. Statistical analyses were performed using the EPI Info 6.04 (CDC, Atlanta, Georgia, USA) and GraphPad Prism 5.01 (San Diego, CA, USA) software. Supplementary information is provided in tables S2 and S3.

Results

Characteristics of the SCD patient groups and the healthy control group

The steady-state SCD patient group consisted of 148 patients (101 HbSS and 47 HbSC), 45.9% (68/148) of whom were female, with a median (50^{th} percentile) age of 9.3 years and an interpercentile range (IPR; 25^{th} - 75^{th} percentiles) of 6.0-12.25 years. The HbSS patient group included 44.5% (45/101) females and had a median age of 8.6 years (IPR: 6.0-12.0 years). The HbSC patient group included 48.9% (23/47) females and had a median age of 10.8 years (IPR: 8.0-15.00 years).

The SCA crisis-state patient group included 23 individuals, 56.0% (13/23) of whom were female, with a median age of 10.4 years (IPR: 8.0-15.0 years). The control group included 131 healthy individuals, 49.0% (64 /131) of whom were female, with a median age of 8.7 years (IPR: 7.0-11.0 years).

As expected, the crisis-state SCA patients had the lowest hematological and chemical marker concentrations (Table 1). The SCD patients (HbSS and HbSC) in the steady state had the lowest Hb and Ht concentrations; the highest reticulocyte, erythroblast, leukocyte and platelet counts; and the greatest chemical and inflammatory systemic biomarker concentration alterations compared with individuals in the control group (Tables 2 and 3).

LTB_4, PGE_2 and TGF-β were increased in steady-state SCA patients, and heme, TIMP-1 and MMP-9 were increased in both crisis- and steady-state SCA patients

The inflammatory state of SCD patients is well known. Several of the molecules involved in this event are associated with the immune response and are part of a complex network in which most mechanisms remain unknown [9]. Thus, the inflammatory mediator and cytokine concentrations were investigated in SCD patients in steady and crisis states and in the healthy control group (Figure 1).

Comparing the inflammatory and vascular remodelling mediators revealed increases in LTB_4, PG_2, and TGF-β in the steady-state SCA patients compared with the patients in the crisis group. However, all of the patients in the SCA groups (steady and crisis state) had similar concentrations of heme, TIMP-1, and MMP-9 (Figure 1A-G).

Inflammatory cytokines IL-1β, IL-6, IL-10 and TNF-α were increased in crisis-state SCA patients, and IL-12 and IL-8 were increased in crisis- and steady-state SCA patients

Cytokine levels are associated with the inflammatory state described in SCD patients [10-14]. Our results show increases in IL-1β, IL-6, IL-10, and TNF-α among SCA patients in the crisis state compared with steady-state patients. In contrast, IL-12 and IL-8 levels were elevated in both the steady-state and crisis-state SCA patient groups (Figure 2A-F).

ROC curves and predictive power demonstrate that LTB_4, TGF-β and PGE_2 are markers of steady-state SCA, whereas TNF-α, IL-1β and IL-10 are markers of crisis-state SCA

Biomarkers for clinical evaluation have been explored in SDC research because patients have heterogeneous clinical outcomes, as demonstrated by specific subphenotypes [3,15]. ROC curves were developed to estimate the predictive power of a group of biomarkers at each stage of SCA. ROC curve analysis was performed using the MMP-9/TIMP-1 ratio and systemic levels of MMP-9, LTB_4, TGF-β and PGE_2. The LTB_4, TGF-β, and PGE_2 levels had the highest sensitivity and specificity for predicting steady-state SCD (Figure 3A). Similarly, TNF-α, IL-1β and IL-10 had the highest sensitivities and specificities for association with crisis-state SCD (Figure 3B) based on the statistical data and the areas under the curves (AUCs).

The highest levels of free heme were associated with altered lipid patterns, increased hemolysis, hepatic dysfunction, and altered hematological markers in steady-state SCD patients

Increased systemic levels of heme have been associated with hemolytic diseases, such as SCD, spherocytosis, and auto-antibody anemia, and are secondary to intravascular hemolysis with an increased pro-inflammatory and pro-oxidant environment [16-20]. Because we found high concentrations of free heme in both steady- and crisis-state SCA patients, and based on the finding of Uzunova et al. [21], which found that a concentration of 66 μM of free heme is associated with an increase in free heme accumulation inside RBCs, we sought to validate our previous results by evaluating whether the free heme concentration was related to hematological and chemical markers of hemolysis, anemia, lipids, the hepatic pattern and inflammation. For this evaluation, the systemic free heme levels of the SCD steady-state patient group were measured, and we performed statistical analyses of these biomarkers in patient groups with values higher and lower than the 50^{th} percentile (54.65 μM) once this value approached the value described by Uzunova et al. [21]. Statistically significant differences were found between the highest free heme concentrations and the lowest levels of high-density lipoprotein cholesterol (HDL-C), as well as the highest very low-density lipoprotein cholesterol (VLDL-C), triglyceride, ferritin, aspartate transaminase (AST), total

bilirubin (TB), indirect bilirubin (IB), direct bilirubin (DB) and lactate dehydrogenase (LDH) concentrations (Figure 4 and Figure 5). Additionally, we performed analyses to compare the systemic concentrations of free heme with hematological marker concentrations and found that the highest levels of free heme were associated with the lowest hematocrit values and with the highest monocyte and platelet counts (Figure 6).

When we analysed the SCA steady-state patient groups with heme values higher and lower than the 50th percentile (62.35 μM), the highest free heme levels were associated with the highest values of VLDL-C, triglycerides, AST, TB, IB and ASLO; with the lowest HDL-C and HbF values; and with a high monocyte count. This group also had a high platelet count associated with high levels of TIMP-1, TGF-β and MMP-9. Moreover, high levels of MMP-9 were associated with a high leukocyte count, a low lymphocyte count, low TNF-α levels and high TIMP-1 levels (Figure S1). In the analysis of HbSC steady-state patients with heme values higher and lower than the 50th percentile (40.9 μM), the highest free heme levels were associated with the highest VLDL-C, triglycerides, albumin, and TGF-β values; high TIMP-1 levels were associated with high alpha-1 antitrypsin levels and with a high monocyte count; and high levels of MMP-9 were associated with a high leukocyte count and elevated TIMP-1 levels (Figure S2).

The highest free heme levels were associated with the highest TGF-β, PGE-$_2$, TIMP-1 and IL-12 levels in a group of steady-state SCD patients

High heme concentration has been associated with increased oxidative stress in steady-state SCD patients, with compromised vascular integrity [18,19] and with the severity of malaria vivax [22]. Therefore, we also analysed the association between free heme concentrations and our studied markers. In our analyses, we found that the highest heme values were associated with the highest levels of TGF-β, PGE_2, TIMP-1 and IL-12 (Figure 7).

Elevated levels of TIMP-1 and MMP-9 were associated with altered lipid patterns, increased hemolysis, hepatic dysfunction, and altered hematological markers in a group of steady-state SCD patients

Matrix metalloproteinase (MMPs) are endopeptidases that are regulated through the tissue inhibitors of metalloproteinase (TIMPs) and are associated with extracellular matrix degradation. However, MMPs also act on cytokines and chemokines and serve as regulators of inflammation and immunity [23-25]. TIMP-1 inhibits MMP-9 activity and can stimulate the growth of several cell types. However, TIMP-1 exhibits diverse activity, some of which is dependent on its MMP-9 inhibitory capacity and some of which is independent [26-29]. We evaluated whether the concentrations of these molecules were related to classical hematological and chemical markers of hemolysis, anemia, and inflammation or to lipid and hepatic patterns in steady-state SCD patients. For this evaluation, systemic levels of MMP-9 and TIMP-1 were measured, and statistical analyses of these biomarkers were established in groups with values higher and lower than the 50th percentile, which was 297.5 ng/mL for TIMP-1 and 444 ng/mL for MMP-9. A statistically significant association was found between the highest TIMP-1 concentration and the lowest HDL-C concentrations; the highest triglyceride, AST, TB, IB, and ferritin concentrations (Figure 8); and low RBC counts, low hemoglobin and hematocrit concentrations, high mean corpuscular hemoglobin concentrations, and high platelet counts (Figure 9). High MMP-9 concentrations were associated with high leukocyte and neutrophil counts and with high TIMP-1 concentrations (Figure 10).

Discussion

This study investigated biomarkers associated with inflammation and vascular remodelling in SCA patients. The highest systemic levels of PGE_2, LTB_4, and TGF-β were found in steady-state patients, and the highest systemic levels of TNF-α, IL-1β and IL-10 were found in crisis-state patients. Free heme, MMP-9 and TIMP-1 levels were increased in both groups of SCA patients (crisis and steady state) and were associated with altered levels of hematological, chemical, and inflammatory markers in a group of steady-state SCD patients, supporting the hypothesis that continued free heme expression may potentiate chronic hyper-hemolysis and that continued MMP-9 and TIMP-1 expression might be associated with the chronic inflammatory process that has been described in SCD.

The altered lipid metabolism described in SCD patients has been associated with phospholipid asymmetry in sickle erythrocytes, contributing to metabolic abnormalities such as dehydration, membrane perturbation, cation imbalance, and hypercoagulability [30-32]. Additionally, eicosanoids and diacylglycerol are released by endothelial cells secondary to sickle red cell stimulation [33], increasing arachidonic acid products such as PGE_2 and LTB_4, whose synthesis has been implicated in the control of the inflammatory response [34,35]. In this report, we found high PGE_2 and LTB_4 levels in steady-state SCA patients compared with crisis-state patients and healthy individuals (Figure 1A and B). Our current data suggest that PGE_2 and LTB_4 are important markers of the chronic inflammation described in SCD and that their synthesis is induced by an abnormal interaction between erythrocytes and endothelial cells and by the presence of a chronic pro-oxidant milieu. Taken together, these factors contribute to the dysfunctional endothelium found in SCD patients. LTB_4 has been reported to be a neutrophil chemoattractant and has been associated with increased neutrophil adhesion to the endothelium [36]. However, Monteiro et al. [37] previously described a heme-dependent neutrophilic inflammation process during hemolysis that was associated with the endogenous activity of LTB_4 originating from macrophages. Graido-Gonzalez et al. [38] reported an increase in PGE_2 levels in crisis-state SCA patients. Additionally, Setty e Stuart [33] described an increase in LTB_4 among steady- and crisis-state SCA patients, and Lanaro et al. [12] described an increase in PGE_2 levels among steady-state SCA patients even after HU administration. Data concerning PGE_2 and LTB_4 levels in SCD patients remain controversial. These mediators can act through a wide range of functions, as previous reports describe [39-44]; thus, further evaluation is needed to clarify their roles in SCD-related inflammation and unravel the complex network of mechanisms involved in the pathogenesis of this disease.

TGF-β is a multipotent cytokine that has been previously associated with Th17 cell differentiation [45]; with myofibroblast development through its receptor (TGFβR), followed by SMAD2 phosphorylation and ROS generation; and, along with endothelin-1, with the induction of extracellular matrix deposition via extracellular signal-regulated kinase 1/2 (ERK1/2) activation [46,47]. Our study demonstrates an increase in systemic TGF-β levels in steady-state SCA patients (Figure 1C), suggesting that TGF-β plays a role in vascular cell remodelling in this disease and most likely contributes to vessel alteration via extracellular matrix deposition. However, TGF-β may act as a ROS generator through its receptor activation, participating in both vascular narrowing and endothelial dysfunction and inflammation.

The systemic levels of MMPs and TIMPs, as well as their ratio, have been associated with normal and pathological events, including tissue remodelling, tumorigenesis, metastasis,

angiogenesis, sepsis, dyslipidemia, hemodialysis, multiple sclerosis, obesity, metabolic syndrome, and atherosclerosis [48-51]. Our results show increased systemic levels of MMP-9 and TIMP-1 in both SCA patient groups (crisis and steady state), with an increased MMP-9/TIMP-1 ratio (Figure 1D-F). These data suggest continuous MMP-9 production in both SCA groups, which may represent active matrix remodelling and the maintenance of tissue destruction and degradation in these patients. The association between high MMP-9 levels and an increased number of leukocytes and neutrophils in the steady-state SCD patient group (Figure 10A, B) indicates the activation of leukocytes, which is likely related to their transmigration into the vascular wall. This leukocyte activation involves the activation of the neutrophil MMP-9 proenzyme, which promotes MMP-9 generation that results in vascular cell changes, an increased inflammatory response, and endothelial dysfunction. The increase in the neutrophil MMP-9 proenzyme and its relationship with increases in environmental inflammation and adhesion have been described for angiogenesis induction and inflammation secondary to venous thrombosis in metabolic syndrome patients [52-55].

Increased systemic TIMP-1 levels in our SCA patients could be associated with an MMP-9-independent function and may be linked to the hypoxic environment found in SCA patients. This finding suggests that TIMP-1 has diverse functions, as elevated MMP-9 levels were associated with high TIMP-1 levels in our analyses (Figure 10C). Our hypothesis could be validated by data demonstrating an association between increased TIMP-1 levels and the chemical markers of hemolysis and inflammation and with the hematological biomarkers of anemia, which is a pathological process associated with hypoxia (Figure 8 and Figure 9).

Our results demonstrate an association between increased TIMP-1 levels and altered lipid patterns and hemolysis markers (Figure 8), suggesting that this molecule is involved with ROS and the presence of heme. High systemic levels of TIMP-1 have been shown to trigger hypoxia-inducible factor-1 (HIF-1), which has been associated with pro-metastatic alterations in the cancer microenvironment. Furthermore, increased TIMP-1 levels are related to the induction of miR210, which regulates a metabolic alteration of a glycolytic pathway [56-58]. Our results also show an association between high levels of TIMP-1 and a high number of platelets, demonstrating that TIMP-1 may be related to corpuscle activation and may be linked to the expression of a heme-mediated tissue factor on endothelial cells, which increases the procoagulant activity. This phenomenon was previously studied by Setty et al. [59] using human umbilical vein endothelial cells (HUVECs) in a simulated hemolytic environment.

The imbalance of the MMP-9/TIMP-1 ratio described in this study suggests that MMP-9 contributes to endothelial dysfunction in SCD and to the inflammatory environment commonly described in these patients [53-55]. This finding was emphasized by our finding that a high MMP-9 concentration is associated with high concentrations of TIMP-1 (Figure 10C).

Systemic free heme levels and ROS are released during hemoglobin (Hb) catabolism following intravascular hemolysis [15-17]. The hazardous effects of heme on the microenvironment have been associated with the programmed necrosis of peritoneal macrophages in knockout and wild type C57BL/6 mouse models and with TNF-α production and increased ROS generation [18]. This study shows an increase in heme in both SCA patient groups (crisis and steady state; Figure 1G), suggesting that high systemic levels of free heme could be directly related to the chronic hyper-hemolytic state previously reported in SCA patients [14]. This relationship would likely be responsible for maintaining the chronic

inflammatory state and for continuous ROS generation, which would lead to progressive endothelial dysfunction [3,16].

Considering the increased systemic levels of free heme in both crisis- and steady-state SCA patients, we evaluated the association between heme and lipid patterns and hematological, chemical and inflammatory markers in these patients (Figures 4-7). The results showed that this product of erythrocyte disruption can interfere with several pathways related to clinical severity in SCD patients. However, our data indicate an association between free heme and lipid marker alterations; hemolysis; some inflammatory and remodelling markers, such as TGF-β, and TIMP-1; the inflammatory mediator PGE_2; and the cytokine IL-12, demonstrating the pivotal role of heme in the chronic hyper-hemolysis and chronic inflammatory process described in this disease [14,21].

Altered levels of pro-inflammatory and anti-inflammatory cytokines have been demonstrated in SCA patients and implicated in the clinical severity of the disease and in the maintenance of the inflammatory state [10–12,60]. We found high levels of IL-1β, IL-6, TNF-α and IL-10 in crisis-state SCA patients and high levels of IL-8 and IL-12 in steady-state SCA patients, although the latter cytokines were also increased in crisis-state SCA patients (Figure 2A-F). These data are consistent with previous studies, including ones performed by our research group [10–13,61–64]. Pro-inflammatory cytokine production in SCD patients was associated with mononuclear cells and with neutrophils, which alter the adhesive characteristics of the environment and contribute to the pathogenesis of this disease. However, these cytokines have been associated with vascular dysfunction, leukocytosis, and the initial involvement of neutrophils, followed by monocytes and endothelial cells, and these cells may serve as a source of these mediators [6,10–12].

The immunomodulatory properties of IL-10 are well known. High amounts of this cytokine have been related to the anti-inflammatory response, decreasing the proinflammatory milieu to resolve inflammation, and recently to protecting age-related vascular dysfunction [65,66]. IL1-β is associated with tissue damage and fibrogenesis and, together with IL-6, has been related to vascular disease [67-69]. The increase in IL-12 in the steady-state patients emphasizes the role of this cytokine and its family members as mediators of the inflammatory response found in sickle cell disease [70].

Using ROC curve analysis, we identified markers associated with steady-state (Figure 3A) and crisis-state SCA patients (Figure 3B). The markers we found exhibited high sensitivity, specificity, and accuracy and showed high predictive values for these biomarkers in the follow-up of SCA patients. These biomarkers also have excellent prospects as targets for further therapeutic modalities in SCD patients.

Conclusions

In summary, consistent with the purpose of this study, we described the roles of LTB_4, PGE_2 and TGF-β as markers of steady-state SCA and of IL-1β, IL-6 and TNF-α as markers of crisis-state SCA. We found high systemic levels of free heme, MMP-9 and TIMP-1 in both SCA states (crisis and steady state), suggesting a possible role of free heme in chronic hyper-hemolysis and of MMP-9 and TIMP-1 in the chronic inflammatory process of this disease. This hypothesis was validated by the association between systemic levels of these molecules with markers of hemolysis and inflammation in the SCD patient group. TIMP-1 likely acts independently of its MMP-9 inhibitory function, as we found high levels of both molecules in both SCA patient groups. The association between TIMP-1 levels and markers of hemolysis and anemia may indicate that this molecule plays a pivotal role in SCD pathogenesis, particularly in responding to the hypoxic milieu that is continuously present in this disease.

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References

1. Ohene-Frempong K, Steinberg MH (2001) Clinical aspects of sickle cell anemia in adults and children. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL , Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. New York: Cambridge University Press. pp. 611-670.

2. Nagel RL, Platt OS (2001) General pathophysiology of sickle cell anemia. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. New York: Cambridge University Press. pp. 494-526.

3. Kato GJ, Gladwin MT, Steinberg MH (2007) Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev 21: 37-47.

4. Okpala I, Daniel Y, Haynes R, Odoemene D, Goldman J (2002) Relationship between the clinical manifestations of sickle cell disease and the expression of adhesion molecules on white blood cells. Eur J Haematol 69: 135-144.

5. Okpala (2004) The intriguing contribution of white blood cells to sickle cell disease - a red cell disorder. Blood Rev 18: 65-73.

6. Okpala I (2006) Leukocyte adhesion and the pathophysiology of sickle cell disease. Curr Opin Hematol 13: 40-44.

7. Miller ST, Sleeper LA, Pegelow CH, Enos LE, Wang WC, *et al.* (2000) Prediction of adverse outcomes in children with sickle cell disease. N Engl J Med. 342:83-89.

8. Azevêdo ES (1980) Subgroup studies of black admixture within a mixed population of Bahia, Brazil. Ann Hum Genet 44: 55-60.

9. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM (2000) Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. Blood 96: 2451-2459.

10. Gonçalves MS, Queiroz IL, Cardoso SA, Zanetti A, Strapazoni AC, *et al.* (2001) Interleukin 8 as a vaso-occlusive marker in Brazilian patients with sickle cell disease. Braz J Med Biol Res 34: 1309-1313.

11. Assis A, Conran N, Canalli AA, Lorand-Metze I, Saad ST, *et al.* (2005) Effect of cytokines and chemokines on sickle neutrophil adhesion to fibronectin. Acta Haematol 113: 130-136.

12. Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad ST, Conran N, *et al.* (2009) Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. J Leukoc Biol 85: 235-242.

13. Cerqueira BA, Boas WV, Zanette AD, Reis MG, Goncalves MS (2011) Increased concentrations of IL-18 and uric acid in sickle cell anemia: contribution of hemolysis, endothelial activation and the inflammasome. Cytokine 56: 471-476.

14. Vilas-Boas W, Cerqueira BA, Zanette AM, Reis MG, Barral-Netto M, *et al.* (2010) Arginase levels and their association with Th17-related cytokines, soluble adhesion molecules (sICAM-1 and sVCAM-1) and hemolysis markers among steady-state sickle cell anemia patients. Ann Hematol 89: 877-882.

15. Taylor JG 6th, Nolan VG, Mendelsohn L, Kato GJ, Gladwin MT, Steinberg MH (2008) Chronic hyper-hemolysis in sickle cell anemia: association of vascular complications and mortality with less frequent vasoocclusive pain. PLoS One. 3:e2095.

16. Ferreira A, Balla J, Jeney V, Balla G, Soares MP (2008) A central role for free heme in the pathogenesis of severe malaria: the missing link? J Mol Med (Berl) 86: 1097-1111.

17. Belcher JD, Beckman JD, Balla G, Balla J, Vercellotti G (2010) Heme degradation and vascular injury. Antioxid Redox Signal 12: 233-248.

18. Fortes GB, Alves LS, de Oliveira R, Dutra FF, Rodrigues D, *et al.* (2012) Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood 119: 2368-2375.

19. Hebbel RP, Morgan WT, Eaton JW, Hedlund BE (1988) Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. Proc Natl Acad Sci U S A 85: 237-241.

20. Kuross SA, Rank BH, Hebbel RP (1988) Excess heme in sickle erythrocyte inside-out membranes: possible role in thiol oxidation. Blood 71: 876-882.

21. Uzunova VV, Pan W, Galkin O, Vekilov PG (2010) Free heme and the polymerization of sickle cell hemoglobin. Biophys J 99: 1976-1985.

22. Andrade BB, Araújo-Santos T, Luz NF, Khouri R, Bozza MT, *et al.* (2010) Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. J Immunol 185: 1196-1204.

23. Reynolds JJ (1996) Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. Oral Dis 2: 70-76.

24. Parks WC, Wilson CL, López-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617-629.

25. Bourboulia D, Stetler-Stevenson WG (2010) Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20: 161-168.

26. Bertaux B, Hornebeck W (1993) Tissue inhibitors of matrix metalloproteinases TIMP 1-2. Structures and functions. C R Seances Soc Biol Fil 187:192-200.

27. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 74: 111-122.

28. Lambert E, Bridoux L, Devy J, Dassé E, Sowa ML, *et al.* (2009) TIMP-1 binding to proMMP-9/CD44 complex localized at the cell surface promotes erythroid cell survival. Int J Biochem Cell Biol 41: 1102-1115.

29. Ould-yahoui A, Tremblay E, Sbai O, Ferhat L, Bernard A, *et al.* (2009) A new role for TIMP-1 in modulating neurite outgrowth and morphology of cortical neurons. PLOS ONE 4: e8289.

30. Connor WE, Lin DS, Thomas G Ey F, DeLoughery T, Zhu N (1997) Abnormal phospholipid molecular species of erythrocytes in sickle cell anemia. J Lipid Res 38:2516-2528.

31. de Jong K, Geldwerth D, Kuypers FA (1997) Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes. Biochemistry 36: 6768-6776.

32. Kuypers FA (2007) Membrane lipid alterations in hemoglobinopathies. Hematology Am Soc Hematol Educ Program 2007:68-73.

33. Setty BN, Stuart MJ (2002) Eicosanoids in sickle cell disease: potential relevance of neutrophil leukotriene B4 to disease pathophysiology. J Lab Clin Med 139: 80-89.

34. Kuehl FA Jr, Egan RW (1980) Prostaglandins, arachidonic acid, and inflammation. Science 210: 978-984.

35. Wong SL, Wong WT, Tian XY, Lau CW, Huang Y (2010) Prostaglandins in action indispensable roles of cyclooxygenase-1 and -2 in endothelium-dependent contractions. Adv Pharmacol 60: 61-83.

36. Setty BN, Chen D, Stuart MJ (1996) Sickle red blood cells stimulate endothelial cell production of eicosanoids and diacylglycerol. J Lab Clin Med 128: 313-321.

37. Monteiro AP, Pinheiro CS, Luna-Gomes T, Alves LR, Maya-Monteiro CM, *et al.* (2011) Leukotriene B4 mediates neutrophil migration induced by heme. J Immunol 186: 6562-6567.

38. Graido-Gonzalez E, Doherty JC, Bergreen EW, Organ G, Telfer M, *et al.* (1998) Plasma endothelin-1, cytokine, and prostaglandin E2 levels in sickle cell disease and acute vaso-occlusive sickle crisis. Blood 92: 2551-2555.

39. Ibe BO, Morris J, Kurantsin-Mills J, Raj JU (1997) Sickle erythrocytes induce prostacyclin and thromboxane synthesis by isolated perfused rat lungs. Am J Physiol 272: L597-L602.

40. Wang J, Limburg D, Carter J, Mbalaviele G, Gierse J, *et al.* (2010) Selective inducible microsomal prostaglandin E(2) synthase-1 (mPGES-1) inhibitors derived from an oxicam template. Bioorg Med Chem Lett 20: 1604-1609.

41. Hegyi B, Kudlik G, Monostori E, Uher F (2012) Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. Biochem Biophys Res Commun 419: 215-220.

42. Konya V, Philipose S, Bálint Z, Olschewski A, Marsche G, *et al.* (2011) Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. Eur J Immunol 41: 2379-2389.

43. Barrie A, Khare A, Henkel M, Zhang Y, Barmada MM, *et al.* (2011) Prostaglandin E2 and IL-23 plus IL-1β differentially regulate the Th1/Th17 immune response of human CD161(+) CD4(+) memory T cells. Clin. Transl Sci 4: 268-273.

44. North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, *et al.* (2007) Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 447: 1007-1011.

45. Han G, Li F, Singh TP, Wolf P, Wang XJ (2012) The pro-inflammatory role of TGFβ1: a paradox? Int J Biol Sci 8: 228-235.

46. Lambers C, Roth M, Zhong J, Campregher C, Binder P, *et al.* (2013) The interaction of endothelin-1 and TGF-β1 mediates vascular cell remodeling. PLOS ONE 28: e73399.

47. Yang Y, Wang Z, Yang H, Wang L, Gillespie SR, *et al.* (2013) TRPV1 potentiates TGFβ-induction of corneal myofibroblast development through an oxidative stress-mediated p38-SMAD2 signaling loop. PLOS ONE 8: e77300.

48. Derosa G, Maffioli P, D'Angelo A, Salvadeo SA, Ferrari I, *et al.* (2009) Evaluation of metalloproteinase 2 and 9 levels and their inhibitors in combined dyslipidemia. Clin Invest Med 32: E124-E132.

49. Derosa G, Maffioli P, D'Angelo A, Salvadeo SA, Ferrari I, *et al.* (2009) Effects of long chain omega-3 fatty acids on metalloproteinases and their inhibitors in combined dyslipidemia patients. Expert Opin Pharmacother 10: 1239-1247.

50. Lorente L, Martín MM, Labarta L, Díaz C, Solé-Violán J, *et al.* (2009) Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. Crit Care 13: R158.

51. Belo VA, Souza-Costa DC, Lana CM, Caputo FL, Marcaccini AM, *et al.* (2009) Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. Clin Biochem 42: 984-990.

52. Aplin AC, Zhu WH, Fogel E, Nicosia RF (2009) Vascular regression and survival are differentially regulated by MT1-MMP and TIMPs in the aortic ring model of angiogenesis. Am J Physiol Cell Physiol 297: C471–C480.

53. Ardi VC, Van den Steen PE, Opdenakker G, Schweighofer B, Deryugina EI, *et al.* (2009) Neutrophil MMP-9 proenzyme, unencumbered by TIMP-1, undergoes efficient activation

in vivo and catalytically induces angiogenesis via a basic fibroblast growth factor (FGF-2)/FGFR-2 pathway. J Biol Chem 284: 25854-25866.

54. Gonçalves FM, Jacob-Ferreira AL, Gomes VA, Casella-Filho A, Chagas AC, *et al.* (2009) Increased circulating levels of matrix metalloproteinase (MMP)-8, MMP-9, and pro-inflammatory markers in patients with metabolic syndrome. Clin Chim. Acta 403: 173-177.

55. Wakefield TW, Myers DD, Henke PK (2008) Mechanisms of venous thrombosis and resolution. Arterioscler Thromb Vasc Biol 28: 387-391.

56. Kondo S, Kubota S, Shimo T, Nishida T, Yosimichi G, *et al.* (2002) Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. Carcinogenesis 23: 769-776.

57. Akhavani MA, Madden L, Buysschaert I, Sivakumar B, Kang N, *et al.* (2009) Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. Arthritis Res Ther 11: R64.

58. Cui H, Grosso S, Schelter F, Mari B, Krüger A (2012) On the pro-metastatic stress response to cancer therapies: evidence for a positive Co-operation between TIMP-1, HIF-1α, and miR-210. Front Pharmacol 3: 134.

59. Setty BN, Betal SG, Zhang J, Stuart MJ (2008) Heme induces endothelial tissue factor expression: potential role in hemostatic activation in patients with hemolytic anemia. J Thromb Haemost 6:2202-2209.

60. Brittain JE, Parise LV (2007) Cytokines and plasma factors in sickle cell disease. Curr Opin Hematol 14: 438-443.

61. Abboud MR, Taylor EC, Habib D, Dantzler-Johnson T, Jackson SM, *et al.* (2000) Elevated serum and bronchoalveolar lavage fluid levels of interleukin 8 and granulocyte colony-stimulating factor associated with the acute chest syndrome in patients with sickle cell disease. Br J Haematol 111: 482-490.

62. Pathare A, Al Kindi S, Alnaqdy AA, Daar S, Knox-Macaulay H, *et al.* (2004) Cytokine profile of sickle cell disease in Oman. Am J Hematol 77: 323-328.

63. Keikhaei B, Mohseni AR, Norouzirad R, Alinejadi M, Ghanbari S, *et al.* (2013) Altered levels of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crises and the steady state condition. Eur Cytokine Netw 24: 45-52.

64. Veiga PC, Schroth RJ, Guedes R, Freire SM, Nogueira-Filho G (2013) Serum cytokine profile among Brazilian children of African descent with periodontal inflammation and sickle cell anaemia. Arch Oral Biol 58: 505-510.

65. Tabas I, Glass CK (2013) Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science 339: 166-172.

66. Kinzenbaw DA, Chu Y, Silva P RA, Didion SP, Faraci FM (2013). Interleukin-10 protects against aging-induced endothelial dysfunction. Physiol Rep 1:e00149.

67. Saxena A, Chen W, Su Y, Rai V, Uche OU, *et al.* (2013) IL-1 induces proinflammatory leukocyte infiltration and regulates fibroblast phenotype in the infarcted myocardium. J Immunol 191:4838-4848.

68. Gieling RG, Wallace K, Han YP (2009) Interleukin-1 participates in the progression from liver injury to fibrosis. Am J Physiol Gastrointest Liver Physiol 296: G1324-G1331.

69. Miwa K, Tanaka M, Okazaki S, Furukado S, Sakaguchi M, *et al.*. (2013) Association between interleukin-6 levels and first-ever cerebrovascular events in patients with vascular risk factors. Arterioscler Thromb Vasc Biol 33: 400-405.

70. Barrie AM, Plevy SE (2005) The interleukin-12 family of cytokines: therapeutic targets for inflammatory disease mediation. Clin Appl Immunol Rev 5: 225-240.

Figure Legends

Figure 1. Inflammatory mediator and tissue remodelling marker concentrations in sickle cell anemia patients and controls. (A-B) Analyses of the inflammatory mediators LTB_4 and PGE_2 indicate a higher concentration of LTB_4 in patients in the steady-state (SP-SCA) group compared with patients in the crisis-state (CP-SCA) group and with individuals in the healthy control (HC) group, as well as a higher concentration of PGE_2 in patients in the SP-SCA group compared with patients in the CP-SCA group and individuals in the HC group. (C-F) Analyses of the tissue remodelling markers TGF-β, MMP-9, and TIMP-1 and the TIMP-1/MMP-9 ratio indicate lower TGF-β concentrations in patients in the CP-SCA group than in patients in the SP-SCA group and individuals in the HC group; a similar MMP-9 concentration in patients in the CP-SCA and SP-SCA groups compared with individuals in the HC group; a similar TIMP-1 concentration in patients in the CP-SCA and SP-SCA groups compared with individuals in the HC group; and a lower TIMP-1/MMP-9 ratio in patients in the SP-SCA group compared with patients in the CP-SCA group and individuals in the HC group. (G) An analysis of free heme indicates a similar concentration in patients in the CP-SCA and SP-SCA groups compared with individuals in the HC group.

Figure 2. Inflammatory and anti-inflammatory cytokine concentrations in sickle cell anemia patients and controls. (A) IL-8 analysis indicates a higher concentration in patients in the steady-state (SP-SCA) group compared with patients in the crisis-state (CP-SCA) group and individuals in the healthy control (HC) group. (B) IL-1β analysis indicates a higher concentration in patients in the CP-SCA group than in patients in the SP-SCA group and individuals in the HC group. (C) IL-6 analysis indicates a higher concentration in patients in the CP-SCA group than in patients in the SP-SCA group and individuals in the HC group. (D) IL-10 analysis indicates a higher concentration in patients in the CP-SCA group than in patients in the SP-SCA group and individuals in the HC group. (E) TNF-α analyses indicate a higher concentration in patients in the CP-SCA group than in patients in the SP-SCA group. (F) IL-12 analysis shows a lower concentration in the CP-SCA group than in patients in the SP-SCA group and individuals in the HC group.

Figure 3. Receiver operating characteristic (ROC) curves investigating inflammatory markers of steady- and crisis-state SCA patients. (A) The ROC curves of the TIMP-1/MMP-9 ratio (lilac solid line), MMP-9 (purple dashed line), leukotriene B4 (red dashed line), TGF-β (black solid line), and PGE_2 (rose dashed line) plasma levels indicate that leukotriene B4, TGF-β, and PGE_2 are markers involved in steady state SCD. (B) The ROC curves of TNF-α (black solid line), IL-1β (red solid line), IL-10 (blue dashed line), IL-12 (gray dashed line), and IL-6 (green dashed line) plasma levels indicate that TNF-α, IL-1β and IL-10 are markers involved in crisis-state SCD, as determined by the statistical data and the areas under the curves (AUCs).

Figure 4. Association of free heme concentration with lipid pattern. Statistical analyses were conducted to compare the chemical and hematological marker concentrations of steady-state SCD patients with free heme concentrations higher and lower than the 50^{th} percentile (54.6 μM). (A-C) The SCD patients with the highest free heme concentrations had the lowest HDL-C concentrations and the highest triglyceride concentrations.

Figure 5. Association of free heme concentration with hemolysis markers. Statistical analyses were conducted to measure the concentration of chemical markers associated with hemolysis in steady-state SCD patients with free heme concentrations higher and lower than the 50^{th}

percentile (54.6 μM). (A-F) The SCD patients with the highest free heme concentrations had the highest concentrations of ferritin, AST, total bilirubin (TB), indirect bilirubin (IB), direct bilirubin (DB) and lactate dehydrogenase (LDH).

Figure 6. Association between free heme concentration and hematological markers. Statistical analyses were conducted to compare the hematological markers associated with hemolysis and anemia in steady-state SCD patients with free heme concentrations higher and lower than the 50th percentile (54.6 μM). (A-C) The SCD patients with the highest free heme concentrations had the lowest hematocrit values and the highest monocyte and platelet counts.

Figure 7. Association between free heme concentration and inflammatory and vascular remodelling markers. Statistical analyses were performed to compare the marker concentrations of steady-state SCD patients with free heme concentrations higher and lower than the 50th percentile (54.65 μM). (A-D) The SCD patients with the highest free heme concentrations had the highest TGF-β, PGE_2, TIMP-1, and IL-12 values.

Figure 8. TIMP-1 concentration is associated with hemolysis and inflammation markers. Statistical analyses were performed to compare chemical markers of hemolysis and inflammation in steady-state SCD patients with TIMP-1 concentrations higher and lower than the 50th percentile (297.5 ng/mL). (A-F) The SCD patients with the highest TIMP-1 concentrations had the lowest HDL-C concentrations and the highest triglyceride, ALT, TB, IB and ferritin concentrations.

Figure 9. TIMP-1 concentration is associated with hematological markers. Statistical analyses were performed to compare hematological markers between steady-state SCD patients with TIMP-1 concentrations higher and lower than the 50th percentile (297.5 ng/mL). (A-E) The SCD patients with the highest TIMP-1 concentrations exhibited the lowest RBC counts and hemoglobin and hematocrit concentrations and the highest mean corpuscular hemoglobin concentrations and platelet counts.

Figure 10. MMP-9 concentration is associated with hematological and inflammatory markers. Statistical analyses were performed to compare hematological markers between steady-state SCD patients with MMP-9 concentrations higher and lower than the 50th percentile (444 ng/mL). (A-C) The SCD patients with the highest MMP-9 concentrations had the highest leukocyte and neutrophil counts and the highest TIMP-1 concentrations.

Supporting information

Figure 1S. Free heme, TIMP1 and MMP9 concentrations are associated with hemolysis, inflammation and vascular remodelling markers in SCA patients. Statistical analyses were performed for steady-state SCD patients with concentrations higher and lower than the 50th percentile for free heme (54.65 μM), TIMP-1 (323.0 ng/mL), and MMP-9 (444.0 ng/mL). (A-I) The SCD patients with the highest free heme concentrations had the highest VLDL-C, triglyceride, AST, total bilirubin, indirect bilirubin, and ASLO concentrations and monocyte counts as well as lower HDL-C and HbF concentrations. (J-M) The patients with the highest TIMP-1 concentrations had increased platelet counts and TGFβ and MMP-9 expression. (N-Q) The patients with MMP-9 concentrations higher than the 50th percentile had increased leucocyte counts and TIMP-1 concentrations as well as decreased TNF-α concentrations and lymphocyte counts.

Figure 2S. Free heme, TIMP-1 and MMP-9 concentrations are associated with hemolysis, inflammation and vascular remodelling markers in HbSC patients. Statistical analyses were performed for steady-state HbSS patients with free heme (43.0 μM), TIMP-1 (247.03 ng/mL) and MMP-9 (530.58 ng/mL) concentrations higher and lower than the 50th percentile. (A-D) The HbSC patients with the highest free heme concentrations had the highest VLDL-C, triglycerides, albumin and TGF-β levels. (E-F) The HbSC patients with TIMP-1 concentrations higher than the 50th percentile had increased monocyte counts and alpha-1 antitrypsin concentrations. (G-H) The HbSC patients with the highest MMP-9 concentrations had increased leucocyte counts and TIMP-1 concentrations.

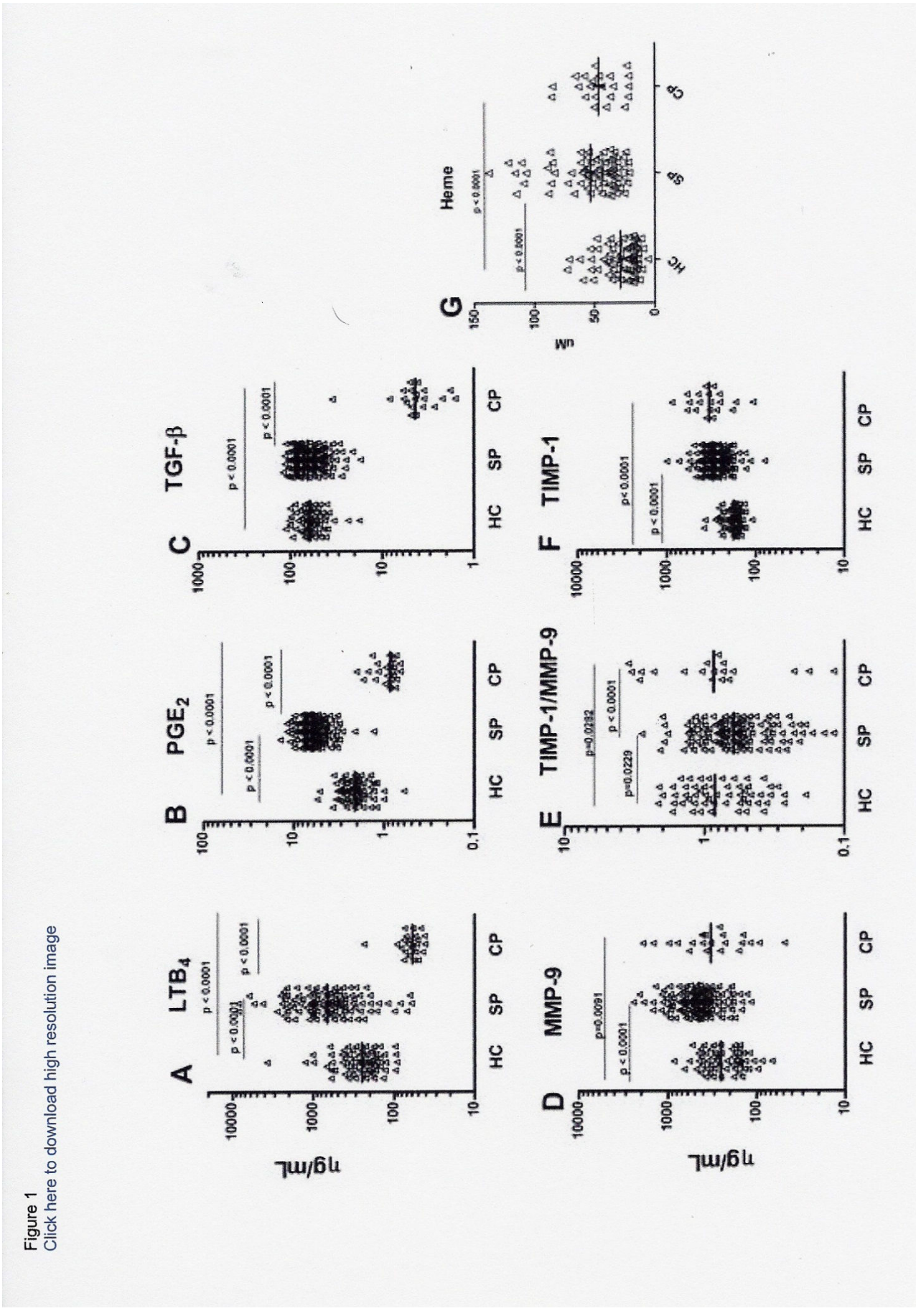

Figure 1
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Figure 2
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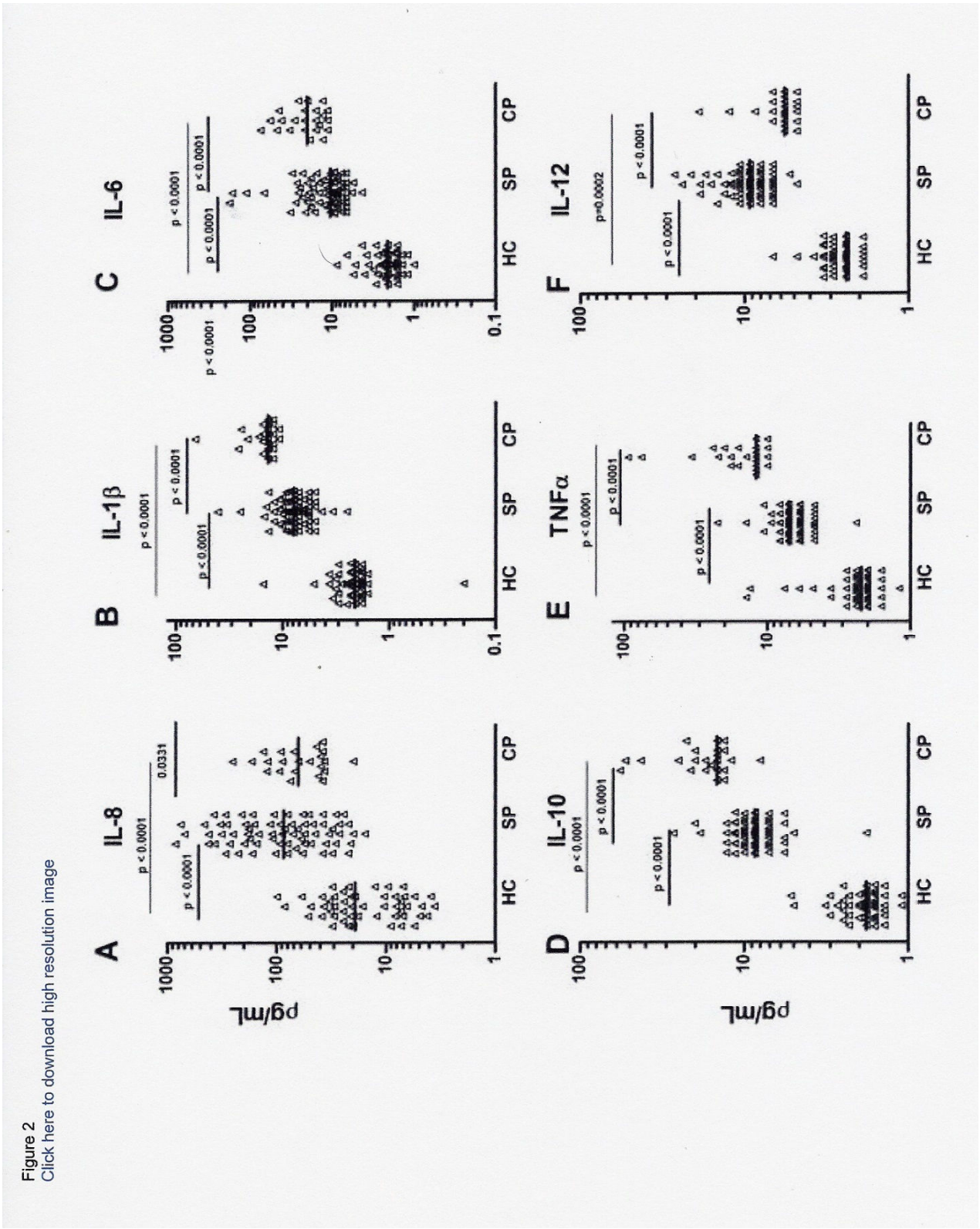

Figure 3
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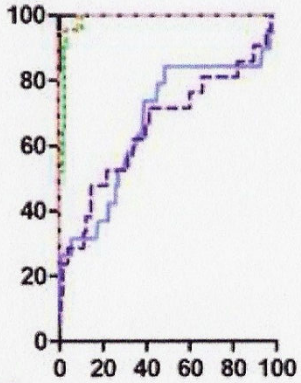
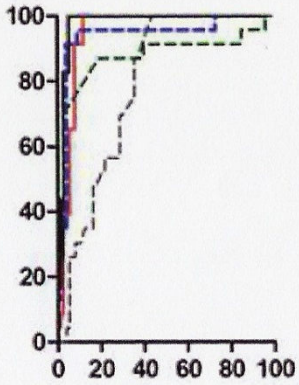
A



	AUC	*p* value	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood ratio	CUT OFF
TIMP-1 /MMP-9	.6682	.1857	63.2% (38.4-83.7)	63.1% (53.9-71.7)	1.71	> 0.755
MMP-9	.6542	.0024	61.9% (38.4-81.9)	65.7% (56.4-73.9)	1.80	< 384.0
LTB4	.9837	<.0001	95.6% (78.0-99.9)	96.3% (98.0-99.9)	25.59	< 94.68
TGF-b	.9955	<.0001	95.0% (75.1-99.9)	99.2% (95.5-99.9)	114.95	< 19.10
PGE2	.9987	<.0001	100% (85.2-100)	99.2% (95.8-99.98)	129.00	< 2.060

B



	AUC	*p* value	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood ratio	CUT OFF
TNF-α	.9765	<.0001	100% (85.2-100)	94.6% (86.7-98.5)	18.50	> 9.700
IL-1β	.9454	<.0001	100% (85.2-100)	87.8% (78.2-94.3)	8.22	> 10.75
IL-10	.9377	<.0001	91.3% (71.9-98.9)	95.9% (88.6-99.2)	25.52	> 13.10
IL-12	.8714	<.0001	86.9% (66.4-97.2)	80.2% (69.9-89.1)	4.53	< 7.350
IL-6	.7877	<.0001	69.6% (47.1-86.8)	71.2% (59.4-81.2)	2.42	> 15.45

Figure 4
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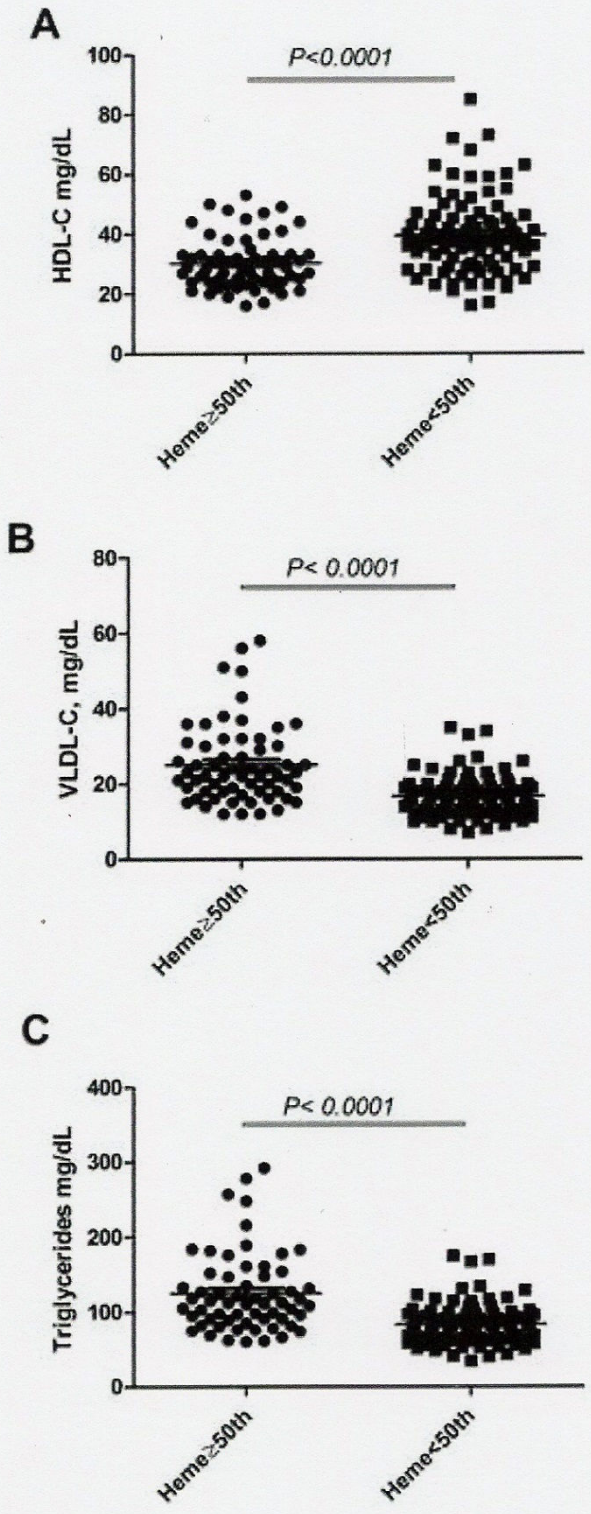

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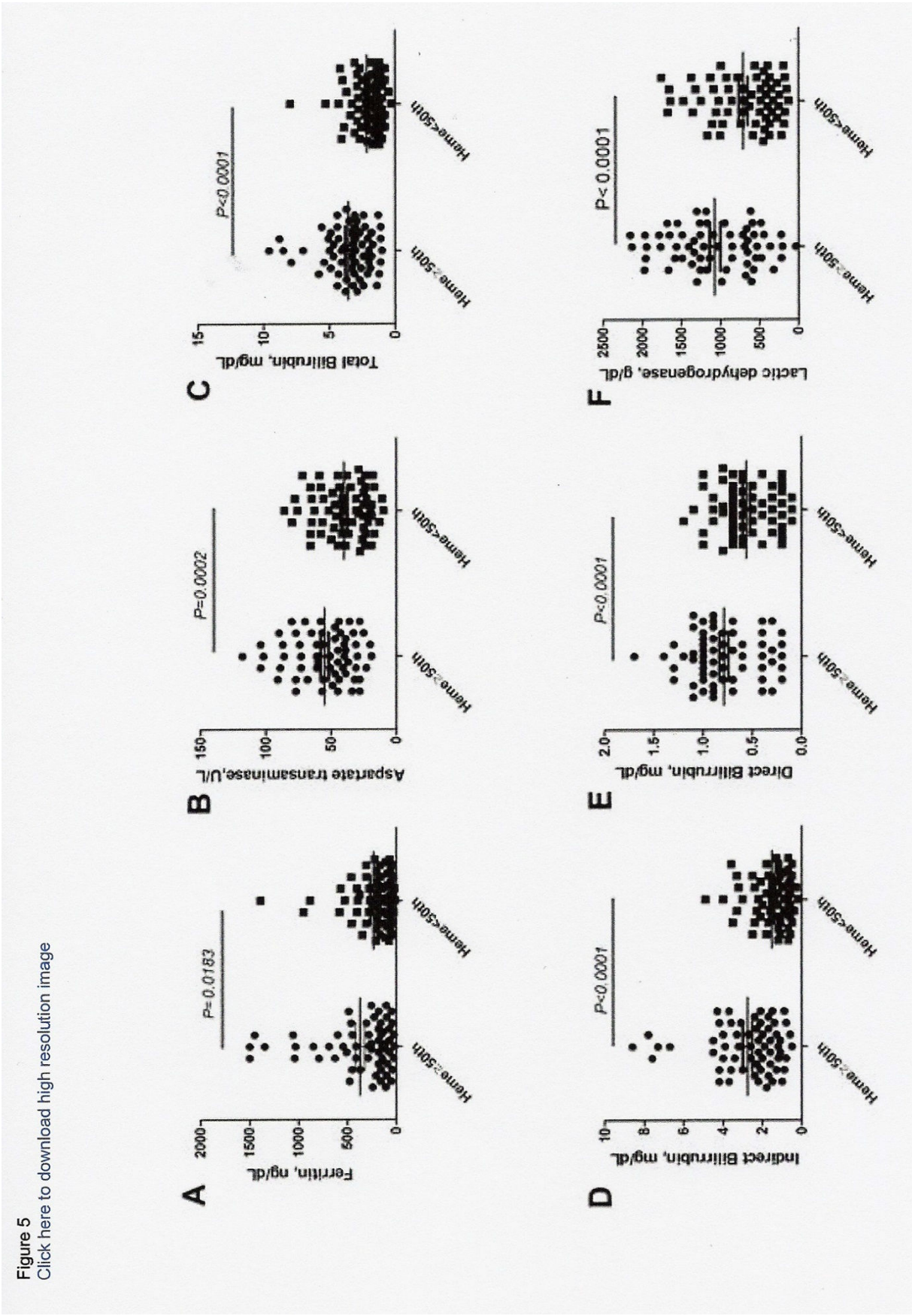

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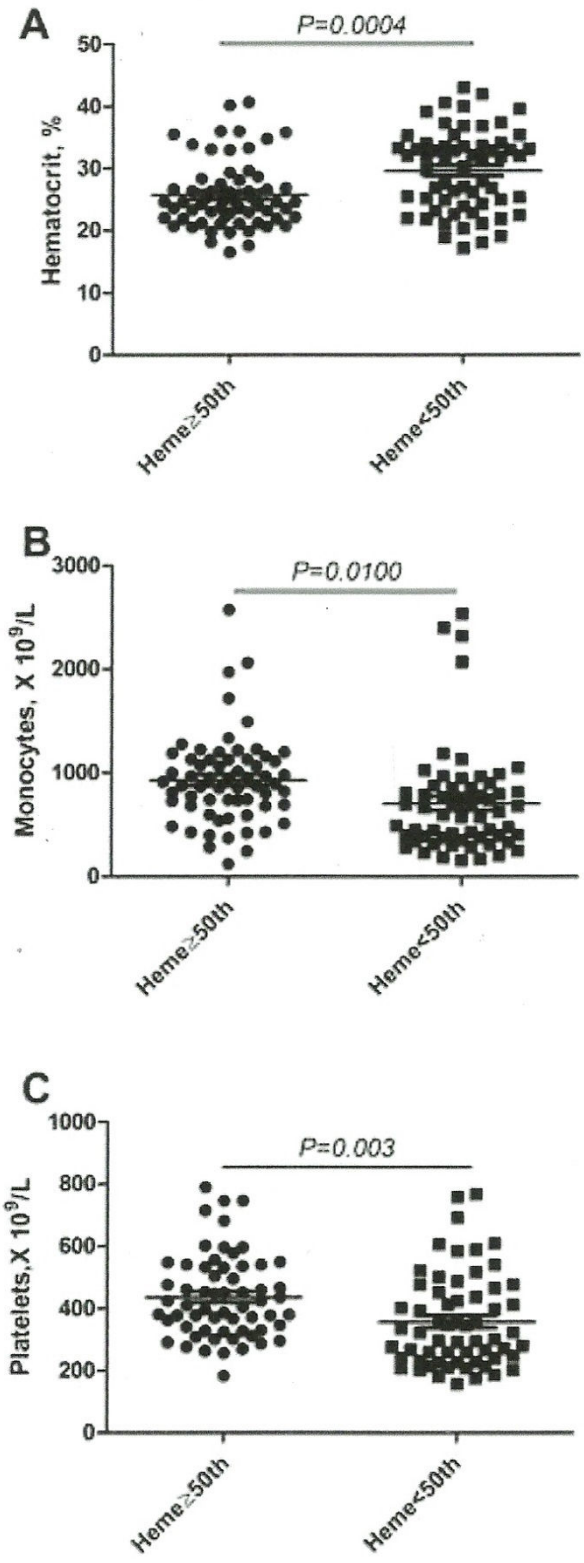

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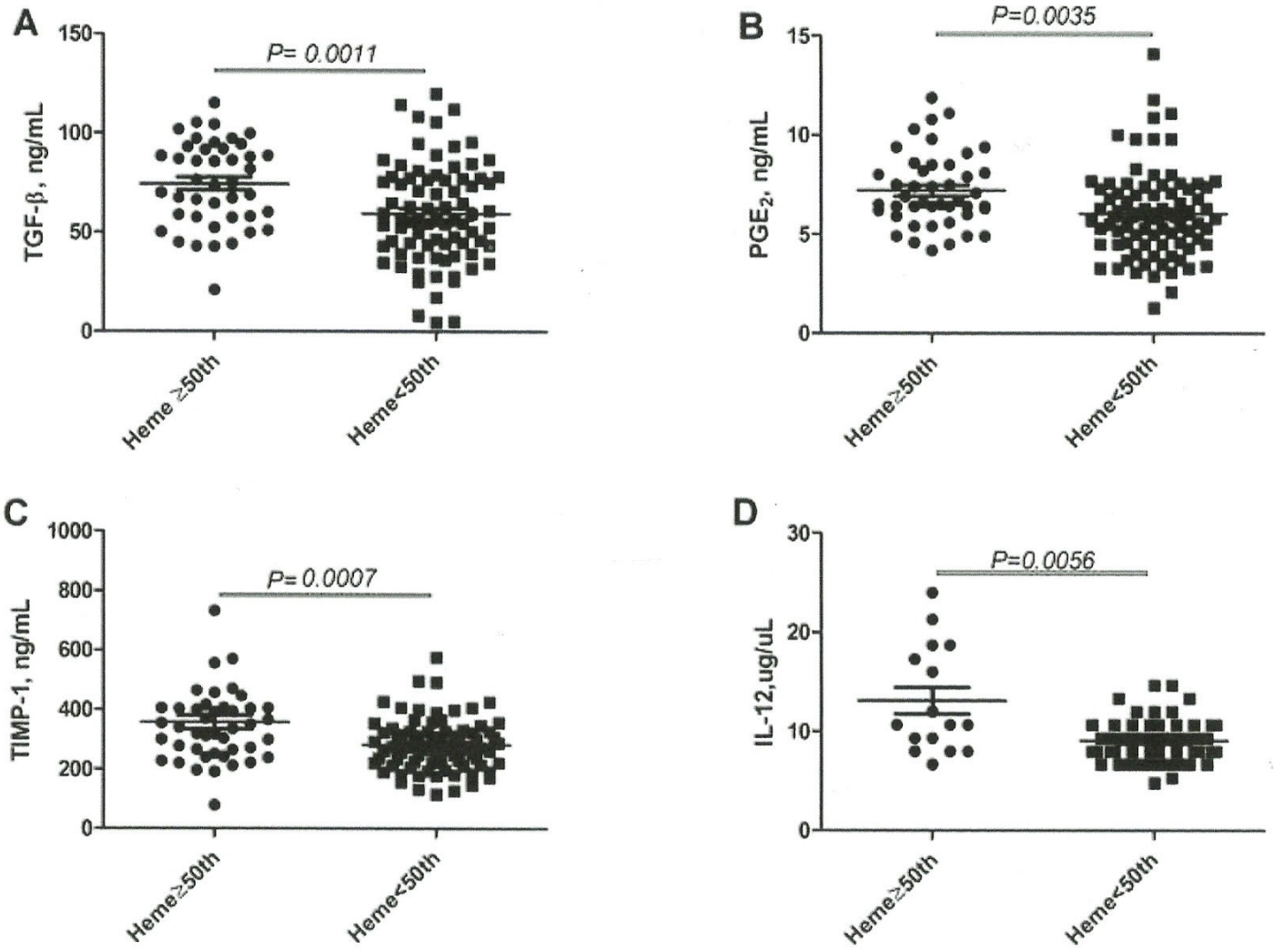

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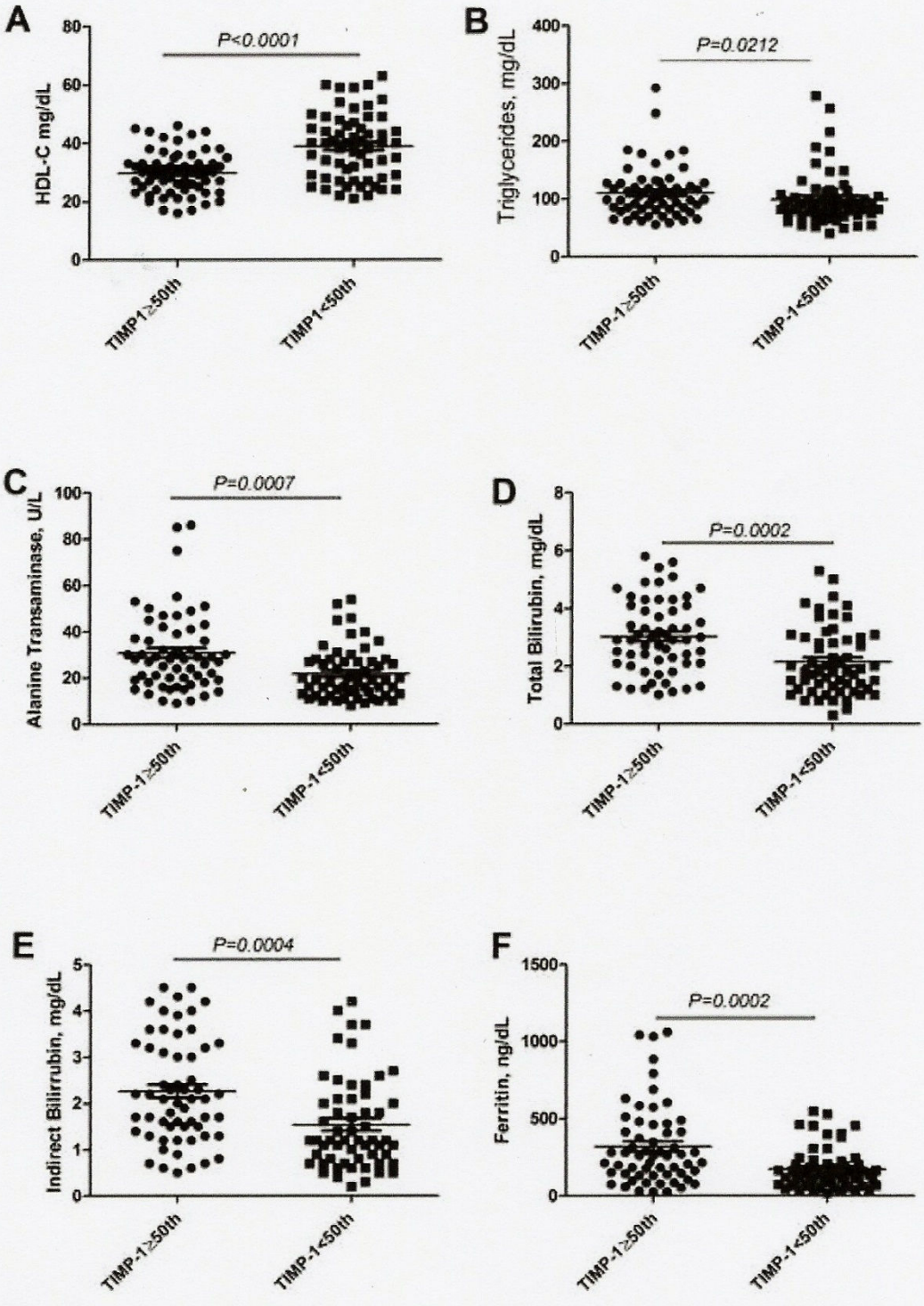

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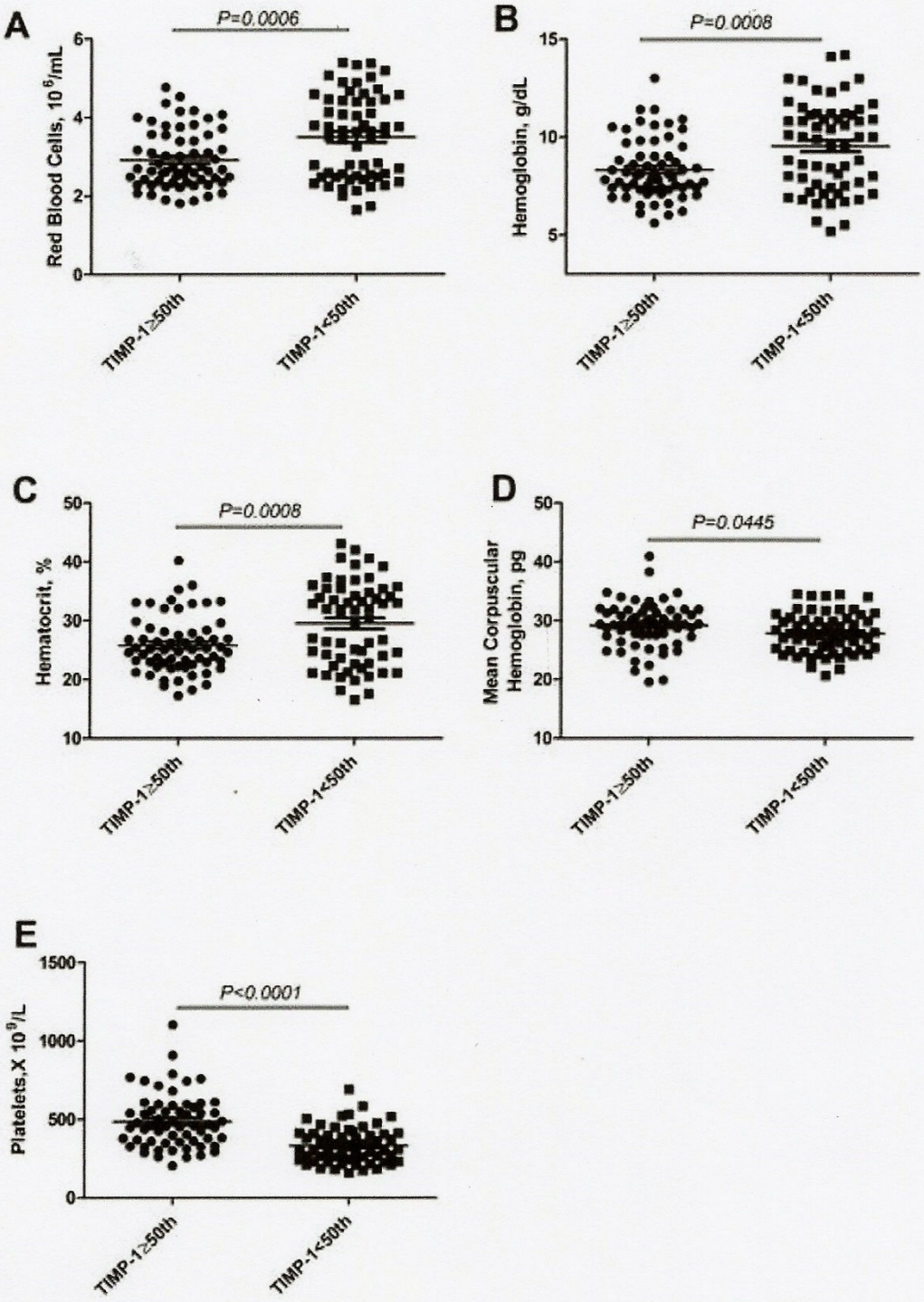

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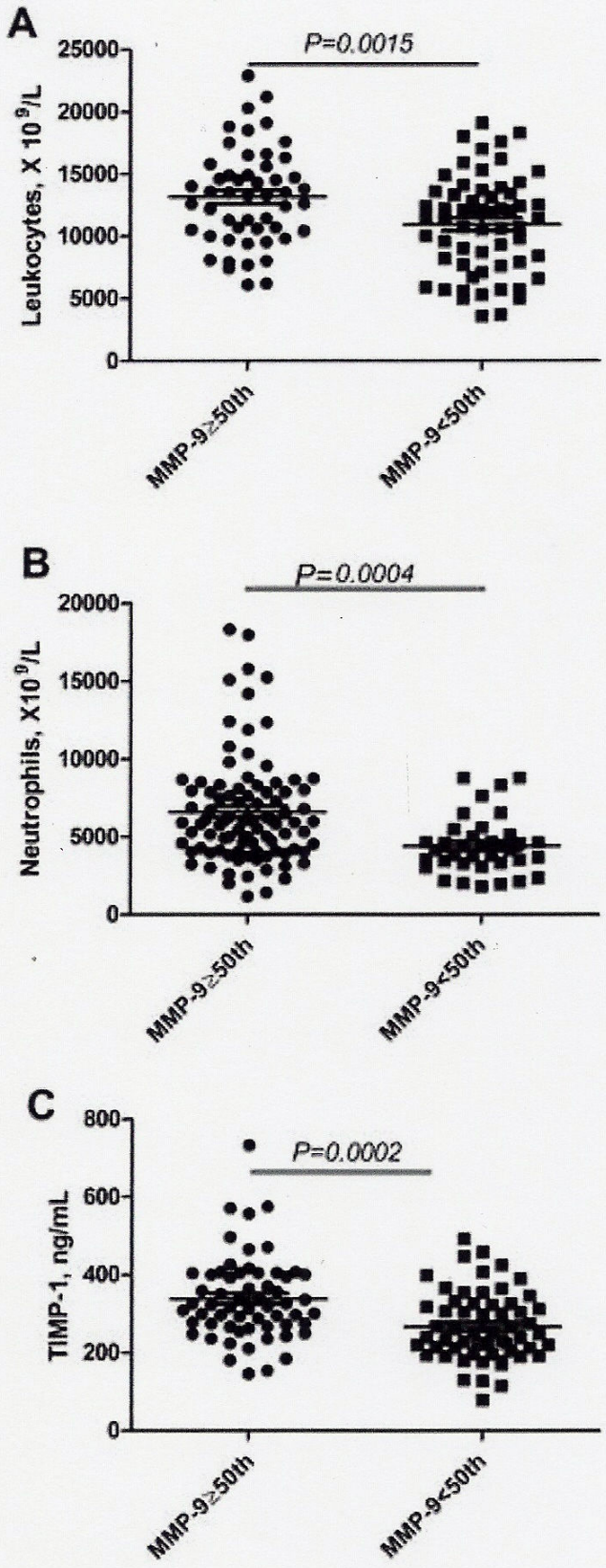

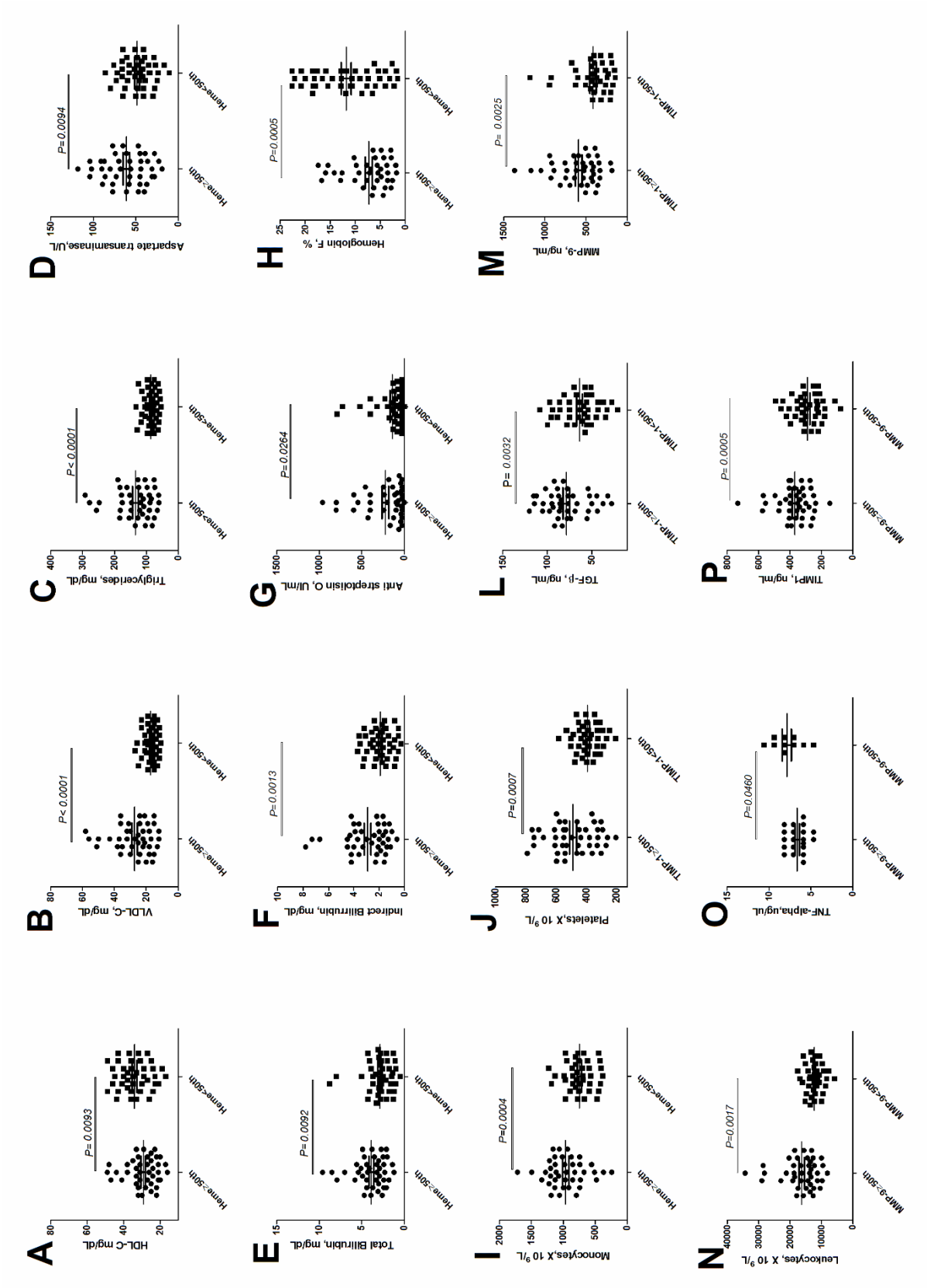
A
HDL-C mg/dL
P= 0.0093
Heme<50th
Heme≥50th
B
VLDL-C, mg/dL
P< 0.0001
Heme<50th
Heme≥50th
C
Triglycerides, mg/dL
P< 0.0001
Heme<50th
Heme≥50th
D
Aspartate Transaminase,U/L
P=0.0094
Heme<50th
Heme≥50th
E
Total Bilirrubin, mg/dL
P= 0.0092
Heme<50th
Heme≥50th
F
Indirect Bilirrubin, mg/dL
P=0.0013
Heme<50th
Heme≥50th
G
Anti streptolisin O, UI/mL
P=0.0264
Heme<50th
Heme≥50th
H
Hemoglobin F, %
P=0.0005
Heme<50th
Heme≥50th
I
Monocytes,X 10 9/L
P=0.0004
Heme<50th
Heme≥50th
J
Platelets,X 10 9/L
P=0.0007
TIMP-1<50th
TIMP-1≥50th
L
TGF-β, ng/mL
P= 0.0032
TIMP-1<50th
TIMP-1≥50th
M
MMP-9, ng/mL
P= 0.0025
TIMP-1<50th
TIMP-1≥50th
N
Leukocytes, X 10 9/L
P=0.0017
MMP-9<50th
MMP-9≥50th
O
TNF-alpha,ug/uL
P=0.0460
MMP-9<50th
MMP-9≥50th
P
TIMP1, ng/mL
P= 0.0005
MMP-9<50th
MMP-9≥50th

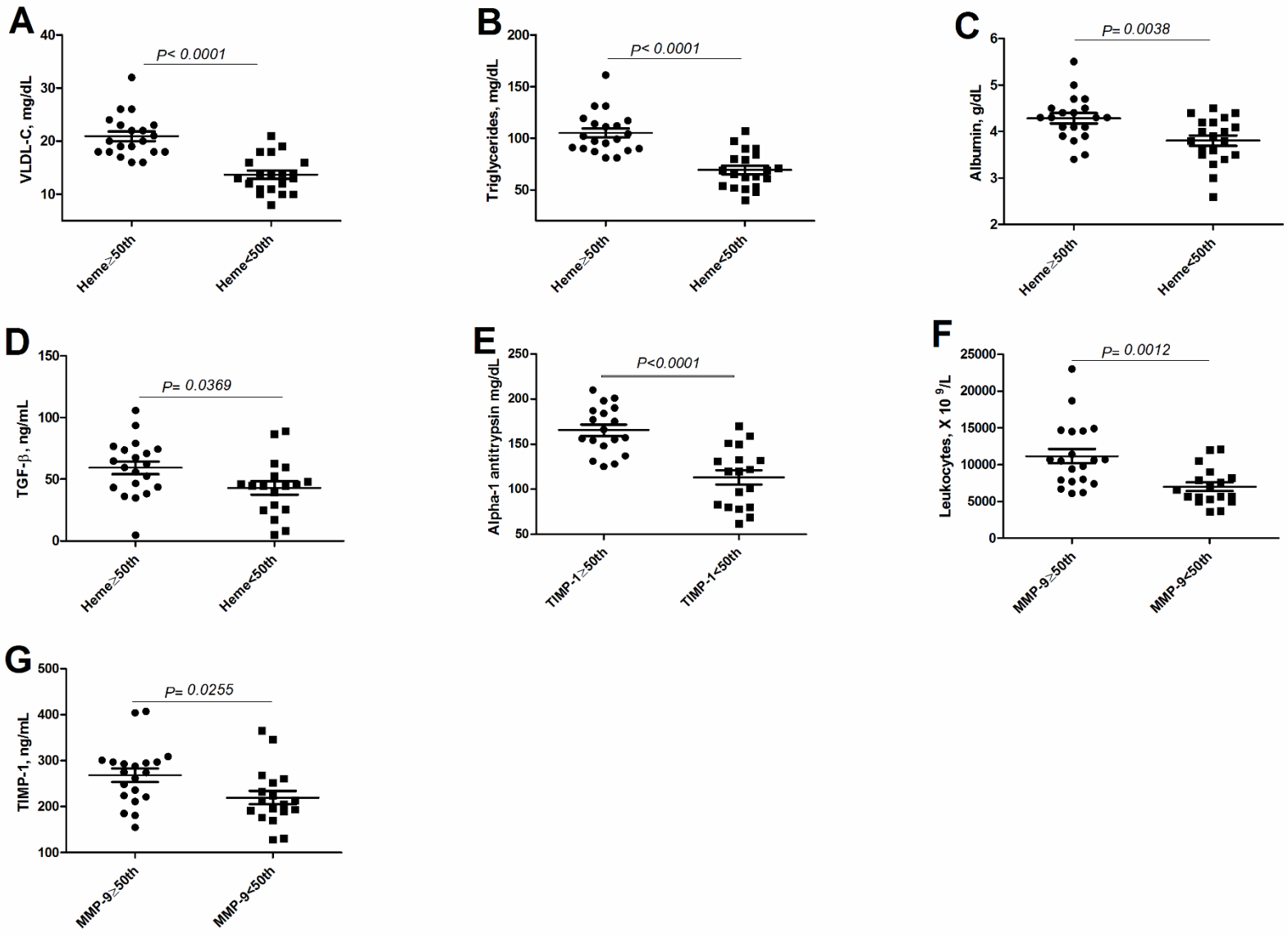
A
VLDL-C, mg/dL
40
30
20
10
P< 0.0001
Heme≥50th
Heme<50th
B
Triglycerides, mg/dL
200
150
100
50
P< 0.0001
Heme≥50th
Heme<50th
C
Albumin, g/dL
6
5
4
3
2
P= 0.0038
Heme≥50th
Heme<50th
D
TGF-β, ng/mL
150
100
50
0
P= 0.0369
Heme≥50th
Heme<50th
E
Alpha-1 antitrypsin mg/dL
250
200
150
100
50
P<0.0001
TIMP-1≥50th
TIMP-1<50th
F
Leukocytes, X 10 9/L
25000
20000
15000
10000
5000
0
P= 0.0012
MMP-9≥50th
MMP-9<50th
G
TIMP-1, ng/mL
500
400
300
200
100
P= 0.0255
MMP-9≥50th
MMP-9<50th

Table 1. Demographic and hematological characteristics of the SCD patient and control groups.

	Healthy controls	HbSC	HbSS	P value
N of patients and controls	148	47	101	-
Age (years) – mean ± SD	8.7±3.2	10.8±4.2	8.6±3.8	0.002
Female, %	51.5	51.1	55.4	NS
Hematological data	Mean±SD			
Red blood cell count (RBC), 10^6/L	4.7±0.4	4.3±0.6	2.7±0.6	<0.0001
Hemoglobin, g/dL	12.8±1.0	11.2±1.3	7.8±1.3	<0.0001
Hematocrit, %	38.5±2.8	34.9±3.8	24.2±3.8	<0.0001
Mean corpuscular volume (MCV), fL	81.4±5.2	80.7±7.5	90.9±10.3	<0.0001
Mean corpuscular hemoglobin (MCH), pg	27.1±2.0	26.0±2.5	29.5±3.6	<0.0001
Mean corpuscular hemoglobin concentration (MCHC), %	33.3±1.4	32.8±1.1	32.4±0.9	<0.0001
Reticulocytes, %	0.8±0.3	4.4±4.9	9.3±4.7	<0.0001
Erythroblasts, %	0	0.5±1.5	2.2±2.5	<0.0001
Hemoglobin S (HbS), %	0	50.9±5.9	86.7±6.3	<0.0001
Fetal hemoglobin (HbF), %	0.5±0.5	2.8±3.3	9.7±6.1	<0.0001
White blood cell count (WBC), $\times 10^9$/L	7027±2179	9353±5599	14753±4997	<0.0001
Neutrophils, $\times 10^9$/L	3240±1686	4790±3260	6714±3488	<0.0001
Monocytes, $\times 10^9$/L	489±205	531±456	945±442	<0.0001
Eosinophils, $\times 10^9$/L	436±397	560±486	980±865	<0.0001
Lymphocytes, $\times 10^9$/L	2775±899	3386±1985	5918±2086	<0.0001
Platelet count, $\times 10^9$/L	308±67.3	321±185.5	444±131.2	<0.0001

SD=standard deviation; NS=Not significant.

Table 2. Chemical characteristics of the SCD patient and control groups.

Biomarkers	Control	HbSC	HbSS	P value
N of patients and controls	146	47	101	
Chemistry profile				
LDH, U/L	409±132	504±273	1035±504	<0.0001
AST, U/L	31±11	33±14	55±26	<0.0001
ALT, U/L	17±7.1	23±12.1	30±24.3	<0.0001
Total bilirubin, mg/dL	0.5±0.2	1.7±1.7	3.3±1.8	<0.0001
Direct bilirubin, mg/dL	0.25±0.1	0.5±0.3	0.8±0.5	<0.0001
Indirect bilirubin, mg/dL	0.2±0.2	1.2±0.9	2.6±1.6	<0.0001
Total plasma protein, g/dL	7.3±0.6	7.3±0.6	7.3±0.9	NS
Albumin, g/dL	4.2±0.5	4.1±0.5	4.1±0.7	NS
Globulin, g/dL	3.1±0.6	3.2±0.6	3.3±0.8	0.042
Urea, g/dL	21.6±5.9	19.5±6.5	17.3±6.5	<0.0001
Creatinine, g/dL	0.5±0.2	0.5±0.1	0.4±0.2	<0.0001
Albumin/globulin ratio	1.5±0.4	1.4±0.4	1.3±0.5	0.029
Total cholesterol, mg/dL	164±34.5	120±27.0	121±26.5	<0.0001
HDL-C, mg/dL	49±13.7	42±14.6	32±9.9	<0.0001
LDL-C, mg/dL	97±33.5	59±21.2	67±22.5	<0.0001
VLDL-C, mg/dL	18±10.4	17±6.3	22±10.2	<0.0001
Triglycerides, mg/dL	88±51.7	88±31.9	110±51.3	<0.0001
Iron, mcg/dL	74±42.4	102±106.2	137±126.7	<0.0001
Ferritin, ng/mL	37±28.3	151±100.5	396±41.4	<0.0001
Total heme, µM	28.5±15.5	43.0±29.5	68.6±29.9	<0.0001
RCP	4.4±19.1	5.8±8.6	7.7±13.2	<0.0001
Alpha 1 antitrypsin, mg/dL	137.5±43.4	138.3±42.9	161.1±45.5	<0.0001
Antistreptolysin O, UI/mL	134±132	163±262	208±297	NS

NS=Not significant; LDH=Lactate dehydrogenase; AST=Aspartate aminotransferase; ALT=Alanine aminotransferase; HDL-C=High-density lipoprotein cholesterol; LDL-C=Low-density lipoprotein cholesterol; VLDL-C=Very low-density lipoprotein cholesterol; RCP=Reactive C protein

Table 3. Demographic and laboratory characteristics of the crisis-state SCA patients.

Characteristics	N (23)	Mean ± Standard Deviation
Age (Years)		10.3±4.34
Gender		
Male, N (%)	10 (44)	
Female, N (%)	13 (56)	
Hematological data		
RBC, $\times 10^6$/mm^3		2.6±0.8
Hemoglobin, g/dL		8.5±2.0
Hematocrit, %		25.9±5.2
Mean cell volume, fL		83.6±8.5
Mean cell hemoglobin, pg		25.8±3.2
Mean corpuscular hemoglobin concentration, g/dL		33.3±1.6
Reticulocyte count, % of RBC		2.5±1.3
WBC, $\times 10^9$/L		15062.4±8006.8
Neutrophil count, $\times 10^9$/L		9428.0±6919.2
Band neutrophil count, $\times 10^9$/L		366.7±179.7
Eosinophils, $\times 10^9$/L		873.6±744.9
Lymphocyte count, $\times 10^9$/L		4238.9±2837.1
Monocyte count, $\times 10^9$/L		421.9±374.5
Platelet count, $\times 10^9$/L		360.6±185.9
Chemistry Profile		
Total cholesterol, mg/dL		134.1±26.7
HDL-C, mg/dL		32.2±7.3
LDL-C, mg/dL		80.3±23.8
VLDL-C, mg/dL		20.4±10.0
Triglycerides, mg/dL		102.3±49.8
Lactate dehydrogenase, U/L		751.3±356.4
Total bilirubin, mg/dL		1.0±0.5
Direct bilirubin, mg/dL		0.4±0.4
Indirect bilirubin, mg/dL		0.7±0.6
Aspartate aminotransferase, U/L		47.7±30.0
Alanine aminotransferase, U/L		27.9±20.2
Albumin, g/dL		4.3±0.4
Urea nitrogen, mg/dL		21.5±6.2
Creatinine, mg/dL		0.4±0.2
Uric acid, mg/dL		3.1±1.2

4.4. MANUSCRITO IV

Prognostic impact of Alpha-1 Antitrypsin levels and SERPINA1 gene polymorphisms on sickle cell disease

Este trabalho avalia os níveis séricos da alfa-1 antitripsina, os polimorfismos no gene da *SERPINA1* e suas possíveis associações com marcadores bioquímicos e manifestações clínicas na doença falciforme.

A patogênese da doença falciforme (DF) envolve diversas vias, sendo a inflamação crônica e a hemólise características importantes na referida patologia. Dessa forma, a busca por marcadores de prognósticos para estabelecer possíveis sub-fenótipos da doença é um aspecto importante. O presente estudo investigou os níveis da alfa-1 antitripsina (AAT), um inibidor de proteases responsáveis por desencadear reações inflamatórias, descrevendo suas associações com polimorfismos no gene *SERPINA1*, biomarcadores hematológicos e bioquímicos e a história clínica em crianças com DF em estado estável. Foram incluídos no presente estudo um total de 356 indivíduos com DF e um grupo controle (GC) composto por 100 indivíduos saudáveis pareados por sexo e idade com a mesma origem geográfica. Os pacientes com níveis de AAT superior ao percentil 50 (158,0 mg/mL) apresentaram contagem de eritrócitos (RBC) ($p = 0,003$), concentração de hemoglobina (Hb) ($p = 0,0002$) e hematócrito (Ht) (0,0002) significativamente menores, e a contagem de leucócitos (WBC) ($p = 0,004$) e os neutrófilos ($p = 0,0001$) maiores, bem como níveis mais elevados de proteína C-reactiva (PCR) e níveis de ureia mais baixos. AAT teve correlação negativa significativa com a RBC ($r = -0,205$, $p = 0,0001$), Hb ($r = -0,203$, $p = 0,0001$), Hct ($r = -0,256$, $p <0,0001$), colesterol liagdo à lipoproteína de alta densidade (HDL-C) ($r = -0,205$, $p = 0,0001$), ureia ($r = -0,184$, $p = 0,0005$), creatinina ($r = -134$, $p = 0,012$), e albumina ($r = -0,135$, $p = 0,011$), e correlação positiva significativa com WBC ($r = 0,18$, 0,0007), neutrófilos ($r = 0,2297$, $p <0,0001$), HbS ($r = 0,2874$, $p <0,0001$), bilirrubina total ($r = 0,137$, $p = 0,01$), bilirrubina direta ($r = 0,136$, $p = 0,001$), bilirrubina indirecta ($r = 0,115$, $p = 0,032$), lactato desidrogenase ($r = 0,159$, $p = 0,003$), ferritina ($r = 0,1353$, $p = 0,011$), PCR ($r = 0,355$, $p <0,0001$). Pacientes com níveis mais elevados de AAT apresentaram mais de três episódios de infecção (OR = 1,71, IC: 1,05-2,65, $p = 0,02$), mais litíase biliar (OR = 1,75, IC: 1,03-2,97, $p = 0,02$), e receberam mais transfusões sanguíneas (OR = 2,35, IC: 1,51-3,65, $p = 0,0001$). Os polimorfismos no gene da *SERPINA1* foram analisados em 126 pacientes com DF e 100 indivíduos do GC. O genótipo selvagem (MM) foi encontrado em 115 (91,3%) pacientes com

DF e 92 (92%) indivíduos do GC; o genótipo SS foi observado em 2 (1,6%) pacientes com DF e em nenhum indivíduo do grupo CG; o genótipo MS foi encontrado em 9 (7,1%) pacientes com DF e em 6 (6%) controles; e o MZ estava presente em 2 (2%) indivíduos controle. Os nossos resultados sugerem que a AAT pode ser considerada como um marcador de prognóstico na DF, sendo associada a marcadores laboratoriais e clínicos importantes na referida patologia. Os resultados relacionados aos genótipos da *SERPINA1* enfatizam o papel do alelo mutante na diminuição da produção de AAT que pode representar um fator de risco para desfechos graves nos pacientes estudados. Estudos adicionais devem ser realizados a fim de investigar os mecanismos pelos quais a AAT se relaciona a eventos importantes na patogênese da DF.

Palavras-chave: Doença falciforme; SERPINA1; Alfa-1 antitripsina; Biomarcadores de prognóstico.

Title: Prognostic impact of Alpha-1 Antitrypsin levels and *SERPINA1* gene polymorphisms on sickle cell disease

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Correspondence: Marilda de S. Goncalves, LaboratBrasil; nd cell disease.ntrol groups individuals with genotype PI*MM had h Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brasil, Rua Valdemar Falcão, 121, Candeal-Salvador-BA-Brasil. CEP: 40.296-710 E-mail: mari@bahia.fiocruz.br

Short title: alpha -1 antitrypsin and *SERPINA1* gene on sickle cell disease

ABSTRACT

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, hematocrit, high-density lipoprotein cholesterol, urea, creatinine, and albumin; and positive correlation with mean corpuscular hemoglobin concentration, white blood cells, neutrophils, hemoglobin 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 blood transfusions (OR=2.35, CI: 1.51-3.65, p=0.0001). Our data on AAT association with others laboratory markers of hemolysis and inflammation suggest that it may be related to 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;* oxidative stress, and inflammation

INTRODUCTION

Clinical symptoms associated with sickle cell disease (SCD) are heterogeneous, with the presence of hemolytic anemia, vaso-occlusive events (VOE), infections, acute chest syndrome (ACS), pulmonary hypertension (PH), stroke, and glomerulopathy, amongst others (Steinberg, 2001; Kato et al., 2009; Rees et al., 2010; Gladwin, 2011; Ataga et al., 2014). SCD has several sub-phenotypes and the search for biomarkers related to the disease severity is very useful to patients' follow-up (Kato et al., 2007; Kato et al., 2009; Rees e Gibson, 2012).

Alpha-1 antitrypsin (AAT) is a glycoprotein of 52-KD with 394 amino acids, secreted and synthesized primarily in hepatocytes, 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 inflammation or tissue injury, and are related to inhibition of proteases that trigger inflammatory reactions (Rudnick e Perlmutter, 2005). AAT controls the tissue degradation promoted by proteases, especially elastase, since it inhibits the pro-inflammatory action of these enzymes on specific tissues, such as lung as well as in neutrophils (Eriksson e Elzouki, 1998; Bals, 2010; Gooptu et al., 2014).

AAT is encoded by the *SERPINA1* gene that is located in the protease inhibitor locus on chromosome 14q32.1. There are more than 500 single nucleotide polymorphisms (SNPs) described in the *SERPINA1* gene, and some are related to AAT expression changes and also with hepatic damage due to the retention of protein in hepatocytes, and occurrence of thrombosis, liver disease, pulmonary edema, emphysema, and chronic obstructive pulmonary disease (COPD) (Lomas e Mahadeva, 2002; Parfrey et al.,2003; Marciniak e Lomas, 2014; Stockley, 2014a; Stockley, 2014b; Stockley e Turner, 2014; Gooptu et al., 2014).

AAT deficiency is defined by the reduced concentration of this glycoprotein in serum and/or the identification of a defective genotype. The *SERPINA1* gene is highly polymorphic (Kohnlein e Welte, 2008; Bals, 2010) and among alleles associated with AAT deficiency, the most common are the S and Z (Brantly et al., 1988; Lucotte e Sesbou, 1999; Stoller e Aboussouan, 2012; Aboussouan e Stoller, 2009; Gooptu et al., 2014). The wild type allele is designated proteinase inhibitor (PI)*M, and the more severe deficient type is associated with the PI*Z allele, which results from a point mutation where adenine replaces guanine in exon 5 of the *SERPINA1* gene and results in the substitution of glutamic acid for lysine in the protein (Glu342Lys). Homozygotes for the PI*Z allele have about 15% of normal levels of AAT and have an increased risk for developing emphysema and to a lesser extent, liver disease in

neonates. PI*MZ heterozygotes produce about 60% of normal levels of AAT when compared to PI*M wild type homozygotes (Lomas et al., 1992; Bals, 2010; Stockley, 2014b).

We demonstrate the association of AAT with other laboratory parameters considered as important prognostic factors in SCD patients' evaluation; the association of decreased AAT levels and *SERPINA1* genotypes among SCD patients and health controls; and association of AAT levels and clinical picture in SCD patients. Our results suggest that AAT may change its function in SCD patients, because of the oxidative and inflammatory milieu.

MATERIALS AND METHODS

Three hundred and three unrelated SCD patients (191 HbSS and 107 HbSC), in steady state, were included in the present study. Patients mean age (± standard deviation) was 13.96 ± 9.91 years, with a median of 12.00, 25th percentile of 8.00 and 75th percentile of 16.00 years. Patients were in the outpatient pediatric hematology unit of the "Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA)" and 47% (142/303) were females. All patients were in steady state, i.e. no blood transfusion in a period of four months and any acute events, hospitalization, or infections three months prior to blood sampling. None of patients took antibiotics or corticosteroids ten days before blood sampling but was on folic acid therapy. None patients were taken hydroxyurea and the medical history was recorded from the patients' files.

The control group consisted of 132 unrelated healthy individuals without clinical and biochemical evidence of SCD; their mean age was 9.96 ± 3.17 years, and 48.5% (64/132) were female, matched for sex and age with the SCD patients group and were from the same geographical origin.

The study protocol was approved by the Research Board of "Centro de Pesquisas Gonçalo Moniz of Fundação Oswaldo Cruz (CPqGM-FIOCRUZ)", and all subjects included in the study had the diagnosis of SCD and the guardians' agreement to participate in the study and to collect biological samples. The study followed ethical principles of the Declaration of Helsinki as well as its revision, informed written consent was signed by each control subject and SCD patient's guardian, and when applicable the child acceptance was recorded. All patients lived in the Brazilian state of Bahia, and the majority was from the city of Salvador and its metropolitan area, a city with a high racial admixture (Azevedo et al., 1980).

After collection, we analyzed biological samples at Laboratory of Hematology, Genetic and Computational Biology (LHGB) of CPqGM-FIOCRUZ and at the Clinical Analyses Laboratory of the School of Pharmacy (LACTFAR), of the "Universidade Federal da Bahia (UFBA)".

Hematological analyses were performed by automated machine ABX Pentra 80 (HORIBA DIAGNOSTICS, Montpellier, FR), and blood smears were stained with Wright's stain and examined with light optic microscopy. We count reticulocytes after stain with brilliant cresyl blue supravital dye (Bain et al., 2012), and confirmed the hemoglobin profile by high performance liquid chromatography (HPLC) (Bio-Rad Variant-I; Bio-Rad, Hercules, CA, USA).

Liver, renal, lipid, inflammation, and hemolysis profiles, including AAT and ferritin serum concentration were analyzed using automated equipment A25 (Biosystems S.A, Costa Brava, Barcelona); Access 2 and Immage (Beckman Coulter, Inc., Fullerton, CA, USA).

Molecular biology analyses were performed on genomic DNA extracted from peripheral leukocytes using Flexigene DNA Kit (QIAGEN Inc., Valencia, CA, USA) and quantified by spectrophotometer (Nanodrop® ND-1000, NanoDrop Technologies, Inc., Wilmington, NC, USA). *SERPINA1* gene variants were investigated by a duplex polymerase chain reaction (PCR), using a combination of specific primers for the detection of variant alleles PI*M, PI*S, and PI*Z in a single reaction, and followed by digestion of PCR products with *TaqI* restriction enzyme (Lucotte e Sesbou, 1999).

We performed statistical analyses with the EPI INFO software version 6.04, SPSS version 18.0 and GraphPad version 5.0, and considered P values <0.05 as significant for analyses. We performed analysis of normal distribution of quantitative variables using the Kolmogorov-Smirnov test, and with the parametric ANOVA or nonparametric Kruskal-Wallis tests. We analyze qualitative or categorical variables with three or more groups by nonparametric chi-square (χ^2) test, corrected by Mantel-Haenszel and Yates tests. In the analysis of less than 4 values, we use the Fisher exact test, and we calculate the confidence interval at 95% and the odds ratio for these variables.

We use the Independent T test and Mann-Whitney for analyses of two variables comparing two groups of values within a given variable, taking into account the distribution of each variable.

RESULTS

Patients and control groups characteristics

Table I shows SCD patients characteristics presenting the mean ± standard deviation (SD) of hematological and biochemical data.

There are differential levels of AAT among SCD patients and control groups, and between SCD patients and control individuals exhibiting different genotypes of the SERPINA1 gene

SCD patients presented higher AAT levels when compared to control individuals (Fig 1). We analyzed the *SERPINA1* gene polymorphisms among 126 SCD patients, and 100 control group individuals. We found the (Pi)*MM or wide type genotype in 115 (91.3%) of SCD patients and 92 (92%) of controls; the PI*SS genotype in 2 (1.6%) SCD patients; the PI*MS genotype in 9 (7.1%) SCD patients and in 6 (6%) controls; and the PI*MZ genotype was found in 2 (2%) controls. We found higher levels of AAT among SCD patients when compared with healthy controls, and changes in AAT levels by the *SERPINA1* polymorphism presence, as previously described (Marciniak e Lomas, 2014; Stockley, 2014a; Stockley, 2014b; Stockley e Turner, 2014; Gooptu et al., 2014). If the hypothesis that AAT may be experiencing changes in its molecule as a result of oxidative stress and inflammation, changes already described in cancer with the presence of an oxidized-alpha-1 antitrypsin (Ox-AAT) as a predictive risk marker (Jamnongkan et al., 2013; Khenjanta et al., 2014), the AAT presence in SCD can be modified independently of the *SERPINA1* allele. However, the presence of some AAT alleles, especially individuals' carrier of genotypes SZ and SS, which may be more sensitive to have severe lung changes in SCD, especially in cases of pulmonary hypertension and recurrent acute chest syndrome.

The AAT is correlated and associated to hematological and biochemistry markers

AAT had negative significant correlation to red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), high-density lipoprotein cholesterol (HDL-C), urea, creatinine, and albumin, and positive significant correlation to mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), neutrophils, HbS, total bilirubin (BT), direct bilirubin (DB), indirect bilirubin (IB), lactate dehydrogenase (LDH), ferritin, and C-reactive protein (CRP) (Fig 2-4). High AAT concentration was higher among SCD patients with altered hematological and biochemistry markers (Fig 5).

Levels of AAT and clinical profile of SCD patients

AAT serum levels were associated with different clinical outcomes among the SCD patients (Fig 6). Increased levels were found in patients that had more than 2 episodes of the following: infection, gallstones, and blood transfusion during follow-up. This would suggest the participation of the molecule in biological responses. We did not find association of patients' clinical history and the presence of *SERPINA1* gene genotypes.

DISCUSSION

SCD presents epidemiological importance due to its high prevalence and high rate of morbidity and mortality worldwide. The clinical variability and the diversity of factors that modulate the disease are still not very well understood (Steinberg, 2001; Kato et al., 2007; Rees e Gibson, 2012).

The present study has demonstrated a correlation between the serum concentration of AAT (in both patients and controls) and the *SERPINA1* genotype, showing higher ATT serum levels in the wild type compared to mutant alleles. AAT is probably an important biomarker among SCD patients, and may have a role in the clinical severity of the disease, especially in those with liver and lung complications, which are prevalent among these patients. AAT levels have been investigated in some few reports in SCD, and have brought controversial results about AAT as a prognostic biomarker of the disease (Omene et al.,1980; Hedo et al., 1993; Bourantas et al., 1998; Tete-Benissan et al., 2000). Also, AAT biochemical genotypes were associated with the clinical course of SCD (Adekile et al.,1984).

Considering the *SERPINA1*, the S and Z alleles are the most frequently associated with reduced serum levels of AAT, with increased risk of developing pulmonary emphysema and liver disease secondary to AAT deficiency and decrease of its immunomodulatory and anti-inflammatory and proteinase inhibitory properties (Lucotte e Sesboüé, 1999; Köknlein e Welte, 2008). In the liver, the presence of the Z allele may facilitate the protein polymerization and hepatocyte accumulation secondary to the altered synthesis and chaperone binding induction (Bals, 2010; Tan et al., 2015). Our results showed that among SCD patients approximately 10.3% had polymorphic alleles. Therefore, AAT levels and the *SERPINA1* genotypes may need to be revisited as markers that should be included in studies related to

SCD and in future therapeutic strategies, especially of pulmonary disease among these patients (Kassim e DeBaun, 2013).

The present study also showed that high serum levels of AAT were associated with a more severe anemia, increased WBC and neutrophil counts, and altered CRP levels. In addition, AAT had significant negative correlation with biochemistry markers of HDL-C, liver and kidney function, such as albumin, creatinine, and urea. On the other hand, we found a significant positive correlation with serum bilirubin (TB, DB, and IB), LDH, ferritin and CRP, both, acute phases proteins.

Based on our results, we hypothesize that the systemic AAT present in SCD patients may change its anti-inflammatory property, once it is exposed to ROS, and it may be altered (Daemen et al., 2000; Nita et al, 2007; O'Dwyer et al, 2012; Jonigk et al., 2013; ; McCarthy et al., 2014Stockley e Turner, 2014). AAT synthesis occurs, preferentially, in the liver; it explains our finding about the positive correlation with hepatic enzymes and other stress proteins, and the negative correlation that may explain its role on renal function. The CRP is a positive acute phase protein that rises rapidly during inflammatory processes, and showed significant positive correlation with AAT levels. However, CRP is an important inflammatory marker in clinical practice due to the ease of determining its serum levels and good clinical-epidemiological correlation (Malm et al., 2013).

With regard to the negative correlation of AAT and renal parameters, our results may emphasize previous hypothesis about a cytoprotective effect of AAT in acute kidney injury (Zager et al., 2014). Our results reinforce the importance in research into prognostic markers in SCD, since they suggest a possible relationship between the levels of AAT and changes in its molecule as result of oxidative stress and inflammation, and suggest association of AAT levels with routine markers, with easy access for monitoring and estimation of severity of the disease.

Genotypes in the *SERPINA1* gene described in this study corroborate the presence of deficient AAT production. Our results suggest a direct correlation between AAT and SCD clinical manifestations, hemolysis, inflammation, and endothelial injury. This hypothesis deserves further studies, which should also focus on the interaction of the *SERPINA1* gene variant and AAT molecule changes and their involvement in the immune response. The role of oxidative stress of SCD on the AAT molecule may also prove quite worthwhile.

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Authorship contributions

MSG and MOSC conceived and designed the study; MOSC, ALCSC, and APASP performed laboratorial analysis; LCR and VML attended patients; MSG, MOSC, MBC, and APASP analyzed the data; MSG contributed reagents/materials/ analysis tools; MSG, ALCSC, AA, and MOSC wrote and gave important contribution of manuscript confection; MOSC, ALCSC, MBC, APASP, LCR, VML, AA and MSG reviewed and approved the manuscript final version.

Competing interest: the authors have no competing interests.

REFERENCES

Aboussouan, L.S., Stoller, J.K. (2009) Detection of alpha-1 antitrypsin deficiency: a review. *Respiratory Medicine*, **103**:335-341.

Adekile, A.D., Olusi, S.O., Ogunye, O.O. (1984) Alpha-I-antitrypsin phenotypes in sickle cell anaemia. *East African Medical Journal*, **61**,136-139.

Ataga, K.I., Derebail, V.K., Archer, D.R. (2014) The glomerulopathy of sickle cell disease. *American Journal of Hematology*, **89**, 907-914.

Azevedo, E.S., Alves, A.F.P., Silva, M.C.B.O., Souza, M.G.F., Lima, A.M.V.M.D., Aevedo, W.C. (1980) Distribution of abnormal hemoglobins and glucose-6-phosphate dehydrogenase variants in 1200 school children of Bahia, Brazil. *American Journal of Physical Anthropology*, **53**, 509-512.

Bals, R. (2010) Alpha-1-antitrypsin deficiency. Best Practice e Research Clinical Gastroenterology, 24, 629-633.

Bourantas, K.L., Dalekos, G.N., Makis, A., Chaidos, A., Tsiara, S., Mavridis, A. (1998) Acute phase proteins and interleukins in steady state sickle cell disease. European Journal of Haematology, 61, 49-54.

Brantly, M., Nukiwa, T., Crystal, R.G. (1988) Molecular basis of alpha 1 antitrypsin deficiency. The American Journal of Medicine, 84, 13-31.

Daemen, M.A., Heemskerk, V.H., van't Veer, C., Denecker, G., Wolfs, T.G., Vandenabeele, P., Buurman, W.A. (2000) Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. Circulation, 102, 1420-1426.

Eriksson, S. e Elzouki, A.N. (1998) Alpha 1-antitrypsin deficiency. *Baillière's Clinical Gastroenterology*, **12**, 257-273.

Gladwin, M.T. (2011) Prevalence, risk factors and mortality of pulmonary hypertension defined by right heart catheterization in patients with sickle cell disease. *Expert Review of hematology*, **4**, 593-596.

Gooptu, B., Dickens, J.A., Lomas, D.A. (2014) The molecular and cellular pathology of □1-antitrypsin deficiency. *Trends in Molecular Medicine*, **20**, 116-127.

Hedo, C.C., Aken'ova, Y.A., Okpala, I.E., Durojaiye, A.O., Salimonu, L.S. (1993) Acute phase reactants and severity of homozygous sickle cell disease. *Journal of Internal Medicine*, **233**, 467-470.

Jamnongkan, W., Techasen, A., Thanan, R., Duenngai, K., Sithithaworn, P., Mairiang, E., Loilome, W., Namwat, N., Pairojkul, C., Yongvanit, P. (2013) Oxidized alpha-1 antitrypsin as a predictive risk marker of opisthorchiasis-associated cholangiocarcinoma. *Tumour Biology*, **34**:695-704.

Jonigk, D., Al-Omari, M., Maegel, L., Müller, M., Izykowski, N., Hong, J., Hong, K., Kim, S.H., Dorsch, M., Mahadeva, R., Laenger, F., Kreipe, H., Braun, A., Shahaf, G., Lewis, E.C., Welte, T., Dinarello, C.A., Janciauskiene, S. (2013) Anti-inflammatory and immunomodulatory properties of α1-antitrypsin without inhibition of elastase. *Proceedings of the National Academy of Sciences of the United States of America*. **110**, 15007-15012.

Kassim, A.A. e DeBaun, M.R. (2012) Sickle cell disease, vasculopathy, and therapeutics. *Annual Review of Medicine*. **64**, 451-466.

Kato, G.J., Gladwin, M.T., Steinberg, M.H. (2007) Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. *Blood Reviews*, **21**, 37-47.

Kato, G.J., Hebbel, R.P., Steinberg, M.H., Gladwin, M.T. (2009) Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. *American Journal of Hematology*, **84**, 618-625.

Khenjanta, C., Thanan, R., Jusakul, A., Techasen, A., Jamnongkan, W., Namwat, N., Loilome, W., Pairojkul, C., Yongvanit, P. (2014) Association of CYP39A1, RUNX2 and Oxidized Alpha-1 Antitrypsin Expression in Relation to Cholangiocarcinoma Progression. *Asian Pacific Journal of Cancer Prevention*, **15**:10187-10192.

Köknlein, T. e Welte, T. (2008) Alpha-1 antitrypsin deficiency: pathogenesis, clinical presentation, diagnosis, and treatment. *The American Journal of Medicine*, **121**, 3-9.
Lomas, D.A., Evans, D.L., Finch, J.T., Carrell, R.W. (1992) The mechanism of Z alpha 1-antitrypsin accumulation in the liver. *Nature*, **357**, 605-607.

Lomas, D.A. e Mahadeva, R. (2002) α1-Antitrypsin polymerization and the serpinopathies: pathobiology and prospects for therapy. The Journal of Clinical Investigation, 110, 1585-1590.

Lucotte, G. e Sesboüé, R. (1999) Polymerase chain reaction detection of S and Z alpha-1-antitrypsin variants by duplex PCR assay. *Molecular and Cellular Probe*s, **13**, 389-391.

Malm, J., Danmyr, P., Nilsson, R., Appelqvist, R., Végvári, A., Marko-Varga, G. (2013) Blood plasma reference material: a global resource for proteomic research. *Journal of Proteome Research*. **12**, 3087-3092.

Marciniak, S.J. e Lomas, D.A. (2014) Genetic susceptibility. *Clinics in Chest Medicine*, **35**, 29-38.

McCarthy, C., Saldova, R., Wormald, M.R., Rudd, P.M., McElvaney, N.G., Reeves, E.P. (2014) The role and importance of glycosylation of acute phase proteins with focus on alpha-1 antitrypsin in acute and chronic inflammatory conditions. *Journal Proteome Research*, **13**, 3131-3143.

Nita, I.M., Serapinas, D., Janciauskiene, S.M. (2007) α1-Antitrypsin regulates CD14 expression and soluble CD14 levels in human monocytes in vitro. *The International Journal of Biochemistry e Cell Biology*, **39**, 1165–1176.

O'Dwyer, C.A., McElvaney, N.G., Reeves, E.P. (2012) WS17.8 Inhibition of leukotriene B4 signaling by alpha-1 antitrypsin: support for the use of aerosolized alpha-1 antitrypsin therapy in cystic fibrosis. Abstracts of the 35th European Cystic Fibrosis Conference. *Journal of Cystic Fibrosis*, **11**, S39.

Omene, J.A., Ihongbe, J.C., Lewis, J.D. (1980) Serum trypsin inhibitory capacity in sickle cell disease. *East African Medical Journal*, **57**,186-189.

Parfrey, H., Mahadeva, R., Lomas, D.A. (2003) Alpha(1)-antitrypsin deficiency, liver disease and emphysema. *The International Journal of Biochemistry e Cell Biology*, **35**:1009-1014.

Rees D.C. e Gibson, J.S. (2012) Biomarkers in sickle cell disease. *British Journal of Haematology*, **156**, 433-445.

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

Rudnick, D.A. e Perlmutter, D.H. (2005) Alpha-1-antitrypsin deficiency: a new paradigm for hepatocellular carcinoma in genetic liver disease. *Hepatology*, **42**, 514-521.
Steinberg, M.H., Forget, B.G., Higgs, D.R., Nagel R.L. (2001) *Disorders of hemoglobin genetics, pathophysiology and clinical management*, Cambridge University Press.

Stockley, R.A. (2014a) Biomarkers in chronic obstructive pulmonary disease: confusing or useful? *International Journal of Chronic Obstructive Pulmonary Disease*, **9**,163-177.

Stockley, R.A. (2014b) Alpha1-antitrypsin review. *Clinics in Chest Medicine*, **35**, 39-50.

Stockley, R.A. e Turner, A.M. (2014) α-1-Antitrypsin deficiency: clinical variability, assessment, and treatment. *Trends in Molecular Medicine*, **20**, 105-115.

Stoller, J.K., Aboussouan, L.S. (2012) A review of α1-antitrypsin deficiency. American Journal of Respiratory and Critical Care Medicine. 185:246-259.Stone, H., Pye, A., Stockley, R.A. (2014) Disease associations in alpha-1-antitrypsin deficiency. *Respiratory Medicine*, **108**, 338-343.

Tan, L., Perez, J., Mela, M., Miranda, E., Burling, K.A., Rouhani, F.N., DeMeo, D.L., Haq, I., Irving, J.A., Ordóñez, A., Dickens, J.A., Brantly, M., Marciniak, S.J., Alexander, G.J., Gooptu, B., Lomas, D.A. (2015) Characterising the association of latency with α(1)-antitrypsin polymerisation using a novel monoclonal antibody. *The International Journal of Biochemistry e Cell Biology*, **58**:81-91.

Tété-Bénissan, A.C., Duriez, P., Parra, H.J. (2000) Study of the protein profile of the Adélé tribe of Togo. *Sante*, **10**, 261-266.

Zager, R.A., Johnson, A.C., Frostad, K.B. (2014) Rapid renal alpha-1 antitrypsin gene induction in experimental and clinical acute kidney injury. *PLoS One*, **9**, e98380, 1-13.

Figure legends

Figure 1. Association of alpha-1 antitrypsin concentrations between heathy controls individuals and sickle cell disease (SCD) patients in steady state, and with polymorphism in *SERPINA1* gene between steady state SCD patients and individuals of control group. (A) SCD patients presented higher AAT levels when compared to control individuals; (B) Association of *SERPINA1* gene polymorphism and AAT concentration among SCD patients. SCD patients with genotype PI*MM had higher AAT levels than patients with polymorphic genotype analysed separately (PI*MS and PI*SS) and together. (C) Association of SERPINA1 gene polymorphism and AAT concentration among control group individuals. Control groups individuals with genotype PI*MM had higher AAT levels than individuals with polymorphic genotype analysed separately (PI*MS and PI*MZ) and together.

Figure 2. Correlation of alpha-1 antitrypsin with hematological markers in steady state sickle cell disease patients. (A) Red blood cells (RBC) are negatively correlated to alpha-1 antitrypsin (AAT); (B) Hemoglobin is negatively correlated to AAT; (C) Hematocrit is negatively correlated to AAT; (D) Mean Corpuscular Hemoglobin Concentration (MCHC) is positively correlated to AAT; (E) WBC count is positively correlated to AAT; (F) Neutrophils count is positively correlated to AAT; (G) Hemoglobin S is positively correlated to AAT.

Figure 3. Correlation of alpha-1 antitrypsin with biochemistry markers in steady state sickle cell disease patients (Part I). (A) Total bilirubin is positively correlated to AAT; (B) Direct Bilirubin is directly correlated to AAT; (C) Indirect Bilirubin is positively correlated to AAT; (D) Lactate dehydrogenase is positively correlated to AAT; (E) Ferritin is positively correlated to AAT; (F) C-Reactive Protein is positively correlated to AAT.

Figure 4. Correlation of alpha-1 antitrypsin with biochemistry markers in steady state sickle cell disease patients (Part II). (A) High Density Lipoprotein of Cholesterol (HDL-C) is negatively correlated to AAT; (B) Urea is negatively correlated to AAT; (C) Creatinine is negatively correlated to AAT; (B) Direct Bilirubin is positively correlated to AAT; (C) Indirect Bilirubin is positively correlated to AAT; (D) Albumin is negatively correlated to AAT.

Figure 5. Association of hematological and biochemistry markers in steady state sickle cell disease patients with alpha-1 antitrypsin concentrations higher and lower than the 50th percentile. Statistical analyses indicate that sickle cell disease (SCD) patients with alpha-1 antitrypsin concentration higher than the 50th percentile (158.0 mg/mL) had: (A) lower count of red blood cells (RBC); (B) lower concentration of hemoglobin (Hb); (C) lower concentration of hematocrit (Hct); (D) higher count of white blood cells (WBC); (E) higher count of neutrophils; (F) higher concentration of C-Reactive Protein (CRP); (G) higher concentration of urea.

Figure 6. Association of alpha-1 antitrypsin concentration between SCD patients and clinical history. (A) Association of AAT concentration among SCD patients with history of infection episodes; (B) Association of AAT concentration among SCD patients with history of gallstones; (C) Association of AAT concentration among SCD patients with history of blood therapy.

Table I. Laboratory characteristics of patients with sickle cell disease.

Laboratory values	N	Mean	Standard Deviation	Percentile values		
				25th	50th	75th
RBC, $x10^{12}$/mL	355	3.25	0.93	2.53	3.00	4.00
Hemoglobin, g/dL	356	9.35	1.95	8.00	9.00	11.00
Hematocrit, %	356	27.18	6.00	22.13	26.00	32.00
MCV, fL	356	85.62	9.21	79.15	85.60	92.00
MCH, ρg	356	29.62	3.78	27.00	29.65	32.00
Reticulocyte Count, %	352	6.07	2.58	4.00	6.00	7.88
Fetal hemoglobin, %	356	7.39	6.57	2.00	5.35	11.00
S hemoglobin, %	356	72.63	17.95	51.32	81.00	88.35
Leukocyte count, x 10^9/mL	356	11955.31	4180.38	8800.00	11450.00	14600.00
Neutrophil count, x 10^9/mL	356	5891.08	2789.25	3870.00	5357.00	7391.00
Eosinophil count, x 10^9/mL	356	768.17	712.07	274.00	521.50	1109.75
Lymphocyte count, x 10^9/mL	356	4327.80	2027.55	2924.50	3922.00	5229.25
Monocyte count, x 10^9/mL	356	826.21	402.89	537.00	741.00	1070.75
Platelet Count, $x10^3$/ uL	356	407.21	158.74	288.50	392.00	507.50
Glucose, mg/dL	356	75.14	19.72	68.00	74.00	79.00
Total Cholesterol, mg/dL	356	130.25	28.39	110.00	126.50	147.00
HDL-c, mg/dL	356	33.21	8.73	27.00	32.00	38.00
LDL-c, mg/dL	356	77.88	24.35	62.00	76.00	92.00
VLDL-c, mg/dL	356	19.23	9.73	13.00	17.00	23.00
Triglycerides, mg/dL	356	96.18	48.47	64.00	86.50	116.00
ALT, U/L	356	22.99	14.72	14.00	20.00	27.00
AST, U/L	356	47.68	22.13	32.00	43.00	60.00
Iron serum, mcg/dL	356	91.67	51.02	60.00	82.00	105.75
Ferritin, ηg/mL	351	305.68	458.89	81.00	167.20	320.60
Total bilirubin, mg/dL	356	1.99	1.35	1.00	1.90	2.50
Direct bilirubin, mg/dL	356	0.51	0.47	0.00	0.40	1.00
Indirect bilirubin, mg/dL	356	1.49	1.23	0.90	1.00	2.00
Total protein, g/dL	356	7.96	0.80	7.50	8.00	8.30
Albumin, g/dL	356	4.45	0.54	4.00	4.40	5.00
Globulin, g/dL	356	3.54	0.82	3.00	3.55	4.00
Uric acid, mg/dL	356	4.12	1.27	3.00	4.00	5.00
Urea nitrogen, mg/dL	356	18.17	8.07	13.00	17.00	21.00
Creatinine, mg/dL	354	0.54	0.51	0.00	1.00	1.00
C-reactive protein, mg/L	353	6.74	9.57	2.47	4.00	7.00
Antistreptolysin-O, UI/mL	353	177.68	339.38	28.50	80.00	165.00
Haptoglobin, mg/dL	353	8.82	15.75	5.83	6.00	6.00
LDH, U/L	356	964.05	531.42	597.25	855.00	1199.75

RBC: Red blood cells; MCV: Mean Cell Volume; MCH: Mean Cell Hemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very Low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase.

FIGURE 1

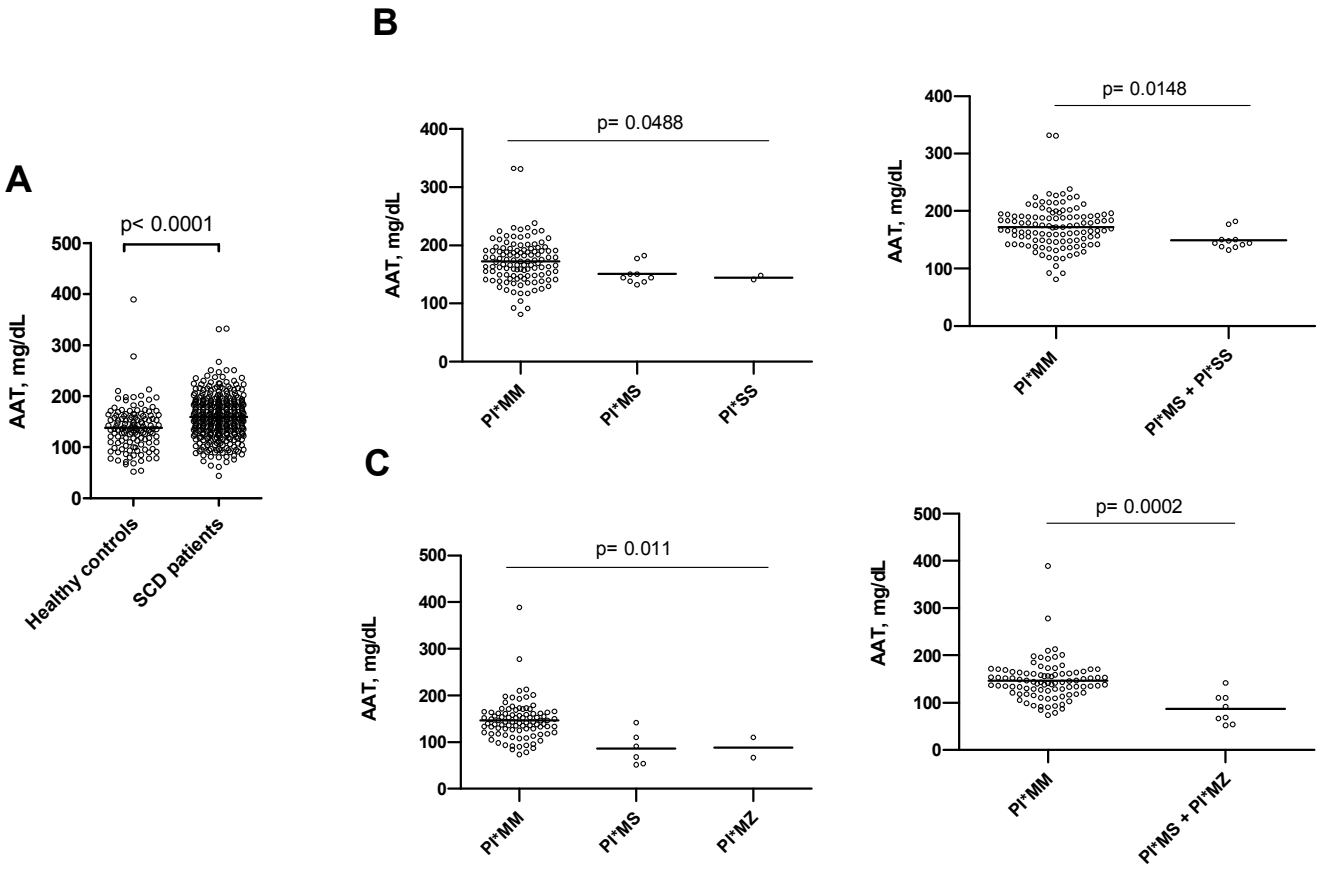

A
p< 0.0001
AAT, mg/dL
500
400
300
200
100
0
Healthy controls
SCD patients
B
p= 0.0488
AAT, mg/dL
400
300
200
100
0
PI*MM
PI*MS
PI*SS
p= 0.0148
AAT, mg/dL
400
300
200
100
0
PI*MM
PI*MS + PI*SS
C
p= 0.011
AAT, mg/dL
500
400
300
200
100
0
PI*MM
PI*MS
PI*MZ
p= 0.0002
AAT, mg/dL
500
400
300
200
100
0
PI*MM
PI*MS + PI*MZ

FIGURE 2

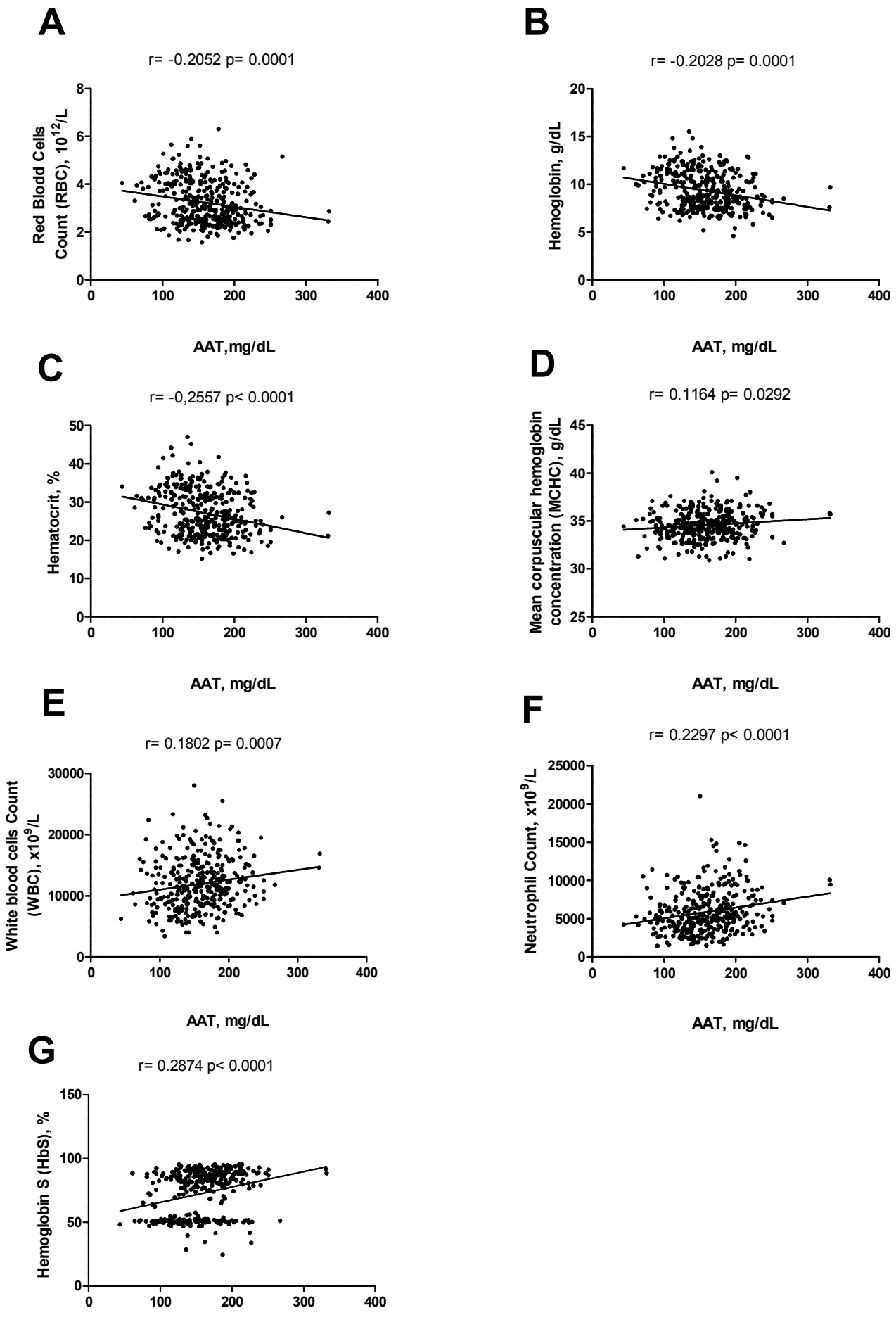

A
r= -0.2052 p= 0.0001
Red Blodd Cells Count (RBC), 10^{12}/L
AAT,mg/dL
B
r= -0.2028 p= 0.0001
Hemoglobin, g/dL
AAT, mg/dL
C
r= -0,2557 p< 0.0001
Hematocrit, %
AAT, mg/dL
D
r= 0.1164 p= 0.0292
Mean corpuscular hemoglobin concentration (MCHC), g/dL
AAT, mg/dL
E
r= 0.1802 p= 0.0007
White blood cells Count (WBC), x10^9/L
AAT, mg/dL
F
r= 0.2297 p< 0.0001
Neutrophil Count, x10^9/L
AAT, mg/dL
G
r= 0.2874 p< 0.0001
Hemoglobin S (HbS), %
AAT, mg/dL

FIGURE 3

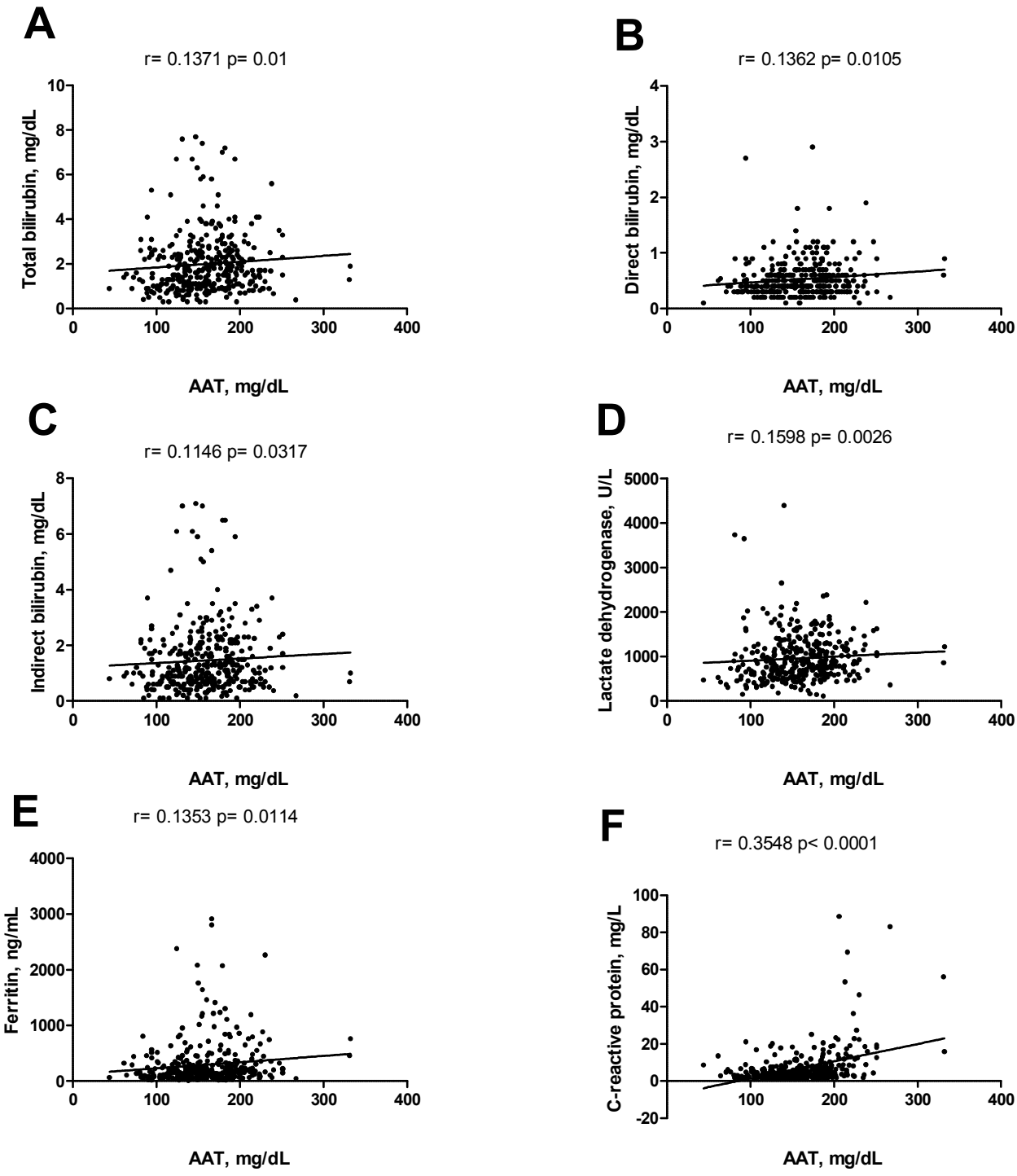

A
r= 0.1371 p= 0.01
Total bilirubin, mg/dL
AAT, mg/dL
B
r= 0.1362 p= 0.0105
Direct bilirubin, mg/dL
AAT, mg/dL
C
r= 0.1146 p= 0.0317
Indirect bilirubin, mg/dL
AAT, mg/dL
D
r= 0.1598 p= 0.0026
Lactate dehydrogenase, U/L
AAT, mg/dL
E
r= 0.1353 p= 0.0114
Ferritin, ng/mL
AAT, mg/dL
F
r= 0.3548 p< 0.0001
C-reactive protein, mg/L
AAT, mg/dL

FIGURE 4

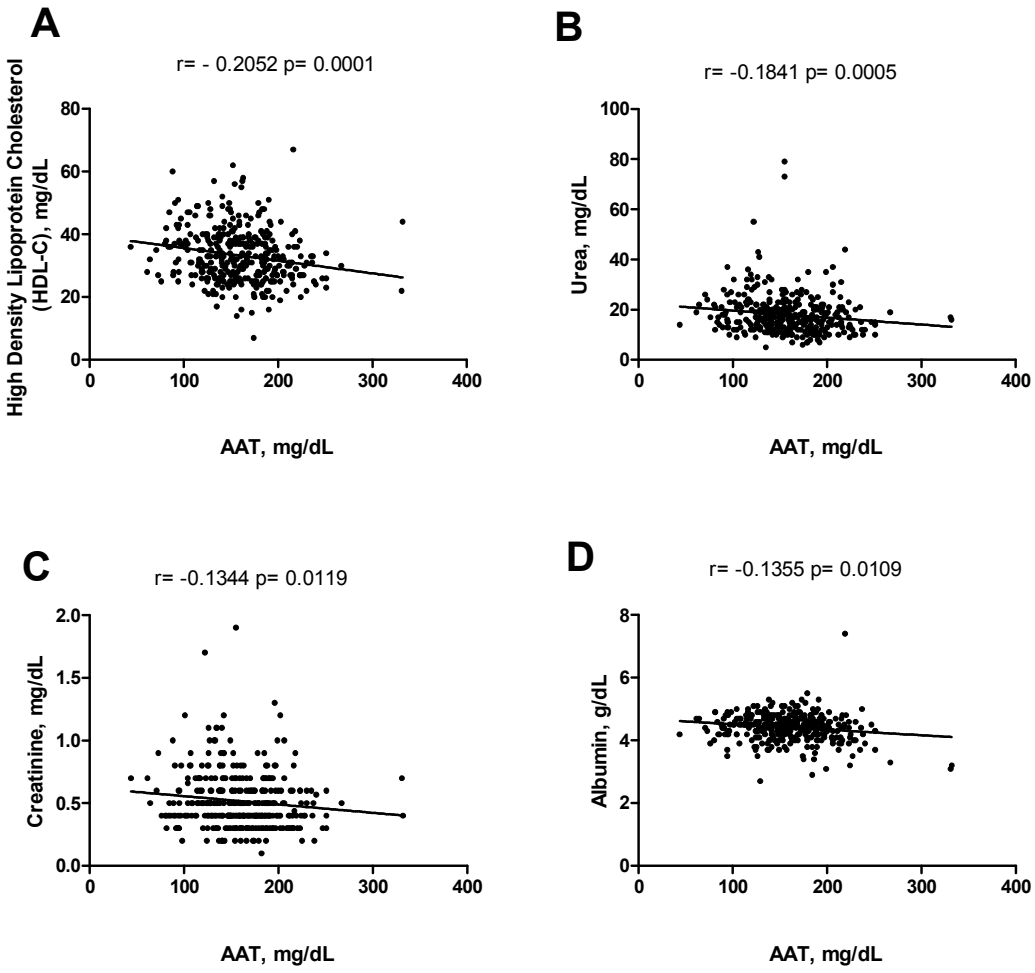

A
r= - 0.2052 p= 0.0001
High Density Lipoprotein Cholesterol (HDL-C), mg/dL
AAT, mg/dL
B
r= -0.1841 p= 0.0005
Urea, mg/dL
AAT, mg/dL
C
r= -0.1344 p= 0.0119
Creatinine, mg/dL
AAT, mg/dL
D
r= -0.1355 p= 0.0109
Albumin, g/dL
AAT, mg/dL

FIGURE 5

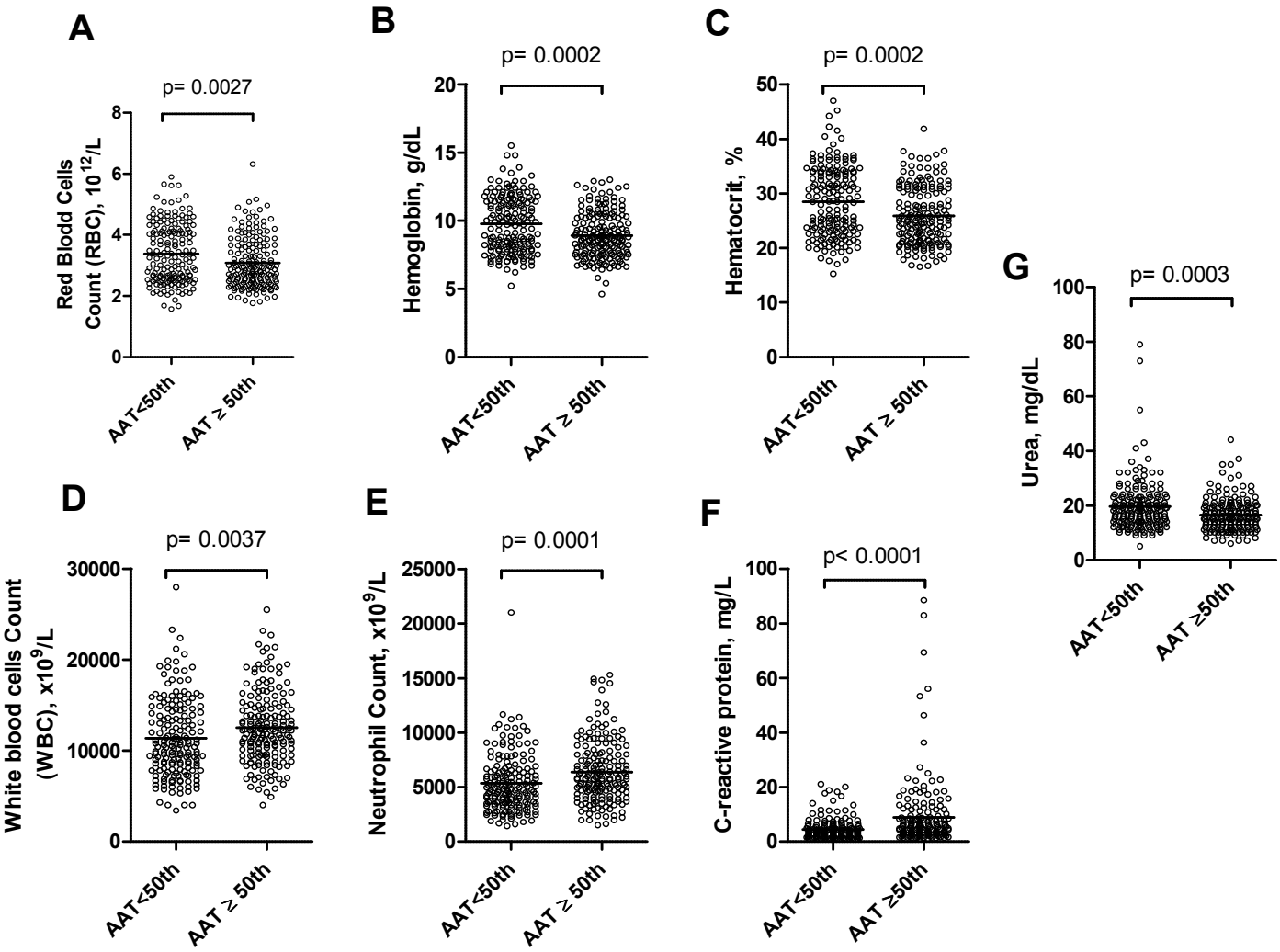
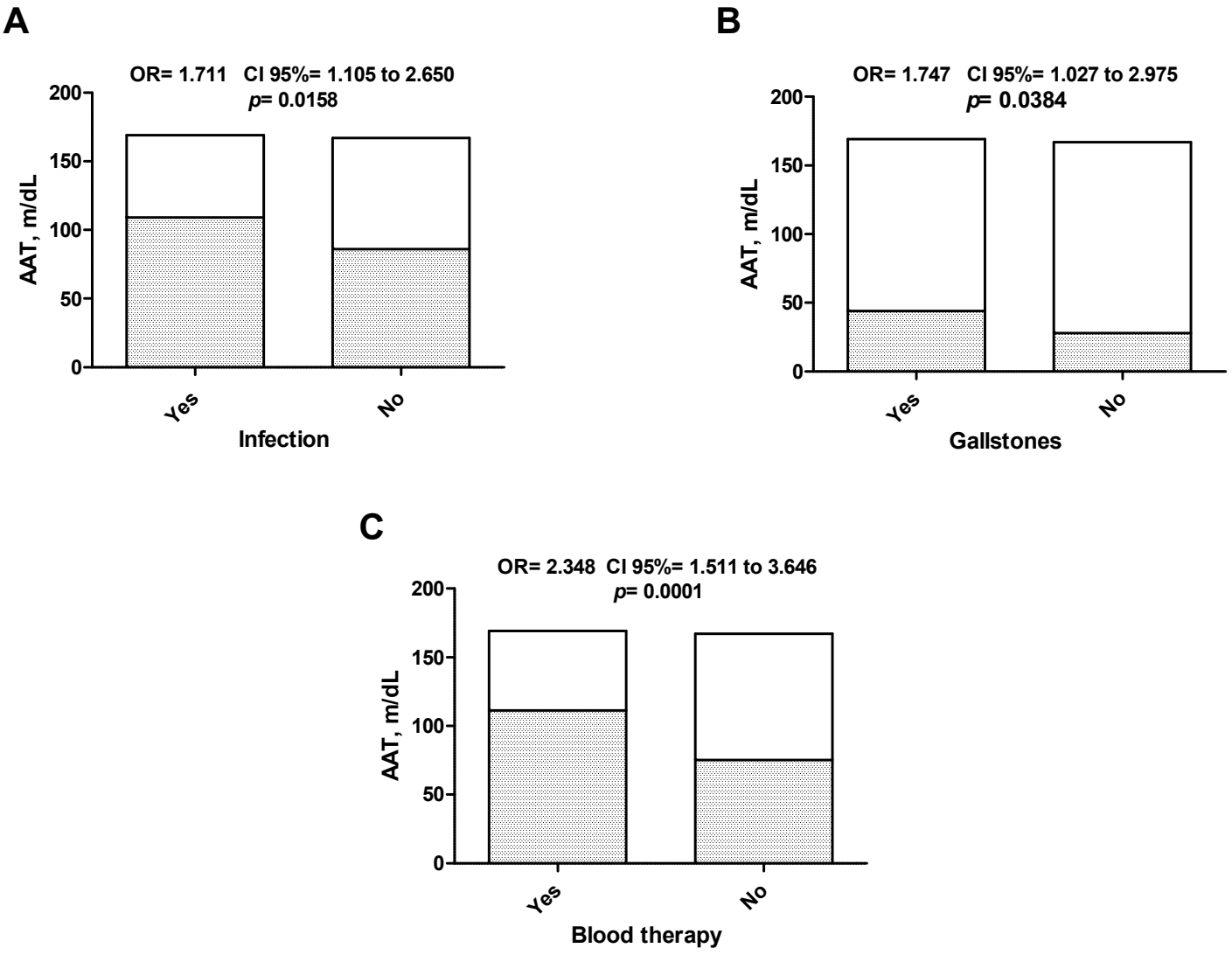

A
p= 0.0027
Red Blodd Cells Count (RBC), 10^12/L
AAT<50th
AAT ≥ 50th
B
p= 0.0002
Hemoglobin, g/dL
C
p= 0.0002
Hematocrit, %
D
p= 0.0037
White blood cells Count (WBC), x10^9/L
E
p= 0.0001
Neutrophil Count, x10^9/L
F
p< 0.0001
C-reactive protein, mg/L
G
p= 0.0003
Urea, mg/dL

FIGURE 6


A
OR= 1.711 CI 95%= 1.105 to 2.650
p= 0.0158
AAT, m/dL
200
150
100
50
0
Yes
No
Infection
B
OR= 1.747 CI 95%= 1.027 to 2.975
p= 0.0384
AAT, m/dL
200
150
100
50
0
Yes
No
Gallstones
C
OR= 2.348 CI 95%= 1.511 to 3.646
p= 0.0001
AAT, m/dL
200
150
100
50
0
Yes
No
Blood therapy

5. DISCUSSÃO

As manifestações clínicas presentes na DF são heterogêneas, com espectro amplo de alterações que compreendem o quadro de anemia hemolítica, eventos vaso-oclusivos e dolorosos, infecções e AVC entre outras (OHENE-FREMPONG e STEINBERG, 2001). A presença de alfa talassemia, os haplótipos ligados a gene da globina β e os níveis elevados de HbF são descritos como moduladores clássicos dos eventos clínicos na DF (NAGEL,1984; STEINBERG, 2001; MARTIN e THOMPSON, 2013). Estudos mais recentes estabeleceram a relação entre a LDH e o NO, por exemplo, com a apresentação de subfenótipos específicos entre os indivíduos com DF, destacando a importância da pesquisa de biomarcadores associados ao prognóstico, com ênfase para os aspectos epistáticos descritos na doença (MORRIS *et al*., 2000; KATO *et al*., 2007).

Na primeira parte deste estudo investigamos indivíduos com DF, em estado estável, provenientes do estado da Bahia, que são acompanhados na fundação HEMOBA e indivíduos saudáveis atendidos no Laboratório de Análises Clínica da Faculdade de Farmácia (LACTFAR) da Universidade Federal da Bahia (UFBA), que compuseram o grupo controle do estudo. Nos pacientes e controles foram avaliados marcadores hematológicos, de função renal, metabolismo lipídico, hemólise e inflamação, sendo que os sujeitos de ambos os grupos foram pareados em relação ao sexo e a idade. No que se refere à comparação entre pacientes estáveis e indivíduos controles, o nosso objetivo foi identificar as possíveis diferenças entre os grupos e eleger biomarcadores, de determinação rápida e rotineira, que estivessem associados as manifestações clínicas mais comuns na DF, na população estudada. Verificamos que a comparação entre os grupos revelou diferenças estatísticas para todos os marcadores estudados, corroborando com o estudo realizado por Isichei (1980), exceto para os níveis séricos de proteínas totais e frações (albumina e globulina), creatinina e antiestreptolisina O, conforme dados da tabela 1 do Manuscrito I. Essas diferenças bioquímicas observadas entre os pacientes e controles podem estar relacionadas aos diferentes mecanismos associados a patogênese da DF, que são precipitados por quadros de infecção, inflamação, bem como pelos eventos vaso-oclusivos presentes entre os pacientes (OHENE-FREMPONG e STEINBERG, 2001; STUART e NAGEL, 2004). Entre os marcadores estudados, o HDL-$_C$ se mostrou promissor como biomarcador de prognóstico, uma vez que a sua presença em níveis reduzidos, foi associada à ocorrência de pneumonia e alterações cardíacas, além de apresentar correlação inversa com marcadores de hemólise, contagem de leucócitos e plaquetas.

As concentrações normais de proteína totais, albumina e globulina, bem como a relação albumina/globulina nos pacientes com DF sugerem que não há lesão hepática precoce no grupo estudado (ISICHEI, 1980). Os níveis séricos de creatinina dentro dos valores de referência entre os pacientes com DF confirmam observações anteriores de que túbulos renais disfuncionais ocasionam o aumento na taxa de secreção de creatinina, contribuindo para a manutenção dos níveis séricos de creatinina e depuração da creatinina falsamente normais, uma vez que os pacientes apresentam hiperfiltração renal. Dessa maneira, a creatinina não é um bom parâmetro para avaliação de comprometimento renal em pacientes pediátricos com DF, sendo necessária a utilização de outros analitos clínicos, como a proteinúria, e, principalmente a avaliação de microalbuminúrica precoce (MAROUF *et al*., 2006).

A HDL é uma lipoproteína composta de várias partículas com diferentes composições e funções, sendo que uma das mais revelantes é a retirada do excesso de colesterol dos tecidos periféricos, transportando-o para o fígado para excreção pela via biliar, processo esse denominado de transporte reverso de colesterol (PRINSEN *et al*., 2003; MIYAZAKI *et al.*, 2009; PEARSON *et al.*, 2009). A redução dos níveis séricos de colesterol tem sido descrita em pacientes com DF, com redução significativa dos níveis de colesterol LDL e do HDL (SASAKI *et al*.,1983; VANDERJAGT *et al*., 2002a; SHORES *et al*., 2003; ZORCA *et al*., 2010). Neste estudo, relacionamos alterações nos lipídios, com ênfase para a redução do colesterol HDL, com manifestações graves na DF, sugerindo o papel importante dessas moléculas como potenciais marcadores de gravidade clínica nos pacientes (SEIXAS *et al*., 2009). O colesterol VLDL é uma molécula rica em triglicerídeos e desempenha função relevante na oxidação lipídica e na gênese da aterosclerose; somado a este fato, nossos dados revelaram a associação inversa entre o colesterol HDL-$_C$ e o VLDL nos pacientes com DF investigados (DJOUMESSI *et al.*, 1994).

A associação negativa observada entre a LDH e o colesterol HDL sugere que este último pode funcionar como marcador de prognóstico da hemólise intravascular e da disfunção endotelial devido as suas propriedades anti-inflamatória, antioxidante, antiagregante, anticoagulante e pró-fibrinolíticas (NOFER *et al.*, 2002). Os pacientes com DF que apresentaram níveis mais elevados do HDL-c apresentaram risco reduzido para a ocorrência de hemólise e para a disfunção endotelial, incluindo contagens menores de reticulócitos e de eritroblastos, bem como, concentração diminuída de HbS, fato que pode estar relacionado ao consumo elevado de colesterol, devido à atividade medular elevada durante a crise hemolítica. Esses indivíduos também apresentaram número reduzido de leucócitos, monócitos e plaquetas, e concentrações diminuídas de marcadores hepáticos e

hemolíticos, concentrações significativamente menores do VLDL-c, triglicerídeos e da AAT, dados que refletem as propriedades anti-inflamatórias e antioxidantes deste biomarcador (NOFER *et al.*, 2002; FREDENRICH e BAYER, 2003).

A confirmação adicional das associações citadas anteriormente foi obtida da comparação entre as concentrações de HDL-c e dos registros clínicos dos pacientes; esta investigação revelou a ocorrência maior de pneumonia e anormalidades cardíacas entre aqueles com concentrações reduzidas de HDL-c. O resultado relacionado ao risco de pneumonia pode ser explicado pela produção de auto-anticorpos específicos contra fosfolipídios oxidados; esses auto-anticorpos têm sido associados à inibição da absorção, pelos macrófagos, do colesterol LDL oxidado e a proteção contra a infecção por pneumococos virulentos (NAVAB *et al.*, 2001). Níveis reduzidos de HDL-c constituem fator de risco cardiovascular importante, sendo que a HDL e a apolipoproteína A1 foram associadas à redução das lesões e melhoria da reatividade vascular em modelos animais e humanos de aterosclerose. Estas alterações podem ser consequência da redução de lipídios oxidados e do incremento do transporte reverso de colesterol (NAVAB *et al.*, 2004).

A presença de hipertensão pulmonar (HP) está associada a uma série de alterações em testes laboratoriais (MINNITI *et al.*, 2009), sendo recentemente demonstrado o papel da via da apolipoproteína A1 e sua associação com a disfunção endotelial em pacientes com DF e HP (MINNITI *et al.*, 2009). Os pacientes com DF que apresentaram níveis reduzidos de HDL-c também apresentaram número maior de transfusões sanguíneas; este fato pode estar relacionado ao curso clínico mais grave da doença, uma vez que esta é uma estratégia terapêutica utilizada para evitar ou minimizar diversos sintomas clínicos presentes na doença, tais como AVC (OHENE-FREMPONG e STEINBERG, 2001).

A litíase biliar em pacientes com anemia hemolítica já é bem descrita como resultante do acúmulo de estruturas compostas por bilirrubinato de cálcio. Tendo em vista os resultados de nossas correlações entre colesterol e triglicerídeos com a hemólise, propomos que os cálculos biliares, em pacientes com DF, podem estar relacionados diretamente à hemólise e geração de bilirrubina e, indiretamente, ao colesterol e aos lipídios, necessitando confirmação através de novas estratégias de investigação. A associação de proteínas de fase aguda e colelitíase pode ser explicada pela resposta ao estresse oxidativo ocorrido em consequência da lesão traumática ou a mecanismos relacionados a infecção, incluindo hipermetabolismo e catabolismo proteico, que contribuem para a ocorrência de resposta inflamatória (STEINBERG, 1999).

No manuscrito II analisamos a associação entre os níveis de PGE_2, LTB_4 e moléculas associadas ao perfil lipídico em crianças em estado estável com DF. As crianças com DF, geralmente, apresentam alterações nos índices hematológicos, com presença de anemia, aumento dos níveis de marcadores de hemólise e alterações metabólicas, quando comparadas com crianças saudáveis, mesmo quando os pacientes estão em estado estável (STEINBERG, 1996; KATO *et al.*, 2007; SEIXAS *et al.*, 2010; MILTON *et al.*, 2012). Essas alterações têm sido associadas a eventos inflamatórios, vaso-oclusivos, dolorosos e com inúmeros eventos clínicos presentes na doença com exacerbação de várias vias de sinalização, com a ativação de leucócitos, hemácias, plaquetas e células endoteliais (OKPALA *et al*., 2002; OKPALA, 2004; OKPALA, 2006). Além disso, a presença de hipóxia e as alterações nos lipídios da membrana eritrocitária contribuem para a manutenção do ambiente inflamatório e de outros distúrbios metabólicos descritos na doença (CONNOR *et al.*, 1997;. SETTY *et al.*, 1996;. DE JONG *et al*., 1997).

A PGE_2 é um componente lipídico, resultante da ação da ciclo-oxigenase (COX) e derivado do metabolismo do ácido araquidônico, que medeia diversos mecanismos fisiológicos, e atua através da ligação de receptores de prostaglandina (PE), caracterizado como receptores acoplados à proteína G (READER *et al*., 2011). O aumento dos níveis de PGE_2 e COX-2 tem sido associado a várias doenças, incluindo doenças inflamatórias, e com a disfunção endotelial (WONG *et al*., 2010). Nosso estudo descreveu a associação de níveis séricos elevados de PGE_2 com alterações nos marcadores relacionados à disfunção hepática e hemólise (aspartato aminotransferase, bilirrubina total e frações, lactato desidrogenase e heme livre); também com alterações no inibidor tecidual da metaloproteinase 1 (TIMP-1), com marcadores de anemia (Hb, Hct) e a ativação de leucócitos e plaquetas. O LTB_4 só foi relacionado a alterações de uréia. Propriedades inflamatórias foram descritas, para a PGE_2, em pacientes com DF; assim como, os níveis elevados de PGE_2 têm sido associados ao aumento de citocinas inflamatórias e a ativação leucocitária (GRAIDO-GONZALEZ *et al.*, 1998; NORTH *et al.*, 2007; KONYA *et al*., 2011; BARRIE *et al.*, 2011; HEGYI *et al.*, 2012), aspectos muito conhecidos na DF e que indicam a PGE_2 como um possível biomarcador de gravidade nesta patologia. No que se refere à associação entre o aumento do LTB_4 e ureia, não há nenhuma descrição na literatura e esse resultado pode estar relacionado a participação dessa molécula nas alterações renais descritas entre pacientes com DF.

Nossos dados sobre a correlação entre o HDL-c, colesterol total e triglicérides e marcadores bioquímicos e hematológicos já foram descritos, e outros os estudos sobre alterações dos lipídios têm demonstrado associações importantes entre a DF e estas desordens

metabólicas. A diminuição do HDL-$_C$ foi descrita não somente quantitativamente, mas também qualitativamente; essa redução foi associada à ação anti-inflamatória e ao uso como potencial biomarcador de gravidade clínica na DF (SASAKI *et al.*, 1983; DJOUMESSI *et al*., 1994; VANDERJAGT *et al.*, 2002a; VANDERJAGT *et al.*, 2002b; SHORES *et al.*, 2003; SEIXAS *et al.*, 2009;. ZORCA *et al.*, 2010). Em relação à clínica dos pacientes, as alterações têm sido associadas à produção de fosfolipídios oxidados, aumentando os distúrbios metabólicos, contribuindo para dano a órgãos e para a disfunção endotelial nos pacientes com DF (NAVAB *et al.*, 2001).

A presença de alterações na membrana lipídica dos eritrócitos falciformes já foi descrita em estudos anteriores (KUCUK *et al.*, 1992; CONNOR *et al.*, 1997;. SETTY *et al.*, 1996; DE JONG *et al.*, 1997), os nossos resultados relativos ao aumento da migração de neutrófilos podem ser explicados com base nas alterações dos lipídios da membrana do eritrócito e da exposição destes produtos metabólicos que contribuem para o ambiente inflamatório presente na doença; estes resultados são confirmados pela constatação da diminuição da migração de neutrófilos após a exposição dos pacientes ao soro com sinvastatina, provavelmente pela ação da mesma sobre o colesterol oxidado. Um achado importante em nosso estudo foi o fato de que a sinvastatina não reduziu a migração de neutrófilos em indivíduos saudáveis. Os dados discutidos até este parágrafo referem-se à primeira parte do estudo e compõem os manuscritos I e II

A próxima etapa do nosso estudo teve como objetivo responder as questões geradas nos dois primeiros manuscritos. Dessa forma, conduzimos um estudo prospectivo que incluiu pacientes com DF em estado estável, atendidos na HEMOBA e pacientes em crise, que estavam internados no Hospital Santo Antônio (HSA) das Obras Sociais Irmã Dulce (OSID). Para tal, investigamos biomarcadores associados à inflamação, hemólise e remodelamento vascular. Nessa investigação encontramos níveis sistêmicos mais elevados de PGE_2, LTB_4 e TGF-β em pacientes com DF em estado estável, e concentrações séricas mais elevadas de TNF-α, IL-1β e IL-10 em pacientes com DF em crise. Os níveis séricos de heme livre, MMP-9 e TIMP-1 aumentaram nos grupos de pacientes com AF, em estado estável e em crise e foram associados a alterações de marcadores hematológicos, bioquímicos e inflamatórios entre pacientes com DF em estado estável, apoiando a hipótese de que a expressão continuada de heme livre, MMP-9 e de TIMP-1 pode estar associada ao processo inflamatório crônico observado nesses pacientes.

A alteração do metabolismo lipídico descrita em pacientes com DF tem sido associada à assimetria de fosfolipídios dos eritrócitos falciformes, contribuindo para alterações

metabólicas, tais como desidratação, perturbação da membrana, desequilíbrio de cátions e hipercoagulabilidade (CONNOR *et al.*, 1997; DE JONG *et al.*, 1997; KUYPERS, 2007). Além disso, eicosanóides e diacilglicerois são liberados, secundariamente, por células endoteliais por estímulo provenientes das hemácias falciformes (SETTY e STUART, 2002), aumentando produtos do ácido araquidônico, tais como PGE_2 e LTB_4, cuja síntese está associada ao controle da resposta inflamatória (KUEHL e EGAN, 1980; WONG *et al.*, 2010). No manuscrio III relatamos níveis elevados de PGE_2 e LTB_4 em pacientes com AF em estado estável em comparação com pacientes em crise e indivíduos sadios (Figura 1A e B). Estes dados sugerem que a PGE_2 e LTB_4 são importantes marcadores de inflamação crônica em pacientes com DF e que a sua síntese é induzida por uma interação anormal entre eritrócitos e células endoteliais e pela presença de um ambiente pro-oxidante crônico presente na doença. Em conjunto, esses fatores contribuem para a disfunção endotelial encontrada nestes pacientes.

O LTB_4 tem sido referido como um quimioatrativo de neutrófilos e associado ao aumento da adesão destes ao endotélio (SETTY *et al.*, 1996). No entanto, Monteiro e cols. (2011) descreveram previamente o processo inflamatório neutrofílico heme dependente durante a hemólise, que foi associado a atividade endógena de LTB_4 proveniente de macrófagos. Graido-Gonzalez e cols. (1998) relataram o aumento nos níveis de PGE_2 em pacientes com AF em crise. Além disso, Setty e Stuart (2002) descreveram o aumento no LTB_4 em pacientes com AF e Lanaro e cols. (2009) descreveram o aumento nos níveis de PGE_2 em pacientes com AF em estado estável, mesmo após a administração de HU. Os dados relativos as concentrações de PGE_2 e LTB_4 em pacientes com DF permanecem controversos. Esses mediadores podem atuar por meio de uma ampla gama de funções, como descrito anteriormente (IBE *et al.*, 1997; NORTH *et al.*, 2007; WANG *et al.*, 2010; BARRIE *et al.*, 2011; KONYA *et al.*, 2011; HEGYI *et al.*, 2012); dessa maneira, são necessárias avaliações mais aprofundadas visando esclarecer o papel do LTB_4 na inflamação associada à DF e desvendar a rede complexa de mecanismos envolvidos na patogênese da doença.

O TGF-β é uma citocina com multiplas funções associada à diferenciação de células Th17 (HAN *et al.*, 2012), ao desenvolvimento de miofibroblastos por meio do seu receptor (TGFβR), seguido pela geração de ROS; e, juntamente com a endotelina-1, induz a deposição de matriz extracelular (LAMBERS *et al.*, 2013; YANG *et al.*, 2013). O nosso estudo demostrou o aumento nos níveis séricos de TGF-β em indivíduos com AF em estado estável (conforme figura 1C do manuscrito III), o que sugere que o TGF-β desempenha papel no remodelamento vascular nesta doença, e que, provavelmente, contribui para a alteração

vascular através da deposição de matriz extracelular. No entanto, o TGF-β pode atuar como um gerador de ROS por meio da ativação do seu receptor, que participa tanto do estreitamento vascular, quanto da disfunção endotelial e da inflamação (YANG *et al.*, 2013).

Os níveis sistêmicos de MMPs e TIMPs, bem como a razão entre eles, têm sido associados a eventos normais e patológicos, incluindo remodelamento tecidual, carcinogênese, metástase, angiogênese, sepse, dislipidemia, hemodiálise, esclerose múltipla, obesidade, síndrome metabólica e aterosclerose (BELO *et al.*, 2009; DEROSA *et al.*, 2009a; DEROSA *et al*., 2009b; LORENTE *et al.*, 2009). Nossos resultados mostram o aumento nos níveis séricos de MMP-9 e TIMP-1 nos dois grupos de pacientes com AF investigados, em estado estável e em crise, com o aumento da razão MMP-9/TIMP-1 (Figura 1E-F do manuscrito III). Estes dados sugerem que a produção contínua de MMP-9, em ambos os grupos, pode representar processos ativos de remodelamento da matriz e manutenção da destruição e degradação tecidual nestes pacientes. A associação entre níveis elevados de MMP-9 e o aumento no número de leucócitos e neutrófilos nos pacientes com DF em estado estável (Figura 10A, B do manuscrito III) sugere uma ativação leucocitária, relacionada, provavelmente, à transmigração dos leucócitos através da parede vascular. Esta ativação leucocitária envolve a ativação da proenzima MMP-9 neutrofílica, que promove a geração de MMP-9 e resulta em mudanças nas células vasculares, aumento da resposta inflamatória e disfunção endotelial. A elevação da MMP-9 nos neutrófilos e a sua relação com o aumento do ambiente inflamatório e adesão vascular foram descritas nos processos de indução a angiogênese e inflamação secundária a trombose venosa em pacientes com síndrome metabólica (WAKEFIELD *et al.*, 2008; APLIN *et al.*, 2009; ARDI *et al.*, 2009; GONÇALVES *et al.*, 2009).

O aumento dos níveis séricos de TIMP-1 no grupo de pacientes com AF incluidos no presente estudo pode estar associado a uma função independente da MMP-9 e ao ambiente hipóxico, encontrado nos pacientes com AF. Nas nossas análises encontramos a associação entre os níveis elevados de MMP-9 e de TIMP-1 (Figura 10C do manuscrito III), sugerindo que o TIMP-1 apresenta diversas funções; sendo que, esta hipótese pode ser validada pela observação das associações entre o aumento do TIMP-1 e os níveis de marcadores bioquímicos de hemólise, inflamação e marcadores hematológicos de anemia, estes últimos associados à hipoxia (Figura 8 e Figura 9 manuscrito III).

Os nossos resultados demonstram a associação entre o aumento do TIMP-1 e alterações nos lípidos e marcadores de hemólise (Figura 8 manuscrito III), sugerindo que esta molécula está envolvida com a geração de ROS e a produção de heme. Os níveis elevados de

TIMP-1 foram descritos como acionadores do fator 1 induzível por hipóxia (HIF-1), que tem sido associado com alterações pró-metastáticas no microambiente cancerígeno. Além disso, o aumento nos níveis de TIMP-1 está relacionado a indução de miRNA210, que regula a alteração metabólica de uma via glicolítica (KONDO *et al.*, 2002; AKHAVANI *et al.*, 2009; CUI *et al.*, 2012). Observamos também a associação entre níveis elevados de TIMP-1 e o aumento da contagem de plaquetas, o que sugere que este marcador pode estar associado a ativação plaquetária e com a expressão de fator tecidual, mediado por heme, em células endoteliais, ocasionando o aumento da atividade procoagulante. Este fenômeno foi estudado anteriormente por Setty e cols. (2008) usando células endoteliais humanas de cordão umbilical (HUVECs) na simulação de um ambiente hemolítico. O desequilíbrio da relação de MMP-9/TIMP-1, descrito neste estudo, sugere que a MMP-9 contribui para a disfunção endotelial na DF e para o ambiente inflamatório comumente descrito nestes pacientes (Wakefield *et al.*, 2008; Ardi *et al.*, 2009; Gonçalves *et al.*, 2009).

O heme livre e as ROS são liberados durante o catabolismo da Hb que se segue à hemólise intravascular (FERREIRA *et al.*, 2008; TAYLOR *et al.*, 2008; BELCHER *et al.*, 2010). Os efeitos nocivos do heme no microambiente vascular têm sido associados a necrose programada de macrófagos peritoneais em modelos de ratos C57BL/6, selvagens e *knockout,* e com a produção de TNF-α e o aumento da produção de ROS (FORTES *et al.*, 2012). Este estudo mostrou o aumento do heme nos paciente com AF, em crise e no estado estável (Figura 1G manuscrito III), sugerindo que níveis sistêmicos elevados de heme livre podem estar diretamente relacionados ao estado de hiper-hemólise crônica anteriormente descrito nesses indivíduos (VILAS-BOAS *et al.*, 2010). Esta relação, provavelmente, é responsável por manter o estado inflamatório crônico e pela geração contínua de ROS, o que levaria a uma disfunção endotelial progressiva (KATO *et al.*, 2007; FERREIRA *et al.*, 2008).

Considerando o aumento nos níveis plasmáticos do heme livre em ambos os grupos de pacientes com AF, avaliamos a associação entre o heme e marcadores lipídios, hematológicos, bioquímicos e inflamatórios nesses pacientes (Figuras 4-7, manuscrito III). Os resultados mostraram que este produto da lise eritrocitária pode interferir com diversas vias associadas à gravidade clínica nos pacientes com DF. Dessa forma, os nossos dados indicam uma associação entre o heme livre e alterações em marcadores lipídicos, de hemólise; alguns inflamatórios e de remodelamento, como o TGF-β, TIMP-1, PGE_2 e a IL-12, o que reforça o papel fundamental do heme na hiper-hemólise crônica e no processo inflamatório crônico descritos na doença (UZUNOVA *et al.*, 2010; VILAS-BOAS *et al.*, 2010).

Níveis alterados de citocinas pró-inflamatórias e anti-inflamatórias têm sido descritos em pacientes com AF com envolvimento na gravidade clínica da doença e na manutenção do estado inflamatório (GONÇALVES *et al.*, 2001; ASSIS *et al.*, 2005; BRITTAIN E PARISE, 2007; LANARO *et al.*, 2009). Nós encontramos níveis elevados de IL-1β, IL-6, TNF-α e IL-10 em pacientes com AF em crise, e níveis elevados de IL-8 e IL-12 em pacientes com AF em estado estável, embora estas últimas citocinas também tenham sido encontradas em concentrações elevadas nos pacientes com AF em crise (Figuras 2A-F, masnuscrito III). Estes dados são consistentes com estudos anteriores, incluindo os realizados por nosso grupo (ABBOUD *et al.*, 2000; GONÇALVES *et al.*, 2001; PATHARE *et al.*, 2004; ASSIS *et al.*, 2005; LANARO *et al.*, 2009; CERQUEIRA *et al.*, 2011; KEIKHAEI *et al.*, 2013; VEIGA *et al.*, 2013). A produção de citocinas pró-inflamatórias em pacientes com DF foi associada a contagem de células mononucleares e aos neutrófilos, com consequente alteração das características adesivas do ambiente, contribuindo para a patogênese da doença. Além disso, estas citocinas têm sido associadas à disfunção vascular, leucocitose, e ao envolvimento inicial de neutrófilos, seguido por monócitos e células endoteliais, sendo que estas podem servir como fonte dos referidos mediadores (GONÇALVES *et al.*, 2001; ASSIS *et al.*, 2005; OKPALA, 2006; LANARO *et al.*, 2009).

As propriedades imunomoduladoras da IL-10 são bem conhecidas, sendo que o aumento nos níveis desta citocina foi associado a resposta anti-inflamatória, diminuição do ambiente pró-inflamatório, e, recentemente, a proteção contra a disfunção vascular (KINZENBAW *et al.*, 2013; TABAS E GLASS, 2013). A IL1-β está associada a danos aos tecidos e a fibrogênese e, em conjunto com a IL-6, foi relacionada com doença vascular (GIELING *et al.*, 2009; MIWA *et al.*, 2013; SAXENA *et al.*, 2013). O aumento da IL-12 nos pacientes em estado estável investigados no presente estudo enfatiza o papel desta citocina como mediador da resposta inflamatória na AF (BARRIE E PLEVY, 2005).

A análise da curva ROC proporcionou a identificação de marcadores associados aos pacientes com AF em em estado estável e em crise (Figuras 3A e 3B, manuscrito III, respectivamente). Os marcadores encontrados exibiram sensibilidade, especificidade e acurácia elevadas, com valor preditivo alto para o acompanhamento de pacientes com AF. Estes biomarcadores apresentam-se como excelentes perspectivas no estudo de possíveis alvos terapêuticos na DF.

No quarto momento do estudo investigamos os níveis séricos da alfa 1 antitripsina (AAT) em pacientes com DF, buscando associá-los a marcadores hematológicas, bioquímicos, a eventos clínicos e com a presença dos polimorfismos no gene *SERPINA1*.

Nossos resultados revelaram que os níveis elevados de AAT foram associados a presença de anemia mais grave e ao aumento das contagens de leucócitos e neutrófilos; além de níveis alterados de CRP e baixos níveis de uréia. Adicionalmente, os resultados obtidos indicam que a AAT teve correlação negativa com marcadores hematológicos referentes à anemia, avaliação das funções hepática e renal, tais como a albumina, creatinina e uréia, e com o HDL-c. Por outro lado, encontramos correlação positiva da AAT com as bilirrubinas (total, direta e indireta), LDH, ferritina e CRP, sendo estas últimas proteínas de fase aguda.

Nossos resultados estão de acordo com estudos anteriores que descreveram as propriedades imunomoduladoras e anti-inflamatórias da AAT, ressaltando as características descritas anteriormente como a alteração da adesão de neutrófilos ao endotélio e seu efeito sobre a redução da expressão de receptores semelhantes a Toll e de citocinas pró-inflamatórias, principalmente no pulmão (DAEMEN *et al.*, 2000; JONIGK *et al.*, 2013; STOCKLEY E TURNER, 2014). Além disso, como a AAT é sintetizada preferencialmente no fígado, esse fato pode explicar os nossos dados sobre a correlação positiva da mesma com enzimas hepáticas e outras proteínas de estresse, bem como a correlação negativa da ATT com marcadores de função renal que pode estar relacionada às propriedades da mesma na regulação dos referidos marcadores. A CRP é uma proteína de fase aguda que aumenta rapidamente durante processos inflamatórios, sendo que esta também apresentou correlação positiva e significativa com os níveis de AAT. Dessa forma, em pacientes com DF, a CRP está aumentada assim como a AAT. A CRP tornou-se um marcador inflamatório importante devido a facilidade de determinação dos seus níveis séricos e a boa correlação clínico-epidemiológica apresentada pela mesma (MALM *et al.*, 2013).

O aumento da produção de AAT tem sido relacionado à disfunção hepática, a resposta inflamatória e ao dano tecidual (ERIKSSON E ELZOUKI, 1998; TRAINA *et al.*, 2007; BALS, 2010). A AAT tem papel importante na regulação de leucócitos, incluindo a expressão de moléculas de superfície em monócitos e a inibição da elastase e outras serina proteases produzidos pelos neutrófilos. Assim, a AAT controla a degradação tecidual promovida por proteases, especialmente pela elastase neutrofílica, e inibe a ação destas enzimas pró-inflamatórias em tecidos específicos, tais como o efeito de elastase neutrofílica nos pulmões, que apresenta papel importante na doença pulmonar obstrutiva crônica e no enfisema (LOMAS *et al.*, 1992; CHURG *et al.*, 2001; JANCIAUSKIENE *et al.*, 2007; NITA *et al.*, 2007; STOCKLEY, 2014a; STOCKLEY, 2014b; STOCKLEY e TURNER, 2014; STONE *et al.*, 2014). A despeito da função inibidora de proteases da AAT, ela também tem características anti-inflamatórias, com atividade imunorreguladora sobre algumas células,

particularmente, os linfócitos, monócitos, macrófagos e neutrófilos, que regulam a síntese de leucotrieno B4 (LTB_4) e de citocinas pró-inflamatórias, tais como a IL-8, TNF-α, IL-1 e IL-6. Curiosamente, foi descrito um efeito citoprotetor da AAT impedindo o acúmulo da elastase neutrofílica nos túbulos renais, em modelos experimental e humano de lesão renal aguda (ZAGER *et al.*, 2014). Os resultados relacionados com marcadores renais podem apoiar a hipótese, anteriormente descrita, da AAT como um biomarcador de doença renal, que pode ser relevante nos pacientes com DF, embora sejam necessários estudos mais aprofundados para confirmação dessa suspeita.

O presente estudo sugere, além disso, que a AAT desempenha função importante na patogênese da DF, uma vez que o aumento dos níveis de bilirrubinas está associado à hemólise, ao aumento da contagem de leucócitos e as alterações nos níveis de CRP, sendo associado, dessa forma, à lesão endotelial e a inflamação crônica descritas nos pacientes com DF (REES E GIBSON, 2012). Em relação aos parâmetros hematológicos, nossos resultados mostram a ocorrência de uma correlação negativa entre a contagem de eritrócitos, a dosagem de hemoglobina e o hematócrito com AAT, o que pode fortalecer a hipótese anteriormente descrita (ZAGER *et al.*, 2014).

No presente estudo, associamos o aumento da concentração da AAT com as manifestações clínicas e as alterações inflamatórias crônicas observadas nos pacientes com DF. As propriedades inibidoras de proteases já descritas para AAT justificam o seu aumento nos episódios de infecção; a ocorrência de cálculos biliares pode ser relacionada com a sua produção hepática e também com a repercussão orgânica da hemólise; o aumento do uso de hemoderivados pode representar uma resposta complementar a hemólise, inflamação e alguma alteração do sistema imune, entre estes pacientes, bem como a associação com a gravidade clínica da doença (KATO *et al.*, 2007).

Por meio dos resultados obtidos no nosso trabalho foi possível correlacionar a concentração de AAT (pacientes e controles) com o genótipo do gene *SERPINA1*, mostrando uma diferença entre os níveis séricos da ATT entre o genótipo selvagem e na presença dos alelos mutantes. Sugerimos que a AAT pode ser um biomarcador importante de doença nos pacientes com DF, estando associada ao aumento da gravidade clínica, em particular das doenças hepática e pulmonar, que apresentam prevalência elevada entre os pacientes. Os níveis de AAT têm sido investigados em alguns relatos esporádicos de DF, mas a ATT sérica não foi considerada como biomarcador de doença ou correlacionada com sintomas clínicos, mas enfatizamos que a mesma pode estar sofrendo alterações conformacionais e assumindo uma natureza inflamatória em decorrência da sua exposição direta a produtos do estresse

oxidativo (OMENE *et al.*, 1980; HEDO *et al.*, 1993; BOURANTAS *et al.*, 1998; TETE-BENISSAN *et al.*, 2000).

Considerando o polimorfismo no gene *SERPINA1*, sabe-se que os alelos mutantes S e Z são os mais frequentemente encontrados e que estão associados a níveis séricos reduzidos de AAT, com gravidade variável da doença de acordo com os genótipos e, consequentemente, com o aumento no risco de enfisema pulmonar, doença hepática secundária a deficiência de AAT e diminuição das suas propriedades imunomoduladora, anti-inflamatória e inibidora de proteases (LUCOTTE E SESBOÖÉ, 1999; KÖKNLEIN E WELTE, 2008). No fígado, a presença do alelo Z pode facilitar a polimerização da proteína e o seu acúmulo nos hepatócitos, secundário a alteração de sua síntese (BALS, 2010). Nossos resultados permitiram avaliar que cerca de 10,3% dos pacientes com DF apresentaram o alelo mutante, embora a grande maioria tenha o genótipo selvagem. Assim, a investigação dos níveis de AAT e a caracterização dos genótipos da *SERPINA1* podem compor uma nova abordagem sobre um antigo marcador que pode ser promissor na compreensão da patogênese da DF e na discussão de estratégias terapêuticas novas para a referida patologia (KASSIM E DEBAUN, 2013).

6. CONCLUSÕES

- Pacientes com DF podem apresentar sub-fenótipo dislipidêmico específico, caracterizado por níveis reduzidos de HDL-C, hipertrigliceridemia e níveis elevados de VLDL-C, associados a biomarcadores, como aqueles relacionados com a inflamação;
- O HDL-c teve destaque como possível biomarcador de prognóstico associado a manifestações clínicas importantes em crianças com DF;
- O aumento na migração dos neutrófilos frente ao estímulo com soro de indivíduos com DF, pode ser explicado pela presença de produtos proveniente da oxidação de lipídios;
- Os níveis das citocinas estudadas são elevados nos indivíduos com DF (estáveis e em crise) quando comparados aos controles saudáveis; IL-1β, IL-6 e TNF-α e são preditores de crise em indivíduos com DF;
- LTB_4, PGE_2 e TGF-β apresentaram elevação em suas concnetrações em indivíduos com DF em estado estável e redução em indivíduos em crise, quando comparados aos controles. LTB_4, PGE_2 e TGF-β são preditores do estado estável da DF;
- O heme livre, MMP-9 e TIMP-1 encontram-se elevados nos pacientes com AF, mesmo aqueles em estado estável da doença, sugerindo um possível papel do heme livre na hemólise crônica; bem como, da MMP-9 e do TIMP-1 no processo inflamatório crônico presente na DF;
- Existe associação entre os níveis sistêmicos de heme livre, MMP-9 e TIMP-1 e marcadores de hemólise e inflamação nos pacientes com DF;
- O TIMP-1 parece desempenhar papel importante na patogênese da DF, especialmente na resposta a hipóxia;
- Os níveis de AAT apresentaram relação com marcadores laboratoriais comumente investigados na rotina laboratorial, incluindo aqueles utilizados no monitoramento e avaliação da gravidade da doença;
- Os diferentes genótipos do gene *SERPINA1* descritos neste estudo mostraram relação com as concentrações de ATT nos pacientes com DF estudados; os dados sugerem a correlação di*reta* entre AAT e as manifestações clínicas naDF;
- Os resultados reforçam a importância da investigação de marcadores novos de prognóstico na DF, visando identificar moléculas alvo a serem utilizadas no monitoramento dos pacientes e em estratégias terapêuticas, com destaque para o desenvolvimento de estudos com abordagens clínicas e de mecanismos.

REFERÊNCIAS

ABBOUD, M.R.; TAYLOR, E.C.; HABIB, D.; DANTZLER-JOHNSON, T.; JACKSON, S.M.; XU, F.; LAVER, J.; BALLAS, S.K. Elevated serum and bronchoalveolar lavage fluid levels of interleukin 8 and granulocyte colony-stimulating factor associated with the acute chest syndrome in patients with sickle cell disease. **Br. J. Haematol.,** v. 111, n. 2, p.482-90, 2000.

ADAMS, R.J. TCD in sickle cell disease: an important and useful test. **Pediatr. Radiol.,** v. 35, n. 3, p. 229-34, 2005.

ADAMS, R.J. Big Strokes in Small Persons. **Arch. Neurol.,** v. 64, n. 11, 2007.

ADORNO, E.V.; COUTO, F.D.; MOURA NETO, J.P.; MENEZES, J.F.; REGO, M.; REIS, M.G; GONÇALVES, M.S. Hemoglobinopathies in newborns from Salvador, Bahia, Northeast Brazil. **Cad. Saude Publ.,** v. 21, p. 292-298, 2005.

AHMED I, GOLDSTEIN BJ. Cardiovascular risk in the spectrum of type 2 diabetes mellitus. **Mt Sinai J. Med.,** v. 73, n. 5, p. 759-68, 2006.

AKHAVANI, M.A.; MADDEN, L.; BUYSSCHAERT, I.; SIVAKUMAR, B.; KANG, N.; PALEOLOG, E.M. Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. **Arthritis Res. Ther.**, v. 11, n. 3, R64, 2009.

AMORIM, T.; PIMENTEL H.; FONTES, M. I. M. M.; PURIFICAÇÃO A.; LESSA, P.; BOA-SORTE, N. Avaliação do Programa de Triagem Neonatal da Bahia entre 2007 E 2009 – As lições da Doença Falciforme**. Gaz. Méd. Bahia**, v. 80, p. 310-13, 2010.

APLIN, A.C.; ZHU, W.H.; FOGEL, E.; NICOSIA, R.F. Vascular regression and survival are differentially regulated by MT1-MMP and TIMPs in the aortic ring model of angiogenesis. **Am. J. Physio.l Cell Physiol.,** v. 297, n. 2, C471-80, 2009.

ARAUJO, M.C.; SERAFIM, E.S.; DE CASTRO JR, W.A.; DE MEDEIROS, T.M. Prevalence of abnormal hemoglobins in newborns in Natal, Rio Grande do Norte, Brazil. **Cad. Saúde Publ.,** v. 20, p. 123-128, 2004.

ARCHER, S, RICH, S. Primary pulmonary hypertension: a vascular biology and translational research "Work in progress". **Circulation,** v. 102, n. 22, p. 2781-91, 2000.

ARDI, V.C.; VAN DEN STEEN, P.E.; OPDENAKKER, G.; SCHWEIGHOFER, B.; DERYUGINA, E.I.; QUIGLEY, J.P. Neutrophil MMP-9 proenzyme, unencumbered by TIMP-1, undergoes efficient activation in vivo and catalytically induces angiogenesis via a basic fibroblast growth factor (FGF-2)/FGFR-2 pathway. **J. Biol. Chem.,** v. 284, n. 38, p. 25854-66, 2009.

ASLAN, M.; FREEMAN, B. A. Oxidant-mediated impairment of nitric oxide signaling in sickle cell disease—mechanisms and consequences. **Cell Mol. Biol.,** n. 50, p. 95-105, 2004.

ASSIS, A.; CONRAN, N.; CANALLI, A.A.; LORAND-METZE, I.; SAAD, S.T.; COSTA, F.F. Effect of cytokines and chemokines on sickle neutrophil adhesion to fibronectin. **Acta Haematol**., v. 113, n. 2, p. 130-136, 2005.

ATWEH GF, SUTTON M, NASSIF I, BOOSALIS V, DOVER GJ, WALLENSTEIN S, WRIGHT E, MCMAHON L, STAMATOYANNOPOULOS G, FALLER DV, PERRINE SP. Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease. **Blood,** v. 93, n. 6, p. 1790-7, 1999.

AZEVEDO, E.S.; ALVES, A.F.P.; SILVA, M.C.B.O.; SOUZA, M.G.F.; LIMA, A.M.V.M.D.; AZEVEDO, W.C. Distribution of abnormal hemoglobins and glucose-6-phosphate dehydrogenase variants in 1200 school children of Bahia, Brazil. **Am. J. Phys. Anthropol.,** v. 53, p. 509-512, 1980.

AZIZ KE, WAKEFIELD D. Modulation of endothelial cell expression of ICAM-1, E-selectin, and VCAM-1 by beta-estradiol, progesterone, and dexamethasone. **Cell Immunol.** v. 167, n. 1, p. 79-85, 1996.

BALLAS, S.K. Sickle cell anaemia: progress in pathogenesis and treatment. **Drugs,** v. 62, n. 8, p. 1143-72, 2002.

BALS, R. Alpha-1-antitrypsin deficiency. **Best Pract. Res. Clin. Gastroenterol.,** v. 24, n. 5, p. 629-33, 2010.

BANDEIRA, F.M.G.C.; LEAL, M.C.;SOUZA, R.R.; FURTADO, V.C.; GOMES, Y.M.; MARQUES, N.M. Características de recém-nascidos portadores de hemoglobina "S" detectados através de triagem em sangue de cordão umbilical. **J. Pediatr.,** v. 75, p. 167-171, 1999.

BARANANO, D. E.; RAO, M.; FERRIS, C. D.; SNYDER, S. H. Biliverdin reductase: amajor physiologic cytoprotectant. **Proc. Natl. Acad. Sci. USA,** v. 99, p.16093-16098, 2002.

BARRIE, A.M.; PLEVY, S.E. The interleukin-12 family of cytokines: therapeutic targets for inflammatory disease mediation. **Clin. Appl. Immunol. Rev.,** v. 5, p. 225-240, 2005.

BARRIE, A.; KHARE, A.; HENKEL, M.; ZHANG, Y.; BARMADA, M.M.; DUERR, R.; RAY, A. Prostaglandin E2 and IL-23 plus IL-1β differentially regulate the Th1/Th17 immune response of human CD161(+) CD4(+) memory T cells. **Clin. Transl. Sci.,** v. 4, n. 4, p. 268-73, 2011.

BARZILAY JI, ABRAHAM L, HECKBERT SR, CUSHMAN M, KULLER LH, RESNICK HE, TRACY RP. The relation of markers of inflammation to the development of glucose disorders in the elderly: the Cardiovascular Health Study. **Diabetes,** v.50, n. 10, p. 2384-9, 2001.

BLASIUS AL, BEUTLER B. Intracellular toll-like receptors. **Immunity,** v. 32, n. 3, p. 305-15, 2010.

BELCHER JD, MAHASETH H, WELCH TE, VILBACK AE, SONBOL KM, KALAMBUR VS, BOWLIN PR, BISCHOF JC, HEBBEL RP, VERCELLOTTI GM. Critical role of

endothelial cell activation in hypoxia-induced vasoocclusion in transgenic sickle mice. **Am J Physiol Heart Circ Physiol**. v. 288, n. 6, p. H2715-25, 2005.

BELCHER, J.D.; BECKMAN, J.D.; BALLA, G.; BALLA, J.; VERCELLOTTI, G. Heme degradation and vascular injury. **Antioxid Redox Signal.** v. 12, n 2, p. 233-48, 2010.

BELO, V.A.; SOUZA-COSTA, D.C.; LANA, C.M.; CAPUTO, F.L.; MARCACCINI, A.M.; GERLACH, R.F.; BASTOS, M.G.; TANUS-SANTOS, J.E. Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. **Clin Biochem. v. 42, n.10-11, p.** 984-90, **2009.**

BENZ, D.J.; MOL, M.; EZAKI, M.; MORI-ITO, N.; ZELÁN, I.;, MIYANOHARA, A.; FRIEDMANN, T.; PARTHASARATHY, S.; STEINBERG**,** D.; WITZTUM, J.L. Enhanced levels of lipoperoxides in low density lipoprotein incubated with murine fibroblast expressing high levels of human 15-lipoxygenase. **J Biol Chem.** v. 270, n.10, p. 5191-7, 1995.

BERTAUX, B.; HORNEBECK, W. Tissue inhibitors of matrix metalloproteinases TIMP 1-2. Structures and functions. **C R Seances Soc Biol Fil1,** v. 87, p. 192-200, 1993.

BOCK, O.; MROWIETZ, U. Development of a new method of analyzing chemotactic deactivation of human neutrophil granulocytes. **J. Biochem. Bioph Methods**, v. 48, p. 257-268, 2001.

BOURANTAS KL, DALEKOS GN, MAKIS A, CHAIDOS A, TSIARA S, MAVRIDIS A. Acute phase proteins and interleukins in steady state sickle cell disease. **Eur J Haematol.** v. 61, n. 1, p. 49-54, 1998.

BOURBOULIA, D.; STETLER-STEVENSON, W. G. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. **Semin Cancer Biol,** v. 20, p. 161-168, 2010.

BRANTLY**,** M.; NUKIRA, T.; CRYSTAL, R.G. Molecular basis of alpha 1 antitrypsin deficiency. **Am J Med.,** v. 84, p. 13, 1988.

BRASIL, Ministério da Saúde. Doença falciforme e outras hemoglobinopatias, http://portal.saude.gov.br/portal/saude, 2008. Acesso em: 11 de novembro de 2014.

BREIT, S. N., WAKEFIELD, D., ROBINSON, J. P. *et al*. The role of alpha 1-antitrypsin deficiency in the pathogenesis of immune disorders. **Clin. Immunol. Immunopathol.**, v. 35, p. 363-380, 1985.

BRITTAIN, J.E.; PARISE, L.V. Cytokines and plasma factors in sickle cell disease. **Curr Opin Hematol.** v. 14, n. 5, p. 438-43, 2007.

BRUGNARA, C.; KOPIN, A. S.; BUNN, H. F.; TOSTESON, D. C. Regulation of cátion content and cell volume in hemoglobin erythrocytes from patients with homozygous hemoglobin C disease. **J Clin Invest,** v. 75, p. 1608-17, 1985.

BRUGNARA, C.; BUNN, H. F.; TOSTESON, D. C. Regulation of erythrocyte cátion and water content in sickle cell anemia**. Science,** v. 232, p. 388-90, 1986.

BUNN, H.F.; FORGET, B.G. **Hemoglobin: Molecular, Genetic and Clinical Aspects**. Philadelphia, PA.: Saunders, 1nd edition, 1986.

BUNN, H.F. Sickle hemoglobin and other hemoglbin mutants. In: STAMATOYANNOPOULOS, G.; NIENHUIS, A.W., MAJERUS, P.W.; VARNUS, H. **The molecular basis of blood diseases**. 2. ed. Philadelphia: W.B. Saunders Company. cap. 6, p. 207-256, 1994.

BUNN, H.F. Pathogenesis and treatment of sickle cell disease. **N. Engl. J. Med**., v. 337, p. 762-769, 1997.

BURNETT AL. Pathophysiology of priapism: dysregulatory erection physiology thesis. **J Urol.** v. 170, n. 1, p. 26-34, 2003.

CAJADO, C.; CERQUEIRA, B. A.; COUTO, F. D.; MOURA-NETO, J. P.; VILAS-BOAS, W.; DOREA, M. J.; LYRA, I. M.; BARBOSA, C. G.; REIS, M. G.; GONCALVES, M. S. TNF-alpha and IL-8: serum levels and gene polymorphisms (-308G>A and -251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia. **Cytokine.** v. 56, n. 2, p. 312-7, 2011.

CANALLI, A.A.; CONRAN, N.; FATTORI, A.; SAAD, S.T.; COSTA, F.F. Increased adhesive properties of eosinophil in sickle cell disease. **Exp. Hematol**., v.32, n.8, p 728-34, 2004.

CANESSA, M.; SPALVINS, A.; NAGEL, R. L. Volume-dependent and NEM-stimulated K_,Cl_ transport is elevated in oxygenated SS, SC and CC human red cells. **FEBS Lett,** v. 200, p. 197-202, 1986.

CASTRO, O.; HOQUE, M.; BROWN, B.D. Pulmonary hypertension in sickle cell disease: cardiac catheterization results and survival. **Blood**. v. 101, n. 4, p. 1257-61, 2003.

CARREL, R.W.; LOMAS, D. A.; SIDEHA, S.; FOREMAN, R. Alpha 1 antitrypsin deficiency. A model for conformational disease. **N. England J Med,** v. 346, p. 45-53, 2002.

CERQUEIRA, B.A.; BOAS, W.V.; ZANETTE, A.D.; REIS, M.G.; GONCALVES, M.S. Increased concentrations of IL-18 and uric acid in sickle cell anemia: contribution of hemolysis, endothelial activation and the inflammasome. **Cytokine**, v. 56, n. 2, p. 471-6, 2011.

CHAMPION, H.C.; BIVALACQUA, T.J.; TAKIMOTO, E.; KASS, D.A.; BURNETT, A.L. Phosphodiesterase-5A dysregulation in penile erectile tissue is a mechanism of priapism. **Proc Natl Acad Sci U S A**, v. 102, n. 5, p. 1661-6, 2005.

CHARNESKI, L.; CONGDON, H.B. Effects of antiplatelet and anticoagulant medications on the vasoocclusive and thrombotic complications of sickle cell disease: A review of the literature. **Am J Health Syst Pharm**, v. 67, p. 895–900, 2010.

CHURG, A.; DAI, J.; ZAY, K.; KARSAN, A.; HENDRICKS, R.; YEE, C.; MARTIN, R.; MACKENZIE, R.; XIE, C.; ZHANG, L.; SHAPIRO, S.; WRIGHT, J.L. Alpha-1-antitrypsin and a broad spectrum metalloprotease inhibitor, RS113456, have similar acute anti-inflammatory effects. **Lab Invest,** v. 81, n. 8, p. 1119-31, 2001.

COKIC VP, SMITH RD, BELESLIN-COKIC BB, NJOROGE JM, MILLER JL, GLADWIN MT, SCHECHTER AN. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. **J Clin Invest.** v. 111, n. 2, p. 231-9, 2003.

CONNOR, W.E.; LIN, D.S.; THOMAS, G.; EY, F.; DELOUGHERY, T.; ZHU, N. Abnormal phospholipid molecular species of erythrocytes in sickle cell anemia. **J Lipid Res**, v. 38, n.12, p.2516-28, 1997.

CONRAN, N.; FATTORI, A.; SAAD, S.T.; COSTA, F.F. Increased levels of soluble ICAM-1 in the plasma of sickle cell patients are reversed by hydroxyurea. **Am. J. Hematol.,** v. 76, p. 343-347, 2004

CONRAN N, ALMEIDA CB, LANARO C, FERREIRA RP, TRAINA F, SAAD ST, COSTA FF. Inhibition of caspase-dependent spontaneous apoptosis via a cAMP-protein kinase A dependent pathway in neutrophils from sickle cell disease patients. **Br J Haematol**. v. 139, n. 1, p. 148-58, 2007.

CORTAS, N. K.; WAKID, N. W. Determination of nitrate in serum and urine by kinetic cadmium-reduction method. **Clinical chemistry,** v. 36, p. 1440-1443, 1990.

COSTA, F.F. Anemia Falciforme. In: ZAGO, M.A.; FALCÃO, R.P.; PASQUINI, R. **Hematologia, Fundamentos e Prática**. **1 ed**, São Paulo: Atheneu, 2001, cap. 7, p. 289-308.

COSTA, R.N.P, Albuquerque D.M. *et al*. – Association of the G-463A myeloperoxidase polymorphism with infection in sickle cell anemia. **Haematologica,** v. 90, n. 7, p. 977,2005.

COVITZ, W.; ESPELAND, M.; GALLAGHER, D.; HELLENBRAND, W.; LEFF, S.; TALNER, N. The heart in sickle cell anemia. The Cooperative Study of Sickle Cell Disease (CSSCD). **Chest**, v. 108, n. 5, p. 1214-9, 1995.

CRYSTAL, R.G.; FERRANS, V.J.; BASSET, F. Biologic basis of pulmonary fibrosis, In: CRYSTAL, R.G.; WEST, J.B.; BARNES, P.J.; CHERNIACK, S (eds). **The lung**: Scientific foundations. Raven Press, New York, 1991.

CUI, H.; GROSSO, S.; SCHELTER, F.; MARI, B.; KRÜGER, A. On the Pro-Metastatic Stress Response to Cancer Therapies: Evidence for a Positive Co-Operation between TIMP-1, HIF-1α, and miR-210. **Front Pharmacol,** v. 3, p.134, 2012.

DAEMEN, M.A.; HEEMSKERK, V.H.; VAN'T VEER, C.; DENECKER, G.; WOLFS, T.G.; VANDENABEELE, P.; BUURMAN, W.A. Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. **Circulation,** v. 19, n. 102(12), p.1420-6, 2000.

DAVIES SC, GILMORE A. The role of hydroxyurea in the management of sickle cell disease. **Blood Rev**. v.17, n.2, p. 99-109, 2003.

DE JONG, K.; GELDWERTH, D.; KUYPERS, F.A. Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes. **Biochemistry,** v. 3, n. 36(22), p. 6768-76, 1997.

DEROSA, G.; MAFFIOLI, P.; D'ANGELO, A.; SALVADEO, S.A.; FERRARI, I.; FOGARI, E.; GRAVINA, A.; MEREU, R.; PALUMBO, I.; RANDAZZO, S.; CICERO, A.F. Evaluation of metalloproteinase 2 and 9 levels and their inhibitors in combined dyslipidemia. **Clin Invest Med,** v. 32, n. 2, p. E124-32, 2009a.

DEROSA, G.; MAFFIOLI, P.; D'ANGELO, A.; SALVADEO, S.A.; FERRARI, I.; FOGARI, E.; GRAVINA, A.; MEREU, R.; RANDAZZO, S.; CICERO, A.F. Effects of long chain omega-3 fatty acids on metalloproteinases and their inhibitors in combined dyslipidemia patients. **Expert Opin Pharmacother**, v. 10, n. 8, p. 1239-47, 2009.

DJOUMESSI, S.; ZEKENG, L.; LANDO, G.; ZEUKENG, D. Serum lipids and atherogenic risk in sickle- cell trait carriers. **Ann Biol Clin, v. 52, p.** 663-665, **1994.**

Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, Hoogeveen R, Folsom AR, Heiss G. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. **Diabetes**. v. 52, n. 7, p.1799-805, 2003.

DUONG, P.T.; COLLINS, H.L.; NICKEL M.; LUND-KATZ, S.; ROTHBLAT, G.H.; PHILLIPS, M.C. Characterization of nascent HDL particles and microparticles formed by ABCA1-mediated efflux of cellular lipids to apoA-I. **Journal of Lipid Research** v. 47, p. 832-843, 2006.

DURANTE, W.; JOHNSON, F. K.; JOHNSON, R. A. Arginase: a critical regulator of nitric oxide synthesis and vascular function. **Clin Exp Pharmacol Physiol,** v. 34, p. 906-911, 2007.

EMBURY, S.H. The not-so-simple process of sickle cell vasoocclusion. Microcirculation. v. 11, n. 2, p. 101-13, 2004.

ENWONWU, C. O. Increased metabolic demand for arginine in sickle cell anaemia. **Med Sci Res,** v. 17, p. 997-998, 1989.

ERIKSSON, S.; ELZOUKI, A.N. Alpha 1-antitrypsin deficiency. **Baillieres Clin Gastroenterol,** v. 12, n. 2, p. 257-73, 1998.

FERREIRA, A.; BALLA, J.; JENEY, V.; BALLA, G.; SOARES, M.P. A central role for free heme in the pathogenesis of severe malaria: the missing link? **J Mol Med (Berl),** v. 86, n. 10, p. 1097-111, 2008.

FIELD, J. J.; GLASSBERG, J.; GILMORE, A.; HOWARD, J.; PATANKAR, S.; YAN, Y.; DAVIES, S. C.; DEBAUN, M. R.; STRUNK, R. C. Longitudinal analysis of pulmonary function in adults with sickle cell disease. **Am J Hematol,** v. 83, p. 574-576, 2008.

FORESTI, R.; HOQUE, M.; BAINS, S.; GREEN, C.J.; MOTTERLINI, R. Haem and nitric oxide: synergism in the modulation of the endothelial haem oxygenase-1 pathway. **Biochemistry Journal,** v. 372, p. 381-390, 2003.

FORTES, G.B.; ALVES, L.S.; DE OLIVEIRA, R.; DUTRA, F.F.; RODRIGUES, D.; FERNANDEZ, P.L.; SOUTO-PADRON, T.; DE ROSA, M.J.; KELLIHER, M.; GOLENBOCK, D.; CHAN, F.K.; BOZZA, M.T. Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. **Blood,** v. 8, n. 119 (10), p. 2368-75, 2003.

FREDENRICH, A.; BAYER, P. Reverse cholesterol transport, high density lipoproteins and HDL cholesterol: recent data. **Diabetes Metab,** v. 29, p. 201–205, 2003.

FRENETTE PS. Sickle cell vasoocclusion: heterotypic, multicellular aggregations driven by leukocyte adhesion. **Microcirculation.** v. 11, n. 2, p. 167-77, 2004.

GASTON, M.H.; VERTER, J.I.; WOODS, G.; PEGELOW, C.; KELLEHER, J.; PRESBURY, G.; ZARKOWSKY, H.; VICHINSKY, E.; IYER, R.; LOBEL, J.S.; et al. Prophylaxis with oral penicillin in children with sickle cell anemia. A randomized trial. **N Engl J Med.** v, 314, n. 25, p. 1593-9,1986.

GIELING, R.G.; WALLACE, K.; HAN, Y.P. Interleukin-1 participates in the progression from liver injury to fibrosis. **Am J Physiol Gastrointest Liver Physiol,** v. 296, n. 6, p. G1324-31, 2009.

GLADWIN, M.T.; SACHDEV, V.; JISON, M.L.; SHIZUKUDA, Y,; PLEHN, J. F.; MINTER, K.; BROWN, B.; COLES, W. A.; NICHOLS, J. S.; ERNST, I.; HUNTER, L. A.; BLACKWELDER, W. C.; SCHECHTER, A. N.; RODGERS, G. P.; CASTRO, O.; OGNIBENE, F. P. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. **N Engl J Med,** v. 350, p. 22-31, 2004.

GLADWIN, M.T. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. **Blood,** v. 107, n. 6, p. 2279-2285, 2006.

GLADWIN, M.T.; VICHINSKY, E. Pulmonary complications of sickle cell disease. **N Engl J Med**, v. 359, p. 2254–65, 2008.

GLADWIN, M.T.; SACHDEV, V. Cardiovascular abnormalities in sickle cell disease. **J Am Coll Cardiol.** v. 59, n. 13, p. 1123-33, 2012.

GOMEZ, D. E.; ALONSO, D. F.; YOSHIJI, H.; THORGEIRSSON, U. P. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. **Eur J Cell Biol,** v. 74, p. 111- 122, 1997.

GONÇALVES, M.S.; QUEIROZ, I.L.; CARDOSO, S.A.; ZANETTI, A.; STRAPAZONI, A.C.; ADORNO, E.; ALBUQUERQUE, A.; SANT'ANA, A.; DOS REIS, M.G.; BARRAL, A.; BARRAL NETTO, M. Interleukin 8 as a vaso-occlusive marker in Brazilian patients with sickle cell disease. **Braz J Med Biol Res,** v. 34, n. 10, p.1309-13, 2001.

GONÇALVES, F.M.; JACOB-FERREIRA, A.L.; GOMES, V.A.; CASELLA-FILHO, A.; CHAGAS, A.C.; MARCACCINI, A.M.; GERLACH, R.F.; TANUS-SANTOS, J.E. Increased circulating levels of matrix metalloproteinase (MMP)-8, MMP-9, and pro-inflammatory markers in patients with metabolic syndrome. **Clin Chim Acta,** v. 403, n. 1-2, p.173-7, 2009.

GORELIK, L.; FLAVELL, R. A. Transforming growth factor-beta in T-cell biology. **Nat Rev Immunol,** v. 2, n. 1, p. 46-53, 2002.

GRAIDO-GONZALEZ E, DOHERTY JC, BERGREEN EW, ORGAN G, TELFER M, MCMILLEN MA. Plasma endothelin-1, cytokine, and prostaglandin E2 levels in sickle cell disease and acute vaso-occlusive sickle crisis. **Blood,** v. 1, n. 92(7), p. 2551-5, 1988.

HAN, G.; LI, F.; SINGH, T.P.; WOLF, P.; WANG, X.J. The pro-inflammatory role of TGFβ1: a paradox? **Int J Biol Sci,** v. 8, n. 2, p. 228-35, 2012.

HARRIS, S. G.; PADILLA, J.; KOUMAS, L.; RAY, D.; PHIPPS, R. P. Prostaglandins as modulators of immunity. **Trends Immunol,** v. 23, n. 3, p. 144-50, 2002.

HEBBEL, R. P.; EATON, J. W.; BALASINGAM, M.; STEINBERG, M. H. Spontaneous oxygen radical generation by sickle erythrocytes. **J Clin Invest,** n. 70, p. 1253-1259, 1982.

HEBBEL RP, VERCELLOTTI GM. The endothelial biology of sickle cell disease**. J Lab Clin Med.** v.129, n. 3, p. 288-93, 1997.

HEDO, C.C.; AKEN'OVA, Y.A.; OKPALA, I.E.; DUROJAIYE, A.O.; SALIMONU, L.S. Acute phase reactants and severity of homozygous sickle cell disease. **J Intern Med,** v. 233, n. 6, p. 467-70, 1993.

HEGYI, B.; KUDLIK, G.; MONOSTORI, E.; UHER, F. Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. **Biochem Biophys Res Commun**, v. 9, n. 419(2), p. 215-20, 2012.

HICKEY, K. A.; RUBANYI, G.; PAUL, R. J.; HIGHSMITH, R. F. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. **Am J Physiol Cell Physiol,** v. 248, n. 5, p. C550, 1985.

HOPPE, C. C. Inflammatory Mediators of Endothelial Injury in Sickle Cell Disease. **Hematol Oncol Clin North Am,** v. 28, p. 265–286, 2014.

HUNTSMAN, R. G,; LEHMANN. H. Treatment of sickle-cell disease. **Br. J. Haematol.** v. 28, n. 4, p. 437-344. 1974.

HSU LL, CHAMPION HC, CAMPBELL-LEE SA, *et al.* Hemolysis in sickle cell mice causes pulmonary hypertension due to global impairment in nitric oxide bioavailability. **Blood,** v. 109, p. 3088-3098, 2007.

IBE, B. O; MORRIS, J; KURANTSIN-MILLS, J; RAJ, J. U. Sickle erythrocytes induce prostacyclin and thromboxane synthesis by isolated perfused rat lungs. **American Journal of Physiology - Lung Cellular and Molecular Physiology**, v. 272, p. 597-602, 1997.

ISICHEI, U. P: Liver function and the diagnosis significance of biochemical changes in the blood of African children with sickle cell disease. **J Clin Pathol**, v. 33, p. 626-630, 1980.

IKUTA T, AUSENDA S, CAPPELLINI MD. Mechanism for fetal globin gene expression: role of the soluble guanylate cyclase-cGMP-dependent protein kinase pathway. **Proc Natl Acad Sci U S A**. v. 98, n. 4, p.1847-52, 2001.

JANCIAUSKIENE, S.M.; NITA, I.M.; STEVENS, T. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. **J Biol Chem,** v. 23, n. 282(12), p. 8573-82, 2007.

JANEWAY, C.A.; TRAVERS, P.; WALPORT, M; SHLOMCHIK, M. **Immunobiology**. 5th ed. New York : Garland Publishing; p 90-100, 2001.

JISON, M. L.; MUNSON, P. J.; BARB, J. J.; SUFFREDINI, A. F.; TALWAR, S.; LOGUN, C.; RAGHAVACHARI, N.; BEIGEL, J. H.; SHELHAMER, J. H.; DANNER, R. L.; GLADWIN, M. T. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. **Blood,** v. 104, p. 270-280, 2004.

JIN, R. C.; VOETSCH, B.; LOSCALZO, J. Endogenous mechanisms of inhibition of platelet function. **Microcirculation, v. 12,** p. 247–258, 2005.

JISON, M.L.; MUNSON, P.J.; BARB, J.J.; SUFFREDINI, A.F.; TALWAR, S.; LOGUN, C.; RAGHAVACHARI, N.; BEIGEL, J.H.; SHELHAMER, J.H.; DANNER, R.L.; GLADWIN, M.T. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. **Blood.** v. 104, n. 1, p. 270-80, 2004.

JONIGK, D.; AL-OMARI, M.; MAEGEL, L.; MÜLLER, M.; IZYKOWSKI, N.; HONG, J.; HONG, K.; KIM, S.H.; DORSCH, M.; MAHADEVA, R.; LAENGER, F.; KREIPE, H.; BRAUN, A.; SHAHAF, G.; LEWIS, E.C.; WELTE, T.; DINARELLO, C.A.; JANCIAUSKIENE, S. Anti-inflammatory and immunomodulatory properties of α1-antitrypsin without inhibition of elastase. **Proc Natl Acad Sci U S A,** v. 10, n. 110(37), p. 15007-12, 2013.

KASSIM, A.A.; DEBAUN, M.R. Sickle cell disease, vasculopathy, and therapeutics. **Annu Rev Med**, v. 64, p. 451-66, 2013.

KATO, G. J.; GLADWIN, M. T.; STEINBERG, M. H. Deconstructing sickle cell disease: Reappraisal of the role of hemolysis in the development of clinical subphenotypes. **Blood Rev,** v. 21, n.1, p. 37-47, 2007.

KATO, G. J.; HEBBEL, R. P., STEINBERG, M. H., GLADWIN, M. T. Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. **Am J Hematol** 84:618–25, 2009.

KAUL, D.K.; FABRY, M.E.; NAGEL, R.L. Microvascular sites and characteristics of sickle cell adhesion to vascular endothelium in shear flow conditions: pathophysiological implications. **Proc Natl Acad Sci U S A.** v. 86, n. 9, p. 3356-60, 1989.

KAUL, D.K.; HEBBEL, R.P. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. **J Clin Invest**, v. 106, p. 411–20, 2000.

KAUL DK, LIU XD, CHOONG S, BELCHER JD, VERCELLOTTI GM, HEBBEL RP. Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice. **Am J Physiol Heart Circ Physiol.** v. 287, n. 1, p, H293-301, 2004.

KEDZIERSKI, R.M.; YANAGISAWA, M. Endothelin system: the double-edged sword in health and disease. **Annu Rev Pharmacol Toxicol.** v. 41, p. 851-76, 2001.

KEIKHAEI B.; MOHSENI, A. R.; NOROUZIRAD, R.;, ALINEJADI, M.; GHANBARI, S.; SHIRAVI, F.; SOLGI, G. Altered levels of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crises and the steady state condition. **Eur Cytokine Netw,** v. 24, n. 1, p. 45-52, 2013.

KHOKHA, R.; MURTHY, A.; WEISS, A. Metalloproteinases and their natural inhibitors in inflammation and immunity. **Nat Rev Immunol,** v. 13, n. 9, p. 649-65, 2013.

KIETZMANN, T.; SAMOYLENKO, A.; IMMENSCHUH, S. Transcriptional regulation of heme oxigenase-1 gene expression by MAP kinases of the JNK and p38 pathways in primary cultures of rat hepatocytes. **The Journal of Biological Chemistry,** v. 278, n. 20, p. 17927-17936, 2003.

KIM, J. M.; LEE, E. K.; KIM, D. H.; YU, B. P.; CHUNG, H. Y. Kaempferol modulates pro-inflammatory NF-kappaB activation by suppressing advanced glycation endproducts-induced NADPH oxidase. **Age (Dordr),** v. 32, n. 2, p. 197-208, 2010.

KINZENBAW, D.A.; CHU, Y.; PEÑA SILVA, R.A.; DIDION, S.P.; FARACI, F.M. Interleukin-10 protects against aging-induced endothelial dysfunction. **Physiol Rep,** v. 1, n. 6, p. e00149, 2013.

KONDO, S.; KUBOTA, S.; SHIMO, T.; NISHIDA, T.; YOSIMICHI, G.; EGUCHI, T.; SUGAHARA, T.; TAKIGAWA, M. Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. **Carcinogenesis,** v. 23, n. 5, p. 769-76, 2002.

KÖHNLEIN, T.; WELTE, T. Alpha-1 antitrypsin deficiency: pathogenesis, clinical presentation, diagnosis, and treatment. **Am J Med,** v. 121, n. 1, p. 3-9, 2008.

KONYA, V.; PHILIPOSE, S.; BÁLINT, Z.; OLSCHEWSKI, A.; MARSCHE, G.; STURM, E.M.; SCHICHO, R.; PESKAR, B.A.; SCHULIGOI, R.; HEINEMANN, A. Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. **Eur J Immunol,** v. 41, n. 8, p. 2379-89, 2011.

KRISTIANSEN, M.; GRAVERSEN, J. H.; JACOBSEN, C.; SONNE, O.; HOFFMAN, H. J.; LAW, S. K.; MOESTRUP, S. K. Identification of the haemoglobin scavenger receptor. **Nature,** v. 409, p. 198-201, 2001.

KUCUK, O.; LIS, L.J.; DEY, T.; MATA, R.; WESTERMAN, M.P.; YACHNIN, S.; SZOSTEK, R.; TRACY, D.; KAUFFMAN, J.W.; GAGE, D.A. *et al.* The effects of cholesterol oxidation products in sickle and normal red blood cell membranes. **Biochim Biophys Acta,** v. 1103, n. 2, p. 296-302, 1992.

KUEHL, F.A. Jr; EGAN, R.W. Prostaglandins, arachidonic acid, and inflammation. **Science,** v. 28, n. 210(4473), p. 978-84, 1980.

KUMAR, V.; KHAN, M. M.; KHANNA, A. K.; SINGH, R. Lipid lowering activity of *Anthocephalus indicus* Root in hiperlipidemic Rats. **eCAM,** february, 2008.

KUTLAR, A. Sickle cell disease: multigenic perspective of a single-gene disorder. **Med. Princ. Prat**., v. 14, p.15-19, 2005.

KUYPERS, F. A. Membrane Lipid Alterations in Hemoglobinopathies. **American Society of Hematology,** p. 68-73, 2007.

LAMBERT, E.; BRIDOUX, L.; DEVY, J.; DASSÉ, E.; SOWA, M. L.; DUCA, L.; HORNEBECK, W.; MARTINY, L. PETITFRÈRE-CHARPENTIER, E. TIMP-1 binding to proMMP-9/CD44 complex localized at the cell surface promotes erythroid cell survival. **Int J Biochem Cell Biol,** v. 41, p. 1102-1115, 2009.

LAMBERS, C.; ROTH, M.; ZHONG, J.; CAMPREGHER, C.; BINDER, P.; BURIAN, B.; PETKOV, V.; BLOCK, L.H. The interaction of endothelin-1 and TGF-β1 mediates vascular cell remodeling. **PLoS One,** v. 28, n. 8(8), p. e73399, 2013.

LANARO, C.; FRANCO-PENTEADO, C.F.; ALBUQUEQUE, D.M.; SAAD, S.T.; CONRAN, N.; COSTA, F.F. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. **J Leukoc Biol,** v. 85, n. 2, p. 235-42, 2009.

LEE, G. R.; BITHELL, T. C.; FOERSTER, J.; ATHENS, J. W.; LUKENS, J. N. **Wintrobe's Clinical Hematology**. Philadelphia: London. Lea e Febirger, 9ed, 1992.

LEE, S. P.; ATAGA, K. I.; ORRINGER, E. P.; PHILLIPS, D. R.; PARISE, L.V. Biologically active CD40 ligand is elevated in sickle cell anemia: potential role for platelet-mediated inflammation. **Arterioscler Thromb Vasc Biol**, v. 26, p. 1626–31, 2006.

LEE, S.K.; DING, J.L. A perspective on the role of extracellular hemoglobin on the innate immune system. **DNA Cell Biol.** v. 32, n. 2, p. 36-40, 2013.

LEY TJ, DESIMONE J, NOGUCHI CT, TURNER PH, SCHECHTER AN, HELLER P, NIENHUIS AW. 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. **Blood.** v. 62, n. 2. P. 370-80, 1983.

LEW, V. L.; HOCKADAY, A.; SEPULVEDA, M. I.; SOMLYO, A. P.; SOMLYO, A. V.; ORTIZ, O. E.; BOOKCHIN, R. M. Compartmentalization of sickle-cell calcium in endocytic inside-out vesicles**. Nature,** v. 315, p. 586-9, 1985.

LEW, V. L.; FREEMAN, C. J.; ORTIZ, O. E.; BOOKCHIN, R. M. A mathematical model of the volume, pH, and ion content regulation in reticulocytes: application to the pathophysiology of sickle cell dehydration. **J Clin Invest,** v. 87, p. 100-12, 1991.

LOMAS, D.A.; EVANS, D.L.; FINCH, J.T.; CARRELL, R.W. The mechanism of Z alpha 1-antitrypsin accumulation in the liver. **Nature,** v. 18, n. 357(6379), p. 605-7, 1992.

LONDON, S.J.; LEHMAN, T. A.; TAYLOR, J.A. Myeloperoxidase genetic polymorfisms and lung cancer risk**. Cancer Res**, v**. 57,** p. 5001-3, 1997.

LOONEY, M.R.; MATTHAY, M.A. Neutrophil sandwiches injure the microcirculation. Nat Med. v. 15, n. 4, p. 364-6, 2009.

LORENTE, L.; MARTÍN, M.M.; LABARTA, L.; DÍAZ, C.; SOLÉ-VIOLÁN, J.; BLANQUER, J.; ORBE, J.; RODRÍGUEZ, J.A.; JIMÉNEZ, A.; BORREGUERO-LEÓN, J.M.; BELMONTE, F.; MEDINA, J.C.; LLIMIÑANA, M.C.; FERRER-AGÜERO, J.M.; FERRERES, J.; MORA, M.L.; LUBILLO, S.; SÁNCHEZ, M.; BARRIOS, Y.; SIERRA, A.; PÁRAMO, J.A. Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. **Crit Care**, v. 13, n. 5, p. R158, 2009.

LUCOTTE, G.; SESBOÜÉ, R. Polymerase chain reaction detection of S and Z alpha-1-antitrypsin variants by duplex PCR assay. **Mol Cell Probes,** v. 13, n. 5, p. 389-91, 1999.

LYRA, I.M.; GONÇALVES, M.S.; BRAGA, J.A.; GESTEIRA, M.D.E.F.; CARVALHO, M. H.; SAAD, S.T.; FIGUEIREDO, M.S.; COSTA, F.F. Clinical, hematological, and molecular characterization of sickle cell anemia pediatric patients from two different cities in Brazil. **Cad Saude Publica**, v. 21, n. 4, p. 1287-90, 2005.

MA, Y.; YABLUCHANSKIY, A.; HALL, M. E.; LINDSEY, M. L. Using plasma matrix metalloproteinase-9 and monocyte chemoattractant protein-1 to predict future cardiovascular events in subjects with carotid atherosclerosis. **Atherosclerosis.** v. 232, n. 1, p. 231-3, 2014.

MACHADO, P.R..L.; ARAÚJO, M.A.S, CARVALHO L., CARVALHO, E.M. Mecanismos de resposta imune às infecções. **An. Bras. Dermatol.,** v.79, n. 6, p. 647-66, 2004.

MAGDA OLIVEIRA SEIXAS, LARISSA ROCHA, MAURICIO CARVALHO, JOELMA MENEZES, ISA LYRA, VALMA NASCIMENTO, RICARDO COUTO, AJAX ATTA, MITERMAYER GALVÃO REIS, MARILDA SOUZA GONCALVES. Lipoprotein Cholesterol and Triglycerides in Children with steady-state sickle cell anemia [Abstract] **Blood, v 114,** s1547, 2009.

MAÎTRE, B.; MEKONTSO-DESSAP, A.; HABIBI, A.; BACHIR, D.; PARENT, F.; GODEAU, B.; GALACTEROS, F. Pulmonary complications in adult sickle cell disease. Rev Mal Respir. v. 28, n. 2, p. 129-37, 2011.

MALAVE, I.; PERDOMO, Y.; ESCALONA, E.; RODRIGUEZ, E.; ANCHUSTEGUI, M.; MALAVE, H.; ARENDS, T. Levels of tumor necrosis factor alpha / cachectin (TNF-alfa) in sera from patients with sickle cell disease. **Acta Haematol.,** v. 90, p. 172-176, 1993.

MALM, J.; DANMYR, P.; NILSSON, R. ; APPELQVIST, R. ; VÉGVÁRI, A.; MARKO-VARGA, G. Blood plasma reference material: a global resource for proteomic research. **J Proteome Res,** v. 5, n. 12(7), p. 3087-92, 2013.

MAROUF, R.; MOJIMINIYI, O.; ABDELLA, N.; KORTOM, M.; AL WAZZAN, H. Comparison of renal function markers in Kuwaiti patients with sickle cell disease. **J Clin Pathol.** v, 59, n 4, p. 345-51, 2006.

MARTIN, A.; THOMPSON, A. A. Thalassemias. **Pediatr Clin North Am,** v. 60, n. 6, p. 1383-91, 2013.

MASAKI, T. The discovery of endothelins. **Cardiovasc Res**. v, 39, n. 3, p. 530-3, 1998.

MEDZHITOV, R.; JANEWAY, C. Jr. Innate immunity. **N. Engl. J. Med.** v 3, n.343, p.338-44. 2000.

MEHTA, S.R.; AFENYI-ANNAN A.; BYRNS P.J.; LOTTENBERG, R. Opportunities to improve outcomes in sickle cell disease. **Am. Fam. Physician**. v. 74, n. 2, p. 303-10, 2006.

MESCHIA, J. F.; PANKRATZ, V. S. Defining stroke risks in sickle cell anemia. **Nature Genetics,** v. 37, n. 4, 2005.

MILLS, C. D.; KINCAID, K.; ALT, J. M.; HEILMAN, M. J.; HILL, A. M. M-1/M-2 macrophages and the Th1/Th2 paradigm. **J Immunol,** v. 164, p. 6166–6173, 2000.

MILTON, J.N.; SEBASTIANI, P.; SOLOVIEFF, N.; HARTLEY, S.W.; BHATNAGAR, P.; ARKING, D.E.; DWORKIS, D.A.; CASELLA, J.F.; BARRON-CASELLA, E.; BEAN, C.J.; HOOPER, W.C.; DEBAUN, M.R.; GARRETT, M.E.; SOLDANO, K.; TELEN, M.J.; ASHLEY-KOCH, A.; GLADWIN, M.T.; BALDWIN, C.T.; STEINBERG, M.H.; KLINGS, E.S. A genome-wide association study of total bilirubin and cholelithiasis risk in sickle cell anemia. **PLoS One,** v. 7, n. 4, p. e34741, 2012.

MINNITI, C.P.; SABLE, C.; CAMPBELL, A.; RANA, S.; ENSING, G.; DHAM, N.; ONYEKWERE, O.; NOURAIE, M.; KATO, G.J.; GLADWIN, M.T.; CASTRO, O.L.; GORDEUK, V.R. Elevated tricuspid regurgitant jet velocity in children and adolescents with sickle cell disease: association with hemolysis and hemoglobin oxygen desaturation. **Haematologica.** v. 94, p. 340–347, 2009.

MIWA, K.; TANAKA, M.; OKAZAKI, S.; FURUKADO, S.; SAKAGUCHI, M.; MOCHIZUKI, H.; KITAGAWA, K. Association between interleukin-6 levels and first-ever cerebrovascular events in patients with vascular risk factors. **Arterioscler Thromb Vasc Biol,** v. 33, n. 2, p. 400-5, 2013.

MIYAZAKI, O.; FUKAMACHI, I.; MORI, A.; HASHIMOTO, H.; KAWASHIRI, M.A.; NOHARA, A.; NOGUCHI, T.; INAZU, A.; YAMAGISHI, M.; MABUCHI, H.; KOBAYASHI, J. Formation of prebeta1-HDL during lipolysis of triglyceride-rich lipoprotein. **Biochem Biophys Res Commun,** v. 379, n. 1, p. 55-9, 2009.

MODELL, B.; DARLISON, M. Global epidemiology of haemoglobin disorders and derived service indicators. **Bull World Health Organ.** v. 86, n. 6, p. 480-7. 2008.

MONTEIRO, A.P.; PINHEIRO, C.S.; LUNA-GOMES, T.; ALVES, L.R.; MAYA-MONTEIRO, C.M.; PORTO, B.N.; BARJA-FIDALGO, C.; BENJAMIM, C.F.; PETERS-GOLDEN, M.; BANDEIRA-MELO, C.; BOZZA, M.T.; CANETTI, C. Leukotriene B4 mediates neutrophil migration induced by heme. **J Immunol,** v. 1, n. 186(11), p. 6562-7, 2011.

MORRIS, C. R.; KUYPERS, F. A.; LARKIN, S.; VICHINSKY, E.; STYLES, L. Patterns of arginine and nitric oxide in sickle cell disease patients with vaso-occlusive crisis and acute chest syndrome. **J Pediatr Hematol Oncol,** v. 22, p. 515-520, 2000.

MORRIS, C. R.; KATO, G. J.; POLJAKOVIC, M.; WANG, X.; BLACKWELDER, W. C.; SACHDEV, V.; HAZEN, S. L.; VICHINSKY, E. P.; MORRIS, S. M. JR.; GLADWIN, M. T. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension and mortality in sickle cell disease. **JAMA,** v. 294, p. 81-90 2005.

MORRIS, C. R.; VICHINSKY, E.; SINGER, S. T. Pulmonary hypertension in thalassemia: association with hemolysis, arginine metabolism dysregulation and a hypercoaguable state. **Advances in Pulmonary Hypertension,** v. 5, p. 31-38, 2007.

MORRIS, C. R.;, SUH, J. H.; HAGAR, W.; LARKIN, S.; BLAND, D. A.; STEINBERG, M. H.; VICHINSKY, E. P.; SHIGENAGA, M.; AMES, B.; KUYPERS, F. A.; KLINGS, E. S. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. **Blood,** v. 140, p. 104-112, 2008.

MORRIS, C. R. Mechanisms of Vasculopathy in Sickle Cell Disease and Thalassemia. **Hematology Am Soc Hematol Educ Program**, p. 177-85, 2008.

NAGEL, R.L.; GIBSON, Q.H. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. **J Biol Chem,** v. 246, p. 69-73, 1971.

NAGEL, R.L., FABRY, M.E. Sickle cell anemia as a multigenetic disease: new insights into the mechanism of painful crisis. **Prog Clin Biol Res,** v**.** 165, p. 93-102, 1984.

NAGEL, R.L.; STEINBERG, M.H.; Role of epistatic (modifier) genes in the modulation of the phenotypic diversity of sickle cell anemia. **Pediatr Pathol Mol Med.** v. 20, n.2, p. 123-36, 2001.

NAQVI, J.; YAP, K. H.; AHMAD, G.; GHOSH, J**.** Transcranial Doppler Ultrasound: A Review of the Physical Principles and Major Applications in Critical Care. **Int J Vasc Med**, ID 629378, 2013.

NAVAB, M.; BERLINER, J.A.; SUBBANAGOUNDER, G.; HAMA, S.; LUSIS, A.J.; CASTELLANI, L.W.; REDDY, S.; SHIH, D.; SHI, W.; WATSON, A.D.; VAN LENTEN, B.J.; VORA, D.; FOGELMAN, A.M. HDL and inflammatory response induced by LDL-derived oxidized phospholipids. **Arterioscler Thromb Vasc Biol,** v. 21, p. 481–488, 2001.

NAVAB, M.; ANANTHRAMAIAH, G.M.; REDDY, S.T. The oxidation hypothesis hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. **J Lipid Res,** v. 45, p. 9932–1007, 2004.

NEIDLINGER, N. A.; LARKIN, S. K.; BHAGAT, A.; VICTORINO, G. P.; KUYPERS, F. A. Hydrolysis of phosphatidylserine-exposing red blood cells by secretory phospholipase A2 generates lysophosphatidic acid and results in vascular dysfunction**. J Biol Chem,** v. 281, p. 775-781, 2006.

NITA, I.M.; SERAPINAS, D.; JANCIAUSKIENE, S.M. alpha1-Antitrypsin regulates CD14 expression and soluble CD14 levels in human monocytes in vitro. **Int J Biochem Cell Biol,** v. 39, n. 6, p. 1165-76, 2007.

NOFER, J. R.; KEHREL, B.; FOBKER, M.; LEVKAU, B.; ASSMANN, G.; ECKARDSTEIN, A. HDL and arteriosclerosis: beyond reverse cholesterol transport. **Atherosclerosis,** v. 161, p. 1-16, 2002.

NORTH, T.E.; GOESSLING, W.; WALKLEY, C.R.; LENGERKE, C.; KOPANI, K.R.; LORD, A.M.; WEBER, G.J.; BOWMAN, T.V.; JANG, I.H.; GROSSER, T.; FITZGERALD, G.A.; DALEY, G.Q.; ORKIN, S.H.; ZON, LI. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. **Nature,** v. 447, n. 7147, p. 1007-11, 2007.

O'CONNELL, M. A.; BENNETT, B. L.; MERCURIO, F.; MANNING, A. M.; MACKMAN, N. Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. **J Biol Chem, v.** 273, n. 46, p. 30410-4, 1998.

ODIÈVRE, M.H.; VERGER, E.; SILVA-PINTO, A.C.; ELION, J. Pathophysiological insights in sickle cell disease. Indian J Med Res. v. 134, p. 532-7, 2011.

OHENE-FREMPONG, K.; WEINER, S.; SLEEPER, L. A.; MILLER, S. T.; EMBURY, S.; MOOHR, J. W.; WETHERS, D. L.; PEGELOW, C. H.; GILL, F. M. Cerebrovascular accidents in sickle cell disease: rates and risk factors. **Blood**, v. 91, n. 1, p. 288–294, 1998.

OHENE-FREMPONG, K.; STEINBERG, M. H. Clinical Aspects of sickle cell anemia in adults and children. In Disorders of Hemoglobin: Genetics, In Patophysiology, and Clinical Management. Edited by: Steinberg MH, Forget BG, Higgs DR, Nagel RL. New York: Cambridge University Press, p. 611-70, 2001.

OKPALA, I.; DANIEL, Y.; HAYNES, R.; ODOEMENE, D.; GOLDMAN, J. Relationship between the clinical manifestations of sickle cell disease and the expression of adhesion molecules on white blood cells. **Eur J Haematol,** v. 69, p. 135-144, 2002.

OKPALA, I. The intriguing contribution of white blood cells to sickle cell disease – a red cell disorder. **Blood,** v. 18, p. 65-73, 2004.

OKPALA, I. Leukocyte adhesion and the pathophysiology of sickle cell disease. **Curr Opin Hematol,** v. 13, n. 1, p. 40-4, 2006.

OMENE, J.A.; IHONGBE, J.C.; LEWIS, J.D. Serum trypsin inhibitory capacity in sickle cell disease. **East Afr Med J,** v. 57, n. 3, p. :186-9, 1980.

OULD-YAHOUI, A. ET AL. A new role for TIMP-1 in modulating neurite outgrowth and morphology of cortical neurons. **PLoS One,** v. 4, ID e8289, 2009.

OZDOGU, H.; BOGA, C.; SOZER, O.; SEZGIN N.; KIZILKILIC, E.; MAYTALMAN, E.; YALCINTAS, D. The apoptosis of blood polymorphonuclear leucocytes in sickle cell disease. **Clinical Cytometry,** v. 72B, p. 276-280, 2007.

PARKS, W. C.; WILSON, C. L.; LÓPEZ-BOADO, Y. S. Matrix metalloproteinases as modulators of inflammation and innate immunity. **Nat Rev Immunol,** v. 4, n. 8, p. 617-29, 2004.

PATHARE, A.; AL KINDI, S.; ALNAQDY, A.A.; DAAR, S.; KNOX-MACAULAY, H.; DENNISON, D. Cytokine profile of sickle cell disease in Oman. **Am J Hematol,** v. 77, n. 4, p. 323-8, 2004.

PEARSON, T.; WATTIS, J.A.; O'MALLEY, B.; PICKERSGILL, L.; BLACKBURN, H.; JACKSON, K.G.; BYRNE, H.M. Mathematical modelling of competitive LDL/VLDL binding and uptake by hepatocytes. **J Math Biol,** v. 58, n. 6, p. 845-80, 2009.

PETERS-GOLDEN, M.; CANETTI, C.; MANCUSO, P.; COFFEY, M. J. Leukotrienes: Underappreciated mediators of innate immune responses. **J Immunol** v. 174, p. 589–594, 2005.

PHILIPPIDIS, P.; MASON, J. C.; EVANS, B. J.; NADRA, I.; TAYLOR, K. M.; HASKARD, D. O.; LANDIS, R. C. Hemoglobin scavenger receptor CD163 mediates interleukin- 10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. **Circ Res,** v. 94, p. 119- 126, 2004.

PLATT OS, ORKIN SH, DOVER G, BEARDSLEY GP, MILLER B, NATHAN DG. Hydroxyurea enhances fetal hemoglobin production in sickle cell anemia. **J Clin Invest.** v. 74, n. 2, p. 652-6,1984.

PLATT, O.S.; BRAMBILLA, D.J.; ROSSE, W.F.; MILNER, P.F.; CASTRO, O.; STEINBERG, M.H.; KLUG, P.P. Mortality in sickle cell disease. Life expectancy and risk factors for early death. **N Engl J Med.** v. 330, n. 23, p. 1639-44, 1994.

PRINSEN, B.H.; ROMIJN, J.A.; BISSCHOP, P.H.; DE BARSE, M.M.; BARRETT, P.H.; ACKERMANS, M.; BERGER, R.; RABELINK, T.J; DE SAIN-VAN DER VELDEN, M.G. Endogenous cholesterol synthesis is associated with VLDL-2 apoB-100 production in healthy humans. **J Lipid Res,** v. 44, n. 7, p. 1341-8, 2003.

PRITCHARD, K. A. JR.; OU, J.; OU, Z.; SHI, Y.; FRANCIOSI, J. P.; SIGNORINO, P.; KAUL, S.; ACKLAND-BERGLUND, C.; WITTE, K.; HOLZHAUER, S.; MOHANDAS, N.; GUICE, K. S.; OLDHAM, K. T.; HILLERY, C. A. Hypoxia-induced acute lung injury in murine models of sickle cell disease. **Am J Physiol Lung Cell Mol Physiol,** v. 286, p. L705-L714, 2004.

POWARS D.R.; CHAN L.S.; HITI A., RAMICONE E.; JOHNSON C. Outcome of sickle cell anemia: a 4-decade observational study of 1056 patients. **Medicine,** v. 84**,** n.6, p.363-76, 2005.

QARI, M. H.; DIER, U.; MOUSA, S. A. Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease. **Clin Appl Thromb Hemost,** v. 18, n. 2, p. 195–200, 2012.

READER, J.; HOLT, D.; FULTON, A. Prostaglandin E2 EP receptors as therapeutic targets in breast cancer. **Cancer Metastasis Rev,** v. 30, n. 3-4, p. 449-63, 2011.

REDDING-LALLINGER, R.; KNOLL, C. Sickle cell disease--pathophysiology and treatment. **Curr. Probl. Pediatr. Adolesc. Health Care**, v. 36, n. 10, p. 346-76, 2006.

REID, M. E.; BADALOO, A.; FORRESTER, T.; JAHOOR, F. In vivo rates of erythrocyte glutathione synthesis in adults with Sickle Cell Disease. **Am J Physiol Endocrinol Metab,** v. 291, p. E73-79, 2006.

REES, D. C.; WILLIAMS, T. N.; GLADWIN, M. T. Sickle cell disease. **Lancet,** v. 376**,** p. 2018–31, 2010.

REES D. C.; GIBSON, J. S. Biomarkers in sickle cell disease. **Br J Haematol,** v. 156, n. 4, p. 433-45, 2011.

REYNOLDS, J. J. Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. **Oral Dis,** v. 2, p. 70-76, 1996.

ROLTHER, R. P.; BELL, L.; HILLMEN, P.; GLADWIN, M. T. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: A novel mechanism of human disease. **JAMA,** v. 293, p. 1653-1662, 2005.

RUF, A.; PICK, M.; DEUTSCH, V.; PATSCHEKE, H.; GOLDFARB, A.; RACHMILEWITZ, E. A.; GUILLIN, M. C.; ELDOR, A. In-vivo platelet activation correlates with red cell anionic phospholipid exposure in patients with betathalassaemia major. **Br.J Haematol**, v. 98, p. 51–56, 1997.

RYTER, S.W.; OTTERBEIN, L.E.; MORSE, D.; CHOI, A.M. Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. **Mol Cell Biochem,** v. 235, p. 249-263, 2002.

SARAF, S. L.; MOLOKIE, R. E.; NOURAIE, M.; SABLE, C. A.; LUCHTMAN-JONES L.; ENSING, G. J.; ANDREW D. CAMPBELL, A. D.; RANA, S.R.; NIU, X. M.; ROBERTO F. MACHADO, R.F.; GLADWIN, M. T.; GORDEUK, V. R. Differences in the clinical and genotypic presentation of sickle cell disease around the world. **Paediatric Respiratory Reviews,** v. 15, p. 4–12, 2014.

SASAKI, J.; WATERMAN, M.R.; BUCHANAN, G.R.; COTTAM, G.L. Plasma and erythrocyte lipids in sickle cell anaemia. **Clin Lab Haematol,** v. 5, n. 1, p. 35-44, 1983.

SATO, H.; IDO, K.; KIMURA, K. Simultaneous separation and quantification of free and metal-chelated protoporphirins in blood by three-dimensional HPLC. **Clinical Chemistry,** v. 40, n. 7, p. 1239-1244, 1994.

SAXENA, A. ; CHEN, W. ; SU, Y. ; RAI, V. ; UCHE, OU. ; LI, N. ; FRANGOGIANNIS, N.G. IL-1 induces proinflammatory leukocyte infiltration and regulates fibroblast phenotype in the infarcted myocardium. **J Immunol**, v. 1, n. 191(9), p. 4838-48, 2013.

SCHAER, D.J.; VINCHI, F.; INGOGLIA, G.; TOLOSANO, E.; BUEHLER, P.W. Haptoglobin, hemopexin, and related defense pathways-basic science, clinical perspectives, and drug development. **Front Physiol.** v. 5, p. 1-13, 2014.

SCHAFER, A.; WIESMANN, F.; NEUBAUER, S.; EIGENTHALER, M.; BAUERSACHS, J.; CHANNON, K. M. Rapid regulation of platelet activation in vivo by nitric oxide. **Circulation,** v. 109, p. 1819–1822, 2004.

SCHMIDT MI, DUNCAN BB, SHARRETT AR, LINDBERG G, SAVAGE PJ, OFFENBACHER S, AZAMBUJA MI, TRACY RP, HEISS G. Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. **Lancet**. v. 353, p. 1649-52, 1999.

SCHNOG, J. J.; HOVINGA, J. A.; KRIEG, S.; AKIN, S.; LAMMLE, B.; BRANDJES, D. P.; MAC GILLAVRY, M. R.; MUSKIET, F. D.; DUITS, A. J. ADAMTS13 activity in sickle cell disease. **Am J Hematol.** v. 81, p. 492–498, 2006.

SEARS, D. A. Disposal of plasma heme in normal man and patients with intravascular hemolysis. **J Clin Invest,** v. 49, p. 5-14, 1970.

SEDLAK, T. W.; SNYDER, S. H. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. **Pediatrics,** v. 113, p. 1776-1782, 2004.

SEIXAS, M.O.; ROCHA, L.; CARVALHO, M.; MENEZES, J.; LYRA, I.; NASCIMENTO, V.; COUTO, R.; ATTA, A.; REIS, M.G.; GONCALVES, M.S. Lipoprotein Cholesterol and Triglycerides in Children with steady-state sickle cell anemia [Abstract]. Blood, v.114, s 1547, 2009.

SEIXAS, M.O.; ROCHA, L.C.; CARVALHO, M.B.; MENEZES, J.F.; LYRA, I.M.; NASCIMENTO, V.M.; COUTO, R.D.; ATTA, A.M.; REIS, M.G.; GONCALVES, M.S. Levels of high-density lipoprotein cholesterol (HDL-C) among children with steady-state sickle cell disease. **Lipids Health Dis,** v. 9, p.91, 2010.

SEIZER, P.; MAY, A. E. Platelets and matrix metalloproteinases. **Thromb Haemost,** v. 110, n. 5, p. 903-9, 2013.

SERJEANT, G.R.; SERJEANT, B.E.; MOHAN, J.S.; CLARE, A. Leg ulceration in sickle cell disease: medieval medicine in a modern world. **Hematol Oncol Clin North Am.** v. 19, n. 5, p, 943-56, 2005.

SETTY, B.N.; CHEN, D.; STUART, M.J. Sickle red blood cells stimulate endothelial cell production of eicosanoids and diacylglycerol. **J Lab Clin Med,** v. 128, n. 3, p. 313-21, 1996.

SETTY, B.N.; STUART, M.J. Eicosanoids in sickle cell disease: potential relevance of neutrophil leukotriene B4 to disease pathophysiology. **J Lab Clin Med,** v. 139, n. 2, p. 80-9, 2002.

SETTY, B.N.; BETAL, S.G.; ZHANG, J.; STUART, M.J. Heme induces endothelial tissue factor expression: potential role in hemostatic activation in patients with hemolytic anemia. **J Thromb Haemost,** v. 6, n. 12, p. 2202-9, 2008.

SHORES, J.; PETERSON, J.; VANDERJAGT, D.; GLEW, R.H. Reduced cholesterol levels in African-American adults with sickle cell disease. **J Natl Med Assoc, v. 95, n.** 9, p. 813-7, **2003.**

SILVA, WDOS. S.; LASTRA, A; DE OLIVEIRA, S.F.; KLAUTAU-GUIMARÃES, N.; GRISOLIA, C.K. Evaluation of coverage by a neonatal screening program for hemoglobinopathies in the Recôncavo region of Bahia, Brazil. **Cad Saude Publica,** v. 22, n. 12, p. 2561-6, 2006.

SKLAR, A.H.; CAMPBELL, H.; CARUANA, R.J.; LIGHTFOOT, B.O.; GAIER, J.G.; MILNER, P. A population study of renal function in sickle cell anemia. **Int J Artif Organs,** v. 13, n. 4, p. 231-6, 1990.

SONATI, M.F. COSTA, F.F. The genetics of blood disorders: the hereditary hemoglobinopaties. **Jornal de Pediatria,** v. 84, 4 suppl, p. S40-S51, 2008.

STEINBERG, M. H.; BENZ JUNIOR, E. J. Hemoglobin synthesis, structure and function. In: HOFFMAN, R.; BENZ JUNIOR, E. J.; SHATTIL, S. J.; FURIE, B.; COHEN, H. J.; SILBERSTEIN, L. E. **Hematology.** 2 ed. New York: Churdhill Livingstonep, p. 458-468, 1995.

STEINBERG, M.H. Modulation of the phenotypic diversity of sickle cell anemia. **Hemoglobin,** v. 20, n. 1, p.1-19, 1996.

STEINBERG, M. H. Management of sickle cell disease. **N Engl J Med**, 340:1021–30, 1999.

STEINBERG, M. H., RODGERS, G. P. Pathophysiology of sickle cell disease: role of genetic cellular modifiers. **Semin Hematol,** v. 38, p. 229–306, 2001.

STEINBERG, M.H. Compound heterozygous and other sickle hemoglobinopathies. In: STEINBERG MH, FORGET BG, HIGGS DR, NAGEL RL, ed. Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management, Cambridge, UK: **Cambridge University Press**, p. 786, 2001.

STEINBERG, M.H. Pathophysiologically based drug treatment of sickle cell disease. **Trends Pharmacol Sci**. v. 27, n. 4, p. 204-10, 2006.

STEINBERG, M. H. Genetic Etiologies for Phenotypic diversity in Sickle Cell Anemia. **The Scientific World Journal.** v. 9, p. 46–67, 2009.

STOCKLEY RA, TURNER AM. α-1-Antitrypsin deficiency: clinical variability, assessment, and treatment. **Trends Mol Med,** v. 20, n. 2, p. 105-15, 2014.

STOCKLEY, R.A. Biomarkers in chronic obstructive pulmonary disease: confusing or useful? **Int J Chron Obstruct Pulmon Dis,** v. 7, n. 9, p. 163-77, 2014a.

STOCKLEY, R.A. Alpha1-antitrypsin review. **Clin Chest Med. 2014b Mar;35(1)**:39-50.
STONE, H.; PYE, A.; STOCKLEY, R.A. Disease associations in alpha-1-antitrypsin deficiency. **Respir Med,** v. 108, n. 2, p. 338-43, 2014b

STROUSE, J J.; LANZKRON, S.; URRUTIA, V. The epidemiology, evaluation and treatment of stroke in adults with sickle cell disease. **Expert Rev Hematol,** v. 4, n. 6, p. 597-606, 2011.

STUART, M.J.; NAGEL, R.L. Sickle cell disease. **Lancet.** v. 364, p.1343–60, 2004.

SWITZER, J.A.; HESS, D.C.; NICHOLS, F.T.; ADAMS, R.J. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. Lancet Neurol. v, 5, n. 6, p. 501-12, 2006.

TABAS, I.; GLASS, C.K. Anti-inflammatory therapy in chronic disease: challenges and opportunities. **Science,** v. 11, n. 339(6116), p. 166-72, 2013.

TABBARA, I. A. Hemolytic anemias: diagnosis and management. **Med Clin NorthAm,** v. 76, p, 649-668, 1992.

TASSEL, C.; ARNAUD, C.; KULPA, M. *et al*. Leukocytosis is a risk fa ctor for lung function deterioration in children with sickle cell disease. **Respir Med,** v. 105, n. 5, p.788–95, 2011.

TAYLOR, G.O.; BAMGBOYE, A.E. Serum cholesterol and diseases in Nigerians. **The American Journal of Clinical Nutrition,** n. 32, p. 2540-2545, 1979.

TAYLOR, G. J.; TANG, C. D.; SAVAGE, A. S.; LEITMAN, F. S.; HELLER, I. S.; SERTEJEANT, R. G.; RODGERS, P. G.; CHANOCK, J. S. Variants in the VCAM-1 gene and risk for symptomatic stroke in sickle cell disease. **Blood**, v. 100, p. 4303-4309, 1997.

TAYLOR, C., CARTER, F., POULOSE, J., ROLLE, S., BABU, S., e CRICHLOW, S. Clinical presentation of acute chest syndrome in sickle cell disease. **Postgraduate Medical Journal,** v. 80, n. 944, p. 346–349, 2004.

TAYLOR, J. G.; NOLAN, V. G.; MENDELSOHN, L.; KATO, G. J.; GLADWIN, M. T.; STEINBERG, M. H. Chronic hyper-hemolysis in sickle cell anemia: association of vascular complications and mortality with less frequent vasoocclusive pain. **PLoS ONE,** v. 3, e2095, 2008.

TETE-BENISSAN, A.C.; DURIEZ, P.; PARRA, H.J. Study of the protein profile of the Adélé tribe of Togo. **Sante,** v. 10, n. 4, p. 261-6, 2000.

TRAINA, F.; JORGE, S.G.; YAMANAKA, A.; DE MEIRELLES, L.R.; COSTA, F.F.; SAAD, S.T. Chronic liver abnormalities in sickle cell disease: a clinicopathological study in 70 living patients. **Acta Haematol,** v. 118, n. 3, p. 129-35, 2007.

TURHAN, A.; WEISS, L.A.; MOHANDAS, N.; COLLER, B.S.; FRENETTE, P.S. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. **Proc Natl Acad Sci U S A.** v. 99, n. 5, p. 3047-51, 2002.

UZUNOVA, V.V.; PAN, W.; GALKIN, O.; VEKILOV, P.G. Free heme and the polymerization of sickle cell hemoglobin. **Biophys J,** v. 22, n. 99(6), p. 1976-85, 2010.

VANDERJAGT, D.J.; HUANG, Y-S.; CHUANG, L-T. Phase angle and n-3 polyunsaturated fatty acids in sickle cell disease. **Arch Dis Child,** v. 87, p. 252-254, 2002a.

VANDERJAGT, D.J.; SHORES, J.; OKORODUDU, A.; OKOLO, S.N.; GLEW, R.H. Hypocholesterolemia in Nigerian children with sickle cell disease. **J. Trop Pediatr,** v. 48: p. 156-161, 2002b.

VEIGA, P.C.; SCHROTH, R.J.; GUEDES, R.; FREIRE, S.M.; NOGUEIRA-FILHO, G. Serum cytokine profile among Brazilian children of African descent with periodontal inflammation and sickle cell anaemia. **Arch Oral Biol,** v. 58, n. 5, p. 505-10, 2013.

VERDUZCO, L.A; NATHAN, D.G. Sickle cell disease and stroke. **Blood**. v. 114, n. 25, p. 5117-25, 2009.

VICHINSKY, E.P. et al.Causes and outcomes of the acute chest syndrome in sickle cell disease. National Acute Chest Syndrome Study Group. **N Engl J Med.** v. 342, n. 25, p. 1855-65, 2000. Erratum in: **N Engl J Med** v. 343, n. 11, p. :824, 2000.

VILAS-BOAS, W.; CERQUEIRA, B.A.; ZANETTE, A.M.; REIS, M.G.; BARRAL-NETTO, M.; GONCALVES, M.S. Arginase levels and their association with Th17-related cytokines, soluble adhesion molecules (sICAM-1 and sVCAM-1) and hemolysis markers among steady-state sickle cell anemia patients. **Ann Hematol.** v. 89, n. 9, p. 877-82, 2010.

VILLAGRA, J.; SHIVA, S.; HUNTER, L. A.; MACHADO, R. F.; GLADWIN, M. T.; KATO, G. J. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension and nitric oxide scavenging by cell-free hemoglobin. **Blood,** v. 110, p. 2166–2172, 2007.

WAKEFIELD, T.W.; MYERS, D.D.; HENKE, P.K. Mechanisms of venous thrombosis and resolution. **Arterioscler Thromb Vasc Biol,** v. 28, n, 3, p. 87-91, 2008.

WANG, C.W.; LUKENS J.N. Sickle cell anemia and other sickling syndromes. In: LEE, G.R.; FOERSTER, J.; LUKENS, J.N.; PARASKEVAS, F.; GREER, J.P.; RODGERS, G.M. **Wintrobe's clinical hematology.** 10th. ed. Baltimore: Williams e Wilkinscap. 1998. cap. 51, p. 1346-1404,

WANG T, ZHANG X, LI JJ. The role of nf-kappab in the regulation of cell stress responses. **Int Immunopharmacol.** v. 2, n. 11, p. 1509-20, 2002.

WESTWICK, J.; WATSON-WILLIAMS, E. J.; KRISHNAMURTHI, S.; MARKS, G.; ELLIS, V.; SCULLY, M. F.; WHITE, J. M.; KAKKAR, V. V. Platelet activation during steady state sickle cell disease. **J Med,** v.14, p. 17–36, 1983.

WEATHERALL, D.J.; PROVAN, A.B. Red cells In: inherited anaemias. **Lancet,** v. 355, p. 1169-1175, 2000.

WORLD HEALTH ORGANIZATION. Genes and Human Disease: Sickle Cell Anemia, 2012. Acesso em janeiro de 2014. Disponível no endereço eletrônico: http://www.who.int/genomics/public/geneticdiseases/en/index2.html#SCA.

WUN, T.; PAGLIERONI, T.; RANGASWAMI, A.; FRANKLIN, P. H.; WELBORN, J.; CHEUNG, A.; TABLIN, F. Platelet activation in patients with sickle cell disease. **Br.J Haematol, 100**:741–749, 1998.

YANAGISAWA, H.; HAMMER, R.E.; RICHARDSON, J.A.; WILLIAMS, S.C.; CLOUTHIER, D.E.; YANAGISAWA, M. Role of Endothelin-1/Endothelin-A receptor-mediated signaling pathway in the aortic arch patterning in mice. J Clin Invest. v. 102, n. 1, p. 22-33, 1998.

YANG, Y.; WANG, Z.; YANG, H.; WANG, L.; GILLESPIE, S.R.; WOLOSIN, J.M.; BERNSTEIN, A.M.; REINACH, P.S. TRPV1 potentiates TGFβ-induction of corneal myofibroblast development through an oxidative stress-mediated p38-SMAD2 signaling loop. **PLoS One,** v. 2, n. 8(10), p. e77300, 2013.

ZAGER, R.A.; JOHNSON, A.C.; FROSTAD, K.B. Rapid renal alpha-1 antitrypsin gene induction in experimental and clinical acute kidney injury. **PLoS One,** v. 21, n. 9(5), p. e98380, 2014.

ZHOU, Z.; HAN, H.; CRUZ, M. A.; LOPEZ, J. A.; DONG, J. F.; GUCHHAIT, P. Haemoglobin blocks von Willebrand factor proteolysis by ADAMTS-13: a mechanism associated with sickle cell disease. **Thromb Haemost,** v. 101, p. 1070–1077, 2009.

ZORCA, S.; FREEMAN, L.; HILDESHEIM, M.; ALLEN, D.; REMALEY, A.T.; TAYLOR, J.G.; KATO, G.J. Lipid levels in sickle-cell disease associated with haemolytic severity, vascular dysfunction and pulmonary hypertension. **Br J Haematol.** v. 149, n. 3, p. 436-45, 2010.

APÊNDICE

A - MANUSCRITOS (Artigos produzidos em colaboração durante o período do doutorado e que não entraram no corpo da tese).

A.1 – MANUSCRITO I

The Leftward deletion 4.2 Kb Alpha-Thalassemia in two sickle cell anemia siblings

A.2 – MANUSCRITO II

Cytokine profiles in sickle cell anemia: Pathways to be unraveled

A.3 – MANUSCRITO III

Sickle cell disease: Only one road, but different pathways for inflammation

A.4 – MANUSCRITO IV

Sickle cell disease retinopathy: characterization among pediatric and teenage patients from Northeast Brazil

B. TERMOS DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

B.1 - PARA MENORES DE 18 ANOS

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C. QUESTIONÁRIO

D. MATERIAS E MÉTODOS

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A - MANUSCRITOS

A.1 – MANUSCRITO I

The Leftward deletion 4.2 Kb Alpha-Thalassemia in two sickle cell anemia siblings

Daniele Takahashi, Silvana S. Paz, Magda O. Seixas, Cynara G. Barbosa, Cyntia Cajado, Nadja J. Gonçalves-Santos, Elisangela V. Adorno, Isa M. Lyra, Larissa C. Rocha, Mitermayer G. Reis, Marilda S. Gonçalves.

Este é um relato de caso de dois irmãos provenientes do Estado da Bahia, Brasil, que têm Anemia Falciforme e são portadores silenciosos da deleção -α 4.2Kb da alfa talassemia. **Gazeta Médica da Bahia, 2010;80:3.**

Resumo: A presença da deleção –α thal 3.7Kb está associada com melhor prognóstico de pacientes que possuem anemia falciforme (AF), contudo não existem estudos na literatura a respeito da associação da –α thal 4.2Kb com a evolução clínica desses pacientes. No presente relato são descritos achados laboratoriais e perfil de hemoglobina de dois irmãos que possuem AF em associação com a –α thal 4.2Kb. Ambos os pacientes apresentam anemia acentuada, baixos índices de volume corpuscular médio (VCM) e contagem de leucócitos elevada. Estudos adicionais são necessários para elucidar a possível associação entre a –α thal 4.2Kb e a gravidade da AF.

Palavras-chave: doença falciforme, doença SC, talassemia, haplótipos.

THE LEFTWARD DELETION $^{4.2\ KB}$ ALPHA-THALASSEMIA IN TWO SICKLE CELL ANEMIA SIBLINGS

DELEÇÃO ^{4,2KB} DA ALFA-TALASSEMIA EM DOIS IRMÃOS COM ANEMIA FALCIFORME

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The presence of –αthal 3.7Kb deletion is associated with better prognosis of Sickle Cell Anemia (SCA) patients, but here are not reports in the literature regarding association of –α thal 4.2Kb and its importance among SCA clinical outcome. In this report, we describe Hemoglobin profile and laboratory findings of two siblings who have SCA and are silent carriers of –α thal 4.2Kb. Both described patients have severe anemia, lower rates of Mean Corpuscular Volume (MCV) and a high leukocytes count. Further studies are required to establish a possible association between –α thal 4.2Kb and SCA severity.

Keywords: Alpha thalassemia, sickle cell anemia, hemoglobin.

A presença da deleção –α thal 3.7Kb está associada com melhor prognóstico de pacientes que possuem anemia falciforme (AF), contudo não existem estudos na literatura a respeito da associação da –α thal 4.2Kb com a evolução clínica desses pacientes. No presente relato são descritos achados laboratoriais e perfil de hemoglobina de dois irmãos que possuem AF em associação com a –α thal 4.2Kb. Ambos os pacientes apresentam anemia acentuada, baixos índices de Volume Corpuscular Médio (VCM) e contagem de leucócitos elevada. Estudos adicionais são necessários para elucidar uma possível associação entre a –α thal 4.2Kb e a gravidade da AF.

Palavras-chave: doença falciforme, doença SC, talassemia, haplótipos.

Alpha-thalassemia, the most common single-gene disease in the world, is characterized by a reduction or complete absence of α-globin gene expression. Many deletions have been described in the Alpha (α)-globin gene located at the short arm of chromosome 16, but the most prevalent are the –α thalassemia with 3.7 kilobases (Kb) deletion (–α thal 3.7Kb) and the –α thalassemia with 4.2 Kb (–α thal 4.2Kb) which are originated by homologous recombination between misaligned chromosomes[4].

Sickle cell anemia (SCA) patients have heterogeneous clinical manifestations, including hemolysis, chronic inflammation and painful crisis. The presence of –α thal 3.7Kb deletion is associated with better prognosis of SCA patients[5]. There are not reports in the literature regarding association of –α thal 4.2Kb and its importance among SCA clinical outcome.

In this report, we describe two siblings from Bahia State in Brazil, who have SCA and are silent carriers of –α thal 4.2Kb.

Whole-blood samples were collected from the *HBSS* patients attending the out-patients clinic in HEMOBA. Hematological analyses were carried out using an electronic cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC / VARIANT I; BIO-RAD, CA, USA). Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain).

DNA was isolated from the white blood cells (WBC) by FlexiGene DNA Kit, Qiagen (USA), according to the manufacturer's recommendations. Beta-globin gene haplotypes were investiged by PCR-RFLP[6]. The alpha-thalassemia was confirmed in the two siblings by a single-tube multiplex PCR method to detect the wide type, the –α thal 3.7Kb, and –α thal 4.2Kb alleles, using primers previously described[3].

The study was approved by the Oswaldo Cruz Research Foundation's Human Research Board (number CAAE 0024.0.225.000-06), and the study was based in accordance with Declaration of Helsinki of 1975, as revised in 2000 and all subjects or official responsible filled out a written informed consent form.

The patient number 1 is a 7-years-old afro-descendent boy, which has had recurrent hospitalizations (more than 3) with many painful crisis, meningitis episode and blood transfusion history. The patient number 2 is a 5-years-old afro-descendent girl and her clinical history indicated recurrent hospitalizations (more than 9), pneumonia episode and blood transfusion history. Moreover, this patient has been submitted to splenectomy surgery.

The laboratory findings and hemoglobin profile of both patients are described in the Table 1, which shows severe anemia, lower rates of Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCHC), a high leukocytes count and an increase of iron and ferritin serum levels that

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Table 1. Hemoglobin profile and laboratory findings of the two -$\alpha^{4.2}$/SCA patients.

	Patient 1	Patient 2
Age (years)	7	5
Gender	Male	Female
Hemoglobins (%)		
S	90.8	86.2
Fetal	5.8	10.6
A2	3.4	3.2
Beta-globin gene Haplotypes	Ben/Car	Ben/Car
Hemolysis		
Erythrocyte, million/mL	2,84	2,27
Hemoglobin, g/dL	6.9	5.5
Hematocrit, %	21.9	18.1
Mean Cell Volume, fL	77.1	79.7
Mean Cell Hemoglobin, pg	24.3	24.2
Reticulocytes Count, %	8.0	5.0
Lactate dehydrogenase,U/L	248	376
Leukocyte		
Leukocyte Count, /mL	16,300	34,300
Platelets		
Platelets cunt, thousand/mm^3	296	268
Iron metabolism		
Iron serum, mcg/dL	715	858
Ferritin, ng/mL	1,391.3	529.50
Lipidic metabolism		
Total Cholesterol, mg/dL	112	129
HDL Cholesterol, mg/dL	29	36
LDL Cholesterol, mg/dL	71	76
VLDL Cholesterol, mg/dL	12	17
Tryglicerides, mg/dL	62	86
Hemolysis plus Hepatic		
Aspartate aminotransferase, U/L	65	66
Total bilirrubin, mg/dL	1.4	0.5
Direct bilirrubin, mg/dL	0.6	0.2
Indirect bilirrubin, mg/dL	0.8	0.3
Hepatic		
Alanine aminotransferase, U/L	73	22
Renal		
Urea nitrogen, mg/dL	12	11
Creatinine, mg/dL	0.4	0.4
Total protein, g/dL	7.1	6.8
Albumin, g/dL	3.8	3.3
Globulin, g/dL	3.3	3.5
Inflammation		
C-reactive protein, mg/mL	39.2	103
Alpha 1 antitrypsin, mg/dL	222	250
ASLO (UI/mL)	132	133

could be associated with an increase of reactive oxygen species (ROS) and consequently with a increase of clinical severity[2]. The coexistence of alpha-talassemia and SCA has been related with a higher survival rates and a decreased hemolysis markers and with a frequent vaso-occlusive episodes and painful crisis[1], but the same approach is not available for the -$\alpha^{4.2Kb}$/SCA association, requiring further study.

References

1. Adorno EV, Couto FD, Moura Neto JP, Menezes JF, Rêgo M, Reis MG, Gonçalves MS. Hemoglobinopathies in newborns from Salvador, Bahia, Northeast Brazil. Cad Saude Publica 21: 292-298, 2005.
2. Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, Fibach E. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. Br J Haematol 132: 108-113, 2006.
3. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of a-thalassemia. Blood 95: 360-362, 2000.
4. Higgs DR, Vickers MA, Wilkie AO, Pretorius IM, Jarman AP, Weatherall DJ. A review of the molecular genetics of the human alpha-globin gene cluster. Blood 73: 1081-1104, 1989.
5. Steinberg MH. Genetic etiologies for phenotypic diversity in sickle cell anemia. ScientificWorldJournal. 18: 46-67, 2009.
6. Sutton M, Bouhassira EE, Nagel RL. Polymerase chain reaction amplification applied to the determination of beta-like globin gene cluster haplotypes. Am J Hematol. 32: 66-69 1989.

A.2 – MANUSCRITO II

Cytokine profiles in sickle cell anemia: Pathways to be unraveled

Thassila Nogueira Pitanga1,2, Wendell Vilas-Boas1,2, Bruno Antônio Veloso Cerqueira1,2,3, Magda Oliveira Seixas1,2,4, Cynara Gomes Barbosa1,2,4, Elisângela Vitória Adorno2,4, Marilda Souza Goncalves1,2,4*

Este trabalho revisa o papel das citocinas nos diversos eventos clínicos associados a anemia falciforme. **Advances in Bioscience and Biotechnology, 2013, 4, 6-12.**

Resumo: A anemia falciforme (AF) é uma doença hemolítica herdada geneticamente, caracterizada por inflamação crônica. A expressão de citocinas afeta as vias centrais que contribuem para a patogênese da doença, mas os mecanismos envolvidos não são bem compreendidos. A AF está associada ao estado pró-inflamatório e a resposta inflamatória aumentada que ocorre durante a crise vaso-oclusiva. O sistema imunitário desempenha, assim, um papel importante nesta doença inflamatória, com vários tipos de células secretoras de citocinas pró-inflamatórias que contribuem para a ocorrência de acontecimentos cíclicos comuns em pacientes com AF, tal como hemólise, oclusão vascular e inflamação. O estudo das citocinas e quimiocinas em pacientes AF tem esclarecido os mecanismos envolvidos e destacam a necessidade de uma melhor compreensão da participação das citocinas na fisiopatologia da AF.

Palavras-chave: Anemia Falciforme; Citocinas; Quimiocinas; Inflamação; Inflamosoma

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Cytokine profiles in sickle cell anemia: Pathways to be unraveled

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ABSTRACT

Sickle cell anemia (SCA) is a genetically inherited hemolytic disorder characterized by chronic inflammation. Cytokine expression affects the pivotal pathways that contribute to disease pathogenesis, but the mechanisms involved are not well understood. SCA is associated with a proinflammatory state, and an enhanced inflammatory response occurs during vaso-occlusive crisis. The immune system thus plays an important role in this inflammatory condition, with several cell types secreting pro-inflammatory cytokines that contribute to the occurrence of common cyclical events in SCA patients, such as hemolysis, vascular occlusion and inflammation. Studies of these cytokines and chemokines in SCA patients have clarified the mechanisms that underlie this disease and highlighted the need for a better understanding of cytokine participation in SCA pathophysiology.

Keywords: Sickle Cell Anemia; Cytokines; Chemokine; Inflammation; Inflammasome

1. INTRODUCTION

Sickle cell anemia (SCA) is an inherited disorder characterized by homozygosis for the mutation that causes hemoglobin S (HbS) production. This point mutation (GAG > GTG) occurs in the sixth codon of the beta globin gene (*HBB*) and causes valine to replace glutamic acid in the sixth amino acid of the beta (β) globin chain of the hemoglobin molecule. SCA patients have a heterogeneous clinical outcome characterized by painful vaso-occlusive crises, stroke, priapism, pulmonary hypertension, acute chest syndrome (ACS) and chronic organ injuries. As a result of this mutation, deoxygenated hemoglobin molecules undergo a polymerization process that is considered the primary event leading to the pathogenesis of SCA [1].

Sickled red blood cells, as well as leukocytes, platelets and the vascular endothelium, are elements that obstruct vessels and trigger vaso-occlusive crises. The hemolysis that occurs in SCA can be both extravascular and intravascular. Intravascular hemolysis occurs when red blood cells (RBCs) rupture and release free hemoglobin into the plasma. Free hemoglobin has inflammatory and oxidant effects that lead to endothelium dysfunction. Other hemolysis products, including heme, reactive oxygen species (ROS) and reactive nitrogen species, are also released into the bloodstream, where they cause increased oxidative stress and decreased plasma levels of the vasodilator nitric oxide (NO) [2]. Increased ROS and RNS levels and decreased NO levels contribute to the activation of RBCs, leukocytes, platelets and endothelial cells. This activation leads to increased production of proinflammatory and anti-inflammatory cytokines, which gives SCA the characteristics of a chronic inflammatory disease [1,3] (**Figure 1**).

Several cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), are associated with the activation of leukocytes, particularly monocytes and neutrophils, in SCA. Several other cytokines are also involved in the chronic inflammatory state that is present in SCA. The activation of cells and the release of cytokines stimulate the NF-κB transcription factor pathway, which regulates the production of interleukin-4 (IL-4), interleukin-6 (IL-6) and interleukin-8 (IL-8). IL-6 and

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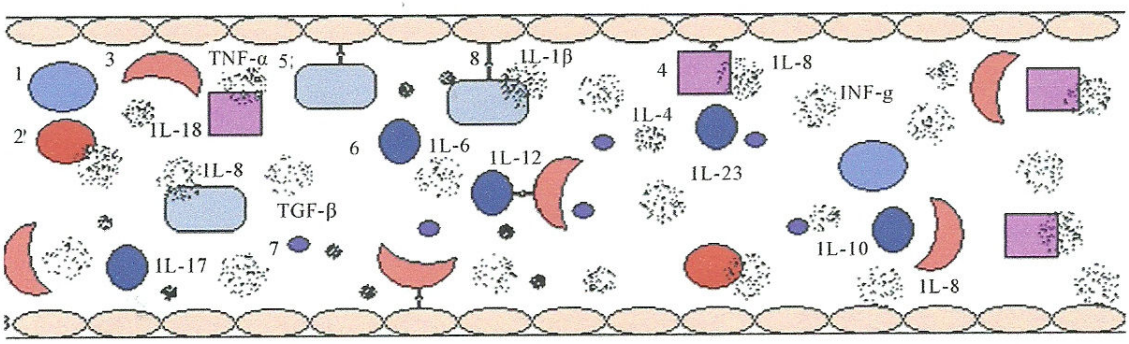

Figure 1. Representation of the vascular inflammatory environment, including the relevant cell types, corpuscles and cytokines. 1 = reticulocytes; 2 = red blood cells; 3 = sickled red blood cells; 4 = neutrophils; 5 = monocytes; 6 = lymphocytes; 7 = platelets and 8 = endothelial cells.

IL-8 production are also enhanced by the STAT3 intracellular pathway and proinflammatory activities [4]. Recently, the involvement of several other cytokines, such as IL-18, IL-17, IL-23, IL-12 and IL10, in inflammatory responses in SCA patients has been described. Because of the extensive participation of cytokines in the inflammatory processes involved in SCA pathology, this review of the cytokines and signaling pathways involved in SCA will contribute to an improved understanding of SCA pathology, especially the pathology of inflammatory vaso-occlusive events.

2. INFLAMMASOME, IL-1β AND IL-18

The junction of many inflammatory molecules is the basis of the inflammasome [5]. This complex forms in response to danger signals [6], and the efficiency of the immune response depends not only on the presence of foreign antigens but also on the release of signaling molecules by stressed or damaged tissues [7]. In SCA, danger signals can be generated by hypoxia and metabolic acidosis, which lead to cell necrosis, hypokalemia and the formation of uric acid and ADP [8].

The imbalance or disruption of cellular integrity can trigger the formation of an inflammasome complex [5]. Inflammasomes activate a class of caspases known as inflammatory caspases [9], whose protease activity promotes the conversion of IL-1 beta (IL-1β) and IL-18 to their active forms [10]. Pro-inflammatory cytokines influence the clinical profile of SCA patients by increasing the adhesion of RBCs and leukocytes to the endothelium. These adhered cells trigger a cycle of increasing aggregation of sickled RBCs, platelets and neutrophils, which causes microvascular occlusion [11]. Qari *et al.* (2012) observed higher plasma concentrations of IL-1β in SCA patients during both the steady state and painful crises than in control subjects. However, higher levels were observed during the steady state than during painful crises. In 2011, Asare *et al.* demonstrated that plasma IL-1β levels are a good predictor of stroke outcome. In addition, high plasma IL-1β levels were associated with protection against stroke development in juvenile SCA patients with altered transcranial Doppler studies [12]. Other studies highlighted the need for an IL-1β-targeted therapeutic approach for the treatment of SCA in patients with a severe clinical profile [13].

Our team was the first to demonstrate that the inflammasome complex plays a role in SCA [14]. We observed increased serum IL-18 levels in SCA patients. Levels of this cytokine were positively correlated with classic danger signals, including uric acid and the hemolysis marker lactic dehydrogenase (LDH), which is indirectly related to oxidative stress. In SCA patients, intravascular hemolysis increases ROS production, which is a highly conserved signal involved in damage and stress sensing. The release of ROS promotes vascular occlusion by 2 mechanisms: classical endothelial activation and ROS-mediated activation of the inflammasome via membrane receptors like NALP3 [15]. In addition, SCA patients with the highest serum concentrations of uric acid displayed increased serum concentrations of IL-18, which strongly suggests that the inflammasome plays a role in SCA. These studies demonstrate that IL-1β and IL-18 are important players in vascular modulation and clinical pathology in SCA patients, but further analysis is needed to understand the interaction of these cytokines with other cytokines, oxidative molecules, classic prognosis markers and epigenetic factors.

3. IL-6 AND IFN-γ

Sickle cell anemia is characterized by painful vaso-occlusive crises (VOC), chronic inflammation and recurrent infections. In the early stages of an infection, CD4+ T cells differentiate into two main classes of effectors: TH1 cells induce cytotoxic CD8+ T cells and the inflammatory response, resulting in the production of cytokines, such as interleukin-12 (IL-12), interleukin-2 (IL-2), interferon gamma (IFN-γ) and TNF-γ; and TH2 cells produce cytokines such as IL-4, IL-5, IL-6 and IL-10, which are

important in the production of antibodies. TH1 and TH2 cells play distinct roles in physiological and pathological conditions [16-18].

The interleukin-6 cytokine family promotes a variety of cellular functions, including differentiation, maturation, proliferation and survival. These cytokines are defined by their common usage of the widely expressed signal-transducing b subunit of the transmembrane receptor glycoprotein 130 (gp 130), which is a member of the class of type I cytokine receptors [19,20].

IFN-γ is a glycoprotein produced by CD4+ and CD8+ T cells after activation by natural killer (NK) cells. The immunoregulatory functions of IFN-γ are diverse and include the activation of mononuclear phagocytes, the upregulation of class I molecules of the major histocompatibility complex (MHC-I), the stimulation of NK cell cytolytic activity and the activation of neutrophils [21].

In a study performed in Oman, Pathare *et al.* (2004) demonstrated that the mean serum level of IFN-γ was higher in SCA patients than in control subjects. This difference was significant in patients during the steady state but not during crises [11]. In this study, the mean serum concentration of IL-6 was higher in SCA patients than in normal controls, and there was also a significant increase in IL-6 levels in crisis patients when compared to steady-state patients. Hibbert *et al.* (2005) showed that the IL-6 levels were significantly higher in the SCA group than in healthy subjects [22]. Veiga *et al.* (2013) used microarrays to investigate cytokine levels in SCA patients with periodontal inflammation and found that the SCA group displayed significantly higher levels of various cytokines ($p < 0.05$), including IFN-γ, than the control group. There was also elevated production of IL-6 in these patients than in control patients, but this difference did not reach statistical significance [23].

SCA patients with a history of chronic transfusion therapy were recruited from the hematology clinic at the Children's Hospital and Research Center Oakland (CHRCO) at the time of a clinically indicated liver biopsy for the evaluation of iron overload. Inflammatory cytokine levels were higher in SCA patients than in control subjects. Although the observed differences in IFN-γ levels were not statistically significant, IL-6 levels were significantly higher in SCA patients than controls [24]. Plasma IL-6 levels were significantly elevated in patients during both painful crises and the steady state when compared to the age-matched control group. Surprisingly, IL-6 levels were significantly higher during the steady state than during painful crises. Plasma levels of IFN-γ showed a slight elevation during painful crises when compared to the steady state, but this difference was not statistically significant when compared to the healthy, age-matched control group [25].

The reported data concerning IL-6 and IFN-γ levels in SCA patients are inconsistent. Some investigators report elevated plasma levels of these proinflammatory cytokines, supporting a role for cytokine driven inflammation. Other investigators report normal or reduced levels of the same proinflammatory cytokines. These conflicting reports highlight the need for further studies regarding the role of these cytokines in SCA.

4. IL-8 AND TNF-α

Interleukin-8 is a pro-inflammatory member of the CXC chemokine family and is involved in both endothelial cell proliferation and angiogenesis [26]. This chemokine is produced by several types of cells, such as neutrophils, endothelial cells, macrophages and fibroblasts [27], and has a number of effects including re-arrangement of the cytoskeleton, changes in intracellular Ca++ levels, activation of integrins and promotion of protein-granule exocytosis and the respiratory burst [28]. The IL-8 receptors CXCR1 and CXCR2, which are expressed mainly by neutrophils, enhance neutrophil recruitment and promote defense against bacterial pathogens [26,29]. The pro-inflammatory cytokine TNF-α is produced mainly by monocytes/macrophages, but other cells, such as T-cells, smooth muscle cells, adipocytes and fibroblasts, can also produce this cytokine. TNF-α is named for its ability to stimulate tumor necrosis and regression *in vivo* [27]. Biological responses to TNF-α are mediated by two groups of receptors, TNFR55 and TNFR 75, which are present on the membrane of several types of cells, excluding RBCs [30].

IL-8 induces leukocyte chemotaxis, and TNF-α stimulates increased expression of adhesion molecules on endothelial cells, contributing to leukocyte adhesion. Both cytokines stimulate RBC adhesion to endothelial cells [11]. In addition, these cytokines induce neutrophil degranulation, capillary leak and vasoconstriction [11], and TNF-α inhibits cell proliferation and induces cell death [27].

IL-8 and TNF-α contribute to the vascular inflammatory state that is present in various inflammatory diseases, including SCA. As mentioned previously, these cytokines induce increased adhesion of RBC and leukocytes to the vascular endothelium, and this adhesion can cause vaso-occlusion and local hypoxia [11]. Several studies have shown that patients display higher levels of a number of cytokines, especially IL-8, during VOC than during the steady state [11,31-34]. In contrast to these findings, other studies showed that the levels of IL-8 were similar between patients in crises and patients in steady-state [35]. In addition, although SCA patients in VOC had higher levels of TNF-α than patients in the steady-state group, this difference was not statistically significant [36]. Hypoxia is a common consequence of

vaso-occlusion. One study reported that hypoxia induces IL-8 expression [37], contributing to increased plasma IL-8 levels and vascular inflammation.

SCA patients who are treated with hydroxyurea (HU) display an altered profile of IL-8 and TNF-α levels. Treatment with HU is associated with increased serum levels of circulating IL-8 [31]. Other studies reported conflicting results, demonstrating that patients undergoing HU therapy displayed significantly lower plasma levels of IL-8 [34,38]. Other studies showed that HU did not significantly increase [36] or decrease [34] plasma TNF-α concentrations.

In addition, IL-8 levels are positively correlated with HbS and negatively correlated with F hemoglobin (HbF) [32]. Tavakkoli *et al.* (2004) showed that an increase in HbF concentration was not associated with a significant change in plasma TNF-α levels [36].

These results demonstrate that increased levels of circulating IL-8 and TNF-α are associated with increased hemolysis, vascular occlusion and inflammation. However, further studies are needed to understand the interrelationships of IL-8 and TNF-α with other proinflammatory cytokines in SCA patients.

5. IL-17 AND TGF-β

Ischemic events that result from the occlusion of major and minor vessels involve interactions between RBCs, leukocytes and the endothelium, and these interactions are regulated by cytokines secreted by leukocytes, adhesion molecules and, consequently, the immune response, which is involved in the initiation and development of crises in SCA [39]. The importance of cytokine IL-17 is well established [40], but recent data has demonstrated that IL-17 is produced by a subset of T cells, named Th17 cells, that is distinct from TH1 and TH2 cells [41]. IL-17 plays an important role in allergic responses and promotes inflammation by inducing the production of inflammatory cytokines and chemokines, the recruitment of neutrophils, the production of antibodies and the activation of T cells [42]. The differentiation of TH17 cells from naïve cells requires transforming growth factor-beta (TGF-β) and the subsequent expansion of the TH17 lineage requires IL-23 [43].

The roles of IL-17 and TGF-β in SCA are not well understood. Keikhaei *et al.* (2013) recently observed higher levels of IL-17 and TGF-β in steady-state patients than in healthy controls, but there was no difference between steady-state and crisis patients. The authors also demonstrated that HU-treated patients displayed lower levels of IL-17 than patients who did not receive this treatment [31]. Our team demonstrated a positive correlation between levels of free serum arginase and the levels of TGF-β in HbSS patients [44]. This finding raises the possibility that TGF-β induces upregulation of the arginase pathway and downregulation of the NO pathway. This switch in arginine metabolism could play a role in vascular activation and the increased serum arginase levels that lead to chronic hemolysis in HbSS individuals.

The role of IL-17 and TGF-β cytokines in HbSS pathophysiology remains unclear. Recent studies have demonstrated interesting changes in serum levels of these cytokines in SCA patients. These results warrant further investigation.

6. IL-12 AND IL-23

IL-12 and IL-23 are proinflammatory cytokines produced by macrophages and dendritic cells in response to microbial pathogens [45,46]. IL-12 regulates both innate and adaptive immunity. A major function of IL-12 is the induction of IFN-γ production by NK cells, T cells, B cells, and even antigen-presenting cells. Thus, IL-12 appears to be the main cytokine that regulates TH1 differentiation. In addition, IL-12 antagonizes TH 2 differentiation and the production of IL-4, IL-5 and IL-13 [45].

Like IL-12, IL-23 induces the production of IFN-γ by human T cells. In addition, IL-23 plays a key role in TH17 development by stabilizing both IL-17 expression and the TH17 phenotype. However, IL-23 is not a differentiation factor for TH17 cells and instead contributes to the induction of a pathogenic phenotype in TH17 cells [47]. IL-12 can also negatively affect the development, homeostasis and function of nTreg cells by limiting IL-2 expression [48].

There are few reports in the literature pertaining to the role of IL-12 in SCA. Taylor *et al.* (1999) [49] investigated the levels of several TH1 cytokines in both steady-state SCA patients and healthy control subjects, but detectable levels of IL-12 were not observed in either group. This result could be because the expression of significant amounts of IL-12 depends on microbial infection or altered immune responses, such as those observed in chronic immune-mediated diseases. Thus, it remains important to investigate IL-12 levels in SCA patients during crises. Hassan *et al.* (2009) performed a similar study of HbAS children who were infected with Plasmodium falciparum. Surprisingly, detectable levels of IL-12 were found in patients with mild malaria, but not in asymptomatic individuals. This finding could be related to the low levels of IL-10 that are typically associated with this infection, as IL-10 is a potent inhibitor of IL-12 [50].

With respect to the relationship between IL-23 and SCA, there is a report that investigated the possible association of IL-23 with arginase levels, an important molecule in the clinical outcome and in the vascular endothelium in inflammatory diseases. Hence, IL-23 was detected in steady-state patients, but it was not correlated

to arginase, which was considered an interesting result [44].

7. ANTI-INFLAMMATORY CYTOKINES

IL-4 is an anti-inflammatory cytokine that participates in the regulation of the immune system at multiple levels. IL-4 promotes TH2 cell differentiation and inhibits TH1 cell differentiation. IL-4 is also a growth and survival factor that protects lymphocytes from apoptosis [51]. This cytokine plays an important role in the pathogenesis of allergic disease, particularly atopic asthma, and is essential for immune responses to parasitic infections [52]. In addition, IL-4 is affected by several alternative pathways of immune regulation, including alternative mRNA splicing. This process yields at least two functional isoforms of IL-4, full-length IL-4 and IL-4δ2. These variants have similar effects, but they can bind different types of receptors on the surface of target cells. Considering the varied functions of IL-4, abnormal regulation of this cytokine may cause immune disease [53].

The role of IL-4 in SCA is controversial. While some studies have reported that IL-4 levels increase during VOC, other studies have demonstrated that IL-4 levels are higher during the steady-state [39,54]. In 2000, an investigation of the TH2 cytokine levels in SCA patients revealed that plasma IL-4 levels were significantly higher among steady-state HbSS patients than HbAA and HbAS individuals. In addition, the ratios of plasma IL-2 to IL-4 and IFN-γ to IL-4 were significantly lower in HbSS patients than in the other groups, suggesting a possible mechanism for the predisposition of SCA patients to bacterial infections [54]. A recent study of SCA patients with asthma in Jamaica and London indicated that IL-4 levels may differ between children in developed countries and children in developing countries [55].

Although IL-10 is mainly produced by activated CD8+ cells, it can also be produced by other types of cells, such as activated TH0, TH1 and TH2 cells, B lymphocytes, mast cells and lipopolysaccharide-activated monocytes. The synthesis of IL-10 is inhibited by IL-4 and by itself [56]. IL-10 is an anti-inflammatory cytokine whose main effect is inhibition of the synthesis of various cytokines, such as TNF-α, GM-CSF, IL-1, IL-6, IL-8 and IL-12, to promote the uptake and retention of iron within monocytes and the reticuloendothelial system. IL-10 also inhibits the proliferation of TH1 cells, decreasing cytolytic function and the secretion of TH1 cytokines and facilitating the development of a TH2 response [57].

There are conflicting reports on the role of IL-10 in SCA patients. One study demonstrated that patients undergoing HU therapy had high levels of IL-10, but the mechanism was not described [34]. In 2009, a Sudanese study of children with malarial infection demonstrated that asymptomatic children with sickle cell trait had significantly lower levels of IL-10 than HbAA children with severe malaria. However, these children had higher IL-10 levels than HbAA children with mild malaria. These results suggest that IL-10 has a protective effect against the occurrence of severe malaria in patients with sickle cell trait [50]. A study of inflammation and iron-overloading observed higher levels of IL-10 and lower levels of non-transferrin bound iron in SCA patients than in thalassemia patients, confirming the contribution of this cytokine to the regulation cellular iron status [24]. Thus, abnormal production of these anti-inflammatory cytokines can affect both cell-mediated and humoral immune responses and increase the risk of morbidity in sickle cell patients.

8. CONCLUSION

SCA is an inherited disorder characterized by homozygosis for HbS, and a number of cytokines have been implicated in disease severity. Understanding the roles of different cytokines in the pathology of SCA is essential for the development of effective therapies for this disease. While some cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, have been extensively studied in the context of SCA, the role of other cytokines in SCA pathology remains less clear. This review highlights several cytokines that are likely to be important in the pathophysiology of SCA.

REFERENCES

[1] Steinberg, M.H. and Rodgers, G.P. (2001) Pathophysiology of sickle cell disease: Role of cellular and genetic modifiers. *Seminars in Hematology*, **38**, 299-306. doi:10.1016/S0037-1963(01)90023-X

[2] Rother, R.P., Bell, L., Hillmen, P. and Gladwin, M.T. (2005) The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: A novel mechanism of human disease. *JAMA*, **293**, 1653-1662. doi:10.1001/jama.293.13.1653

[3] Frenette, P.S. (2002) Sickle cell vaso-occlusion: Multistep and multicellular paradigm. *Current Opinion Hematology*, **9**, 101-106. doi:10.1097/00062752-200203000-00003

[4] Levy, D.E. and Darnell Jr., J.E. (2002) Stats: Transcriptional control and biological impact. *Nature Reviews. Molecular Cell Biology*, **3**, 651-662. doi:10.1038/nrm909

[5] Martinon, F., Mayor, A. and Tschopp, J. (2009) The inflammasomes: Guardians of the body. *Annual Review of Immunology*, **27**, 229-265. doi:10.1146/annurev.immunol.021908.132715

[6] Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annual Review of Immunology*, **12**, 991-1045. doi:10.1146/annurev.iy.12.040194.005015

[7] Kono, H. and Rock, K.L. (2008) How dying cells alert the immune system to danger. *Nature Reviews Immunol-*

ogy, **8**, 279-289. doi:10.1038/nri2215

[8] Shi, Y., Evans, J.E. and Rock, K.L. (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*, **425**, 516-521. doi:10.1038/nature01991

[9] Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J. and Golenbock, D.T. (2008) The Nalp3 inflammasome is involved in the innate immune response to amyloid-beta. *Nature Immunology*, **9**, 857-865. doi:10.1038/ni.1636

[10] Martinon, F., Burns, K. and Tschopp, J. (2002) The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proil-beta. *Molecular Cell*, **10**, 417-426. doi:10.1016/S1097-2765(02)00599-3

[11] Pathare, A., Al Kindi, S., Alnaqdy, A.A., Daar, S., Knox-Macaulay, H. and Dennison, D. (2004) Cytokine profile of sickle cell disease in Oman. *American Journal of Hematology*, **77**, 323-328. doi:10.1002/ajh.20196

[12] Asare, K., Gee, B.E., Stiles, J.K., Wilson, N.O., Driss, A., Quarshie, A., Adams, R.J., Kutlar, A. and Hibbert, J.M. (2010) Plasma interleukin-1beta concentration is associated with stroke in sickle cell disease. *Cytokine*, **49**, 39-44. doi:10.1016/j.cyto.2009.10.002

[13] Wanderer, A.A. (2009) Rationale for Il-1beta-targeted therapy to minimize hypoxic-ischemic encephalopathy. *Journal of Perinatology*, **29**, 785-787. doi:10.1038/jp.2009.114

[14] Cerqueira, B.A., Boas, W.V., Zanette, A.D., Reis, M.G. and Goncalves, M.S. (2011) Increased concentrations of il-18 and uric acid in sickle cell anemia: Contribution of hemolysis, endothelial activation and the inflammasome. *Cytokine*, **56**, 471-476. doi:10.1016/j.cyto.2011.08.013

[15] Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T. and Tschopp, J. (2008) Innate immune activation through nalp3 inflammasome sensing of asbestos and silica. *Science*, **320**, 674-677. doi:10.1126/science.1156995

[16] Machado, P.R.L., Araújo, M.A.S., Carvalho L. and Carvalho, E. M. (2004) Mecanismos de resposta imune às infecções. *Anais Brasileiro de Dermatologia*, **79**, 647-662. doi:10.1590/S0365-05962004000600002

[17] Frenette P.S. (2004) Sickle cell vaso-occlusion: Heterotypic, multicellular aggregations driven by leukocyte adhesion. *Microcirculation*, **11**, 167-177.

[18] Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M. (2001) *Immunobiology.* 5th Edition, Garland Publishing, New York, 90-100.

[19] Heinrich, P.C., Behrmann, I., Haan, S., Hermanns. H.M., Müller-Newen, G. and Schaper, F. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochemistry Journal*, **374**, 1-20. doi:10.1042/BJ20030407

[20] Mansell, A. and Jenkins, B.J. (2013) Dangerous liaisons between interleukin-6 cytokine and toll-like receptor families: A potent combination in inflammation and cancer. *Cytokine & Growth Factor Reviews.* doi:10.1016/j.cytogfr.2013.03.007

[21] Abbas, A.K., Lichtman, A.H. and Pober, J.S. (2000) Section III—Effector mechanisms of the immune response. *Celular and Molecular Immunology*.

[22] Hibbert, J.M., Hsu, L.L., Bhathena, S.J., Irune, I., Sarfo, B., Creary, M.S., Gee, B.E., Mohamed, A.I., Buchanan, I.D., Al-Mahmoud, A. and Stiles, J.K. (2005) Proinflammatory cytokines and the hypermetabolism of children with sickle cell disease. *Experimental Biology and Medicine*, **230**, 68-74.

[23] Veiga, P.C., Schroth, R.J., Guedes, R., Freire, S.M. and Nogueira-Filho, G. (2013) Serum cytokine profile among Brazilian children of African descent with periodontal inflammation and sickle cell anaemia. *Archives Oral Biology*, **58**, 505-510. doi:10.1016/j.archoralbio.2012.11.006

[24] Walter, P.B., Fung, E.B., Killilea, D.W., Jiang, Q., Hudes, M., Madden, J., Porter, J., Evans, P., Vichinsky, E. and Harmatz, P. (2006) Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. *British Journal of Haematology*, **135**, 254-263. doi:10.1111/j.1365-2141.2006.06277.x

[25] Qari, M.H., Dier, U. and Mousa, S.A. (2012) Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease. *Clinical and Applied Thrombosis Hemostasis*, **18**, 195-200. doi:10.1177/1076029611420992

[26] Li, A., Varney, M.L., Valasek, J., Godfrey, M., Dave, B.J. and Singh, R.K. (2005) Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis*, **8**, 63-71. doi:10.1007/s10456-005-5208-4

[27] Ikram, N., Hassan, K. and Tufail, S. (2004) Cytokines. *International Journal of Pathology*, **2**, 47-58.

[28] Graves, D.T. and Jiang, Y. (1995) Chemokines, a family of chemotactic cytokines. *Critical Reviews in Oral Biology and Medicine*, **6**, 109-118. doi:10.1177/10454411950060020101

[29] Wolf, M., Delgado, M.B., Jones, S.A., Dewald, B., Clark-Lewis, I. and Baggiolini, M. (1998) Granulocyte chemotactic protein 2 acts via both IL-8 receptors, CXCR1 and CXCR2. *European Journal of Immunology*, **28**, 164-170. doi:10.1002/(SICI)1521-4141(199801)28:01<164::AID-IMMU164>3.0.CO;2-S

[30] Popa, C., Netea, M.G., van Riel, P.L., van der Meer, J.W. and Stalenhoef, A.F. (2007) The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *Journal of Lipid Research*, **48**, 751-762. doi:10.1194/jlr.R600021-JLR200

[31] Keikhaei, B., Mohseni, A.R., Norouzirad, R., Alinejadi, M., Ghanbari, S., Shiravi, F. and Solgi, G. (2013) Altered levels of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crises and the steady state condition. *European Cytokine Network*.

[32] Cajado, C., Cerqueira, B.A., Couto, F.D., Moura-Neto, J.P., Vilas-Boas, W., Dorea, M.J., Lyra, I.M., Barbosa, C.G., Reis, M.G. and Goncalves, M.S. (2011) TNF-alpha and IL-8: Serum levels and gene polymorphisms (-308G >A and -251A>T) are associated with classical biomarkers and medical history in children with sickle

cell anemia. *Cytokine*, **56**, 312-317. doi:10.1016/j.cyto.2011.07.002

[33] Gonçalves, M.S., Queiroz, I.L., Cardoso, S.A., Zanetti, A., Strapazoni, A.C., Adorno, E., Albuquerque, A., Sant'Ana, A., dos Reis, M.G., Barral, A. and Barral Netto, M. (2001) Interleukin 8 as a vaso-occlusive marker in Brazilian patients with sickle cell disease. *Brazilian Journal of Medical and Biology Research*, **34**, 1309-1313.

[34] Lanaro, C., Franco-Penteado, C.F., Albuqueque, D.M., Saad, S.T., Conran, N. and Costa, F.F. (2009) Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *Journal of Leukocyte Biology*, **85**, 235-242. doi:10.1189/jlb.0708445

[35] Michaels, L.A., Ohene-Frempog, K., Zhao, H. and Douglas, S.D. (1998) Serum levels of substance P are elevated in patients with sickle cell disease and increase further during vaso-occlusive crisis. *Blood*, **92**, 3148-3151.

[36] Tavakkoli, F., Nahavandi, M., Wyche, M.Q. and Perlin, E. (2004) Plasma levels of TNF-alpha in sickle cell patients receiving hydroxyurea. *Hematology*, **9**, 61-64. doi:10.1080/1024533032000158869

[37] Kim, K.S., Rajagopal, V., Gonsalves, C., Johnson, C. and Kalra, V.K. (2006) A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. *Journal Immunology*, **177**, 7211-7224.

[38] Saleh, A.W., Hillen, H.F. and Duits, A.J. (1999) Levels of endothelial, neutrophil and platelet-specific factors in sickle cell anemia patients during hydroxyurea therapy. *Acta Haematologica*, **102**, 31-37. doi:10.1159/000040964

[39] Musa, B.O., Onyemelukwe, G.C., Hambolu, J.O., Mamman, A.I. and Isa, A.H. (2010) Pattern of serum cytokine expression and T-cell subsets in sickle cell disease patients in vaso-occlusive crisis. *Clinical and Vaccine Immunology*, **17**, 602-608.

[40] Kolls J.K. and Linden, A. (2004) Interleukin-17 family members and inflammation. *Immunity*, **21**, 467-476. doi:10.1016/j.immuni.2004.08.018

[41] Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M. and Weaver, C.T. (2005) Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*, **6**, 1123-1132. doi:10.1038/ni1254

[42] Iwakura, Y., Nakae, S., Saijo, S. and Ishigame, H. (2008) The roles of IL-17A in inflammatory immune responses and host defense against pathogens. *Immunological Review*, **226**, 57-79.

[43] Stockinger, B. and Veldhoen, M. (2007) Differentiation and function of Th17 cells. *Current Opinion in Immunology*, **19**, 281-286. doi:10.1016/j.coi.2007.04.005

[44] Vilas-Boas, W., Cerqueira, B.A., Zanette, A.M., Reis, M.G., Barral-Netto, M. and Goncalves M.S. (2010) Arginase levels and their association with Th17-related cytokines, soluble adhesion molecules (sICAM-1 and sVCAM-1) and hemolysis markers among steady-state sickle cell anemia patients. *Annals of Hematolology*, **89**, 877-882. doi:10.1007/s00277-010-0954-9

[45] O'Shea, J.J and Paul W.E. (2002) Regulation of T(H)1 differentiation-controlling the controllers. *Nature Immunology*, **3**, 506-508. doi:10.1038/ni0602-506

[46] Hunter, C.A. (2005) New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nature Reviews Immunology*, **5**, 521-531. doi:10.1038/nri1648

[47] Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L. and Kuchroo, V.K. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 andregulatory T cells. *Nature*, **11**, 235-238. doi:10.1038/nature04753

[48] Vignali, D.A. and Kuchroo, V.K. (2012) IL-12 family cytokines: Immunological playmakers. *Nature Immunology*, **13**, 722-728. doi:10.1038/ni.2366

[49] Taylor, S.C., Shacks, S.J. and Qu, Z. (1999) *In vivo* production of type 1 cytokines in healthy sickle cell disease patients. *Journal of the National Medical Association*, **91**, 619-624.

[50] Hassan, D.A., Marques, C., Santos-Gomes, G.M., do Rosario, V.E., Mohamed H.S., Elhussein, A.M., Ibrahim, M.E. and Abdulhadi, N.H. (2009) Differential expression of cytokine genes among sickle-cell-trait (HbAS) and normal (HbAA) children infected with Plasmodium falciparum. *Annals of Tropical Medicine and Parasitology*, **103**, 283-295. doi:10.1179/136485909X435049

[51] Taylor, SC., Shacks, S.J., Qu, Z. and Wiley, P. (1997) Type 2 cytokine serum levels in healthy sickle cell disease patients. *Journal of the National Medical Association*, **89**, 753-757.

[52] Oh, C.K., Geba, G.P. and Molfino, N. (2010) Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. *European Respiratory Review*, **115**, 46-54. doi:10.1183/09059180.00007609

[53] Luzina, I.G., Keegan, A.D., Heller, N.M., Rook, G.A., Shea-Donohue, T. and Atamas, S.P. (2012) Regulation of inflammation by interleukin-4: A review of "alternatives". *Journal of Leukocyte Biology*, **92**, 753-764. doi:10.1189/jlb.0412214

[54] Raghupathy, R., Haider, M.Z., Azizieh, F., Abdelsalam, R., D'Souza, T.M. and Adekile, A.D. (2000) Th1 and Th2 cytokine profiles in sickle cell disease. *Acta Haematologica*, **103**, 197-202. doi:10.1159/000041049

[55] Knight-Madden, J., Vergani, D., Patey, R., Sylvester, K., Hussain, M.J., Forrester, T. and Greenough, A. (2012) Cytokine levels and profiles in children related to sickle cell disease and asthma status. *Journal of Interferon & Cytokine Research*, **32**, 1-5. doi:10.1089/jir.2011.0030

[56] Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A. and Mosmann, T.R. (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science*, **248**, 1230-1234. doi:10.1126/science.2161559

[57] Barbosa, M.C., Dos Santos, T.E., de Souza, G.F., de Assis, L.C., Freitas, M.V. and Gonçalves, R.P. (2013) Impact of iron overload on interleukin-10 levels, biochemical parameters and oxidative stress in patients with sickle cell anemia. *Revista Brasileira de Hematologia e Hemoterapia*, **35**, 29-34. doi:10.5581/1516-8484.20130011

A.3 – MANUSCRITO III

Sickle cell disease: Only one road, but different pathways for inflammation

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Este trabalho revisa os diversos processos envolvidos com a inflamação na doença falciforme.
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Resumo: A doença falciforme (DF) é uma doença genética caracterizada por um processo inflamatório crônico. Biomarcadores novos têm sido estudados como moléculas promissoras para a compreensão da inflamação e da fisiopatologia da DF. A hemólise e a liberação de moléculas associadas ao catabolismo da hemoglobina (Hb), como a Hb livre, ferro e heme, geram um ambiente oxidante com a produção de espécies reativas de oxigênio e nitrogênio. O sistema imunológico desempenha papel importante na inflamação e há, também, um estado de resistência ao óxido nítrico (NO), com redução da bioatividade do NO, que contribui para a disfunção vascular, ativação de plaquetas, leucócitos, eritrócitos e células endoteliais, com a expressão de moléculas de adesão e os seus ligantes, e vários receptores, que, em conjunto, participam do processo inflamatório. Durante a inflamação, há o aumento de células dendríticas (DCs) expressando receptores tipo toll (TLR), mas o papel de DCs e dos TLR na patogênese da DF não é claro. Além disso, existem moléculas que contribuem para o aumento da disfunção endotelial, tais como a homocisteína, que tem sido associada a complicações vasculares em outras patologias e pode contribuir para as mesmas complicações na DF. Micropartículas (MPs) circulantes apresentam-se em níveis aumentados em várias doenças e têm sido descritas na DF, sendo que compostos da membrana celular estão associados à trombose e a ativação da coagulação, tais como fator tecidual e a fosfatidilserina (PS), o que pode contribuir para a disfunção endotelial. O conhecimento de todos estes biomarcadores pode contribuir para descoberta de abordagens terapêuticas novas, melhorando a qualidade de vida do paciente DF.

Palavras-chave: Doença Falciforme; Inflamação; Estresse Oxidativo; Ativação Celular.

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Sickle cell disease: Only one road, but different pathways for inflammation

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ABSTRACT

Sickle cell disease (SCD) is a genetic disorder characterized by a chronic inflammatory process, and new biomarkers have been studied as promising molecules for understanding the inflammation in its pathophysiology. The hemolysis and the release of molecules associated to the hemoglobin (Hb) catabolism, such as free Hb, iron, and heme, generate an oxidant environment with production of reactive oxygen and nitrogen species. The immune system plays a very important role in the inflammation, with cells secreting pro-inflammatory cytokines and chemokines. There is also a nitric oxide (NO) resistance state, with an impaired NO bioactivity, leading to a vascular dysfunction; activation of platelet, leukocytes, erythrocytes, and endothelial cells, with expression of adhesion molecules and its ligands, and several receptors, that altogether participate at inflammatory process. During inflammation, there is an increase of dendritic cells (DCs) expressing toll like receptors (TLR), but the role of DCs and TLR in SCD pathogenesis is unclear. Also, there are molecules contributing for enhance the endothelium dysfunction, such as homocysteine that has been associated with vascular complications in the pathology of other diseases and it may contribute to the vascular complications presented by SCD patients. Circulating microparticules (MPs) levels are augmented in several diseases and have been described in SCD, where cells membrane compounds are associated to cell's thrombotic and coagulation state, such as tissue factor and phosphatidylserine (PS), which may contribute to endothelial dysfunction. The knowledge of all these biomarkers may contribute to new therapeutic approach discover, improving SCD patient life quality.

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Keywords: Sickle Cell Disease; Inflammation; Oxidative Stress; Cells Activation

1. INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder, and the sickle cell anemia (HbSS) is the more severe genotype. The disease is characterized by the presence of the hemoglobin S (HbS), where valine replace glutamic acid ($\beta^{S\ 6\ Glu\rightarrow Val}$) at the beta globin chain, that has a single point mutation (GAG →GTG) at the sixth codon of the β-globin (*HBB*) gene [1].

Sickle cell disease clinical outcome vary widely from mild to severe and has been associated with multi-organ damage and risk of early mortality [1], with acute and chronic clinical manifestations, including vaso-occlusive episodes (VOE), painful crisis, tissue ischemia/reperfusion injury, hemolysis, impaired blood flow as a result of intravascular sickling in capillary and vessels, inflammation processes and high susceptibility to infection, encephalic vascular accident (EVA), dactylitis, leg ulceration, pulmonary hypertension, acute chest syndrome, and priapism [1,2].

Moreover, the disease pathogenesis comprehends a complex network of mechanisms, involving the vaso-occlusive phenomenon and tissue ischemia, with surface and ligands molecules activation from stressed reticulocytes, sickled erythrocytes, leukocytes, platelets and endothelial cells [1-3]; there is also an increase of oxidative stress, secondary to the hemolysis episodes and heme cytotoxicity, electron donation from the iron atom when yet inside the protoporphyrin IX ring, with the generation via Fenton reaction of reactive oxygen and nitrogen species (ROS; RNS), that has a very strong pro-oxidant capacity. Also, there is an increase of nitric oxide (NO) scavenger molecules, a vasodilator that play important

role as regulators of vascular homeostasis in SCD pathogenesis [1,4].

Acute and chronic inflammatory phenomenon's can contribute to activate several cells types and may play important role in the steady- and crisis-states of SCD patients. The immune system pathway, has several mechanisms and needs to be better understood, including the participation of inflammation and cells markers, activation of molecules related to hemolysis, nitric oxide resistance, and some very important inflammatory mediators involved in the arachidonic acid pathway, including the synthesis of molecules such as, prostaglandin E2 (PGE2), thromboxane, and leukotrienes B4 (LTB4) [1].

There are many chemistry and genetic markers, which can modulate symptoms presented by SCD patients, such as alpha-thalassemia presence, reticulocytes count, lactate dehydrogenase (LDH) and bilirubin serum levels [5]. Currently, new biomarkers have been studied as promising molecules for understanding inflammation process in SCD, and have been highlighted the role of lipids metabolism and its participation in vascular injury, C-reactive protein (CRP) and inflammation, and the myeloperoxidase (MPO), a enzyme that had been related with patients susceptibility to infection [6-8].

Bilirubins are resulted of protoporphyrin IX metabolism, which in turn is a heme component. Sickle cell disease patients, particularly those HbSS, are at risk for bile pigment cholelithiasis due to the association of this disease with hemolysis, which produces an unconjugated hyperbilirubinemia [9]. Cholecystitis presents with abdominal pain, nausea and vomiting, fever, and/or jaundice, a constellation of symptoms that has multiple possible etiologies in SCD [10].

C-reactive protein (CRP), an acute-phase protein, increases significantly in inflammatory disorders and nowadays CRP has been used for evaluation of cardiac risk. CRP is produced not only by the liver but also in atherosclerotic lesions by vascular smooth muscle cells and macrophages in response to stimulation by the pro-inflammatory cytokine interleukin-6 (IL-6) [11]. SCD is associated with elevated cardiac output and cardiomegaly to partly compensate for the reduced oxygen-carrying capacity associated to hemolysis and oxidative stress. The combination of these events has been associated with increased levels of CRP in SCD since the childhood [12-14].

Myeloperoxidase is a lysosomal enzyme and plays an important role in the host defense system. MPO deficiency was associated with a higher occurrence of severe and chronic inflammatory processes in SCD patients, and the −463G > A *MPO* gene polymorphism may be a significant genetic modulator that makes HbSS patients more susceptible to infection [13].

An association between increased low-density lipoprotein cholesterol (LDL-C) and low plasma levels of high-density lipoprotein cholesterol (HDL-C) is an important risk factor for coronary disease. Actually it has been showed an association between coronary heart disease, high levels of LDL-C and MPO, since MPO catalyses the conversion of chloride and hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl), resulting in LDL-C oxidation and conversion into high-uptake forms, such as ox-LDL for macrophages, leading to cholesterol deposition and foam cell formation *in vivo* [14]. Data from our research group showed that some SCD patients can have a specific dyslipidemic subphenotype, characterized by low HDL-C with hypertriglyceridemia and high very low density lipoprotein cholesterol (VLDL-C) in association with other biomarkers, including those related to inflammation like ferritin and CRP [15]. These biomarkers may help in understanding the inflammatory mechanism associated with SCD as well as be used as predictor tests for severe events.

2. VASCULAR DYSFUNCTION AND INFLAMMATION: NITRIC OXIDE SCAVENGING AND ARGININE METABOLISM

In regard to the vascular complication of the HbSS, the decrease of nitric oxide (NO) bioavailability is now associated with the intravascular hemolysis [16], that participate in several important complication of HbSS patients, including pulmonary hypertension, leg ulcers, priapism and different types of stroke [16-18].

The nitric oxide is a diatomic gas produced by vascular endothelial cells that act as a potent vasodilator on smooth muscle cells. The NO synthesis is from the amino acid L-arginine, via an oxidation reaction catalyzed by the enzyme nitric oxide synthase (NOS) [19]. Nitric oxide also tonically inhibits platelet activation and the expression of endothelial adhesion molecules, thus participating in health endothelial function and in the maintenance of blood flow [20,21]. The reaction involveing vascular NO can have a beneficial antioxidant effect. Moreover, NO has been demonstrated to have a cytoprotective effect by scavenging reactive oxygen species (ROS) [22]. Solovey *et al.* [23] examined the hypothesis that enhanced endothelial tissue factor (TF) expression is modulated by endogenous NO produced by endothelial nitric oxide synthase (eNOS) in animal models. The mechanism by which NO exerts its inhibiting effect on TF have not been completely defined, although it is accompanied by parallel changes in amount of TF mRNA. Because NO exerts the same regulatory influence on vascular cellular adhesion molecule-1 (VCAM-1) expression, current results also have implications beyond inflammation and coagulation system, but also with VCAM-1 inflammatory expression.

Chronic elevated level of cell-free hemoglobin in SCD patients with intravascular hemolysis range from 2 to 20 uM per heme during steady-state and increase to approximately 20 to 40 uM during vaso-occlusive crises [24]. HbSS patients present a NO resistance state, associated with an impaired NO bioactivity that can be due by cell-free hemoglobin accumulation in plasma, intensifying the NO endogenous consumption that contribute for several cellular dysfunction, such as vasoconstriction and inflammation by leukocytes, platelets and endothelial cells activation [25]. Also, ROS are generated by hemolysis, and can react with NO, limiting its bioavailability and contributing to its state of resistance in HbSS patients [18]. The heme that is released from hemolysis also induces the expression of adhesion molecules from leukocytes, such as intercellular adhesion molecules-1 (ICAM-1), and from endothelium, such as VCAM-1.

Another mechanism of NO depletion during the hemolysis is the release of arginase-1 from lysed RBC that converts arginine to ornithine, which competes with the substrate, the eNOS, for L-arginine synthesis [26]. In recent research by our team, we confirm an increased levels of serum arginase-1 in HbSS patients when compared to health controls, as well as an association of serum arginase-1 with biochemical hemolysis markers and cytokines involved in Th17 response, levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) [27]. A very new insight in this metabolism lays on a shift in arginine catabolism, where transforming grow factor beta (TGF-beta) may induces the arginase pathway instead of the NO pathway, with a possible involvement of the vascular activation and the increase of serum arginase in chronic hemolysis among HbSS patients.

3. HOMOCYSTEINE AND SICKLE CELL DISEASE

Homocysteine (Hcy), a sulfur-containing amino acid, is found at low concentration in blood and cells and is an important intermediate molecule involved in the biosynthesis of methionine and cysteine [28]. The high plasma concentration of Hcy is a well-established risk factor for several disorders, including cardiovascular disease (CVD) and stroke [29], venous thrombosis and arteriosclerosis [30].

Hyperhomocysteinemia play an important role in vascular disorders and may act through increase cytotoxic activity, especially for endothelial cells; elevating H_2O_2 levels and decreasing NO synthesis, with pro-inflammatory cytokines synthesis, pro-coagulant factors activetion, and lipid metabolism dysregulation, characterized by LDL-C oxidative modification, enhancing of atherogenesis [31]. High levels of Hcy have also been implicated in changes in the rheological properties of blood, such as decreasing antithrombin III and tissue plasminogen activator (TPA), and increasing factor VII and CRP [32]. Additionally, Hcy is reported to enhance endothelial leukocyte interactions [33].

A chronic inflammatory state in vascular tissue is recognized to contribute to thrombotic and vaso-occlusive events in HbSS patients [34]. Since Hcy has been associated with vascular complications in the pathophysiology of other disease, it may contribute to vascular complications presented by HbSS patients.

The possibility that Hcy may contribute to the ischemic phenomena present in HbSS has attracted some interest in plasma total Hcy. Lowenthal *et al.* [31] showed that the median plasma concentration of Hcy among HbSS subjects was approximately 1.5-fold higher than that of healthy controls. Additionally, SCD patients have higher plasma Hcy concentration in spite of elevated plasma folate levels and vitamin B12 concentration similar to those observed in controls. In a recent study (2012) from our team, we found significant associations between Hcy levels and increased expression of pro-inflammatory cytokines and adhesion molecules (data not published) in HbSS patients, supporting the hypothesis that Hcy levels contribute to the vascular activation and with the inflammatory state presented by HbSS patients, and probably has an important role in vaso-occlusive mechanism.

4. INNATE IMMUNITY AND INFLAMMATION IN SCD

Individuals with SCD have transient and periodic painful vaso-occlusive episodes with exposure of organ to ischemia and reperfusion, which may activate inflammatory response in other organs, leading to multiple organ failure [35,36]. Despite ischemia and reperfusion occur in a sterile environment, and activation of innate and adaptive immune responses contribute to injury, including activetion of pattern-recognition receptors, such as Toll like receptors (TLRs) and inflammatory cell trafficking into the damaged organ [37]. Moreover, is well known that the presence of immunodeficiency should be associated with SCD, but no directly deficiency has been related to the immune system was observed to explain the amount of recurrent infections presented by these individuals [34]. On the basis of these, the immune response system seems to be related with health and inflammation in SCD.

The innate immune system is the first line of protection against invading microbial pathogens and of responses to inflammatory stimuli that are mediated by phagocytes, polymorphonuclear leukocytes (PMN), monocytes, macrophages, dendritic cells (DCs), and inflammatory T cell subsets (Th1 cells and Natural Killer T cells). This immune response relies on recognition of

evolutionarily conserved structures on pathogens, named pathogen associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs), of which the family of TLRs has been studied most extensively [38].

TLRs are integral glycoprotein characterized by an extracellular or luminal ligand binding domain containing leucine rich repeat (LRR) motifs and a cytoplasm signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain [38]. Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. To date, 10 TLRs have been identified in humans, and they can recognize distinct PAMPs derived from various microbial pathogens, including viruses, bacteria, fungi, and protozoa. TLRs can be divided into subfamilies primarily recognizing related PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids [39].

Cell types expressing TLRs are APCs, including macrophages, DCs, and B lymphocytes. TLRs have been identified in most cell types, expressed either constitutively or in an inducible manner in the course of infection [39]. During inflammation, increased numbers of DCs are rapidly recruited and efficiently capture antigens due to their high phagocytic ability. Subsequent to antigen capture DCs become activated and the mature DCs by pathogens can migrate into draining lymph nodes or the spleen and transforming to powerful antigen-presenting cells that are capable of activating naive T cells. The transition from immature to mature state is followed by production of several cytokines and chemokines by the DCs that regulate their ability to interact with naïve T cells to direct T cell differentiation [40].

Two major DCs subsets can be detected in the peripheral blood, with distinct, but overlapping functions. Myeloid DCs (mDCs) express human leukocyte antigens (HLA) DR, CD11c, and CD1c and are the main producers of interleukin-12 (IL-12), while plasmacytoid DCs (pDCs) express HLA DR, CD123, and blood dendritic cell antigen 2 (BDCA2), and are the main producers of interferon-α (IFN-α) [41]. However, HbSS patients in steady-state seems to have a particular CD1-positive phenotype expression of CD1a, b, and c molecules at DCs, while the classical phenotype found among the general population, in which only 15% of the individuals express the CD1 molecules at the surface of their monocytes [42]. According to Sloma *et al.* [43], the elevated concentration of cytokines associated with monocyte activation in HbSS patients can contribute for their activated status and it may be hypothesized that CD1 expression on DCs from HbSS patients is a consequence of the elevated plasma levels of endothelin.

The role of DCs in malaria affected population and its association with hemoglobinopathies have been described and show that the activation of mDCs and pDCs during acute malaria may be faster or deeper in children with α+thalassemia than in children with normal hemoglobin profile [44]. Other studies shows the *Plasmodium falciparum* glycosylphosphatidylinositol (GPI) anchors can bind to TLR2 and TLR4 expressed on mDCs and monocytes [45], whereas a component of schizont lysate as well as hemozoin can bind to TLR9 and activate pDCs [46].

Single-nucleotide polymorphisms (SNPs) have been described for *TLR*-4 and *TLR*-9 genes. For *TLR*-4, the polymorphism (Asp299Gly) has been related to Gram-negative infections susceptibility and septic shock [47]. More recently, the Asp299Gly has been involved as a protective allele against malaria, explaining its high prevalence in sub Saharan Africa [41].

The immune response in SCD is poorly understood. It is knows that the immune system has a close relationship with health and morbidity in SCD, although the complex network involved in the mechanisms of pathogenesis present in this disease, is difficult to understand, once it is a chronic inflammatory condition. Additional studies need to be warranted to elucidate the immunologic processes in SCD.

5. OXIDATIVE STRESS AND INFLAMMATION IN SICKLE CELL ANEMIA

Oxidative stress is a physiological condition that occurs when there is imbalance between the amount of free radicals (ROS; RNS) generated by physiological processes and antioxidant mechanisms. Free radicals are defined as chemical species that contains a pair of electrons unpaired, and this gives the high-capacity reactive free radical [48]. The reactive oxygen of species includes free radicals and non-free radicals, such as hydroxyl, superoxide (O_2^-) and H_2O_2. In biological systems, the most common source of free radicals is oxygen, and ROS that can be produced from both endogenous and exogenous cellular products [49,50].

The endogenous sources of ROS include mitochondria, cytochrome P450, peroxisomes, and inflammatory cells activated [51]. Mitochondria generates significant quantities of H_2O_2 and use ~90% of cellular O_2. During the process of reducing mitochondrial oxygen for production of water, several short-lived intermediates are produced, including H_2O_2, O_2^- and the hydroxyl radical [OH], which are toxic to the cell. Another molecule is the peroxynitrite ($ONOO^-$), an anion and an unstable isomer of nitrate (NO_3^-). The peroxynitrite can be formed in vivo by the reaction of the free O_2^- with free NO, and is a potentially cytotoxic molecule [52]. Cell destruction also causes further free radical generation [53]. Neutrophils,

eosinophils and macrophages are additional endogenous sources of cellular ROS. Activated macrophages initiate increase in oxygen uptake and give rise to a variety of ROS, including O_2, NO and H_2O_2 [54].

In addition, intracellular formation of free radicals can occur by environmental sources including ultraviolet light, ionizing radiation, and pollutants such as paraquat and ozone. All of these sources of free radicals, both enzymatic and non-enzymatic have the potential to inflict oxidative damage on a wide range of biological macromolecules [55]. Membranes are target of free radicals forming due to its lipid composition, lipid peroxide, thus compromising the characteristics of fluidity and elasticity, leading to cell rupture. Other target tissues to ROS are proteins, which may lose their functionality enzyme and cell signaling and DNA; the interaction of ROS with DNA can lead to the DNA strand breaks, point mutations, gene deletions, or gene rearrangement, such changes can be lethal to the cell, with DNA lesion, that accumulate with age, and can be an important etiology of aging processes [56]. The endogenous antioxidant system responsible for neutralize free radicals, include enzymes, such as glutathione peroxidase, superoxide dismutase (SOD) and catalase. The non-enzymatic antioxidants that participate in oxidative stress defense include ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione (GSH), carotenoid, and flavonoids [57-59]. The ROS occur under physiological conditions and in many diseases cause direct or indirect damage in different organs; thus, it is known that oxidative stress (OS) is involved in pathological processes such as obesity, diabetes, cardiovascular disease, and atherogenic processes [60].

Inflammation is an immune system reaction aiming to contain and eliminate pathogens or foreign elements to the body [61,62]. After activation, innate immune system cells secrete pro-inflammatory cytokines and chemokines that induce ROS/RNS production [63]. In the innate immune system, macrophages generate ROS, including O_2^-, NO, H_2O_2, hydroxyl radical, $ONOO^-$ and HOCl to play pathogen elimination [62,63]. Chronic inflammation can lead to cellular damage, hyperplasia and, consequently, to the overproduction of ROS by inflammatory cells [61].

In the HbSS, the chronic inflammatory state promotes the production of ROS and predicts the disease severity [64-68]. In the HbSS some events contribute to the maintenance of oxidative stress such as the excessive levels of cell-free hemoglobin with its catalytic action on oxidative reactions; the characteristic recurrent ischemia-reperfusion injury, a chronic pro-inflammatory state with higher autoxidation of HbS [69-71].

Iron is a chemical element that participates in the reaction of electron transfer between molecules in the process of cellular respiration (redox reactions), and it is deposited in the form of ferritin and hemosiderin. Patients receiving multiple blood transfusions, such as patients with chronic anemia, thalassemia, and HbSS, they can exceed the storage and detoxification capacity of ferritin. Consequently, the free iron begins to accumulate into tissues; this can catalyze the formation of very injurious compounds, such as [OH] by Fenton reaction [71]. Compared with normal red blood cells (RBC) membranes, those from sickle RBC have abnormally increased Fenton reactivity, once that the instability of HbS results in generation of $.O^-$ and H_2O_2, the combination of which potentially forms the .OH [70,71]. The phenomenon of sickling and vaso-occlusive events in HbSS are directly associated with its pathogenesis, and there is evidence that several inflammatory events occur with increased levels of inflammatory and anti-inflammatory cytokines, such as IL1β, IL4, IL6, TNFα, the expression of cell adhesion molecules, such as ICAM-1, VCAM-1, P-selectin and integrins; the adhesion of activated PMN to the endothelium; the participation of activated platelets, and the presence of inflammatory biomarkers, such as CRP and prostaglandins. These factors contribute to the occurrence of vaso-occlusion and chronic organ damage, favoring an increased production of ROS [65,72-77].

6. CYTOKINE IN SICKLE CELL ANEMIA: BREAK THE BALANCE

Pro-inflammatory cytokines mainly expressed by monocytes from stimulation of bacterial components, ROS and growth factors have important role in immune innate response and inflammatory state [78]. These effects are modulated by anti-inflammatory cytokines, such as IL-4 and IL-10, necessary to down regulate leukocytes and the vascular endothelium activation [79]. This delicate balance is broken when inflammatory cytokine increase oxidative stress and overcoming antioxidant barrier by activation of complex transporter of electron in mitochondria [78,80,81], stimulating the transcription of the trans factor NF-kB with degradation of IkB, up regulating the expression of selectins, integrins, ICAM-1, VCAM-1, resulting on pre-activation of leukocytes and with the interaction of these molecules with the activated endothelium [81-83].

Cytokine levels in HbSS patients are elevated not only during crisis-state, but also in different pathophysiologic mechanisms of the disease, like hypoxia and reperfusion rate; beyond clinical inflammatory history generate an increased concentration of these molecules also in steady-state patients. Pro-inflammatory cytokines like IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-alpha are increased on basal state and during vascular occlusion events unaccompanied of an increased level of anti-inflammatory cytokines,

such as IL-4 and IL-10 [3,20,79,84-88]. This inflammatory profile is not consensus; maybe it is associated with a high inter-individual variation, mainly due difference of genetic background and environmental aspects that can generate these molecules plasma levels fluctuation.

Soluble vascular cell adhesion molecule-1 (sVCAM-1) amount increase in plasma and change accord to the profile expression of selectins and integrins on leukocytes, such as CD62L and CD11b indirectly reflecting the rate of inflammation, and oxidative stress and have been associated as promising markers of HbSS prognosis [27, 89-91]. Based on these, oxidative stress and inflammatory profile may have complementary and symbiotic effects, mainly in a chronic inflammatory and oxidative disease, such as HbSS.

Inflammatory and oxidative profile, therefore, are extremely connect. This link is also confirmed when it is analyzed the conversion of purines to uric acid by the xanthine oxidase action [92,93]. This reaction generate free radicals, like intermediary products, and is up regulated by pro-inflammatory cytokines due many potential cytokine responsive elements in the *xanthine oxidase* (*XO*) gene regulatory region [94].

Therefore, up regulation of *XO* gene generates more uric acid and contact of this molecule with free sodium driving the monosodium urate that is consider to be a biologically active structure [95]. The monosodium urate is an active form act as a danger signal by activation of inflammasome, resulting on the production of pro-inflammatory cytokines, such as IL-1 and IL-18 [96]. In recent research by our team, inflammasome pathway was observed in HbSS patients, where uric acid, considered as a danger signal was associated with high serum levels of IL-18, further connect to cytokines levels and oxidative stress markers [91]. These effects, accordingly, are amplified on a chronic hemolytic and inflammatory disease, like HbSS, where the intravascular hemolysis contribute to release of cell free hemoglobin, heme and iron, consequently increase of oxygen radicals, limiting NO bioavailability, attracting more leukocytes, activating endothelial cells, contributing to the vascular occlusion [2,15,17,27,91].

7. PLATELETS AND INFLAMMATION IN SICKLE CELL DISEASE

Platelets are small enucleated structures derivatives from megakaryocyte fragmentation and are important to homeostasis process, primary function originally described to platelets. Platelets undergo activation, adhesion and aggregation binding to damage blood vessel, producing a platelet plug and contributing to the generation of thrombin. Furthermore, platelets produce and store a variety of molecules that affect platelet function and modified the vascular tone, the fibrinolysis [97]. Leukocytes and endothelial cells are associated as a critical player in the microvascular alteration induced by inflammation. Actually, have been thought about the role of platelets in inflammatory states through leukocytes interaction, release proteins, chemokines and endothelial disfunction [98].

Some studies suggest that circulating platelets in HbSS patients are chronically activated, both during steady-state and vaso-occlusive crises, which may result of the overall hypercoagulable state or with the vaso-occlusive process or with the pro-inflammatory characteristics of the microvasculature in HbSS [20,99,100].

Platelets have important organelles to performance its function, such as alpha-granules, lysosomes, peroxisomes, dense bodies and a complex membranous system that contribute to store and rapidly release several factors and proteins [99,101]. Among released products by platelets, there are secretion of adhesion proteins, such as fibrinogen, von Willebrand factor (vWF), thrombospondin, P-selectin, GPIIb/IIIa; there are important chemokines, including RANTES and platelet factor-4; cytokine-like factors as IL1-β, CD40L, β-thromboglobulin or factors essentials for the coagulation process, as plasminogen activator inhibitor (PAI-1), protein S and factors V and XI, and also expression of innate receptors of the Toll-like receptor family, such as TLR2 and TLR4 [102-105].

During inflammatory process, activated endothelial cells and others perivascular cells and leukocytes, release several soluble mediators, such as lipids, IFN-γ, IL-2, and CXCL12, which bind to platelet receptors, leading to degranulation of platelets dense and alpha-granules, promoting self adhesion and activation. Furthermore, the OS actives phospholipase A2 and the generation of the arachidonic acid pathway metabolites and platelet activator factor (PAF), and also contribute for platelet activation [103,105]. On the other hand, the platelet activetion can induce several inflammatory responses in monocytes, neutrophils, endothelial cells or endothelial progenitor cells; product released by platelets are potent inflammatory and mitogenics substances, modifying the chemostatic, adhesive and proteolytic properties of cellular microenvironment, mainly of endothelial cells and leukocytes, resulting in an increase of transmigration of leukocytes to the site of inflammation, suggesting that platelets-leukocytes interaction may be a key role in the initiation of inflammation [98,103,104].

Many mechanisms of platelets-leukocytes interactions have been described, but the initial interaction appears to be mediated by P-selectin expressed on the surface activated platelets and P-selectin ligand glycoprotein-1 (PSGL-1) on the surface of neutrophils and monocytes and, subsequently, firmly adhere by binding of Mac-1 to GPIb or other receptors of the platelet membrane [98, 104]. The P-selectin was found in sickle cell transgenic mice with high constitutive levels, which could be attri-

buted to platelet activation, contributing to inflammatory response [98,104]; study suggest the P-selectin-mediated platelet-neutrophil aggregate formation, which activates neutrophils in SCD mouse model and human been carriers [104]. The sCD40L level is increased and biologically active in HbSS patients due to platelet activation, mainly, in patients in crises and positively correlates with an increase of TF and ICAM-1 expression, suggesting that an increase of CD40L may contribute to the chronic inflammation and with the increased pro-coagulant activity in HbSS patients [105].

Thus, several studies suggest that in addition to pro-coagulant role, platelets contribute directly to constant vascular inflammation state in HbSS patients by activateing neutrophils and monocytes and further research about therapies targeting function and interaction of platelets and endothelial cells and leukocytes may help to control inflammation and vaso-occlusive events among these patients.

8. CIRCULATING MICROPARTICLES: NEW INFLAMMATION BIOMARKERS IN HBSS

Microparticles (MPs) are heterogeneous group of membrane-bound vesicles described as vesicles smaller than 10^{-15} m in diameter [106]. MPs are shedding from plasma membranes, after cell activation or apoptosis of several cellular types. Essentially any cell type (e.g., leucocytes, and endothelial cells), but also platelets and RBC can release MPs [107,108]. They have been implicated to play a role in inflammation, coagulation and vascular function.

During blebbing, the lipid bilayer forms cytoplasmatic protrusions, culminating in the release of MPs [109]. This process involve an increase in intracellular calcium which affects many important enzymes for the maintaining of the cytoskeletal and membrane structure, such as gelsolin, calpain, flippase, floppase and scramblase [108].

As the lipid and protein composition of the MPs membrane resembles that from the releasing cell, analysis of MPs surface markers by flow cytometry can identify the MPs origin. Internally, MPs contain a variety of cytoplasmatic and nuclear components of their precursor cells [110]. Circulating MPs levels result from the balance between their rates of release from cells and their clearance from the circulation.

MPs levels are augmented in several diseases, such HbSS. Patients have elevated MPs, both in steady-state and crisis, implying that even patients in steady-state are fundamentally different from healthy subjects [111,112]. Total circulating MPs are more elevated in the crisis phase of the disease than in steady-state [111,113]. During crisis, endothelial damage and coagulation activation increase dramatically, and those condition are accompanied by an increases of circulating MPs [113]. Thus, it is worth considering circulating MPs as biomarkers of HbSS.

Sickle cell anemia patients have an increased risk of vascular thrombotic occlusion [114]. In addition, there is strong *in vitro* evidence that circulating MPs are involved in the coagulation system activation in HbSS [115,112]. The pro-coagulant activity depends of some molecules presence, such as phosphatidylserine (PS) and TF, both exposed on several MPs types outer membranes [110]. Therefore, this pro-coagulant activity may be relevant clinically as MPs concentrations with this phenotype are elevated in HbSS.

The majority of circulating MPs in HbSS has RBC and platelets origin [116]. The report by van Beers *et al.* [116] showed a strong association between erythrocyte-derived MPs and markers of *in vivo* coagulation and fibrinolysis activation status as well as endothelial activation [116]. In addition, MPs may support coagulation activation by exposure of PS [115,116], which offers multiple binding sites for the coagulation factors II, Va, and Xa [117]. Sickle cell anemia patients have elevated plasma levels of annexin A5- and PS-exposing MPs [111,112]. Thus, MPs can provide a platform for the assembly of the prothrombinase complex and accelerate the conversion of prothrombin into thrombin.

Importantly to vascular homeostasis has been the discovery of TF, the principal initiator of coagulation, in MPs from HbSS. Sickle blood contains a fraction of MPs originating from platelets [114], endothelial cells and monocytes [113], which are TF positive. Furthermore, once initiated by TF, thrombin generation is greatly accelerated in the presence of PS [67] and co-expression of these molecules can contribute to thrombotic events frequently observed in patients with HbSS [118]. Importantly, MPs could be capable to initiate blood coagulation.

Microparticles are emerging as important biomarker of inflammation, coagulation and thrombosis in HbSS. Linked to crucial steps of HbSS, MPs can now be viewed "partners in disease", especially in patients in crisis-state. MPs provide a vehicle to couple inflammation and coagulation, contributing to thrombotic tendencies in this disease. This increasingly close relationship between MPs and HbSS demonstrates the need for more studies on this subject. Thus, it is necessary additional research to define the precise role of circulating MPs in HbSS and allow the development of new therapeutic strategies either blocking the release of MPs or modifying their activity.

9. CONCLUSION

It is very well known that SCD is a group of genetic disorders, with 101 years of its first medical relate, but still has several pieces of the puzzle to be solved. The search for pathways and biomarkers involved in the pathophysiology of the disease are still need to be exhausted search, and it will bring the knowledge of molecules that

may contribute to increase SCD patients life quality, given opportunity for new therapeutic approaches and clinical management modalities. This review just point several mechanisms associated with SCD, and may contribute to give some ideas about the very complex molecules network involved in the inflammatory process associated with the disease pathogenesis.

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REFERENCES

[1] Steinberg, M.H. (2008) Sickle cell anemia, the first molecular disease: Overview of molecular etiology, pathophysiology, and therapeutic approaches. *The Scientific World Journal*, **8**, 1295-1324. doi:10.1100/tsw.2008.157

[2] Steinberg, M.H. (2005) Predicting clinical severity in sickle cell anaemia. *British Journal of Haematology*, **129**, 465-481. doi:10.1111/j.1365-2141.2005.05411.x

[3] Lanaro, C., Franco-Penteado, C.F., Albuqueque, D.M., Saad, S.T., Conran, N. and Costa, F.F. (2009) Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *Journal of Leukocyte Biology*, **85**, 235-242. doi:10.1189/jlb.0708445

[4] Bender, M.A. and Hobbs, W. (1993) Sickle cell disease. In: Pagon, R.A., Bird, T.D., Dolan, C.R., Stephens, K. and Adam, M.P., Eds., *Genereviews*, Seattle, WA.

[5] Morris, C.R. (2011) Vascular risk assessment in patients with sickle cell disease. *Haematologica*, **96**, 1-5. doi:10.3324/haematol.2010.035097

[6] Medina-Urrutia, A., Juarez-Rojas, J.G., Cardoso-Saldana, G., Jorge-Galarza, E., Posadas-Sanchez, R., Martinez-Alvarado, R., Caracas-Portilla, N., Mendoza Perez, E. and Posadas-Romero, C. (2011) Abnormal high-density lipoproteins in overweight adolescents with atherogenic dyslipidemia. *Pediatrics*, **127**, e1521-1527.

[7] Elkind, M.S. (2006) Inflammation, atherosclerosis, and stroke. *The Neurologist*, **12**, 140-148. doi:10.1097/01.nrl.0000215789.70804.b0

[8] Thanoon, I.A., Abdul-Jabbar, H.A. and Taha, D.A. (2012) Oxidative stress and C-reactive protein in patients with cerebrovascular accident (ischaemic stroke): The role of ginkgo biloba extract. *Sultan Qaboos University Medical Journal*, **12**, 197-205.

[9] Milton, J.N., Sebastiani, P., Solovieff, N., Hartley, S.W., Bhatnagar, P., Arking, D.E., Dworkis, D.A., Casella, J.F., Barron-Casella, E., Bean, C.J., Hooper, W.C., DeBaun, M.R., Garrett, M.E., Soldano, K., Telen, M.J., Ashley-Koch, A., Gladwin, M.T., Baldwin, C.T., Steinberg, M.H. and Klings, E.S. (2012) A genome-wide association study of total bilirubin and cholelithiasis risk in sickle cell anemia. *PloS One*, **7**, e34741. doi:10.1371/journal.pone.0034741

[10] Ebert, E.C., Nagar, M. and Hagspiel, K.D. (2010) Gastrointestinal and hepatic complications of sickle cell disease. *Clinical Gastroenterology and Hepatology: The Official Clinical Practice Journal of the American Gastroenterological Association*, **8**, 483-489; quiz e470.

[11] Wakugawa, Y., Kiyohara, Y., Tanizaki, Y., Kubo, M., Ninomiya, T., Hata, J., Doi, Y., Okubo, K., Oishi, Y., Shikata, K., Yonemoto, K., Maebuchi, D., Ibayashi, S. and Iida, M. (2006) C-Reactive protein and risk of first-ever ischemic and hemorrhagic stroke in a general japanese population: The hisayama study. *Stroke; A journal of Cerebral Circulation*, **37**, 27-32. doi:10.1161/01.STR.0000194958.88216.87

[12] Voskaridou, E., Christoulas, D. and Terpos, E. (2012) Sickle-cell disease and the heart: Review of the current literature. *British Journal of Haematology*, **157**, 664-673.

[13] Costa, R.N., Conran, N., Albuquerque, D.M., Soares, P.H., Saad, S.T. and Costa, F.F. (2005) Association of the G-463a myeloperoxidase polymorphism with infection in sickle cell anemia. *Haematologica*, **90**, 977-979.

[14] Liu, C., Xie, G., Huang, W., Yang, Y., Li, P. and Tu, Z. (2012) Elevated serum myeloperoxidase activities are significantly associated with the prevalence of ACS and high Ldl-C Levels in Chd patients. *Journal of Atherosclerosis and Thrombosis*, **19**, 435-443. doi:10.1111/j.1365-2141.2012.09143.x

[15] Seixas, M.O., Rocha, L.C., Carvalho, M.B., Menezes, J.F., Lyra, I.M., Nascimento, V.M., Couto, R.D., Atta, A.M., Reis, M.G. and Goncalves, M.S. (2010) Levels of high-density lipoprotein cholesterol (Hdl-C) among Children with steady-state sickle cell disease. *Lipids in Health and Disease*, **9**, 91. doi:10.1186/1476-511X-9-91

[16] Wood, K.C., Hsu, L.L. and Gladwin, M.T. (2008) Sickle cell disease vasculopathy: A state of nitric oxide resistance. *Free Radical Biology & Medicine*, **44**, 1506-1528. doi:10.1016/j.freeradbiomed.2008.01.008

[17] Reiter, C.D., Wang, X., Tanus-Santos, J.E., Hogg, N., Cannon, R.O., 3rd, Schechter, A.N. and Gladwin, M.T. (2002) Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nature Medicine*, **8**, 1383-1389. doi:10.1038/nm1202-799

[18] Ryter, S.W. and Tyrrell, R.M. (2000) The heme synthesis and degradation pathways: Role in oxidant sensitivity. heme oxygenase has both pro- and antioxidant properties. *Free Radical Biology & Medicine*, **28**, 289-309. doi:10.1016/S0891-5849(99)00223-3

[19] Akinsheye, I. and Klings, E.S. (2010) Sickle cell anemia and vascular dysfunction: The nitric oxide connection. *Journal of Cellular Physiology*, **224**, 620-625. doi:10.1002/jcp.22195

[20] Villagra, J., Shiva, S., Hunter, L.A., Machado, R.F., Gladwin, M.T. and Kato, G.J. (2007) Platelet activation in

patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin. *Blood*, **110**, 2166-2172. doi:10.1182/blood-2006-12-061697

[21] Spiecker, M., Darius, H., Kaboth, K., Hubner, F. and Liao, J.K. (1998) Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. *Journal of Leukocyte Biology*, **63**, 732-739.

[22] Aslan, M., Ryan, T.M., Adler, B., Townes, T.M., Parks, D.A., Thompson, J.A., Tousson, A., Gladwin, M.T., Patel, R.P., Tarpey, M.M., Batinic-Haberle, I., White, C.R. and Freeman, B.A. (2001) Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 15215-15220. doi:10.1073/pnas.221292098

[23] Solovey, A., Kollander, R., Milbauer, L.C., Abdulla, F., Chen, Y., Kelm, R.J. Jr. and Hebbel, R.P. (2010) Endothelial nitric oxide synthase and nitric oxide regulate endothelial tissue factor expression *in vivo* in the sickle transgenic mouse. *American Journal of Hematology*, **85**, 41-45.

[24] Jain, S. and Gladwin, M.T. (2010) Arginine metabolism and nitric oxide bioavailability in sickle cell disease. *Journal of Pediatric Hematology/Oncology*, **32**, e247-248. doi:10.1097/MPH.0b013e3181ec0b00

[25] Zhou, Z., Behymer, M. and Guchhait, P. (2011) Role of extracellular hemoglobin in thrombosis and vascular occlusion in patients with sickle cell anemia. *ANEMIA*, article ID: 918916. doi:10.1155/2011/918916

[26] Morris, C.R., Kato, G.J., Poljakovic, M., Wang, X., Blackwelder, W.C., Sachdev, V., Hazen, S.L., Vichinsky, E.P., Morris, S.M. Jr. and Gladwin, M.T. (2005) Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA: The Journal of the American Medical Association*, **294**, 81-90. doi:10.1001/jama.294.1.81

[27] Vilas-Boas, W., Cerqueira, B.A., Zanette, A.M., Reis, M.G., Barral-Netto, M. and Goncalves, M.S. (2010) Arginase levels and their association with th17-related cytokines, soluble adhesion molecules (Sicam-1 and Svcam-1) and hemolysis markers among steady-state sickle cell anemia patients. *Annals of Hematology*, **89**, 877-882. doi:10.1007/s00277-010-0954-9

[28] Bialecka, M., Robowski, P., Honczarenko, K., Roszmann, A. and Slawek, J. (2009) Genetic and environmental factors for hyperhomocysteinaemia and its clinical implications in Parkinson's disease. *Neurologia i Neurochirurgia Polska*, **43**, 272-285.

[29] Refsum, H., Ueland, P.M., Nygard, O. and Vollset, S.E. (1998) Homocysteine and cardiovascular disease. *Annual Review of Medicine*, **49**, 31-62. doi:10.1146/annurev.med.49.1.31

[30] Clarke, R., Daly, L., Robinson, K., Naughten, E., Cahalane, S., Fowler, B. and Graham, I. (1991) Hyperhomocysteinemia: An independent risk factor for vascular disease. *The New England Journal of Medicine*, **324**, 1149-1155. doi:10.1056/NEJM199104253241701

[31] Lowenthal, E.A., Mayo, M.S., Cornwell, P.E. and Thornley-Brown, D. (2000) Homocysteine elevation in sickle cell disease. *Journal of the American College of Nutrition*, **19**, 608-612.

[32] Siniscalchi, A., Mancuso, F., Gallelli, L., Ferreri Ibbadu, G., Biagio Mercuri, N. and De Sarro, G. (2005) Increase in plasma homocysteine levels induced by drug treatments in neurologic patients. *Pharmacological Research: The Official Journal of the Italian Pharmacological Society*, **52**, 367-375.

[33] Dudman, N.P., Temple, S.E., Guo, X.W., Fu, W. and Perry, M.A. (1999) Homocysteine enhances neutrophil-endothelial interactions in both cultured human cells and rats *in vivo*. *Circulation Research*, **84**, 409-416. doi:10.1161/01.RES.84.4.409

[34] Chies, J.A. and Nardi, N.B. (2001) Sickle cell disease: A chronic inflammatory condition. *Medical Hypotheses*, **57**, 46-50. doi:10.1054/mehy.2000.1310

[35] Wallace, K.L. and Linden, J. (2010) Adenosine A2a receptors induced on Inkt and Nk cells reduce pulmonary inflammation and injury in mice with sickle cell disease. *Blood*, **116**, 5010-5020. doi:10.1182/blood-2010-06-290643

[36] Park, S.W., Kim, M., Brown, K.M., D'Agati, V.D. and Lee, H.T. (2011) Paneth cell-derived interleukin-17a causes multiorgan dysfunction after hepatic ischemia and reperfusion injury. *Hepatology*, **53**, 1662-1675. doi:10.1002/hep.24253

[37] Chen, G.Y. and Nunez, G. (2010) Sterile inflammation: Sensing and reacting to damage. Nature reviews. *Immunology*, **10**, 826-837. doi:10.1038/nri2873

[38] Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, **124**, 783-801. doi:10.1016/j.cell.2006.02.015

[39] Iwasaki, A. and Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. *Nature Immunology*, **5**, 987-995. doi:10.1038/ni1112

[40] Pulendran, B. (2005) Variegation of the immune response with dendritic cells and pathogen recognition receptors. *Journal of Immunology*, **174**, 2457-2465.

[41] Ferwerda, B., McCall, M.B., Alonso, S., Giamarellos-Bourboulis, E.J., Mouktaroudi, M., Izagirre, N., Syafruddin, D., Kibiki, G., Cristea, T., Hijmans, A., Hamann, L., Israel, S., ElGhazali, G., Troye-Blomberg, M., Kumpf, O., Maiga, B., Dolo, A., Doumbo, O., Hermsen, C.C., Stalenhoef, A.F., van Crevel, R., Brunner, H.G., Oh, D.Y., Schumann, R.R., de la Rua, C., Sauerwein, R., Kullberg, B.J., van der Ven, A.J., van der Meer, J.W. and Netea, M.G. (2007) Tlr4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 16645-16650. doi:10.1073/pnas.0704828104

[42] Gregory, S., Zilber, M., Charron, D. and Gelin, C. (2000) Human Cd1a molecule expressed on monocytes plays an accessory role in the superantigen-induced activation of T lymphocytes. *Human Immunology*, **61**, 193-201. doi:10.1016/S0198-8859(99)00129-9

[43] Sloma, I., Zilber, M.T., Charron, D., Girot, R., Tamouza, R. and Gelin, C. (2004) Upregulation and atypical ex-

166, 963-972. doi:10.1016/S0002-9440(10)62318-8

[69] Nagababu, E., Fabry, M.E., Nagel, R.L. and Rifkind, J.M. (2008) Heme degradation and oxidative stress in murine models for hemoglobinopathies: Thalassemia, sickle cell disease and hemoglobin C disease. *Blood Cells, Molecules & Diseases*, **41**, 60-66. doi:10.1016/j.bcmd.2007.12.003

[70] Nur, E., Biemond, B.J., Otten, H.M., Brandjes, D.P., Schnog, J.J. and Group, C.S. (2011) Oxidative stress in sickle cell disease; Pathophysiology and potential implications for disease management. *American Journal of Hematology*, **86**, 484-489. doi:10.1002/ajh.22012

[71] Repka, T. and Hebbel, R.P. (1991) Hydroxyl radical formation by sickle erythrocyte membranes: Role of pathologic iron deposits and cytoplasmic reducing agents. *Blood*, **78**, 2753-2758.

[72] Setty, B.N., Rao, A.K. and Stuart, M.J. (2001) Thrombophilia in sickle cell disease: The red cell connection. *Blood*, **98**, 3228-3233. doi:10.1182/blood.V98.12.3228

[73] Francis, R.B. Jr. and Haywood, L.J. (1992) Elevated immunoreactive tumor necrosis factor and interleukin-1 in sickle cell disease. *Journal of the National Medical Association*, **84**, 611-615.

[74] Aslan, M., Thornley-Brown, D. and Freeman, B.A. (2000) Reactive species in sickle cell disease. *Annals of the New York Academy of Sciences*, **899**, 375-391. doi:10.1111/j.1749-6632.2000.tb06201.x

[75] Gladwin, M.T., Schechter, A.N., Ognibene, F.P., Coles, W.A., Reiter, C.D., Schenke, W.H., Csako, G., Waclawiw, M.A., Panza, J.A. and Cannon, R.O. 3rd (2003) Divergent nitric oxide bioavailability in men and women with sickle cell disease. *Circulation*, **107**, 271-278. doi:10.1161/01.CIR.0000044943.12533.A8

[76] Selvaraj, S.K., Giri, R.K., Perelman, N., Johnson, C., Malik, P. and Kalra, V.K. (2003) Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. *Blood*, **102**, 1515-1524. doi:10.1182/blood-2002-11-3423

[77] Musa, B.O., Onyemelukwe, G.C., Hambolu, J.O., Mamman, A.I. and Isa, A.H. (2010) Pattern of serum cytokine expression and t-cell subsets in sickle cell disease patients in vaso-occlusive crisis. *Clinical and Vaccine Immunology: CVI*, **17**, 602-608. doi:10.1128/CVI.00145-09

[78] Goossens, V., Grooten, J., De Vos, K. and Fiers, W. (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 8115-8119. doi:10.1073/pnas.92.18.8115

[79] Muhl, D., Woth, G., Drenkovics, L., Varga, A., Ghosh, S., Csontos, C., Bogar, L., Weber, G. and Lantos, J. (2011) Comparison of oxidative stress & leukocyte activation in patients with severe sepsis & burn injury. *The Indian Journal of Medical Research*, **134**, 69-78.

[80] Read, M.A., Whitley, M.Z., Williams, A.J. and Collins, T. (1994) Nf-kappa B and I kappa B alpha: An inducible regulatory system in endothelial activation. *The Journal of Experimental Medicine*, **179**, 503-512. doi:10.1084/jem.179.2.503

[81] Patel, S.J., Jindal, R., King, K.R., Tilles, A.W. and Yarmush, M.L. (2011) The inflammatory response to double stranded DNA in endothelial cells is mediated by Nfkappab and Tnfalpha. *PloS One*, **6**, e19910. doi:10.1371/journal.pone.0019910

[82] Croizat, H. (1994) Circulating cytokines in sickle cell patients during steady state. *British Journal of Haematology*, **87**, 592-597. doi:10.1111/j.1365-2141.1994.tb08318.x

[83] Raghupathy, R., Haider, M.Z., Azizieh, F., Abdelsalam, R., D'Souza, T.M. and Adekile, A.D. (2000) Th1 and Th2 cytokine profiles in sickle cell disease. *Acta Haematologica*, **103**, 197-202. doi:10.1159/000041049

[84] Goncalves, M.S., Queiroz, I.L., Cardoso, S.A., Zanetti, A., Strapazoni, A.C., Adorno, E., Albuquerque, A., Sant'Ana, A., dos Reis, M.G., Barral, A. and Barral Netto, M. (2001) Interleukin 8 as a vaso-occlusive marker in Brazilian patients with sickle cell disease. *Brazilian Journal of Medical and Biological Research*, **34**, 1309-1313.

[85] Pathare, A., Al Kindi, S., Alnaqdy, A.A., Daar, S., Knox-Macaulay, H. and Dennison, D. (2004) Cytokine profile of sickle cell disease in Oman. *American Journal of Hematology*, **77**, 323-328. doi:10.1002/ajh.20196

[86] Akohoue, S.A., Shankar, S., Milne, G.L., Morrow, J., Chen, K.Y., Ajayi, W.U. and Buchowski, M.S. (2007) Energy expenditure, inflammation, and oxidative stress in steady-state adolescents with sickle cell anemia. *Pediatric Research*, **61**, 233-238. doi:10.1203/pdr.0b013e31802d7754

[87] Lard, L.R., Mul, F.P., de Haas, M., Roos, D. and Duits, A.J. (1999) Neutrophil activation in sickle cell disease. *Journal of Leukocyte Biology*, **66**, 411-415.

[88] Okpala, I. (2002) Steady-state platelet count and complications of sickle cell disease. *The Hematology Journal: The Official Journal of the European Haematology Association/EHA*, **3**, 214-215.

[89] Schnog, J.B., Rojer, R.A., Mac Gillavry, M.R., Ten Cate, H., Brandjes, D.P. and Duits, A.J. (2003) Steady-state svcam-1 serum levels in adults with sickle cell disease. *Annals of Hematology*, **82**, 109-113.

[90] Dworkis, D.A., Klings, E.S., Solovieff, N., Li, G., Milton, J.N., Hartley, S.W., Melista, E., Parente, J., Sebastiani, P., Steinberg, M.H. and Baldwin, C.T. (2011) Severe sickle cell anemia is associated with increased plasma levels of Tnf-R1 and Vcam-1. *American Journal of Hematology*, **86**, 220-223. doi:10.1002/ajh.21928

[91] Cerqueira, B.A., Boas, W.V., Zanette, A.D., Reis, M.G. and Goncalves, M.S. (2011) Increased concentrations of Il-18 and uric acid in sickle cell anemia: Contribution of hemolysis, endothelial activation and the inflammasome. *Cytokine*, **56**, 471-476. doi:10.1016/j.cyto.2011.08.013

[92] Berry, C.E. and Hare, J.M. (2004) Xanthine oxidoreductase and cardiovascular disease: Molecular mechanisms and pathophysiological implications. *The Journal of Physiology*, **555**, 589-606. doi:10.1113/jphysiol.2003.055913

[93] Xu, P., Huecksteadt, T.P. and Hoidal, J.R. (1996) Molecular cloning and characterization of the human xanthine dehydrogenase gene (Xdh). *Genomics*, **34**, 173-180. doi:10.1006/geno.1996.0262

[94] Zhang, C., Hein, T.W., Wang, W., Ren, Y., Shipley, R.D. and Kuo, L. (2006) Activation of Jnk and xanthine oxidase by Tnf-alpha impairs nitric oxide-mediated dilation of coronary arterioles. *Journal of Molecular and Cellular Cardiology*, **40**, 247-257. doi:10.1016/j.yjmcc.2005.11.010

[95] Martinon, F., Mayor, A. and Tschopp, J. (2009) The inflammasomes: Guardians of the body. *Annual Review of Immunology*, **27**, 229-265. doi:10.1146/annurev.immunol.021908.132715

[96] Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. and Tschopp, J. (2006) Gout-associated uric acid crystals activate the Nalp3 inflammasome. *Nature*, **440**, 237-241. doi:10.1038/nature04516

[97] Vieira-de-Abreu, A., Campbell, R.A., Weyrich, A.S. and Zimmerman, G.A. (2012) Platelets: Versatile effector cells in hemostasis, inflammation, and the immune continuum. *Seminars in Immunopathology*, **34**, 5-30. doi:10.1007/s00281-011-0286-4

[98] Maugeri, N., Baldini, M., Ramirez, G.A., Rovere-Querini, P. and Manfredi, A.A. (2012) Platelet-leukocyte deregulated interactions foster sterile inflammation and tissue damage in immune-mediated vessel diseases. *Thrombosis Research*, **129**, 267-273. doi:10.1016/j.thromres.2011.12.001

[99] Stokes, K.Y. and Granger, D.N. (2012) Platelets: A critical link between inflammation and microvascular dysfunction. *The Journal of Physiology*, **590**, 1023-1034.

[100] Chiang, E.Y. and Frenette, P.S. (2005) Sickle cell vaso-occlusion. *Hematology/Oncology Clinics of North America*, **19**, 771-784. doi:10.1016/j.hoc.2005.08.002

[101] Charneski, L. and Congdon, H.B. (2010) Effects of antiplatelet and anticoagulant medications on the vasoocclusive and thrombotic complications of sickle cell disease: A review of the literature. *American Journal of Health-System Pharmacy: AJHP: Official Journal of the American Society of Health-System Pharmacists*, **67**, 895-900.

[102] May, A.E., Seizer, P. and Gawaz, M. (2008) Platelets: Inflammatory firebugs of vascular walls. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **28**, s5-10. doi:10.1161/ATVBAHA.107.158915

[103] Gawaz, M., Langer, H. and May, A.E. (2005) Platelets in inflammation and atherogenesis. *The Journal of Clinical Investigation*, **115**, 3378-3384. doi:10.1172/JCI27196

[104] Polanowska-Grabowska, R., Wallace, K., Field, J.J., Chen, L., Marshall, M.A., Figler, R., Gear, A.R. and Linden, J. (2010) P-selectin-mediated platelet-neutrophil aggregate formation activates neutrophils in mouse and human sickle cell disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **30**, 2392-2399. doi:10.1161/ATVBAHA.110.211615

[105] Lee, S.P., Ataga, K.I., Orringer, E.P., Phillips, D.R. and Parise, L.V. (2006) Biologically active Cd40 ligand is elevated in sickle cell anemia: Potential role for platelet-mediated inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **26**, 1626-1631. doi:10.1161/01.ATV.0000220374.00602.a2

[106] Wolf, P. (1967) The nature and significance of platelet products in human plasma. *British Journal of Haematology*, **13**, 269-288. doi:10.1111/j.1365-2141.1967.tb08741.x

[107] Amabile, N., Guerin, A.P., Leroyer, A., Mallat, Z., Nguyen, C., Boddaert, J., London, G.M., Tedgui, A. and Boulanger, C.M. (2005) Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *Journal of the American Society of Nephrology: JASN*, **16**, 3381-3388. doi:10.1681/ASN.2005050535

[108] Piccin, A., Murphy, W.G. and Smith, O.P. (2007) Circulating microparticles: Pathophysiology and clinical implications. *Blood Reviews*, **21**, 157-171. doi:10.1016/j.blre.2006.09.001

[109] Cocucci, E., Racchetti, G. and Meldolesi, J. (2009) Shedding microvesicles: Artefacts no more. *Trends in Cell Biology*, **19**, 43-51. doi:10.1016/j.tcb.2008.11.003

[110] Ardoin, S.P., Shanahan, J.C. and Pisetsky, D.S. (2007) The role of microparticles in inflammation and thrombosis. *Scandinavian Journal of Immunology*, **66**, 159-165. doi:10.1111/j.1365-3083.2007.01984.x

[111] van Tits, L.J., van Heerde, W.L., Landburg, P.P., Boderie, M.J., Muskiet, F.A., Jacobs, N., Duits, A.J. and Schnog, J.B. (2009) Plasma annexin A5 and microparticle phosphatidylserine levels are elevated in sickle cell disease and increase further during painful crisis. *Biochemical and Biophysical Research Communications*, **390**, 161-164. doi:10.1016/j.bbrc.2009.09.102

[112] Noubouossie, D.C., Le, P.Q., Rozen, L., Debaugnies, F., Ferster, A. and Demulder, A. (2011) Evaluation of the procoagulant activity of endogenous phospholipids in the platelet-free plasma of children with sickle cell disease using functional assays. *Thrombosis Research*. doi:10.1016/j.thromres.2011.10.016

[113] Shet, A.S., Aras, O., Gupta, K., Hass, M.J., Rausch, D.J., Saba, N., Koopmeiners, L., Key, N.S. and Hebbel, R.P. (2003) Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood*, **102**, 2678-2683. doi:10.1182/blood-2003-03-0693

[114] Ataga, K.I., Brittain, J.E., Desai, P., May, R., Jones, S., Delaney, J., Strayhorn, D., Hinderliter, A. and Key, N.S. (2012) Association of coagulation activation with clinical complications in sickle cell disease. *PloS One*, **7**, e29786. doi:10.1371/journal.pone.0029786

[115] Mahfoudhi, E., Lecluse, Y., Driss, F., Abbes, S., Flaujac, C. and Garcon, L. (2012) Red cells exchanges in sickle cells disease lead to a selective reduction of erythrocytes-derived blood microparticles. *British Journal of Haematology*, **156**, 545-547. doi:10.1111/j.1365-2141.2011.08897.x

[116] Van Beers, E.J., Schaap, M.C., Berckmans, R.J., Nieuwland, R., Sturk, A., van Doormaal, F.F., Meijers, J.C., Biemond, B.J. and Group, C.S. (2009) Circulating erythrocyte-derived microparticles are associated with

coagulation activation in sickle cell disease. *Haematologica*, **94**, 1513-1519. doi:10.3324/haematol.2009.008938

[117] Michelson, A.D., Rajasekhar, D., Bednarek, F.J. and Barnard, M.R. (2000) Platelet and platelet-derived microparticle surface factor V/Va binding in whole blood: Differences between neonates and adults. *Thrombosis and Haemostasis*, **84**, 689-694.

[118] Adedeji, M.O., Cespedes, J., Allen, K., Subramony, C. and Hughson, M.D. (2001) Pulmonary thrombotic arteriopathy in patients with sickle cell disease. *Archives of Pathology & Laboratory Medicine*, **125**, 1436-1441.

LIST OF ABBREVIATIONS

BDCA 2: blood dendritic cell antigen 2;
CRP: C-reactive protein;
CVD: cardiovascular disease;
DC: dendritic cells;
EVA: encephalic vascular accident;
GPI: glycosylphosphatidylinositol;
GSH: glutathione;
Hb: hemoglobin;
HBB: β-globin gene;
HBSS: sickle cell anemia;
Hcy: Homocysteine;
HDL-C: high-density lipoprotein cholesterol;
HLA: human leukocyte antigen;
H_2O_2: hydrogen peroxide;
HOCl: hypochlorous acid;
ICAM-1: intercellular adhesion molecule-1;
IL-1: interleukin-1;
IL-12: interleukin-12;
IL-6: interleukin-6;
INF-α: interferon-α;
LDH: lactate dehydrogenase;
LDL-C: low-density lipoprotein cholesterol;
LRR: leucine rich repeat;
LTB4: leukotriene B4;
mDC: myeloid dendritic cells;
MPO: myeloperoxidase;
MPs: Microparticles;
NO: nitric oxide;
NO_3^-: nitrate;
NOS: nitric oxide synthase;
[OH]: hydroxyl radical;
$ONOO^-$: peroxynitrite;
OS: oxidative stress;
O_2^-: superoxide;
PAF: platelet activator factor;
PAI: plasminogen activator inhibitor;
PAMP: pathogen associated molecular patterns;
pDC: plasmacytoid dendritic cells;
PGE2: prostaglandin E2;
PMN: polymorphonuclear leukocytes;
PRR: pattern recognition receptors;
PS: phosphatidylserine;
PSGL-1: P-selectin ligand glycoprotein-1;
RBC: red blood cells;
RNS: reactive nitrogen species;
ROS: reactive oxygen species;
SCD: sickle cell disease;
SNP: Single-nucleotide polymorphisms;
SOD: superoxide dismutase;
sICAM-1: soluble intercellular adhesion molecule-1;
sVCAM-1: soluble vascular cellular adhesion molecule-1;
TF: tissue factor;
TGF-beta: transforming grow factor beta;
TLR: toll like receptor;
TPA: tissue plasminogen activator;
VCAM-1: vascular cellular adhesion molecule-1;
VLDL-C: very low density lipoprotein cholesterol;
VOE: vaso-occlusive episodes;
vWF: von Willebrand factor;
XO: xanthine oxidase.

A.4 - MANUSCRITO IV

Sickle cell disease retinopathy: characterization among pediatric and teenage patients from Northeast Brazil

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Este trabalho avalia a retinopatia falciforme em crianças e adolescentes com foco no papel do exame oftalmológico para a prevenção da gravidade desta alteração. **Brazilian Journal of Hematology and Hemotherapy.**

Resumo: A doença falciforme (DF) é caracterizada por uma variedade de alterações clínicas que estão ligadas à anemia hemolítica e aos eventos vaso-oclusivos associados a crises dolorosas e outras características clínicas, incluindo retinopatia. O objetivo do presente estudo foi caracterizar retinopatia em crianças e adolescentes com DF da Bahia, estado do nordeste brasileiro com incidência e prevalência elevada da doença. Foi realizado um estudo de corte transversal que envolveu um grupo de 51 pacientes com DF (36 HbSS e 15 HbSC) com idade variando entre 4 a 18 anos. O exame oftalmológico foi realizado e para os pacientes com DF com mais de 10 anos de idade, o exame foi acompanhado por angiofluoresceinografia. As lesões oculares mais frequentes foram "tortuosidade vascular", que foi encontrado em 9 (25%) pacientes HbSS, e "black sunburst", que foi encontrado em 3 (20%) pacientes HbSC. Alterações arteriais periféricas foram observadas em cinco (13,9%) pacientes HbSS e 3 (13,3%) HbSC. Anastomoses arteriovenosas estavam presentes em 6 (16,5%) pacientes HbSS e 6 (37,5%) pacientes HbSC. A neovascularização não foi identificada em nenhum dos pacientes com DF. Este estudo demonstra a importância da utilização do exame oftalmológico precoce em pacientes jovens com DF para prevenir a progressão da retinopatia falciforme para uma doença grave ou até mesmocegueira.

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ELSEVIER

Original article

Sickle cell disease retinopathy: characterization among pediatric and teenage patients from northeastern Brazil

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ABSTRACT

Objective: The aim of the present study was to characterize sickle cell disease retinopathy in children and teenagers from Bahia, the state in northeastern Brazil with the highest incidence and prevalence of sickle cell disease.

Methods: A group of 51 sickle cell disease patients (36 hemoglobin SS and 15 hemoglobin SC) with ages ranging from 4 to 18 years was studied. Ophthalmological examinations were performed in all patients. Moreover, a fluorescein angiography was also performed in over 10-year-old patients.

Results: The most common ocular lesions were vascular tortuosity, which was found in nine (25%) hemoglobin SS patients, and black sunburst, in three (20%) hemoglobin SC patients. Peripheral arterial closure was observed in five (13.9%) hemoglobin SS patients and in three (13.3%) hemoglobin SC patients. Arteriovenous anastomoses were present in six (16.5%) hemoglobin SS patients and six (37.5%) hemoglobin SC patients. Neovascularization was not identified in any of the patients.

Conclusions: This study supports the use of early ophthalmological examinations in young sickle cell disease patients to prevent the progression of retinopathy to severe disease and further blindness.

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Introduction

Sickle cell disease (SCD) is characterized by the presence of the hemoglobin S (Hb S) variant due to a single nucleotide change (GAG → GTG) in the *β-globin* gene (*HBB*) that replaces a glutamic acid with a valine at the sixth position of the β-globin chain. The hemoglobin C (Hb C) variant is characterized by a point mutation at the sixth position of the *HBB* gene, wherein a lysine replaces a glutamic acid at the sixth position of the β-globin chain.[1]

Hb S has a high overall frequency worldwide; in Brazil its distribution is heterogeneous. In northeastern Brazil, in particular in Bahia, the population has a tri-racial mixture of Europeans (mostly Portuguese), Africans and indigenous Brazilians.[2] The frequency of Hb S in the state of Bahia is the highest in Brazil, varying from 4.5 to 14.7% in different population groups.[3]

Sickle cell disease is characterized by a variety of clinical abnormalities that are frequently linked to hemolytic anemia and the vaso-occlusive processes often responsible for the pain and other clinical features described by patients, including retinopathy.[1,4]

Methods

The present study was approved by the Human Research Ethics Board of the Fundação Oswaldo Cruz (FIOCRUZ), and informed consent was obtained from the guardians of participants in accordance with ethical principles and in accord with the Helsinki Declaration of 1975 and its revisions. Ophthalmologic examinations were carried out and peripheral blood samples were collected only after signed informed consent has been obtained.

This cross-sectional study involved a group of 51 SCD patients (36 Hb SS and 15 Hb SC) from the state of Bahia in northeastern Brazil and was carried out during the period of January 2010 to December 2012.

Patients were randomly selected among those attending the Hematology Outpatient Clinic at the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), a referral center for SCD patients who are seen in routine visits. Patients were then sent to the Instituto Brasileiro de Oftalmologia e Prevenção da Cegueira (IBOPC) for an eye examination, including fundus biomicroscopy and indirect binocular ophthalmoscopy in all patients and fluorescein angiography in over 10-year-old patients. The following findings were observed in the fundoscopic examination: vascular tortuosity, black sunburst pattern, salmon patches and iridescent spots. The pathologic classification of fundoscopic alterations as proliferative was based on Goldberg's five stage grouping: stage I – peripheral arteriolar occlusions; stage II – peripheral arteriovenous anastomoses; stage III – preretinal neovascularization; stage IV – vitreous hemorrhage and stage V – retinal detachment.

Patients with proliferative retinopathy were stratified by age and were classified as severe when displaying stages III–V. The hemoglobin pattern was confirmed in the Hematology, Genetic and Computacional Biology Laboratory of FIOCRUZ and the Universidade Federal da Bahia (UFBA) using high performance liquid chromatography (HPLC – Variant I/BIO-RAD, CA, USA).

SPSS version 18.0 and GraphPad version 5.0 were used to store and analyze the data. Pearson's Chi-squared or Fisher's exact tests were used to compare the two groups of SCD patients as necessary. A *p*-value of less than 0.05 was considered statistically significant.

Results

A total of 51 SCD patients were enrolled in this study: 36 (70.6%) with sickle cell anemia (SCA) or Hb SS and 15 (29.4%) with Hb SC disease. Overall, the mean age of the patients was 11.76 ± 3.69 years. The Hb SS group had a mean age of 11.39 ± 3.76 years, and the Hb SC group had a mean age of 13.29 ± 2.8 years. Overall 23 (45%) SCD patients were female and 28 (55%) were male. In the Hb SS group, 19 patients (52.78%) were male and 17 (47.22%) were female, and in the Hb SC group, nine (60%) were male and six (40%) were female.

Visual acuity was 20/20 or 20/25 for the best eyes of all patients, corresponding to 90% of the total number of cases.

Age-related ocular lesions, both non-proliferative and proliferative (Goldberg) (Figures 1–4), were very frequent in both

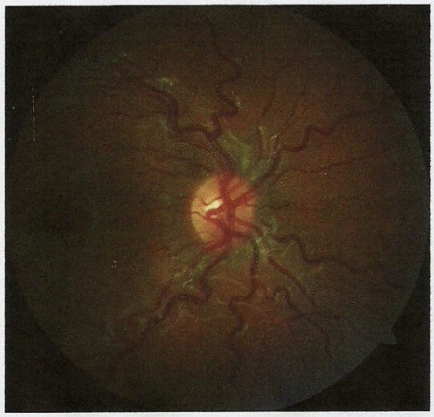

Figure 1 – Tortuous vessels in an 11-year-old female with hemoglobin SS.

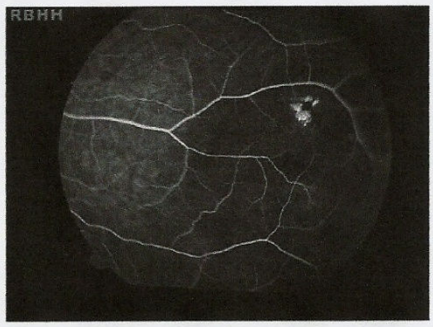

Figure 2 – Black sunburst. Hyperpigmented lesions observed in the retinal periphery of an 18-year-old female with hemoglobin SC.

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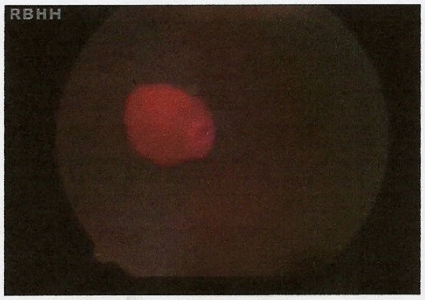

Figure 3 – Salmon patches in a 13-year-old male with hemoglobin SC.

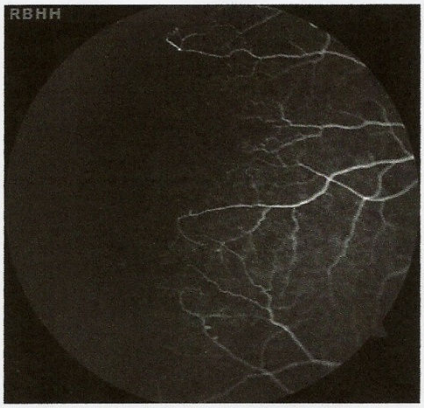

Figure 4 – Peripheral arteriolar occlusions and arteriovenous anastomoses in a 14-year-old male with hemoglobin SC.

groups of patients (Hb SS and Hb SC). Table 1 shows the distribution of ocular lesions across the two age groups. The Hb SS group had more ocular changes in the age range 0–9 years old. In the Hb SC group of patients, ocular changes were more frequent in the age range 10–18 years old.

Vascular tortuosity and black sunbursts were the most common fundoscopic ocular lesions found in both SCD groups (Hb SS and Hb SC). Table 2 shows that vascular tortuosity lesions were most frequent in the Hb SS group. However, black sunburst lesions were more frequent in the Hb SC group. Iridescent spots were found in both groups of SCD patients.

Table 3 shows the distribution of proliferative ocular lesions across Goldberg stages I–V in the Hb SS and Hb SC patient groups. There were patients in both groups with proliferative ocular lesions in Goldberg stages I and II; however, the Hb SS group had more proliferative ocular lesions in the first stage than the Hb SC group; this increased with age in the Hb SC group. There were no cases of proliferative ocular lesions at stages III–V in either of the groups of SCD patients.

Discussion

Several ocular changes were observed in the two SCD patient groups; however, no change in visual acuity was found, as has previously been reported.[5–10]

Fundoscopic lesions, such as vascular tortuosity and black sunbursts, were the most frequent changes, in accordance with previous Brazilian reports.[7–11] The overall frequency of vascular tortuosity was most frequently in SCA patients,

Table 1 – The distribution of retinal vessel occlusion lesions across two age groups of sickle cell disease patients.

	Total ($n=51$)		Hemoglobin SC ($n=15$)		Hemoglobin SS ($n=36$)		p-value[a]
	n	%	n	%	n	%	
Age (years)							
0–9	4	36.4	–	–	4	20.0	0.02
10–18	7	17.5	9	22.2	16	80.0	0.008

[a] Fisher's exact test

Table 2 – Fundoscopic ocular non-proliferative lesions in both sickle cell disease groups.

	Hemoglobin SS ($n=36$)		Hemoglobin SC ($n=15$)		Total ($n=51$)		p-value[a]
	n	%	n	%	n	%	
Vascular tortuosity	9	25.0	1	6.7	10	19.6	0.95
Black sunburst	2	5.6	3	20.0	5	9.8	
Salmon patches	–	–	1	6.7	1	2.0	
Angioid streaks	–	–	–	–	–	–	
Disk sign	–	–	–	–	–	–	
Iridescent spots	3	8.3	1	6.7	4	7.8	
Total	14	38.9	6	40	20	39.3	

[a] Chi-square.

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Table 3 – The distribution of proliferative ocular lesions for Goldberg stages I–V in the hemoglobin SS and hemoglobin SC groups.

	Hemoglobin SS		Hemoglobin SC		*p*-value	Total	
	n	%	*n*	%		*n*	%
Normal	25	69.4	6	40.0	0.09[a]	31	60.8
Ocular Lesions (Goldberg)	11	30.6	9	60.0		20	39.2
Stage I	5	13.9	3	13.3		08	15.7
Stage II	6	16.7	6	37.5		12	23.5

[a] Yates corrected Chi-square test.

as previously described.[10–12] The black sunburst lesion was observed in both groups, although its frequency in Hb SC patients was much lower than that described in previously published studies.[10–13] Salmon patch lesions were present only in the Hb SC group; however, their occurrence has been reported in both groups albeit more frequent in the Hb SC group.[10–13] Iridescent spots were found in both groups, but they were less common than previously reported, which is probably because these alterations are at sites of salmon patch resolution and appear less in younger patients.[14,15]

This study indicates that retinal vascular disease occurs in both Hb SS and Hb SC patients and proliferative retinopathies (Goldberg stages) begin early in the Hb SS group and are more common in both groups with older ages.[11,12]

Proliferative retinopathy (stages III–V) was not observed in our study, probably as the patients were very young rather than old.[16–20] Fox et al.[19] studied Jamaican patients and observed that ocular proliferative lesions in both genotypes increase with age.

Conclusions

The ocular lesions described herein may help to define standardized protocols involving the clinical and ophthalmologic follow-up of Hb SS and Hb SC patients especially for younger patients. Multiple ocular complications exist for SCD patients, and continuous assessment is required to detect lesions early enough for effective prophylactic therapy.

It is necessary to recommend that SCD patients receive periodic ophthalmological examinations at early ages to prevent progression of the disease and early blindness, establish a more effective treatment, and assure a better quality of care for patients' eyes.

Funding

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Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Ohene-Frempong K, Steinberg MH. Clinical aspects of sickle cell anemia in adults and children. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, editors. Disorders of hemoglobin: genetics, phatophysiology and clinical management. New York: Cambridge University Press; 2001. p. 611–70.
2. Azevêdo ES, Fortuna CM, Silva KM, Sousa MG, Machado MA, Lima AM, et al. Spread and diversity of human populations in Bahia, Brazil. Hum Biol. 1982;54(2):329–41.
3. Azevêdo ES, Alves AF, Da Silva MC, Souza MG, Muniz Dias Lima AM, Azevedo WC. Distribution of abnormal hemoglobins and glucose-6-phosphate dehydrogenase variants in 1200 school children of Bahia, Brazil. Am J Phys Anthropol. 1980;53(4):509–12.
4. Vilela RQ, Bandeira DM, Silva MA. Alterações oculares nas doenças falciformes. Rev Bras Hematol Hemoter. 2007;29(3):285–7.
5. Goldberg MF. Classification and pathogenesis of proliferative sickle cell retinopathy. Am J Ophthalmol. 1971;71(3): 649–65.
6. Condon PI, Serjeant GR. Ocular findings in hemoglobin SC disease in Jamaica. Am J Ophthalmol. 1972;74(5): 921–31.
7. Bonanomi MT, Cunha SL, de Araújo JT. Funduscopic alterations in SS and SC hemoglobinopathies. Study of a Brazilian population. Ophthalmologica. 1988;197(1):26–33.
8. Popma SE. Ocular manifestation of sickle hemoglobinopathies. Clin Eye Vis Care. 1996;8(2):111–7.
9. Akinsola FB, Kehinde MO. Ocular findings in sickle cell disease patients in Lagos. Niger Postgrad Med J. 2004;11(3):203–6.
10. Garcia CA, Fernandes MZ, Uchôa UB, Cavalcante BM, Uchôa RA. Achados fundoscópicos em crianças portadoras de anemia falciforme no Estado do Rio Grande do Norte. Arq Bras Oftalmol. 2002;65(6):615–8.
11. Cury D, Boa Sorte N, Lyra IM, Zanette AD, Castro-Lima H, Galvão-Castro B, et al. Ocular lesions in Sickle Cell disease patients from Bahia, Brazil. Rev Bras Oftalmol. 2010;69(4):259–63.
12. Talbot JF, Bird AC, Serjeant GR, Hayes RJ. Sickle cell retinopathy in young children in Jamaica. Br J Ophthalmol. 1982;66:149–54.
13. Moriarty BJ, Acheson RW, Condon PI, Serjeant GR. Patterns of visual loss in untreated sickle cell retinopathy. Eye (Lond). 1988;2(Pt. 3):330–5.
14. Welch RB, Goldberg MF. Sickle-cell hemoglobin and its relation to fundus abnormality. Arch Ophthalmol. 1966;75(3):353–62.

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15. Goldberg MF. Retinal vaso-occlusion in sickling hemoglobinopathies. Birth Defects Orig Artic Ser. 1976;12(3):475–515.
16. Goldbaum MH, Jampol LM, Goldberg MF. The disc sign in sickling hemoglobinopathies. Arch Ophthalmol. 1978;96(9):1597–600.
17. Gagliano DA, Goldberg MF. The Evolution of salmon-patch hemorrhages in sickle cell retinopathy. Arch Ophthalmol. 1989;107(12):1814–5.
18. Oliveira FV, Aihara T, Cançado RD. Alterações fundoscópicas nas hemoglobinopatias SS e SC. Arq Bras Oftalmol. 1996;59(3):234–8.
19. Moraes Junior HV, Araújo PC, Brasil OF, Oliveira MV, Cerqueira V, Turchetti R. Achados oculares em doença. Rev Bras Oftalmol. 2004;63(5/6):299–302.
20. Fox PD, Dunn DT, Morris JS, Serjeant GR. Risk factors for proliferative sickle retinopathy. Br J Ophthalmol. 1990;74(3):172–6.

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B. TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

B.1. PARA MENORES DE 18 ANOS

Você está sendo convidado a consentir com a participação do menor_____, em pesquisas realizadas no Centro de Pesquisas Gonçalo Moniz – FIOCRUZ - BA, cujo título está especificado a seguir, uma vez que oficialmente é o seu representante legal.

"AVALIAÇÃO DO PAPEL DE LIPOPROTEÍNAS DO COLESTEROL E TRIGLICÉRIDES E BIOMARCADORES CLÁSSICOS NA DOENÇA FALCIFORME: ASSOCIAÇÃO COM PROCESSOS INFLAMATÓRIOS E VASO-OCLUSIVOS"

A doença 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 alterações clínicas, como alteração nos olhos, rins, coração, pulmão e cérebro.

Nessa pesquisa estudaremos a doença falciforme, 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 das células 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. Os resultados obtidos nesta pesquisa poderão posteriormente servir para estudos futuros de medicamentos novos para a doença.

A sua participação é totalmente voluntária e a sua permissão para participar do estudo pode ser retirada a qualquer momento, não resultando em punições.

O objetivo deste trabalho é investigar aspectos epidemiológicos, clínicos e laboratoriais da população atendida na fundação HEMOBA.

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. As amostras coletadas serão identificadas por código, bem como os dados individuais dos exames e testes, que 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 CNS 340/2004, item V.1.e.

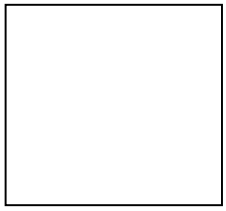
A permissão para que o menor participe deste estudo não implicará na retirada de sangue adicional, de modo que será utilizada uma quantidade remanescente da mesma amostra coletada para a realização dos exames solicitados pelo médico. Também queremos que você concorde que as amostras colhidas sejam armazenadas 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 196/96 e 347/05 do Conselho Nacional de Saúde.

Comunicamos que o sangue será colhido do braço e poderá acarretar em riscos e desconfortos, como sangramento e dor. Entretanto, a coleta de sangue será realizada por pessoal habilitado e especializado, visando diminuir esses 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, mas possibilitará a obtenção de dados que poderão ser utilizados futuramente no acompanhamento de indivíduos que apresentam alguma doença conhecida e na implantação de políticas de saúde.

Assinatura do responsável _____
Data ___/___/___
RG:_____



Nome do responsável (letra de forma) _____
Endereço_____
_____

Nome Testemunha 1_____
RG: _____

Nome Testemunha 2 _____
RG: _____

Por favor, entre em contato com uma das pessoas abaixo caso você necessite de maiores esclarecimentos:

Dra Marilda de Souza Gonçalves - Coordenadora do projeto - Laboratório de Pesquisa em Anemias da Faculdade de Farmácia/UFBA e Laboratório de Hematologia e Genética Biocomputacional (LHGB)/Centro de Pesquisas Gonçalo Moniz (CPqGM) – Fiocruz-Bahia. Fone (71) 3176-2226.

Magda Oliveira Seixas – Responsável pelo desenvolvimento do projeto – Laboratório de Hematologia e Genética Biocomputacional (LHGB)/Centro de Pesquisas Gonçalo Moniz (CPqGM) – Fiocruz-Bahia. Fone (71) 3176-2226.

B.2. PARA MAIORES DE 18 ANOS

Você está sendo convidado a participar de um projeto de pesquisa realizado no Centro de Pesquisas Gonçalo Moniz – FIOCRUZ - BA, cujo título está especificado a seguir:

"AVALIAÇÃO DO PAPEL DE LIPOPROTEÍNAS DO COLESTEROL E TRIGLICÉRIDES E BIOMARCADORES CLÁSSICOS NA DOENÇA FALCIFORME: ASSOCIAÇÃO COM PROCESSOS INFLAMATÓRIOS E VASO-OCLUSIVOS"

A doença 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 alterações clínicas, como alteração nos olhos, rins, coração, pulmão e cérebro.

Nessa pesquisa estudaremos a doença falciforme, 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 das células 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. Os resultados obtidos nesta pesquisa poderão posteriormente servir para estudos futuros de medicamentos novos para a doença.

A sua participação é totalmente voluntária e a sua permissão para participar do estudo pode ser retirada a qualquer momento, não resultando em punições.

O objetivo deste trabalho é investigar aspectos epidemiológicos, clínicos e laboratoriais da população atendida na fundação HEMOBA.

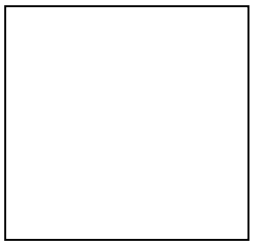
Os registros da sua participação no estudo serão mantidos confidencialmente, sendo do conhecimento apenas da equipe participante do projeto e do médico que o acompanha. As amostras coletadas serão identificadas por código, bem como os dados individuais dos exames e testes, que serão do conhecimento somente 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 CNS 340/2004, item V.1.e.

Sua participação não implicará na retirada de sangue adicional, de modo que será utilizada uma quantidade remanescente da mesma amostra coletada para a realização dos exames solicitados pelo médico. 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 196/96 e 347/05 do Conselho Nacional de Saúde.

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

A sua participação no estudo não trará benefícios, mas possibilitará a obtenção de dados que poderão ser utilizados futuramente no acompanhamento de indivíduos que apresentam alguma doença conhecida e na implantação de políticas de saúde.

Assinatura do voluntário_____
Data ___/___/___
RG:_____



Nome do responsável (letra de forma) _____
Endereço_____
_____
_____

Nome Testemunha 1_____
RG: _____

Por favor, entre em contato com uma das pessoas abaixo caso você necessite de maiores esclarecimentos:

Dra Marilda de Souza Gonçalves - Coordenadora do projeto - Laboratório de Pesquisa em Anemias da Faculdade de Farmácia/UFBA e Laboratório de Patologia e Biologia Molecular do Centro de Pesquisa Gonçalo Moniz - FIOCRUZ. Fone (71) 3176-2226.

Magda Oliveira Seixas – Mestranda responsável pelo desenvolvimento do projeto – Laboratório de Patologia e Biologia Molecular do Centro de Pesquisa Gonçalo Moniz – FIOCRUZ. Fone (71) 3176-2265.

C. QUESTIONÁRIO

"AVALIAÇÃO DO PAPEL DE LIPOPROTEÍNAS DO COLESTEROL E TRIGLICÉRIDES E BIOMARCADORES CLÁSSICOS NA DOENÇA FALCIFORME: ASSOCIAÇÃO COM PROCESSOS INFLAMATÓRIOS E VASO-OCLUSIVOS"

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 {AATP} ( ) 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 [8]
( ) 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+[3]
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+[3]
Quando foi a última crise? {ULTCRISDOR} ( ) <1mês [0] ( ) 1-3m [1] ( ) 4m ou+[2]
Usa medicação para a dor? {MDOR} ( ) NÃO [0] ( ) SIM [1]
Prescrita por um médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]
Assistido por especialista em dor? {ESPECDOR} ( ) NÃO [0] ( ) SIM [1]

Faz tratamento com hidroxiuréia? {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. MATERIAS E MÉTODOS

D.1 - CASUÍSTICA E INSTITUIÇÕES PARTICIPANTES

Para a realização desta tese foi conduzido um estudo de corte transversal com a coleta de dados de pacientes com DF em estado estável da doença (PE), internados (PI) e controles saudáveis (CS). Fizeram parte do estudo 356 pacientes com DF e foram realizadas 503 coletas; O número de crianças com DF foi 306, sendo as crianças em estado estável da doença (287) acompanhadas na Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA) e as em crise (23) estavam internadas no Hospital da Criança/Obras Sociais Irmã Dulce (HC-OSID). Além destes, foram incluídos no estudo 69 pacientes adultos com DF acompanhados regularmente na HEMOBA. Como grupos de comparação, também foram incluídos no estudo 150 crianças e 88 adultos saudáveis atendidos Laboratório de Análises Clínicas e Toxicológicas da Faculdade de Farmácia da Universidade Federal da Bahia (LACTFAR- UFBA), com o perfil de hemoglobina Hb AA. No dia da coleta todos os participantes e/ou responsáveis que aceitaram participar assinaram o termo de consentimento livre e esclarecido (TCLE – apêndices B1 e B2) e responderam a um questionário (apêndice C) para obtenção de dados individuais, socioeconômicos (escolaridade, número de irmãos, contatos sociais) e referentes a comorbidades, tais como, pneumonias, problemas cardíacos, renais, hepáticos e possíveis problemas familiares. As coletas de sangue e de dados clínicos, as análises laboratoriais e moleculares foram realizadas entre os anos de 2009 a 2013. Os experimentos moleculares e celulares foram realizados no Centro de Pesquisas Gonçalo Moniz (CPqGM) – Fiocruz-Bahia. A seguir seguem algumas características dos grupos de pacientes e controles:

Pacientes

1) Estáveis: indivíduos acompanhados regularmente no ambulatório de hematologia da Fundação HEMOBA que não haviam recebido hemoderivados ou apresentado qualquer evento grave associado a DF (pneumonia, internação, sequestro esplênico, AVC, úlcera maleolar, crise hemolítica, priaprismo, entre outros) nos três meses que precederam a coleta de amostras para o estudo. A média de idade destes pacientes foi de 13,95 ± 9,09 anos, sendo 191 homens (53,65%) e 165 mulheres (46,35%).

2) Em crise: pacientes admitidos para internamento no HC-OSID devido a complicações associadas a DF, descritas anteriormente no corpo desta tese. A média de idade neste grupo foi de 10,34 ± 4,36 anos, sendo destes 12 (52,2%) homens e 11 (47,8 %) mulheres.

A coleta de sangue dos pacientes estáveis foi realizada durante a consulta ambulatorial e os dados clínicos informados pelos pacientes foram confirmados através da avaliação dos prontuários médicos dos mesmos. Os pacientes internados realizaram coleta de amostras no momento da admissão, antes de realizar transfusão ou passar por qualquer procedimento; os dados clínicos foram obtidos por entrevista, com os pais ou responsáveis, utilizando o questionário padronizado para o estudo (apêndice C).

Controles

Esse grupo foi formado por crianças e adultos saudáveis, sem históricos de qualquer patologia ou processo infeccioso no último mês, atendidos no LACTFAR- UFBA. Do total de participantes, 54 (32,3%) eram homens e 113 (67,7%) mulheres. A média de idade deste grupo foi de 22,49 ± 16,29 anos.

D.2 - MÉTODOS

Coleta de amostras: Foram obtidos 15mL de sangue venoso dos participantes, sendo que 5 mL coletados em anticoagulante (EDTA) e 10 mL sem aditivos. As amostras dos pacientes coletadas na HEMOBA e Hospital da Criança – OSID foram adequadamente transportadas para o Laboratório de Pesquisa em Anemias (LPA) e Laboratório de Análises Clínicas (LACTFAR) da Faculdade de Farmácia (FACFAR) da Universidade Federal da Bahia (UFBA), onde foram realizadas todas as determinações hematológicas e bioquímicas.

Análise Hematológica e de hemoglobinas: A confirmação do perfil de hemoglobinas foi realizada para todos os participantes do estudo, pelo método automatizado de cromatografia líquida de alto desempenho (HPLC) utilizando o equipamento *Variant II (*Bio-Rad, Hercules, CA, USA). O hemograma foi realizado pelo método automatizado utilizando-se o

contador eletrônico (*Coulter Corporation*, Miami, FL, USA); a contagem de reticulócitos realizada pela técnica manual, utilizando o corante azul de cresil brilhante (Figura 1A).

Análises Bioquímicas: As análises bioquímicas incluíram a determinação da glicemia em jejum, do perfil lipídico (colesterol total e frações e triglicerídeos), dosagem de proteínas totais e frações, bilirrubinas totais e frações, desidrogenase lática (LDH), transaminases (ALT e AST), perfil renal (ureia e creatinina), ferro e ácido úrico, sendo realizadas pelo método automatizado, utilizando técnicas de imunoquímica e imunoensaio (*A25 system*, BIOSYSTEMS SA, Barcelona, *Spain*). As dosagens de ferritina e haptoglobina foram realizadas em sistema de imunoensaio (Access® 2 *Immunoassay system* X2, Beckman Coulter, Fullerton, CA, *USA*) e as dosagens de PCR, alfa 1 antitripsina, e antiestreptolisina O (ASLO) pelo método imunoquímico automatizado (Immage® 800 system, Beckman Coulter Fullerton, CA, *USA*) conforme figura 2A. A determinação da insulinemia foi realizada no laboratório da Associação de Pais e Amigos dos Excepcionais (APAE) em colaboração com o Dr. Gildásio Carvalho.

Dosagem do CBA inflamatório

Os níveis plasmáticos de TNF-α, IFN-γ, IL-10, IL-1β , IL-6 e IL-8 foram quantificados pelo kit *Cytometric Bead Array - CBA* (BD Biosciences Pharmingen , EUA), de acordo com o protocolo do fabricante. O ensaio de citometria de fluxo foi realizado e analisado por um único operador e foram obtidas curvas padrão para cada citocinas avaliadas.

Mediadores inflamatórios

Os níveis de PGE_2 foram determinados por enzima imunoensaio, de acordo com as instruções do fabricante (Cayman Chemical, Ann Arbor, MI, *USA*). As concentrações plasmáticas de MMP-9, TIMP-1 e TGF-β foram avaliadas no soro dos pacientes, utilizando kits de ELISA específicos para cada molécula, de acordo com as instruções do fabricante (R e D Systems, Minneapolis, MI, USA).

Determinação do Heme total

A dosagem do heme livre total foi realizada em soro pelo Kit *QuantiChrom Heme Assay Kit* (BioAssay Systems, Hayward, CA, *USA*), de acordo com o protocolo do fabricante.

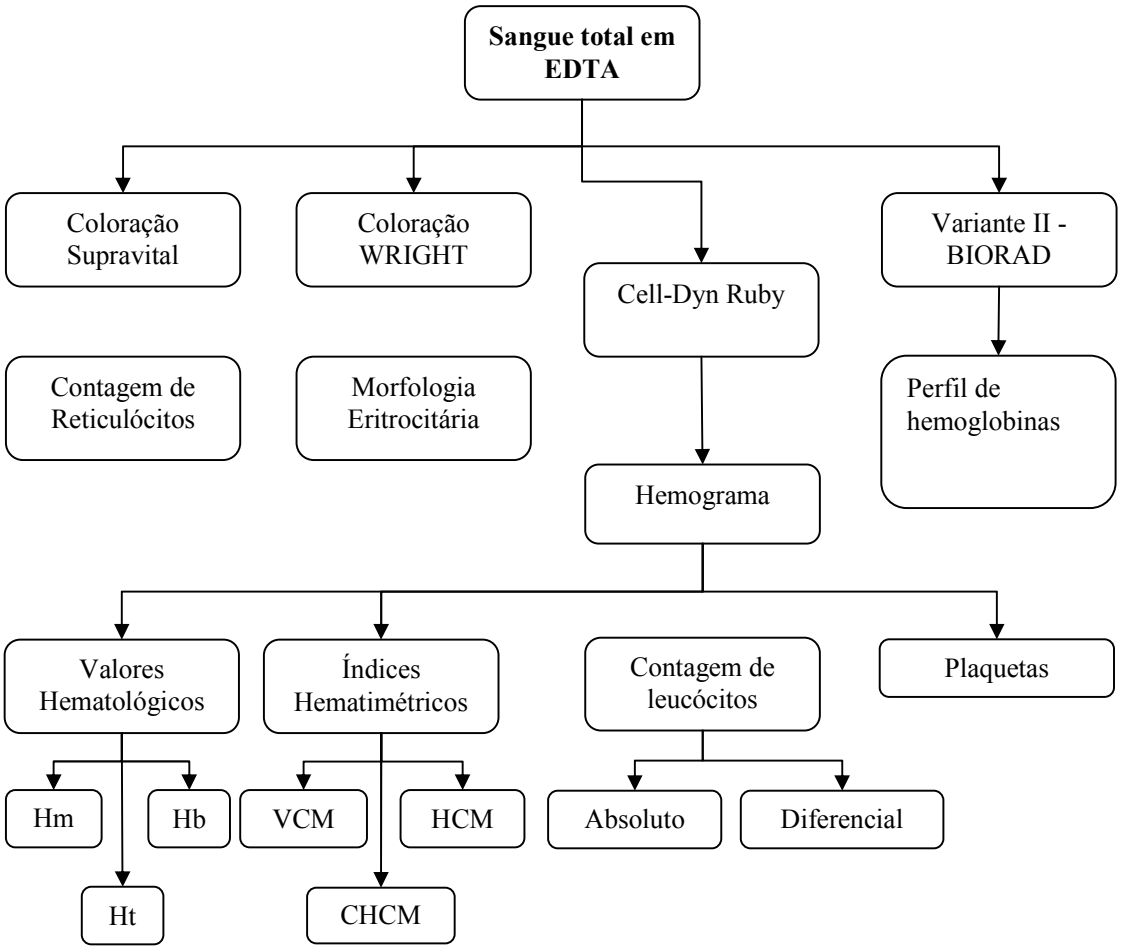


Figura 1A. Desenho experimental da realização das determinações hematológicas. Hm: contagem de hemácias; Hb: concentração de hemoglobina; Ht: Hematócrito; VCM: volume corpuscular médio; HCM: Hemoglobina corpuscular média; CHCM: concentração de hemoglobina corpuscular média.

.

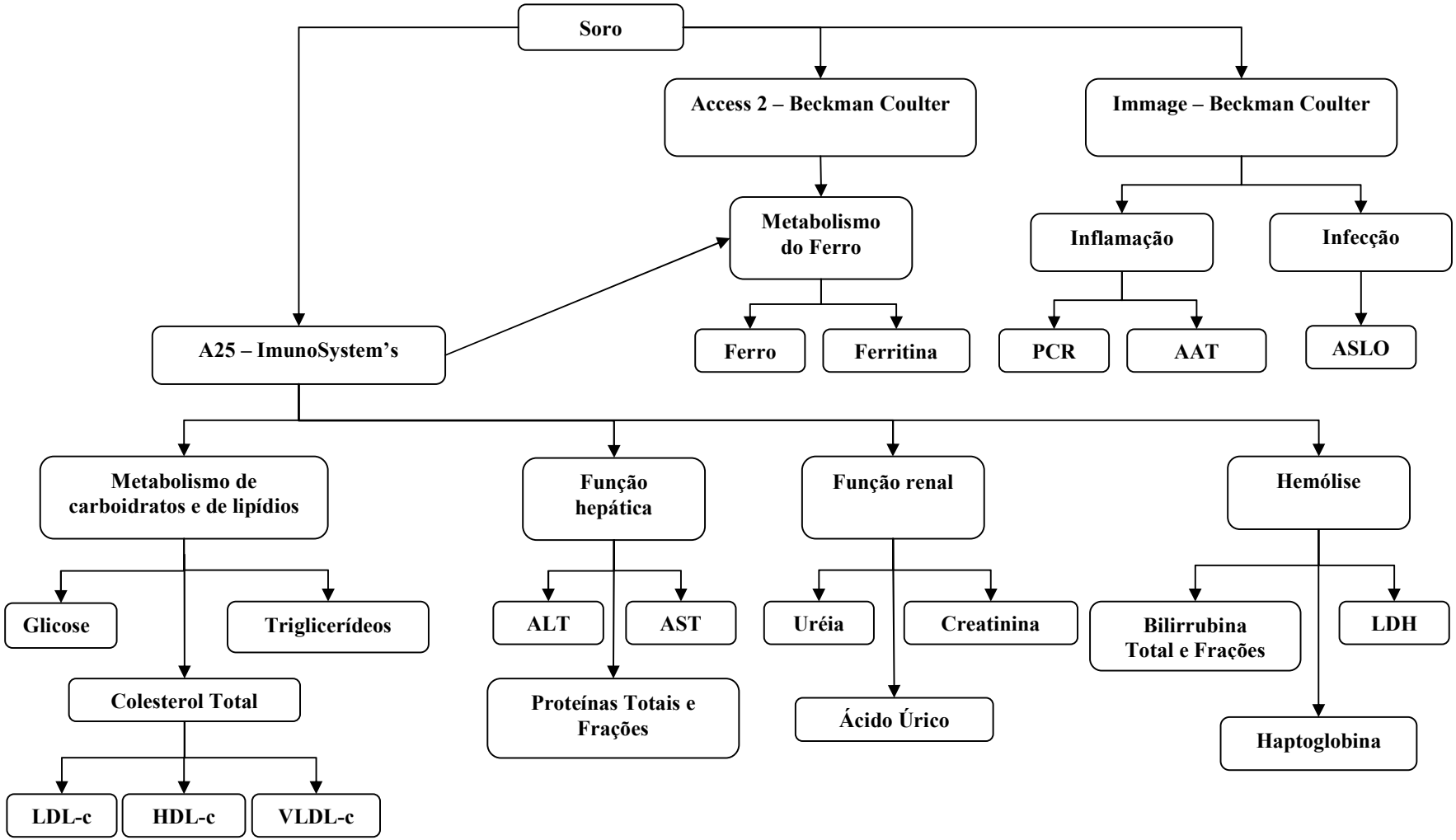


Figura 2A. Desenho experimental das determinações bioquímicas. PCR: Proteína C reativa; AAT: Alfa 1 Anti-tripsina; ASLO: Antiestreptolisina O; HDL-c: Colesterol HDL; LDL-c: Colesterol LDL; VLDL-c: Colesterol VLDL; ALT: Alanina transaminase; AST: Aspartato transaminase; LDH: Desidrogenase Lática.

Análise de Biologia Molecular

O DNA genômico foi extraído em 200uL de sangue periférico, utilizando o kit *QIAamp® DNA Mini Kit (Qiagen)* de acordo com as recomendações do fabricante. A caracterização dos polimorfismos gênicos no gene gene *SERPINA1* (alelos S e Z) e dos haplótipos ligados ao grupo de genes da globina beta foi realizada pela amplificação de fragmentos de DNA pela reação de polimerase em cadeia (PCR), utilizando oligonucleotídeos sintéticos (*primers*) específicose avaliação do tamanho dos fragmentos gerados pelo corte com enzimas de restrição (*Restriction Fragment Lenght Polymorphism*-RFLP (SEITZER *et al*., 1997; TAYLOR *et al*., 2002; HEINZMANN *et al*., 2004). A talassemia alfa deleção de 3,7 kilobases (Kb) (Tal alfa$^{-3,7kb}$) foi investigada por PCR.

Isolamento de neutrófilos dos pacientes e controles

Para essa etapa foram convidados pacientes e controles com níveis de HDL-c normais e diminuídos. Os neutrófilos foram obtidos por separação em gradiente de Ficoll *(*Histopaque 1077 Sigma*,* St. Louis, Estados Unidos*)* a partir de sangue venoso, recém-coletado em EDTA, sendo que as células separadas foram lavadas duas vezes em PBS e centrifugadas a 800 rpm durante 5 minutos. Após as lavagens as células foram diluídas 1:20 em meio RPMI 1640 (Gibco, Grand Island, NY) e, em seguida, foram feitas outras diluições, no mesmo meio, para obter a quantidade de 10^5 células/mL (ASSIS *et al.,* 2005; O'MAHONY *et al.*, 2008). A população celular foi confirmada através da observação de esfregaços sanguíneos corados pelo Wright (Merck, Darmstadt, Alemanha) e observadas ao microscópio óptico a um aumento de 400 vezes.

Quimiotaxia de neutrófilos

Foram definidos como estímulos para o ensaio de quimiotaxia, o uso de soro fresco; suspensão de sinvastatina em soro dos pacientes a 10mg/mL; suspensão de hemácias em salina fisiológica a 5%; suspensão de *S. pneumoniae* realizada de acordo com a escala de Mac Farlland a 0,5, que equivale a uma densidade bacteriana de 150 milhões/mL. Todos os testes foram utilizados sob as mesmas condições para indivíduos com DF e controles saudáveis. Os estímulos foram adicionados a microplaca de quimiotaxia de 96 poços do sistema ChemoTx (Neuro Probe, Gaithersburg, MD, EUA). Os neutrófilos foram obtidos

conforme método descrito no item 4.5.9 e foram diluídos em meio RPMI, antes da adicão a parte superior dos poços (10^5 células/poço); em seguida e placa foi incubada a 37°C a 5% de CO_2 durante 1,5 h. Após a incubação, as células que migraram para os poços inferiores foram contadas em hemocitômetro. A migração dos neutrófilos frente ao estímulo com meio RPMI puro (quimiotaxia radômica) foi utilizada como controle negativo e a migração frente ao lipopolissacarídeo (LPS a 75ng/mL), como controle positivo. Os índices de quimiotaxia foram calculados como a razão entre o número de neutrófilos que migraram sobre os estímulos descritos e número destes leucócitos que migraram com o meio RPMI puro. A sinvastatina é um agente redutor do colesterol, cujo principal metabólito é um inibidor da 3-hidróxi-3-metilglutaril-coenzima A (HMG-CoA) redutase, enzima que catalisa um passo precoce e limitante da taxa de biossíntese do colesterol; por essa razão, esse fármaco foi escolhido para avaliar o efeito *in vitro* dos lipídios sobre a quimiotaxia dos neutrófilos.

Definição de Eventos Clínicos

Os dados clínicos foram coletados dos prontuários médicos e os dados demográficos foram obtidos por meio de entrevistas aos paciente, pais ou responsáveis legais. Os pacientes foram considerados em estado estável da doença quando não apresentavam febre, infecção, internamento no período de 15 dias ou uso de hemocomponentes nos últimos 3 meses, antes da coleta de sangue. Os pacientes em crise foram aqueles que estavam hospitalizados por crise vaso-oclusiva ou infecção no momento da coleta da amostra.

D.3 - ANÁLISE ESTATÍSTICA

Os dados dos questionários e dos resultados obtidos dos experimentos realizados foram analisados estatisticamente em banco de dados gerado no Software EPI INFO versão 6.04, *Graph pad Prism 5.0 (Graphpad Software, San Diego, CA-USA)* e SPSS versão 10, de acordo com o tipo de variável. A análise de normalidade da distribuição das variáveis foi realizada pelo teste de D'Agostino e Pearson. A partir desta informação utilizou-se o teste paramétrico ANOVA ou não paramétrico de Kruskal-Wallis, sendo os resultados confirmados pelo pós-teste de Bonfferoni. A análise de variáveis qualitativas ou categóricas foi realizada pelo teste não paramétrico do Qui-quadrado (χ^2), devidamente corrigido pelos

testes de Mantel-Haenszel e Yates. Na análise de parâmetros menores que 5 para variáveis categóricas, utilizou-se o teste exato de Fisher. Os intervalos de confiança em 95% e a razão de prevalência foram calculados para essas variáveis. As análises de correlação foram realizadas utilizando os coeficientes de Pearson´s para os dados de distribuição normal e os coeficientes de Kendall´s tau-b e Spearman para os dados com distribuição não normal. O teste de Mann-Whitney e teste T independente foram utilizados para a análise de duas variáveis numéricas, na comparação de dois grupos de valores dentro de uma mesma variável para variáveis de distribuição não normal e normal, respectivamente. Os valores de P <0,05 foram considerados significativos para as análises realizadas.

D.4. CONSIDERAÇÕES ÉTICAS E DE BIOSSEGURANÇA

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa (CEP) do CPqGM/FIOCRUZ-BA sob o CAAE 0022.0.225.000-09 (136/2009) e 0016.0.225.000-09 (131/2009), este último também aprovado pelo CEP/OSID. Todas as informações obtidas através das análises das amostras coletadas foram e serão mantidas sob sigilo, com acesso permitido apenas para a equipe clínica e pesquisadores. Os resultados dos testes laboratoriais foram fornecidos ao médico, para que pudessem auxiliá-lo no acompanhamento clínico dos pacientes. Todo o trabalho foi desenvolvido de acordo com os critérios da Regulamentação de Bioética no Brasil, Resolução n° 196/96, posteriormente revogada pela resolução n° 466/2012 do Conselho Nacional de Saúde. Os experimentos foram realizados com base nas normas de Biossegurança, de acordo com a Lei no. 11.105/2005, seguindo as normas técnicas existentes no manual de biossegurança da FIOCRUZ (Comissão Técnica de Biossegurança da FIOCRUZ – Ministério da Saúde, 1998).

Os indivíduos foram convidados a participar do presente estudo e assinaram o TCLE e autorizaram a utilização dos dados e dos materiais coletados. Todos os participantes da pesquisa concordaram que suas amostras fizessem parte do biorrepositório e fossem utilizadas em estudos futuros, desde que os estudos adicionais fossem analisados por um Comitê de Ética em Pesquisa em Seres Humanos e seguissem os aspectos éticos determinados nas resoluções 466/2012 e 347/05 do Conselho Nacional de Saúde.