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FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

FIOCRUZ

Curso de Pós-Graduação em Patologia Humana e Experimental

TESE DE DOUTORADO

FATOR DE TRANSFORMAÇÃO DO CRESCIMENTO BETA E POLIMORFISMOS NO GENE DO RECEPTOR 3 DO FATOR DE TRANSFORMAÇÃO DO CRESCIMENTO BETA NA DOENÇA FALCIFORME

RAYRA PEREIRA SANTIAGO

Salvador – Bahia

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RESUMO

INTRODUÇÃO: o fator de transformação do crescimento beta (TGF-β) é uma citocina com papel importante em processos biológicos, como disfunção endotelial e vascular, inflamação e homeostase hematopoiética. **OBJETIVO:** o presente estudo buscou investigar a associação dos níveis plasmáticos de TGF-β1 e de polimorfismos no gene do receptor 3 do fator de transformação do crescimento beta (*TGFBR3*) com biomarcadores genéticos, hematológicos, bioquímicos e imunológicos em indivíduos com doença falciforme (DF) e com as complicações clínicas da doença. **MATERIAL E MÉTODOS:** para tanto, foi conduzido um estudo transversal, onde foram investigados 175 indivíduos com DF (120 HbSS e 55 HbSC). Os níveis plasmáticos do TGF-β, inibidor tecidual de metaloproteases-1 (TIMP-1) e da metaloproteinase da matriz 9 (MMP-9) foram determinados pela técnica de ELISA e os marcadores hematológicos, bioquímicos e imunológicos foram determinados por métodos automatizados. A genotipagem dos polimorfismos no gene *TGFBR3* foi realizada utilizando *TaqMan SNP Genotyping Assays*. **RESULTADOS:** os indivíduos HbSS apresentaram concentrações elevadas de TGF-β1 quando comparados a indivíduos controles saudáveis e HbSC. Nos indivíduos HbSS, o TGF-β1 esteve positivamente correlacionado com as hemácias, plaquetas, hemoglobina, hematócrito e TIMP-1. Além desses marcadores, os indivíduos HbSS com concentrações de TGF-β1 ≥72.29 ng/mL apresentaram contagem elevada de monócitos e níveis diminuídos de albumina. Os indivíduos HbSC apresentaram correlação positiva entre o TGF-β1 e leucócitos, eosinófilos, linfócitos, monócitos, plaquetas, TIMP-1, lipoproteínas de muito baixa densidade (VLDL-C), triglicérides, heme e aspartato aminotransferase (AST). Os indivíduos HbSC com concentrações de TGF-β1 ≥ 47.80 ng/mL apresentaram contagens elevadas de leucócitos e plaquetas e concentrações elevadas de triglicérides, VLDL-C, MMP-9 e TIMP-1 e concentrações diminuídas de lipoproteína de alta densidade (HDL-C). Nos indivíduos HbSS, o alelo variante A do polimorfismo *rs1805110* no gene *TGFBR3* esteve associado a concentrações elevadas de hemoglobina, hematócrito, lipoproteína de baixa densidade (LDL-C), ácido úrico e endotelina; contagem elevada de reticulócitos e *platelet distribution width* (PDW) diminuídos e estiveram associados à ocorrência de alterações ósseas. O alelo variante T do polimorfismo *rs7526590* no gene *TGFBR3* esteve associado a concentrações elevadas de *red cell distribution width* (RDW), PDW, fosfatase alcalina, AST, bilirrubina indireta e lactato desidrogenase e concentrações diminuídas de ferritina e a ocorrência de úlceras de pernas. Os indivíduos com DF portadores do haplótipo

GG no gene *TGFBR3* apresentaram níveis mais elevados de colesterol total (T-CHOL), LDL-C, triglicérides, colesterol não HDL (não-HDL-C), proteínas totais e globulina que aqueles com o haplótipo não-GG. Indivíduos com o haplótipo CGG apresentaram níveis elevados de plaquetócrito, T-CHOL, LDL-C e não-HDL-C. Ambos os haplótipos GG e CGG estiveram associados à ocorrência de pneumonia e os indivíduos com haplótipo não-GG apresentaram ocorrência maior de colelitíase. **CONCLUSÃO:** nossos dados sugerem que o TGF-β1 desempenha papel importante no remodelamento vascular, vasculopatia, angiogênese, inflamação e hemólise na DF e que polimorfismos no gene *TGFBR3* podem estar ligados ao estado inflamatório, hemólise e a complicações clínicas. Além disso, os indivíduos portadores dos haplótipos GG e CGG no gene *TGFBR3* apresentaram alterações importantes no perfil lipídico e apresentaram ocorrência maior de pneumonia.

Palavras chaves: Doença falciforme, Fator de transformação do crescimento Beta, Receptor 3 do fator de transformação crescimento Beta.

SANTIAGO, Rayra Pereira. Transforming growth factor beta and transforming growth factor beta receptor 3 gene polymorphisms in sickle cell disease. 2020. 169 f. il. Tese (Doutorado em Patologia) - Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2020.

ABSTRACT

INTRODUCTION: transforming growth factor beta (TGF-β) is a cytokine that plays an important role in biological process, such as endothelial and vascular dysfunction, inflammation and hematopoietic homeostasis. **OBJECTIVE:** this study aimed to investigate associations of TGF-β1 levels and transforming growth factor beta receptor 3 (*TGFBR3*) gene polymorphisms with genetic, hematological, biochemical and immunological biomarkers in individuals with sickle cell disease (SCD), as well as clinical complications. **MATERIALS AND METHODS:** a cross-sectional study was conducted, which were investigated 175 individuals with SCD (120 HbSS and 55 HbSC genotype). TGF-β, tissue inhibitor of metalloproteases-1 (TIMP-1) and matrix metalloproteinase 9 (MMP-9) plasma measurements were performed by ELISA technique. Hematological, biochemical and immunological markers were carried out by automated methods and *TGFBR3* polymorphisms genotyping was performed using TaqMan SNP Genotyping Assays. **RESULTS:** TGF-β1 plasma levels were higher in HbSS individuals than in HbSC and health controls. In HbSS individuals, TGF-β1 levels were positively correlated with red blood cells (RBC), hemoglobin, hematocrit, platelets and TIMP-1. In addition, HbSS individuals with TGF-β1 levels above the median (≥72.29 ng/mL) also presented increased monocyte counts and decreased albumin levels. In patients with HbSC, TGF-β1 levels were positively correlated with leukocytes, eosinophils, lymphocytes, monocytes, platelets, TIMP-1, VLDL-C, triglycerides, heme and AST. Additionally, HbSC individuals with TGF-β1 levels above the median (≥47.80 ng/mL) presented increased leukocyte and platelet counts, as well as increased levels of triglycerides, VLDL-C, MMP-9 and TIMP-1, and decreased HDL-C. In HbSS individuals, the minor allele (A) of the *TGFBR3 rs1805110* polymorphism was associated with increased hemoglobin, hematocrit, reticulocyte counts, low density lipoprotein, uric acid and endothelin levels, as well as decreased platelet distribution width (PDW) and the occurrence of bone alterations. The minor allele (T) of *TGFBR3 rs7526590* was associated with increased red cell distribution width, PDW, alkaline phosphatase, aspartate aminotransferase, indirect bilirubin and lactate dehydrogenase levels, as well as lower ferritin levels and the occurrence of leg ulcers. Our data suggest that the minor allele (A) of *TGFBR3 rs1805110* is associated with inflammation and bone alterations, while the minor allele (T) of *TGFBR3 rs7526590* is related to hemolysis and the occurrence of leg ulcers. SCD individuals carries of GG haplotype presented higher levels of total cholesterol (T-CHOL), low density lipoprotein cholesterol (LDL-C), triglycerides, non-HDL cholesterol, total proteins and globulin than individuals with non-GG haplotypes. SCD individuals with the CGG haplotype presented increased plateletcrit, T-CHOL,

LDL-C levels and non-HDL cholesterol. Both haplotypes were associated with a previous history of pneumonia. In addition, the GG haplotype was associated with a previous history of pneumonia. **CONCLUSION:** our findings suggest the importance of TGF-β1 in vascular remodeling, vasculopathy, angiogenesis and inflammation in pediatric patients with SCD. *TGFBR3* polymorphisms were associated to inflammatory status, hemolysis as well as clinical complications, bone alterations and leg ulcers occurrence. In addition, individuals with the GG and CGG haplotypes of *TGFBR3* present significant lipid profile alterations and could be associated with the occurrence of pneumonia.

Key words: Sickle cell disease, Transforming growth factor beta, Transforming growth factor beta receptor 3.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AF	Anemia falciforme
AST	Aspartato aminotransferase
AVC	Acidente vascular cerebral
BMP6	Proteína óssea morfogenética 6, do inglês *Bone Morphogenetic Protein 6*
BMPR1A	Receptor de proteína morfogenética óssea, tipo 1A, do inglês *Bone Morphogenetic Protein Receptor Type 1A*
CS	Controles sadios
DF	Doença falciforme
eDAMPS	Padrões moleculares associados a microrganismos ou perigo
Enos	Óxido nítrico sintase endotelial
GMCSF	Fator de estimulação de colônias de macrófagos granulócitos
Hb	Hemoglobina
HBB	Gene da globina beta
HbC	Hemoglobina C
HbF	Hemoglobina Fetal
HbS	Hemoglobina S
HbSC	Hemoglobinopatia SC
HbSS	Anemia falciforme
HDL-C	Lipoproteína de alta densidade, do inglês *High density lipoprotein*
ICAM	Molécula de adesão intercelular, do inglês *Intercellular adhesion molecule*
IL	Interleucina
LDH	Lactato Desidrogenase
LDL-C	Lipoproteína de baixa densidade, do inglês *Low density lipoprotein*
MMP-2	Metaloproteinase da matriz 2, do inglês *matrix metalloproteinase 2*
MMP-9	Metaloproteinase da matriz 9, do inglês *matrix metalloproteinase 9*
Não-HDL-C	Colesterol não HDL
NO	Óxido Nítrico
NOS	Óxido Nítrico Sintase
PDW	Amplitude de distribuição de plaquetas do inglês *Platelet Distribution*

	Width
RDW	Amplitude de distribuição dos eritrócitos, do inglês *Red Cell Distribution Width*
ROS	Espécies reativas de oxigênio, do inglês *Reactive oxygen species*
SMAD	*Mothers Against Decapentaplegic Homolog*
SNP	Polimorfismos de único nucleotídeo, do inglês *Single Nucleotide Polymorphism*
STA	Síndrome torácica aguda
T-CHOL	Colesterol total
TIMP-1	Inibidor tecidual de metaloproteases-1, do inglês *tissue inhibitor of metalloproteases-1*
TNF-α	Fator de necrose tumoral alfa
TGF- β	Fator de transformação do crescimento beta
TGFBR1	Receptor 1 do fator de transformação do crescimento beta
TGFBR2	Receptor 2 do fator de transformação do crescimento beta
TGFBR3	Receptor 3 do fator de transformação do crescimento beta
TβRI	Receptor 1 do fator de transformação do crescimento beta
TβRII	Receptor 2 do fator de transformação do crescimento beta
TβRIII	Receptor 3 do fator de transformação do crescimento beta
VCAM	Molécula de adesão de células vascular, do inglês *Vascular cell adhesion molecule*
VLDL-C	Lipoproteína de muito baixa densidade, do inglês *Very low density lipoprotein*

LISTA DE SÍMBOLOS

β^S	Alelo beta S
β^6	Sexto códon do gene da globina beta
α	Alfa
β	Beta

SUMÁRIO

1 INTRODUÇÃO

A doença falciforme (DF) é uma doença genética de herança autossômica recessiva caracterizada por uma mutação de ponto no sexto códon do gene da globina beta (*HBB*), que codifica a cadeia da globina beta da molécula da hemoglobina, ocasionando alteração na cadeia polipeptídica e desencadeando a formação da hemoglobina S (HbS) responsável pela modificação conformacional do eritrócito que adquire o formato de foice (drepanócito). O termo DF abrange um conjunto de doenças decorrentes da presença da HbS associada a hemoglobinopatias de síntese ou a outras hemoglobinas variantes (C e D, por exemplo), como na hemoglobinopatia SC (HbSC), ou a própria HbS gerando a condição de homozigose denominada anemia falciforme (AF) (STEINBERG, 2001).

Os indivíduos com a DF possuem complicações clínicas diversas que podem afetar quase todos os órgãos e sistemas, com morbidade elevada, redução da capacidade laboral e da expectativa de vida (STEINBERG, 2001; RAPHAEL, 2005). As principais manifestações clínicas encontradas em indivíduos com DF são as crises dolorosas, acidente vascular cerebral (AVC), infarto cerebral silencioso, retinopatia proliferativa, cardiomegalia, cardiomiopatia, síndrome torácica aguda (STA), hipertensão pulmonar, colelitíase, sequestro hepático, priapismo, sequestro esplênico agudo, úlceras de pernas e osteopenia (BALLAS et al., 2010).

As manifestações clínicas encontradas na DF ocorrem em resposta aos processos fisiopatológicos da doença, sendo eles a polimerização da HbS, vaso-oclusão, disfunção endotelial e inflamação estéril (TAYLOR et al., 2008; BALLAS et al., 2010; DOMINGOS et al., 2014). A polimerização da HbS desencadeia a vaso-oclusão resultante da interação entre os eritrócitos, reticulócitos, leucócitos, plaquetas e o endotélio vascular que geram a oclusão microvascular com isquemia, seguida de reperfusão. Ciclos repetidos de isquemia e reperfusão geram estresse oxidativo devido à ativação de oxidases vasculares e inflamação devido ao aumento da expressão de moléculas de adesão pelo endotélio vascular e da produção de fatores quimiotáticos e citocinas inflamatórias que auxiliam no recrutamento de leucócitos (PIEL et al., 2017; GUARDA et al., 2017; KATO et al., 2018; SUNDD et al., 2018). Os indivíduos com DF apresentam quadro pró-inflamatório, no qual as células endoteliais e monócitos produzem citocinas de forma exacerbada, como por

exemplo, interleucina (IL) 1 e fator de necrose tumoral alfa (TNF-α). Estudos recentes têm demonstrado a participação do fator de transformação do crescimento beta (TGF-β), IL-17 e IL-18 no processo inflamatório da DF (CERQUEIRA et al., 2011; VILAS-BOAS et al., 2016; GUARDA et al., 2017).

A via do TGF-β está presente em todas as células do corpo humano e exerce papel importante na regulação do sistema imunológico, inflamação, fibrose pulmonar, proliferação celular, apoptose em resposta a lesão tecidual, infecção, homeostase óssea, crescimento endotelial, nefropatia diabética e síntese de matriz extracelular. Além disso, essa é uma via suscetível a modulação farmacológica, o que reforça a importância da investigação da mesma (MIYAZONO et al, 2001; ATTISANO e WRANA, 2002; MOUSTAKAS et al, 2002; ZIYADEH, 2004; NOLAN et al., 2006).

Genes que participam da via do TGF-β têm sido associados a manifestações clínicas na DF, em especial, os genes dos três receptores de TGF-β, sendo o principal deles o gene do receptor do fator de transformação do crescimento beta 3 (*TGFBR3*). Polimorfismos no gene *TGFBR3* têm sido associados a várias complicações clínicas na DF, como por exemplo, AVC, úlceras de perna, bacteremia, priapismo, a taxa de filtração glomerular e ao risco de nefropatia (SEBASTIANI et al., 2005; NOLAN et al., 2006; ADEWOYE et al., 2006; ELLIOTT et al., 2007; ASHLEY-KOCH et al., 2008). Contudo, nenhum estudo prévio buscou verificar se esses genes também apresentam associação com marcadores laboratoriais, mostrando que eles podem fazer parte do mecanismo fisiopatológico da DF, além da sua possível influência nas manifestações clínicas. Assim, há a necessidade de se investigar a associação dos níveis plasmáticos de TGF-β1 e de polimorfismos no gene *TGFBR3* com manifestações clínicas, marcadores laboratoriais e genéticos em indivíduos com DF.

2 REVISÃO DE LITERATURA

2.1 DOENÇA FALCIFORME

O termo DF é utilizado para definir um grupo de doenças genéticas de herança autossômica recessiva, caracterizada pela presença da HbS, que é decorrente de mutação de ponto no gene da subunidade globina beta (*HBB*) (rs334) localizado no cromossomo 11 p.15.5, incluindo a anemia falciforme (AF) (HbSS), hemoglobinopatia SC (HbSC), HbSβ-talassemia, entre outras (STEINBERG, 2001; KATO et al., 2018). A DF é caracterizada pela presença da HbS associada a outra HbS como na AF, a outras Hbs variantes (C e D, por exemplo), como na HbSC, ou a hemoglobinopatias de síntese, como a HbSβ-talassemia (STEINBERG, 2001).

O alelo beta S (β^S) é resultado da mutação de ponto no sexto códon do gene *HBB,* onde a adenina substitui a timina (G<u>A</u>G→G<u>T</u>G). Na cadeia polipeptídica β, gerada a partir do alelo β^S, ocorre a substituição de aminoácido, onde o ácido glutâmico é substituído por valina na posição β^6, formando a hemoglobina S (HbS) (NAGEL e STEINBERG, 2003; HANNEMANN et al., 2011). A presença do alelo β^S, em homozigose (β^S/β^S), caracteriza o genótipo HbSS e, consequentemente, a AF, forma mais grave da DF (Figura 1) (KATO et al., 2018).

A hemoglobina C (HbC), por sua vez, resulta na mutação de ponto no sexto códon do gene *HBB,* onde a base nitrogenada guanina é substituída por adenina (<u>G</u>AG → <u>A</u>AG), ocasionando a substituição na posição β^6 da cadeia polipeptídica β, onde o aminoácido ácido glutâmico é substituído por lisina (NAGEL et al., 2003; HANNEMANN et al., 2011; SANTIAGO et al., 2018). A HbSC ocorre devido a heterozigose dupla das hemoglobinas S e C, sendo a segunda DF em gravidade depois da AF, uma vez que os indivíduos apresentam concentração intracelular reduzida de HbS (Figura 1) (NAGEL et al., 2003; STEINBERG e SEBASTIANI, 2012; SANTIAGO et al., 2018).

De acordo com as análises realizadas pelo *Global Burden of Disease Study*, 3,2 milhões de pessoas no mundo possuem DF, 43 milhões apresentam o traço falciforme (HbAS) e 176.000 pessoas morrem anualmente em decorrência de complicações relacionadas a doença (ABUBAKAR et al., 2015). O Brasil apresenta uma prevalência elevada da DF e a incidência da doença varia entre os diferentes

estados brasileiros; na Bahia, a incidência é de 1 recém-nascido com DF a cada 650 nascidos vivos; no Rio de Janeiro, a incidência é de 1 a cada 1300 e em Santa Catarina, 1 a cada 13500 (BRASIL, 2014). Essa variação reflete a heterogeneidade étnica da população, visto que o Brasil recebeu ao longo do seu processo civilizatório grande quantidade de imigrantes resultando numa população com diversidade genética, étnica, social e cultural elevada (GONÇALVES et al.,2003; SANTIAGO, et al., 2017). Os genótipos da DF mais prevalentes no território brasileiro são a AF e a HbSC, enquanto as hemoglobinas variantes mais frequentes são as HbS e HbC que podem ter sido introduzidas durante o tráfico de escravos vindos da África (SANTIAGO, et al., 2017).

A HbS sofre modificações em situações de tensão baixa de oxigênio, devido à interação de natureza hidrofóbica da valina com a fenilalanina na posição 85 e com a leucina da posição 88, desencadeando assim, a formação de polímeros no processo de nucleação (STEINBERG, 2001; SILVA e SHIMAUTI, 2006). A polimerização da HbS modifica o formato e as propriedades físicas dos eritrócitos, resultando na anemia hemolítica e bloqueio do fluxo sanguíneo, particularmente em pequenos vasos, o que pode ocasionar lesão em órgãos. Os polímeros de HbS levam a outras alterações no nível celular que contribuem para o mecanismo fisiopatológico geral da DF (STEINBERG, 2001; WARE et al., 2017).

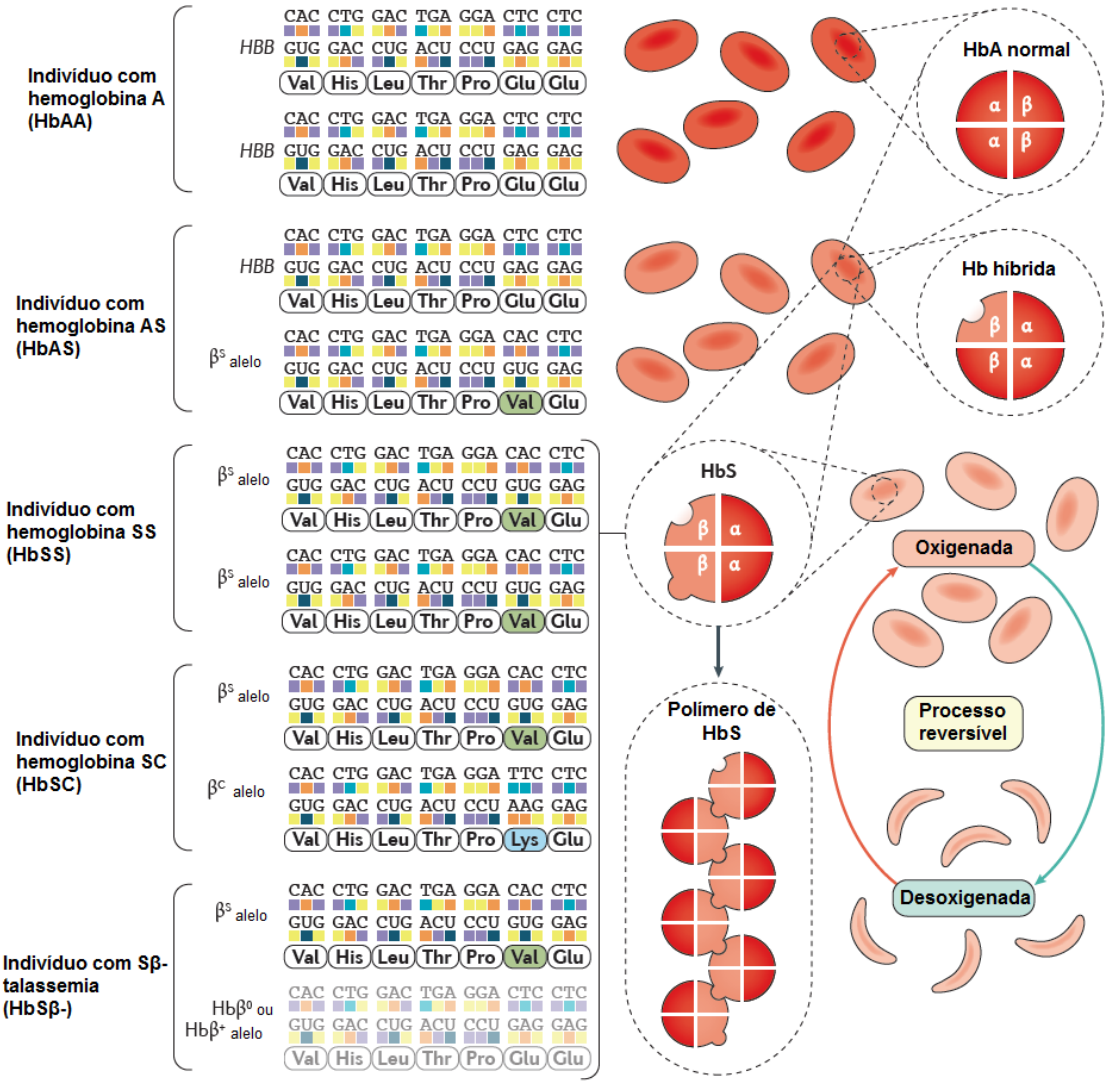


Figura 1. Alterações genéticas no gene *HBB*, que são responsáveis pelos diferentes genótipos associados a DF, bem como influencia na polimerização da HbS e no processo de falcização. Fonte: adaptado de KATO et al., 2018.

2.2 FISIOPATOLOGIA DA DF

Os diferentes genótipos da DF experimentam fisiopatologia comum, as variações existentes decorrem de diferenças fenotípicas ou da gravidade da doença. A polimerização da HbS está diretamente correlacionada com a concentração de HbS presente nos eritrócitos e com a composição de outras hemoglobinas que podem participar na formação dos polímeros (KATO et al., 2018).

A desoxigenação intraeritrocitária da HbS ocasiona a formação de polímeros da HbS que geram alterações na membrana eritrocitária e distorcem os eritrócitos, alterando o seu formato para o formato de foice. Além disso, esse processo desencadeia insuficiência energética celular, estresse, desidratação, alteração na reologia e lise prematura dos eritrócitos. Os ciclos recorrentes de oxigenação e desoxigenação causam deformações na membrana do eritrócito que resultam na formação do eritrócito irreversivelmente falcizado que não apresenta mais a capacidade de reverter o seu formato (Figura 2a) (NETO e PITOMBEIRA, 2003; MARENGO-ROWE, 2006; KATO et al., 2018; SUNDD et al., 2018).

A polimerização da HbS desencadeia dois processos fisiopatológicos de extrema importância para a DF, são eles a vaso-oclusão e a hemólise.

Os eventos vaso-oclusivos que ocasionam a isquemia tecidual são a principal fisiopatologia responsável pelas crises dolorosas. Esses eventos vaso-oclusivos são resultantes da interação entre os eritrócitos falcizados e não falcizados, reticulócitos, leucócitos, plaquetas e o endotélio vascular, que contribuem para a oclusão microvascular com isquemia, sendo esse processo seguido da restauração do fluxo sanguíneo, o que gera lesão tecidual ocasionada pela reperfusão. Os ciclos repetidos de isquemia e reperfusão geram estresse oxidativo devido à ativação de oxidases vasculares e inflamação devido ao aumento da expressão de moléculas de adesão pelo endotélio vascular e da produção de fatores quimiotáticos e citocinas inflamatórias que auxiliam no recrutamento de leucócitos (Figura 2b e 2d) (PIEL et al., 2017; GUARDA et al., 2017; KATO et al., 2018; SUNDD et al., 2018).

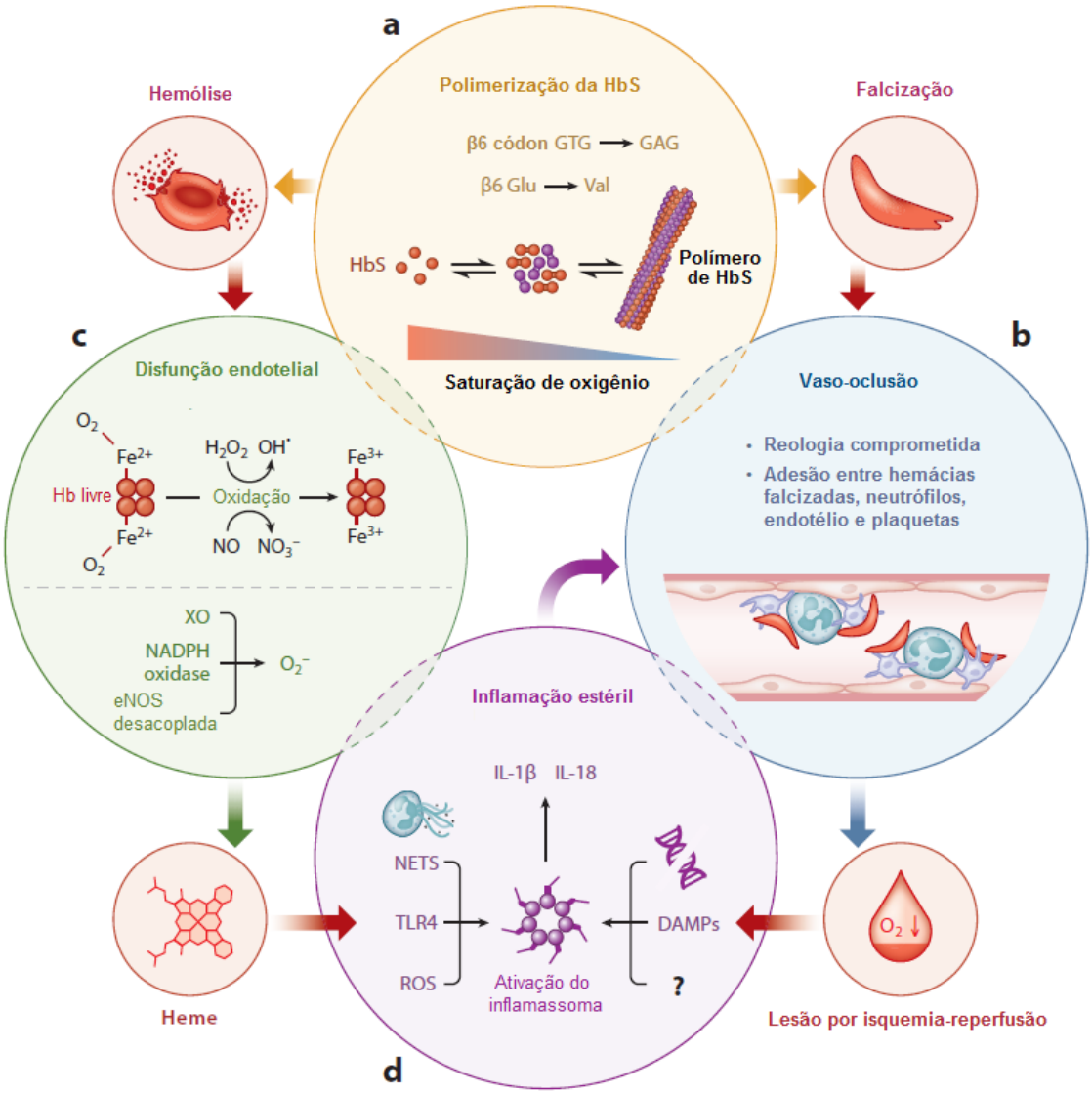


Figura 2. Representação dos mecanismos fisiopatológicos responsáveis pela patogenia da DF. a) Polimerização da HbS; b) Vaso-oclusão; c) Disfunção endotelial e d) Inflamação estéril. Fonte: adaptado de SUNDD *et al*., 2018.

A hemólise, o segundo processo de maior importância na DF, é ocasionada pela ruptura do eritrócito que libera no espaço vascular hemoglobina (Hb) livre, heme e arginase, que consome a arginina, promovendo o estresse oxidativo e respostas inflamatórias como, por exemplo, vasculopatia, ativação plaquetária, leucocitária e endotelial (Figura 2c) (SMITH et al., 2015; PIEL et al., 2017; GUARDA et al., 2017; SANTIAGO et al., 2018). Devido à hemólise intensa os indivíduos com DF apresentam concentrações diminuídas de Hb, o que desencadeia anemia crônica (KATO et al., 2006; SUNDD et al., 2018). Além da anemia, a hemólise intravascular desencadeia a injúria vascular e a disfunção endotelial. Com a hemólise, a arginase que é liberada dos eritrócitos consome a arginina disponível,

reduzindo o seu aporte para as células endoteliais, que a utilizam para produção de óxido nítrico (NO) através da oxido nítrico sintase endotelial (eNOS). Além disso, há o aumento da produção de nitrato, meta-hemoglobina e ferro-nitrosil hemoglobina devido à reação da Hb livre com o NO. A diminuição da biodisponibilidade do NO reduz a vasodilatação, enquanto causa ativação e proliferação endotelial, levando a disfunção endotelial (PIEL et al., 2017; GUARDA et al., 2017; SANTIAGO et al., 2017).

Na DF, um terço da hemólise ocorre no compartimento intravascular enquanto dois terços ocorrem no compartimento extravascular (CROSBY, 1995; KATO e GLADWIN, 2009). A hemólise extravascular consiste na remoção de eritrócitos irreversivelmente falcizados da corrente sanguínea pelo sistema fagocitário mononuclear no fígado e baço. O estresse oxidativo desencadeia lesões na membrana e citoesqueleto dos eritrócitos falcizados expondo, por exemplo, a fosfatidilserina na superfície celular, permitindo a ligação da imunoglobulina à membrana, promovendo endocitose mediada pelo receptor Fc do eritrócito danificado por macrófagos reticuloendoteliais (KATO e GLADWIN, 2009; GUARDA et al., 2017). Diversas proteínas relacionadas a degradação do heme e metabolismo do ferro participam da regulação da hemólise extravascular, sendo elas: heme oxigenase, receptor de transferrina 1, transportador do metal divalente 1, ferroportina exportadora de ferro 1 e hepcidina. Esse fenômeno contribui com a gravidade da DF devido à redução do tempo de vida dos eritrócitos de 120 dias para aproximadamente 31 dias (KONG et al., 2013; QUINN et al., 2016; GUARDA et al., 2017).

Conforme mostrado na figura 2d, a inflamação estéril também é parte importante do mecanismo fisiopatológico da DF. Essa inflamação é desencadeada pela vaso-oclusão, que contribui para a lesão por isquemia e reperfusão, combinada com a liberação de padrões moleculares associados a microrganismos ou perigo (eDAMPS) e pela hemólise que libera heme e hemina. Esse estado pró-inflamatório é marcado pela ativação celular, mais especificamente de leucócitos, plaquetas, células endoteliais, pela produção de fator tecidual, citocinas e espécies reativas de oxigênio (ROS) e diminuição dos níveis de NO (BECKER et al., 2000; HEBBEL et al., 2004; GUARDA et al., 2017; SUNDD et al., 2018).

No contexto pró-inflamatório identificado na DF, as células endoteliais e monócitos produzem citocinas de forma exacerbada, como por exemplo, interleucina

(IL) 1, fator de necrose tumoral alfa (TNF-α), endotelina-1 e fator de estimulação de colônias de macrófagos granulócitos (GMCSF). Além dessas citocinas, estudos recentes têm demonstrado a participação do fator de transformação do crescimento beta (TGF-β), IL-17 e IL-18 no processo inflamatório da DF (CERQUEIRA et al., 2011; VILAS-BOAS et al., 2016; GUARDA et al., 2017).

Os mecanismos fisiopatológicos descritos aqui são os responsáveis por desencadear as manifestações clínicas que ocorrem na DF.

2.3 MANIFESTAÇÕES CLÍNICAS NA DOENÇA FALCIFORME

Os indivíduos com DF apresentam quadro clínico heterogêneo, com grande variabilidade nas manifestações clínicas. Essa variabilidade pode ser explicada pelos genótipos, modificadores genéticos, fatores ambientais, como por exemplo, ambiente doméstico, status socioeconômico, nutrição e acesso a cuidado médico e diferenças geográficas (LYRA et al., 2005; KATO et al., 2018).

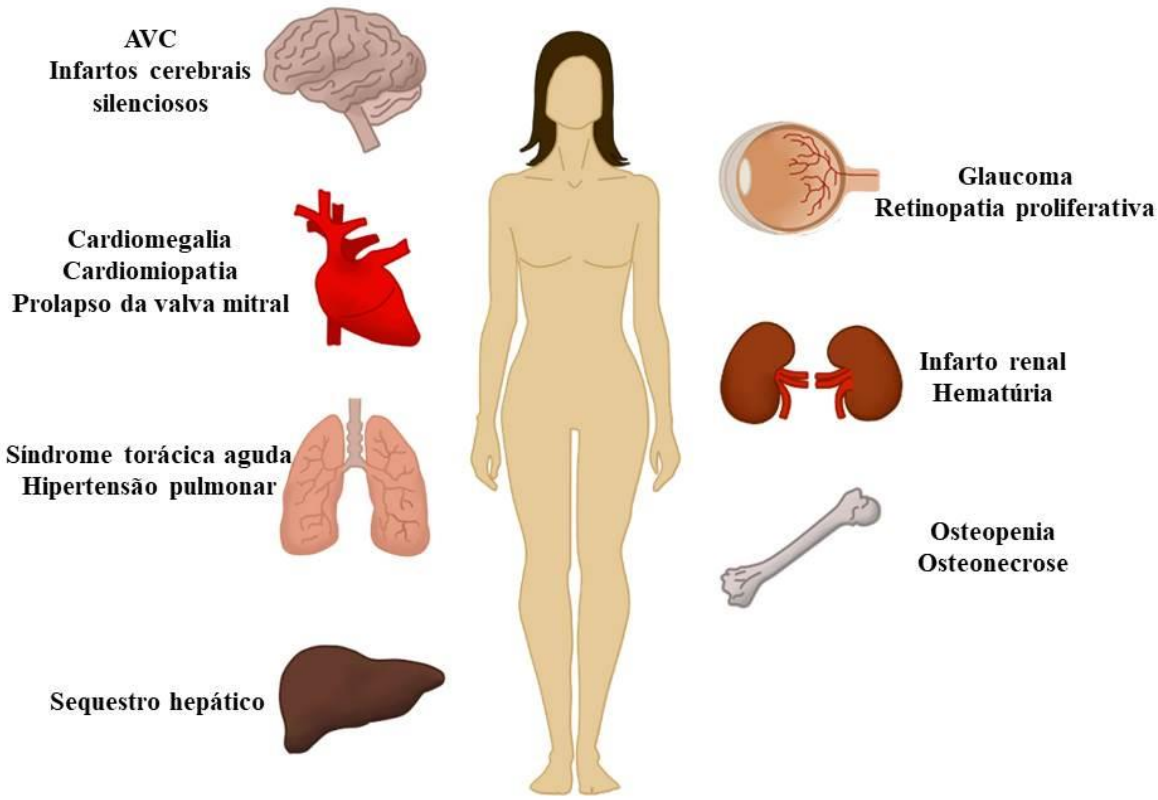
Diversas manifestações clínicas já foram descritas em indivíduos com DF, sendo que as complicações encontradas em indivíduos com AF podem ocorrer em grau menor nos indivíduos HbSC (NAGEL et al., 2003; LIONNET et al., 2012).

Entre as manifestações clínicas associadas à hemólise encontramos, por exemplo, anemia aguda exacerbada, hiper hemólise, sequestro esplênico agudo, crises aplásticas, complicações relacionadas à transfusão, síndrome de hiper viscosidade, hemólise imune e hemossiderose transfusional. Entre as síndromes dolorosas mais frequentes estão os quadros de dor, episódios vaso-oclusivos e neuropatias (BALLAS et al., 2010; KATO et al., 2018). Além das manifestações já mencionadas, os indivíduos com DF podem ser acometidos pelo acidente vascular cerebral (AVC) e o infarto cerebral silencioso, que ocasionam alterações neurológicas importantes e debilitantes; por complicações gastrointestinais, como colelitíase e sequestro hepático; complicações pulmonares, como síndrome torácica aguda (STA) e hipertensão pulmonar; complicações musculoesqueléticas, como necrose avascular, úlceras de pernas e osteopenia; complicações cardíacas, como prolapso da válvula mitral, cardiomegalia e cardiomiopatia; complicações esplênicas, como sequestro esplênico agudo e asplenia funcional; complicações oftalmológicas, como glaucoma e retinopatia proliferativa e por complicações renais e geniturinárias,

como falha renal aguda, hematúria e priapismo (Figura 3) (STEINBERG et al., 2001; BALLAS et al., 2010; KATO et al., 2018).

Os indivíduos HbSC apresentam episódios menos frequentes e graves de aplasia e colelitíase, visto que a hemólise é menos intensa nos indivíduos desse genótipo, apesar de ser relatado que os indivíduos HbSC possuem risco aumentado para a ocorrência de STA, osteonecrose óssea e retinopatia proliferativa (Figura 3) (NAGEL et al., 2003; REES et al., 2015).

A fim de estabelecer a relação entre a fisiopatologia e as manifestações clínicas encontradas em indivíduos com DF foram propostos os subfenótipos clínicos.



Fonte: Elaborada por Luciana Fiuza

Figura 3. Manifestações clínicas encontradas nos indivíduos com doença falciforme.

2.4 SUBFENÓTIPOS DA DOENÇA FALCIFORME

Indivíduos com DF apresentam subfenótipos que normalmente se sobrepõem, mas ainda assim eles são úteis para entender os mecanismos fisiopatológicos da doença. Kato e colaboradores (2007) descreveram dois subfenótipos, o viscoso vaso-oclusivo e o hemolítico-disfunção endotelial; além desses, em 2017, Aleluia e colaboradores (2017) adicionaram a essa lista o subfenótipo dislipidêmico.

Os indivíduos com o subfenótipo viscoso vaso-oclusivo apresentam concentrações elevadas de Hb quando comparados com indivíduos dos demais subfenótipos, além disso possuem frequência elevada de complicações decorrentes da polimerização da HbS, fato que resulta na alteração do formato eritrocitário que adquire o formato de foice e apresenta maior adesão, o que propicia a ocorrência dos fenômenos vaso-oclusivos. A presença de talassemia alfa, vem sendo associada ao aumento nas concentrações de Hb e, assim, ao subfenótipo viscoso vaso-oclusivo. As manifestações clínicas mais identificadas nesse subfenótipo são crises vaso-oclusivas dolorosas, STA e osteonecrose (KATO et al., 2007; ALELUIA et al., 2017).

No subfenótipo hemolítico-disfunção endotelial os indivíduos com DF apresentam concentrações diminuídas de Hb, mas níveis elevados de marcadores de hemólise, como a lactato desidrogenase (LDH), reticulócitos, hemoglobina plasmática e arginase, que impactam diretamente na biodisponibilidade do NO, conforme referido anteriormente. Nesse subfenótipo, as manifestações clínicas mais comuns estão associadas à vasculopatia proliferativa e desregulação da função vasomotora, como por exemplo, AVC, priapismo, úlceras maleolares e hipertensão pulmonar (KATO et al., 2007; ALELUIA et al., 2017).

O sub-fenótipo dislipidêmico é marcado pelos níveis diminuídos de NO, colesterol total, *high density lipoproteins cholesterol* (HDL-C) e *low density lipoproteins cholesterol* (LDL-C) e níveis elevados de *very low density lipoprotein cholesterol* (VLDL-C) e triglicérides (ALELUIA et al., 2017). Os lipídios desempenham papel importante na resposta inflamatória, isso ocorre devido às propriedades anti-inflamatórias encontradas na fração HDL-C e a propriedades pró-inflamatórias da fração LDL-C (SEIXAS et al., 2010; ALELUIA et al., 2017).

Outras moléculas apresentam papel importante na resposta inflamatória, como é o caso do TGF-β, que será objeto de estudo desse trabalho, uma vez que este pode também estar modulando a inflamação na DF.

2.5 FATOR DE TRANSFORMAÇÃO DO CRESCIMENTO BETA

O TGF-β é expresso em todas as células do corpo humano e exerce papel importante em diversos processos celulares como, por exemplo, proliferação, apoptose em resposta a lesão tecidual, crescimento endotelial, inflamação, regulação do sistema imunológico e síntese de matriz extracelular. Além disso, se encontra associado a homeostase óssea, nefropatia diabética, fibrose pulmonar e infecção, sendo suscetível a modulação farmacológica (MIYAZONO et al, 2001; ATTISANO e WRANA, 2002; MOUSTAKAS et al, 2002; ZIYADEH, 2004; NOLAN et al., 2006).

Existem ao todo três isoformas de TGF-β (TGF-β1, 2 e 3), elas apresentam atividade biológica similar e participam, principalmente, de processos envolvidos na regulação da proliferação, migração, inflamação, diferenciação celular e apoptose. Para dar início a via de sinalização, as isoformas de TGF-β se ligam aos receptores de TGF-β, que apresentam três isoformas TGFBR1, 2 e 3, também conhecidos como TβRI, TβRII e TβRIII (DERYNCK e ZHANG, 2003; ARK et al.,2018).

A via de sinalização do TGF-β regula diversos processos celulares e tem papel importante na resposta imune, nefropatia, angiogênese, osteogênese, hematopoese, inflamação, proliferação celular e fibrose (STEINBERG e SEBASTIANI, 2012). A maioria das complicações clínicas encontradas em indivíduos com DF sofre influências desses processos, então é razoável suspeitar que alterações na via do TGF-β podem modular o seu desenvolvimento, progressão e resolução.

Os níveis de TGF-β1 vem sendo cada vez mais estudados na DF. Um estudo realizado em adultos com DF identificou que os indivíduos com AF, forma mais grave da DF, apresentaram níveis elevados de TGF-β1 quando comparados com indivíduos com HbSC e HbSβ-talassemia (TORRES et al., 2016). Keikhaei e colaboradores (2013) também observaram níveis elevados de TGF-β em indivíduos com DF, em estado estável, quando comparados com indivíduos sadios. Carvalho e colaboradores (2018) demonstraram que os níveis de TGF-β estavam aumentados

em indivíduos com AF em estado estável quando comparados com aqueles em crise, mostrando que o TGF-β pode ser um preditor do estado estável da DF.

Na DF, os genes que codificam partes da via do TGF-β têm sido associados a várias complicações clínicas, como por exemplo, AVC, úlceras de perna, bacteremia e priapismo; além disso, vêm sendo associados à taxa de filtração glomerular diminuída e ao risco de nefropatia (SEBASTIANI et al., 2005; NOLAN et al., 2006; ADEWOYE et al., 2006; ELLIOT et al., 2007; ASHLEY-KOCH et al., 2008). Entre os genes que codificam os receptores do TGF-β, o gene *TGFBR3*, que codifica o receptor 3, tem sido associado a variedade ampla de manifestações clínicas em indivíduos com DF e, por isso, será o objeto do presente estudo (SEBASTIANI et al., 2005; BALDWIN et al., 2005; NOLAN et al., 2006; ADEWOYE et al., 2006; ELLIOTT et al., 2007; MARTINEZ-CASTALDI et al., 2007; ASHLEY-KOCH et al., 2008; FLANAGAN et al., 2011).

2.6 GENE DO RECEPTOR 3 DO FATOR DE TRANSFORMAÇÃO DO CRESCIMENTO BETA

O gene do receptor 3 do fator de transformação do crescimento beta (*TGFBR3*), também conhecido como betaglicano, está localizado no braço curto do cromossomo 1, na posição 22.1, e codifica o TβRIII, um dos três tipos de receptores do TGF-β (Figura 4) (ELLIOTT et al., 2007; GAO e LEE, 2016).

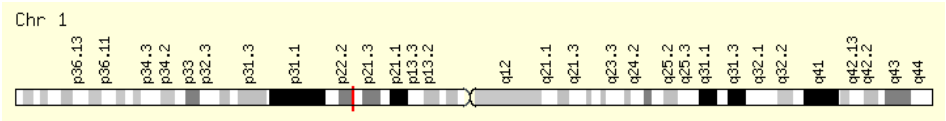


Figura 4. Localização do gene do fator de transformação do crescimento beta receptor 3.
Fonte: *GeneCards human gene database*.

O TβRIII é um receptor proteoglicano com 849 aminoácidos e com um domínio citoplasmático pequeno, com 41 aminoácidos, sendo o receptor do TGF-β mais expresso e capaz de se ligar com afinidade alta a todas as três formas de TGF-β (TGF-β1, TGF-β2 e TGF-β3) (GATZA et al., 2012; GAO e LEE, 2016; ARK et al., 2018; ARK et al., 2018).

Polimorfismos de único nucleotídeo, do inglês *single nucleotide polymorphism* (SNP) no gene *TGFBR3* vêm sendo associados a diversas doenças. Os SNPs rs1805113 e rs1805117 foram associados a hepatite B crônica (KIM et al., 2011), o rs1192415 a danos no nervo ótico e glaucoma (KHOR et al., 2011), o rs1805110 à doença de Behcet's (CHANG et al., 2001; CHEN et al., 2012; BARRY et al., 2015) e os SNPs rs284875, rs2038931, rs10874940, rs284176, rs7526590, rs284157 e rs2765888 têm sido associados a manifestações clinicas que acometem os indivíduos com DF (SEBASTIANI et al., 2005; BALDWIN et al., 2005; ADEWOYE et al., 2006; NOLAN et al., 2006; ELLIOTT et al., 2007; MARTINEZ-CASTALDI et al., 2007; ASHLEY-KOCH et al., 2008; FLANAGAN et al., 2011).

No presente estudo buscou-se avaliar seis SNPs no *TGFBR3*, sendo eles rs1805110, rs2038931, rs2765888, rs284157, rs284875 e rs7526590. Cinco desses polimorfismos foram previamente estudados e associados a manifestações clínicas na DF e um deles, o rs1805110, não foi previamente estudado na doença; contudo, a literatura descreve a sua associação com a doença de Behcet's. A doença de Behcet's é uma doença inflamatória multissistêmica que pode levar ao desenvolvimento de osteonecrose e infarto ósseo, complicações clínicas encontradas em indivíduos com DF (CHANG et al., 2001; CHEN et al., 2012; BARRY et al., 2015).

2.7 POLIMORFISMOS NO GENE *TGFBR3* NA DF

Alguns estudos têm associado os polimorfismos no gene *TGFBR3* com manifestações clínicas que acometem os indivíduos com DF (Quadro 1).

Quadro 1. Polimorfismos no gene *TGFBR3* associados as manifestações clínicas na DF.

Manifestação clínica	**Polimorfismo**	**Referência**
Úlceras de perna	**rs2038931**	NOLAN et al., 2006
Hipertensão pulmonar	rs10874940 *rs284176* *rs7526590*	ASHLEY-KOCH et al., 2008

Infecção	**rs2765888** rs6662385	ADEWOYE et al., 2006 DARBARI et al., 2008
AVC	rs1555889 **rs284875** rs2038931 rs901917 rs2148322 **rs2765888** rs2007686 rs284874	SEBASTIANI et al, 2005 FLANAGAN et al., 2011
Priapismo	**rs7526590**	ELLIOTT et al., 2007
Síndrome torácica aguda	**rs284157**	MARTINEZ-CASTALDI et al., 2007
Osteonecrose	**rs284157**	BALDWIN et al., 2005

Os polimorfismos investigados nesse estudo estão marcados em negrito.
Fonte: adaptado de FERTRIN e COSTA (2010).

Os polimorfismos no gene *TGFBR3* vêm sendo associados com manifestações clínicas relacionadas aos sub-fenótipos da DF, mais especificamente aos sub-fenótipos hemolítico e viscoso vaso-oclusivo (KATO et al., 2006). Acredita-se que as manifestações clínicas, incluindo AVC, úlceras de perna, hipertensão pulmonar e priapismo estejam associadas ao sub-fenótipo hemolítico.

Utilizando uma rede bayesiana, Sebastiani e colaboradores (2005) encontraram 31 SNPs que interagem com a hemoglobina fetal (HbF) para modular o risco do AVC em indivíduos com DF, sendo alguns deles no gene *TGFBR3*, envolvidos na via do TGF-β. Em 2011, um estudo desenvolvido por Flanagan e colaboradores (2011), estudou 103 indivíduos com AF evidenciou que o polimorfismo *rs284875* no gene *TGFBR3* estava associado ao AVC.

Nolan e colaboradores (2006) encontraram associação entre o polimorfismo *rs2038931* no gene do *TGFBR3* com a ocorrência de úlceras de perna em indivíduos com DF. O estudo de Ashley-Koch e colaboradores (2008) conseguiu estabelecer a associação entre os polimorfismos *rs10874940*, *rs284176* e *rs7526590* no gene

TGFBR3 com o risco para o desenvolvimento de hipertensão pulmonar em indivíduos com DF. O polimorfismo *rs7526590* também esteve associado à ocorrência de priapismo, em estudo realizado em indivíduos com AF e HbSβ-talassemia (ELLIOTT et al., 2007).

Os polimorfismos em *TGFBR3* foram também associados a manifestações clínicas que fazem parte do sub-fenótipo viscoso vaso-oclusivo (KATO et al., 2006). O polimorfismo *rs284157* foi associado à osteonecrose e a STA em indivíduos com AF (BALDWIN et al., 2005; MARTINEZ-CASTALDI et al., 2007).

Além de estar associado às manifestações clínicas presentes nos sub-fenótipos hemolíticos e viscoso vaso-oclusivo, o polimorfismo *rs2765888* no gene *TGFBR3* foi associado à bacteremia em indivíduos com AF (ADEWOYE et al., 2006).

É importante ressaltar que estudos sobre esses polimorfismos só foram realizados em indivíduos com DF e não foram caracterizados em outras populações saudáveis ou em outras doenças. O desenho desses estudos foi de corte-transversal não permitindo assim aos autores estabelecer marcadores de prognóstico da doença, mas podem servir de guias para estudos de coorte.

Apesar de estar sendo associado às manifestações clínicas na DF, a investigação das associações entre os polimorfismos no gene do *TGFBR3* com marcadores laboratoriais relacionados ao perfil lipídico, renal, hepático, hemolítico e inflamatório, ainda não está completamente esclarecida. Dessa forma, estabelecer uma relação entre esses polimorfismos e os processos fisiopatológicos e clínicos da DF é importante, principalmente do ponto do manejo clínico dos pacientes.

O estudo de Kim e colaboradores (2010), realizado em indivíduos asmáticos, identificou que polimorfismos no gene *TGFBR3* são mais informativos quando avaliados em haplótipo e não de forma individual. Na DF esses polimorfismos nunca foram avaliados como haplótipo, então no presente estudo, além de avaliar os polimorfismos individualmente, serão identificados os haplótipos e a associação destes a marcadores laboratoriais e manifestações clínicas presentes em indivíduos com DF.

3 JUSTIFICATIVA

A DF é uma doença genética com prevalência elevada na população brasileira, especialmente no estado da Bahia, visto que as Hbs variantes, HbS e HbC, apresentam frequência elevada (SANTIAGO et al., 2017b). Estima-se que nesse estado, a incidência é de 1 indivíduo com DF a cada 650 recém-nascido (BRASIL, 2014).

Os indivíduos com DF apresentam quadro clínico heterogêneo, no qual é possível verificar a presença de todas as complicações encontradas em indivíduos com AF, embora na HbSC elas ocorram em gravidade e frequência menores e mais tardiamente em relação a AF (NAGEL et al., 2003; LIONNET et al., 2012). Esses indivíduos apresentam manifestações clínicas que vão desde crises vaso oclusivas e dolorosas, AVC e priapismo até lesões crônicas em órgãos diversos (STEINBERG et al., 2002).

As complicações clínicas são importantes causas de mortalidade entre os indivíduos com DF, sendo que em avaliações epidemiológicas sobre a mortalidade, foi observado que no estado da Bahia 42% das mortes ocorrem entre adultos jovens, de 20 a 39 anos de idade (RAMOS et al., 2015). Durante o período de 2008 a 2014, apenas no estado da Bahia, houve registro de 8103 internações decorrentes da DF, onde 34% correspondia a faixa etária de 4-15 anos de idade, sendo estimado um gasto público de aproximadamente R$ 2.894.556,63 com as internações dos pacientes em todo o estado (MARTINS e TEIXEIRA, 2017). Esses dados reforçam a importância de se investigar marcadores de prognóstico da DF na população pediátrica, pois à medida que os pacientes envelhecem as manifestações tendem a ser mais crônicas e debilitantes.

A via de sinalização do TGF-β regula diversos processos celulares, desempenhando papel importante na resposta imune, nefropatia, angiogênese, osteogênese, hematopoese, inflamação, proliferação celular e fibrose (STEINBERG e SEBASTIANI, 2012).

Os genes que codificam receptores da via do TGF-β, em especial do *TGFBR3*, têm sido associados a várias manifestações clínicas na DF, como por exemplo, AVC, úlceras de perna, bacteremia e priapismo; além disso, vêm sendo associados à taxa de filtração glomerular e ao risco de nefropatia (SEBASTIANI et al., 2005; NOLAN et al., 2006; ADEWOYE et al., 2006; ELLIOTT et al., 2007;

ASHLEY-KOCH et al., 2008). As manifestações clínicas foram associadas aos polimorfismos no gene *TGFBR3* em indivíduos com DF, embora nenhum estudo tenha realizado a associação a marcadores laboratoriais.

Os marcadores genéticos são fonte importante de informação sobre o quadro clínico dos indivíduos com DF. A presença de determinados polimorfismos genéticos tem sido associada a possíveis interferências no curso da DF, sendo potencialmente uteis como marcadores de prognóstico, podendo ainda influenciar a seleção de terapias personalizadas, com possibilidades de serem alvos novos de tratamento terapêuticos (STEINBERG e SEBASTIANI, 2012).

Os pacientes com DF apresentam manifestações clínicas heterogêneas. Contudo, a grande maioria das manifestações é afetada de forma direta ou indireta pelos processos nos quais o TGF-β e sua via de sinalização estão envolvidos. Dessa forma, a identificação precoce de biomarcadores laboratoriais associados aos processos hemolíticos, vaso-oclusivos e inflamatórios na DF podem impactar positivamente no manejo clínico e contribuir com o desenvolvimento de novas estratégias terapêuticas, que podem auxiliar a qualidade de vida dos pacientes.

4 HIPÓTESE

Alterações nos níveis de TGF-β1 e polimorfismos no gene *TGFBR3* estão associados aos processos fisiopatológicos da DF.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Investigar a associação dos níveis plasmáticos de TGF-β1 e dos polimorfismos no gene *TGFBR3* com biomarcadores genéticos, hematológicos, bioquímicos e imunológicos, bem como nas complicações clínicas, em indivíduos com DF.

5.2 OBJETIVOS ESPECÍFICOS

- Avaliar os marcadores hemolíticos, renais, hepáticos, lipídicos, inflamatórios, bem como marcadores relacionados à disfunção endotelial em indivíduos com DF;
- Quantificar os níveis plasmáticos de TGF-β em indivíduos com DF;
- Associar os níveis plasmáticos de TGF-β com biomarcadores associados à hemólise, inflamação e disfunção endotelial nesses indivíduos;
- Investigar a frequência dos polimorfismos rs1805110 e rs7526590 no gene *TGFBR3* em indivíduos com AF;
- Investigar a frequência dos polimorfismos rs1805110, rs2038931, rs2765888, rs284157, rs284875 e rs7526590 no gene *TGFBR3* em indivíduos com DF;
- Investigar as associações entre polimorfismos no gene *TGFBR3* e os biomarcadores investigados, bem como as complicações clínicas apresentadas por estes indivíduos;
- Identificar os haplótipos no gene *TGFBR3* e associá-los a biomarcadores hematológicos, bioquímicos, genéticos, imunológicos e a complicações clínicas apresentadas pelos indivíduos com DF.

6 MANUSCRITOS

6.1 MANUSCRITO 1

Título: Associations between TGF-β1 levels and markers of hemolysis, inflammation and tissue remodeling in pediatric sickle cell patients

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Situação: Submetido na *Mediators of Inflammation* – ID: 4651891

Objetivos: (referente aos três objetivos específicos da tese):

- Avaliar os marcadores hemolíticos, renais, hepáticos, lipídicos, inflamatórios, bem como marcadores relacionados à disfunção endotelial em indivíduos com DF;
- Quantificar os níveis plasmáticos de TGF-β em indivíduos com DF;
- Associar os níveis plasmáticos de TGF-β com biomarcadores associados à hemólise, inflamação e disfunção endotelial nesses indivíduos.

Principais resultados: Os indivíduos HbSS apresentaram concentrações plasmáticas elevadas de TGF-β1 quando comparados a indivíduos controles saudáveis e HbSC. Nos indivíduos HbSS, as concentrações de TGF-β1 estiveram positivamente correlacionadas a contagem de hemácias e plaquetas e aos níveis de hemoglobina, hematócrito e TIMP-1. Além desses marcadores, os indivíduos HbSS possuidores de concentrações de TGF-β1 ≥72.29 ng/mL apresentaram contagem elevada de monócitos e níveis diminuídos de albumina. Em indivíduos HbSC, as concentrações de TGF-β1 estiveram positivamente correlacionadas com as contagens de leucócitos, eosinófilos, linfócitos, monócitos e plaquetas e as concentrações de TIMP-1, VLDL-C, triglicérides, heme e AST. Os indivíduos HbSC

com concentrações de TGF-β1 ≥47.80 ng/mL apresentaram contagem elevada de leucócitos e plaquetas e concentrações elevadas de triglicérides, VLDL-C, MMP-9 e TIMP-1, além de concentrações diminuídas de HDL-C. Nossos dados reforçam o papel do TGF-β no remodelamento vascular, vasculopatia, angiogênese e inflamação na DF.

Associations between TGF-β1 levels and markers of hemolysis, inflammation and tissue remodeling in pediatric sickle cell patients

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ABSTRACT

Transforming growth factor beta (TGF-β) is a cytokine with important involvement in biological processes related to the pathogenesis of sickle cell disease (SCD), including endothelial and vascular dysfunction, inflammation, and hematopoietic homeostasis. This study aimed to investigate associations between levels of TGF-β1 and classical laboratory biomarkers and inflammatory mediators, as well as the tissue inhibitor of metalloproteases-1 (TIMP-1) and matrix metalloproteinase-9 (MMP-9), in pediatric patients (n=123) with SCD in steady state: 84 with sickle cell anemia (HbSS) and 39 with hemoglobin SC disease (HbSC). A healthy control (HC) group of 59 individuals was also included. Hematological and biochemical analyses were carried out using electronic methods. TGF-β1, TIMP-1, and MMP-9 plasma quantifications were performed by ELISA. TGF-β1 plasma levels were higher in HbSS individuals than in HbSC and HC. In individuals with HbSS, TGF-β1 levels were positively correlated with red blood cells, hemoglobin, hematocrit, platelets, and TIMP-1. In addition, HbSS individuals with TGF-β1 levels above the median (≥72.29 ng/mL) also presented increased monocyte counts and decreased albumin levels. In patients with HbSC, TGF-β1 levels were positively correlated with leukocytes, eosinophils, lymphocytes, monocytes, and platelets, as well as levels of TIMP-1, VLDL-C, triglycerides, heme and AST. Additionally, HbSC individuals with TGF-β1 levels above the median (≥47.80 ng/mL) presented increased leukocyte and platelet counts, as well as increased levels of triglycerides, VLDL-C, MMP-9 and TIMP-1, and decreased HDL-C. Our findings suggest the importance of TGF-β in vascular remodeling, vasculopathy, angiogenesis and inflammation in pediatric patients with SCD.

KEY WORDS: sickle cell disease, transforming grow factor-beta 1, tissue inhibitor of metalloproteases-1, matrix metalloproteinases 9, inflammation and vascular dysfunction, pediatrics

INTRODUCTION

Sickle cell disease (SCD) is a group of disorders characterized by the presence of the hemoglobin variant S (HbS). Sickle cell anemia (SCA), the most severe type of SCD, represents homozygosity in the inheritance of the beta allele S (HbSS). Hemoglobin SC disease (HbSC) is characterized by the association of HbS with another hemoglobin variant, hemoglobin C (HbC), and is considered a milder phenotype of SCD [1-3].

Individuals with SCD exhibit an acute and chronic inflammatory status associated with recurrent infections and increased leukocyte counts, as well as the activation of leukocytes, red blood cells, reticulocytes, and endothelial cells. These individuals often experience clinical events related to vascular dysfunction, such as priapism, pulmonary hypertension, vaso-occlusive crisis (VOC) and stroke [4,5].

Vascular dysfunction is a common feature in SCD, mainly caused by nitric oxide (NO) imbalance in association with chronic hemolysis [4,6]. During intravascular hemolysis, free hemoglobin and heme react with NO, causing the degradation of this free radical. The hemolytic process also induces the release of arginase, which consumes arginine, the main substrate for NO production. These events result in reactive oxygen and nitrogen species (ROS and RNS) production, which contribute to cell damage and vascular dysfunction by way of nitrosative stress [5].

The chronic inflammatory status observed in SCD has also been linked to increased levels of inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor-alpha (TNF-α) and endothelin-1, which produced by activated endothelial cells [7]. SCA patients have been described to present increased plasma levels of TNF-α, IL-8 and prostaglandin E_2 (PGE_2) [8]. In addition, transforming growth factor beta 1 (TGF-β1), IL-17 and IL-18 are capable of activating the vascular endothelium [7,9]. In SCD, patterns of cytokine production vary during steady and crisis state [10], suggesting that the inflammatory response is capable of modulating clinical events.

TGF-β is a pleiotropic family of cytokines produced by many cell types, such as immune cells (T cells and macrophages), tumor cells and stromal cells. These growth factors have been implicated in the regulation of cell growth, proliferation, differentiation, adhesion and migration, as well as Th17 response polarization, ROS production and apoptosis [11-15]. In the context of SCD, TGF-β is involved in several processes, including wound healing/ulceration, proliferative vasculopathy,

inflammation, immune response and ROS production [9,16], all of which have been linked to clinical events.

TGF-β can up-regulate the expression and activity of matrix metalloproteinases 9 (MMP-9), as well as its antagonist, tissue inhibitor of metalloproteases-1 (TIMP-1) [17,18]. MMP-9, a zinc-dependent endopeptidase that acts in the extracellular matrix, promotes tissue remodeling in response to physiological and pathological conditions [17-20] Additionally, MMP-9 can also activate cytokine and chemokine production and is a regulator of inflammation and immunity [9]. Interplay between TIMP-1 and MMP-9 is involved in angiogenesis, cell growth and apoptosis [21,22].

Considering the immunomodulatory role of TGF-β together with the plethora of effects exerted by this superfamily, we hypothesized that TGF-β1 may play a key role in the pathogenesis of SCD. Hence, we sought to investigate associations between plasma levels of TGF-β1 and classical laboratory biomarkers, as well as TIMP-1 and MMP-9, in individuals with HbSS and HbSC.

MATERIAL AND METHODS

Subjects

Pediatric patients with SCD (n=123) were recruited from the Bahia State Hematology and Hemotherapy Foundation (HEMOBA), located in Salvador, Brazil. Of these, 84 were HbSS and 39 were HbSC; 37 (44.04%) and 19 (48.71%) were female, respectively. All individuals were in steady state, signifying the absence of acute events in the three months prior to inclusion, and none were undergoing hydroxyurea therapy. The mean age of patients with HbSS and HbSC was 8.76 ± 3.78 and 10.72 ± 4.24 years, respectively.

A healthy control (HC) group consisting of 59 individuals was included, 30 (50.85%) of whom were female, with mean age of 8.38 ± 3.50 years. These individuals were recruited from the Laboratory of Clinical and Toxicological Analysis, College of Pharmaceutical Sciences, Federal University of Bahia (LACTFAR-UFBA).

This research protocol was approved by the institutional research board of the Gonçalo Moniz Institute (protocol number: 0016.0.225.000-09) and was conducted in accordance with the 1964 Declaration of Helsinki and its subsequent revisions. All

individuals were informed regarding the purpose and procedures of this study and informed written consent was obtained from each patient's legal guardian.

Hematological and Biochemical parameters

Hematological parameters were quantified using a Coulter Count T-890 electronic cell counter (Coulter Corporation, Hialeah, Florida, USA). Reticulocytes were counted after staining supravitally with brilliant cresyl blue dye. Hemoglobin profiles and fetal hemoglobin (HbF) levels were determined by high-performance liquid chromatography using an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA).

Biochemical parameters were measured using an automated A25 chemistry analyzer (Biosystems S.A., Barcelona, Catalunya, Spain). Serum ferritin was measured by immunoassay using an Access® 2 immunoassay system (Beckman Coulter Inc., Pasadena, California, USA). In addition, alpha 1 antitrypsin (AAT) levels were determined using an IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). Total systemic free heme was measured in plasma samples using the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, California, USA) following the manufacturer's protocol.

Laboratory analysis was performed at the Laboratory of Genetic Investigation and Translational Hematology at the Gonçalo Moniz Institute-FIOCRUZ (LIGHT-IGM/FIOCRUZ) and at LACTFAR-UFBA.

TGF-β1, TIMP-1 and MMP-9 plasma measurement

TGF-β1, TIMP-1 and MMP-9 plasma levels were measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA) in accordance with the manufacturer's protocol.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 20.0 (IBM, Armonk, New York, USA) and GraphPad Prism version 6.0 (Graphpad Software, San Diego, California, USA), which was also used for graph assembly. Significance was considered when $p<0.05$. Variable values were summarized as means. To perform comparisons among SCD phenotypes, subgroups of individuals were formed according to TGF-β1 median

values. The Shapiro-Wilk test was used to determine the distribution of quantitative variables. Depending on distribution, comparisons of two numerical variables were performed using the independent t-test and Mann-Whitney U test. Ordinary one-way ANOVA or Kruskal-Wallis test were performed to compare three numerical variables depending on distribution. Spearman's rank correlation coefficient or Pearson's correlation coefficient were used to measure the strength of linear relationships between paired variables.

RESULTS

Hematological and biochemical laboratory parameters

Table 1 lists the hematological and biochemical laboratory parameters of the investigated HbSS, HbSC and HC individuals. Comparisons among the hematological and biochemical parameters in these individuals revealed statistically significant differences in hemolytic and inflammatory markers, leukocyte counts, as well as biomarkers of lipid and iron metabolism, and hepatic and renal function.

In comparison to individuals with HbSC and HC, patients with HbSS presented significantly decreased red blood cell (RBC) counts, hemoglobin (Hb), hematocrit (Ht), high-density lipoprotein cholesterol (HDL-C), urea and creatinine levels, in addition to increased mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total bilirubin, indirect bilirubin, direct bilirubin, lactate dehydrogenase (LDH), fetal hemoglobin (HbF), iron, very low-density lipoprotein cholesterol (VLDL-C), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha 1 antitrypsin (AAT) and ferritin levels, as well as counts of reticulocytes, leukocytes, neutrophils, eosinophils, lymphocytes, monocytes and platelets.

HbSS individuals present increased plasma levels of TGF-β1

HbSS individuals presented higher plasmatic TGF-β1 levels than HC and HbSC individuals (SS>HC>SC). Individuals with HbSS presented mean TGF-β1 levels of 70.80 ± 23.11 ng/mL, with median values of 72.29 ng/mL (IQR: 54.23 – 87.68 ng/mL), versus HC: mean 62.63 ± 19.44 ng/mL, and median 61.90 ng/mL (IQR: 49.10 – 73.60 ng/mL). Individuals with HbSC presented mean TGF-β1 levels of

51.43 ± 23.76 ng/mL and a median value of 47.80 ng/mL (IQR: 38.24 – 67.51 ng/mL) (Figure 1).

Correlations between TGF-β1 plasma levels and laboratory parameters

In individuals with HbSS, TGF-β1 was positively correlated with RBC (*r*= 0.282*; p* =0.0094), Hb (*r*= 0.254*; p* =0.0197), Ht (*r*= 0.284*; p* <0.0089), platelets (*r*= 0.663*; p* <0.0001) and TIMP-1 (*r*= 0.381*; p* =0.0005) (Figure 2.A). In addition, in HbSC individuals, TGF-β1 was positively correlated with leukocytes (*r*= 0.5168*; p* =0.0008), eosinophils (*r*= 0.3619*; p*=0.0236), lymphocytes (*r*= 0.6575*; p* <0.0001), monocytes (*r*= 0.4421*; p =0*.0048), platelets (*r*= 0.5318*; p*= 0.0005), AST (*r*= 0.326*; p* =0.0425), VLDL-C (*r*= 0.424*; p* =0.0072), triglycerides (*r*= 0.439*; p* =0.0051), heme (*r*= 0.426*; p* =0.0076) and TIMP-1 (*r*= 0.408*; p*= 0.0110) (Figure 2.B).

TGF-β1 plasma levels are associated with laboratory parameters in SCD

Considering the fact no standard or normal clinical range exists with respect to plasma levels of TGF-β1 in humans, we endeavored to perform association analyses by creating subgroups of individuals with HbSS and HbSC according to the median values of TGF-β1 obtained for each SCD genotype.

In individuals with HbSS, TGF-β1 levels above the median (TGF-β1 ≥ 72.29) were associated with increased RBC (p=0.0208) (Figure 3.A); hemoglobin (p=0.0192) (Figure 3.B), hematocrit (p=0.0080) (Figure 3.C), monocytes (p=0.0401) (Figure 3.D), platelets (p<0.0001) (Figure 3.E) and TIMP-1 (p<0.0001) (Figure 3.F), as well as decreased albumin (p=0.0444) (Figure 3.G). Among individuals with HbSC, TGF-β1 levels above the median (TGF-β1 ≥ 47.80) were associated with increased leukocytes (p=0.0049) (Figure 4.A), eosinophils (p=0.0207) (Figure 4.B), lymphocytes (p=0.0002) (Figure 4.C), monocytes (p=0.0062) (Figure 4.D), platelets (p=0.0067) (Figure 4.E), ferritin (p=0.0125) (Figure 4.F), TIMP-1 (p=0.0065) (Figure 4.G), MMP-9 (p=0.0191) (Figure 4.H), triglycerides (p=0.0102) (Figure 5.A) and VLDL-C (p=0.0454) (Figure 5.B), as well as decreased HDL-C levels (p=0.0425) (Figure 5.C).

DISCUSSION

Despite the fact that several laboratory and genetic biomarkers have been associated with sub-phenotypes of SCD, in which individuals present a greater

propensity of clinical events, the search for prognostic biomarkers of SCD remains challenging [4,6,23].

Our analyses showed that HbSS individuals presented increases in biomarkers of hemolysis, leukocytosis, and inflammation, together with decreased levels of HDL-C, in comparison to HbSC and HC individuals. These findings are consistent with previous reports describing HbSS as the most severe form of SCD [24-26]. As individuals with HbSS present more intense hemolysis, anemia tends to be more severe, and hemolytic complications occur more frequently [23,25].

Herein, higher levels of TGF-β1 were found in individuals with HbSS than in those with HbSC and HC. Despite the fact that our case series consisted of pediatric individuals, our results were nonetheless similar to a previous report in steady-state adults with HbSS, HbSC and HC [16]. The elevated TGF-β levels found in HbSS may be associated with endothelial remodeling, [11] since the endothelium controls the release of relaxing and contracting factors that regulate localized vascular tone. Vasculopathy, in addition to endothelial dysfunction, are the main chronic events described in SCD, and are also involved in the pathogenesis of stroke, renal disease and pulmonary hypertension [23]. Thus, it is possible that TGF-β could be directly involved in the modulation of vasculopathy in SCD individuals. In addition, elevated TIMP-1 levels were detected in individuals with HbSS and HbSC who presented TGF-β1 levels above the median. TIMP-1, a protein that modulates cell growth, apoptosis, and angiogenesis, is known to inhibit the catalytic activity of MMP-9 [18]. As TGF-β1 stimulates the expression of MMP-2, MMP-9 and TIMP-1, increases in TIMP-1 levels driven by TGF-β1 may positively impact angiogenesis [27]. Our results also show that individuals with HbSC who presented TGF-β1 levels above the median value also exhibited higher levels of MMP-9, which reinforces the role of TGF-β in angiogenesis, vasculopathy and endothelial dysfunction.

The individuals with HbSS and HbSC who presented TGF-β1 levels above the median also exhibited higher platelet counts than those below the median. This finding is supported by previous studies reporting that more TGF-β is produced by platelets than by other cells types; moreover, after activation, platelets rapidly release TGF-β [20,28,29]. Platelet counts were previously shown to be correlated with TGF-β levels in both HbSS and HbSC individuals [16], which corroborates the present correlation analysis.

The positive correlation demonstrated herein between TGF-β1 and levels of heme and AST in individuals with HbSC provides evidence of the participation of TGF-β1 in hemolysis. Interestingly, genes involved in TGF-β/BMP signaling pathway were previously associated with the clinical manifestations of a hemolytic subphenotype, such as leg ulcers [11].

Our association analysis revealed that individuals with HbSC and TGF-β1 levels above the median presented increased leukocyte counts. In addition, our correlation analysis found a positively correlation between TGF-β1 levels and leukocyte, eosinophil, lymphocyte, and monocyte counts, indicating the presence of an inflammatory response. Similarly, a previous study reported a correlation between TGF-β levels and total leukocyte counts in individuals with HbSβ–thalassemia [16]. TGF-β is involved in neutrophil and monocyte chemotaxis, which influences leukocyte recruitment to inflammatory sites [30]. In the inflammatory state seen in SCD individuals, markedly high expression of adhesion molecules, as well as the production of chemotactic factors and inflammatory cytokines, all promote leukocyte recruitment [3,7,31].

Regarding lipid profile, HbSC individuals with TGF-β1 levels above the median presented decreased HDL-C. In addition, TGF-β1 levels were also found to be positively correlated with VLDL-C and triglycerides in these individuals. HDL-C exerts important anti-inflammatory activity in vascular diseases [32,33] In SCD, decreased HDL-C levels have been associated with an inflammatory state [6,32]. Triglycerides and VLDL-C have been shown to induce inflammatory events in atherosclerosis, supporting the notion that these molecules participate in proinflammatory activity [34].

Our findings suggest that TGF-β1 levels bear relations to vasculopathy, endothelial dysfunction and inflammation in individuals with SCD. Collectively, our results highlight the relevance of investigating novel biomarkers of disease severity in the clinical management of individuals with HbSS and HbSC. To the best of our knowledge, the present study was the first to investigate associations and correlations between TGF-β1 levels and hematological and biochemical parameters in a pediatric population with HbSS and HbSC. We emphasize that the identification of markers indicative of a worse prognosis of SCD in children can greatly aid in the improved clinical management of patients.

CONCLUSION

The results presented herein suggest that pediatric patients with HbSS present higher levels of TGF-β1 than those with HbSC or healthy individuals. The fact that TGF-β1 was associated with TIMP-1 in both genotypes indicates that these molecules may play an important role in vascular remodeling and vasculopathy through extracellular matrix deposition. We suggest that since TGF-β was found to be associated with hemolysis, leukocytes, platelets, and lipid metabolism, this provides evidence that this immunomarker likely modulates the inflammatory response in SCD.

In sum, the full spectrum of biological effects provoked by TGF-β in SCD warrant further investigation, as evidence points to the involvement of this molecule in the pathogenesis of vascular disease.

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CONFLICTS OF INTEREST

The authors declare that no conflict of interest exist regarding this publication.

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Table 1. Laboratory profile of pediatric patients with sickle cell anemia (HbSS), hemoglobin SC disease (HbSC) and healthy controls (HC).

Parameter	HbSS N=84 (Mean ± SD)	HbSC N=39 (Mean ± SD)	HC N=59 (Mean ± SD)	*p* value
Hemoglobin pattern				
Fetal hemoglobin, %	9.79 ± 6.17	2.69 ± 1.42	0.63 ± 0.51	**< 0.0001**
S hemoglobin, %	86.64 ± 6.41	51.17 ± 6.31	-	**< 0.0001***
Hematological parameters				
RBC, $x10^6$/Ml	2.68 ± 0.59	4.30 ± 0.57	4.70 ± 0.34	**<0.0001**
Hemoglobin, g/Dl	7.83 ± 1.30	11.14 ± 1.23	12.90 ± 0.94	**< 0.0001**
Hematocrit, %	24.19 ± 4.00	34.73 ± 3.61	38.79 ± 2.59	**< 0.0001**
MCV, Fl	91.70 ± 10.54	80.67 ± 7.80	82.62 ± 4.77	**< 0.0001**
MCH, ρg	29.73 ± 3.66	25.89 ± 2.53	27.48 ± 1.79	**< 0.0001**
MCHC, %	32.39 ± 0.97	32.08 ± 0.94	33.25 ± 0.68	**< 0.0001**
Reticulocyte count, %	9.25 ± 4.63	4.06 ± 2.50	0.86 ± 0.28	**< 0.0001**
Leukocyte count, /mL	14734.52 ± 5090.44	9802.56 ± 5988.29	7313.56 ± 2448.04	**< 0.0001**
Neutrophil count, /mL	5972.00 ± 473.00	4349.00 ± 2095.00	3430.15 ± 1937.21	**< 0.0001**
Eosinophil count, /mL	751.70 ± 423.40	562.00 ± 462.20	452.36 ± 467.08	**< 0.0001**
Lymphocyte count, /mL	5953.00 ± 1973.00	3076.00 ± 1241.00	2889.17 ± 957.95	**< 0.0001**
Monocyte count, /mL	882.00 ± 311.10	459.90 ± 254.80	510.16 ± 221.06	**< 0.0001**
Platelet count, $x10^3$/ mL	439.18 ± 128.10	319.33 ± 175.30	313.73 ± 67.59	**< 0.0001**
Biochemical parameters				
Total Cholesterol, mg/dL	122.00 ± 25.38	119.00 ± 25.96	157.85 ± 33.41	**< 0.0001**
HDL-C, mg/dL	32.20 ± 9.40	41.62 ± 12.62	48.19 ± 14.75	**< 0.0001**
LDL-C, mg/dL	67.39 ± 22.50	59.77 ± 21.03	88.87 ± 33.30	**< 0.0001**
VLDL-C, mg/dL	22.36 ± 10.01	17.44 ± 5.20	19.60 ± 10.34	**0.0180**
Triglycerides, mg/dL	111.96 ± 50.02	87.87 ± 26.16	97.75 ± 51.47	**0.0150**
Total bilirubin, mg/dL	3.39 ± 1.81	1.75 ± 1.05	0.57 ± 0.23	**< 0.0001**
Direct bilirubin, mg/dL	0.81 ± 0.51	0.47 ± 0.27	0.23 ± 0.07	**< 0.0001**
Indirect bilirubin, mg/dL	2.58 ± 1.66	1.27 ± 0.97	0.30 ± 0.20	**< 0.0001**
LDH, U/L	1054.20 ± 522.09	518.38 ± 293.41	420.90 ± 87.15	**< 0.0001**
ALT, U/L	24.31 ± 15.90	20.17 ± 11.78	18.22 ± 8.12	**< 0.0001**
AST, U/L	56.79 ± 26.92	33.15 ± 14.94	33.78 ± 12.16	**< 0.0001**
Total protein, g/dL	7.31 ± 0.99	7.24 ± 0.67	7.08 ± 0.65	0.1450
Albumin, g/dL	3.99 ± 0.77	4.06 ± 0.54	4.19 ± 0.49	0.577
Globulin, g/dL	3.31 ± 0.86	3.17 ± 0.67	2.89 ± 0.55	**0.0080**
Ferritin, ηg/mL	265.20 ± 209.10	152.20 ± 102.90	31.16 ± 13.57	**< 0.0001**
Urea, mg/dL	17.55 ± 6.73	19.00 ± 6.49	21.87 ± 6.63	**< 0.0001**
Creatinine, mg/dL	0.43 ± 0.18	0.54 ± 0.15	0.58 ± 0.18	**< 0.0001**
A1AT, mg/dL	166.92 ± 40.75	139.95 ± 42.45	148.50 ± 44.04	**< 0.0001**

RBC: Red blood cells; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; Mean Corpuscular Hemoglobin Concentration; 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; A1AT: Alpha-1 antitrypsin; N: number; SD: Standard Deviation. All p-values obtained using the Kruskal-Wallis test, with the exception of (*), which indicates a p-value obtained from the ordinary one-way ANOVA.

FIGURE 1

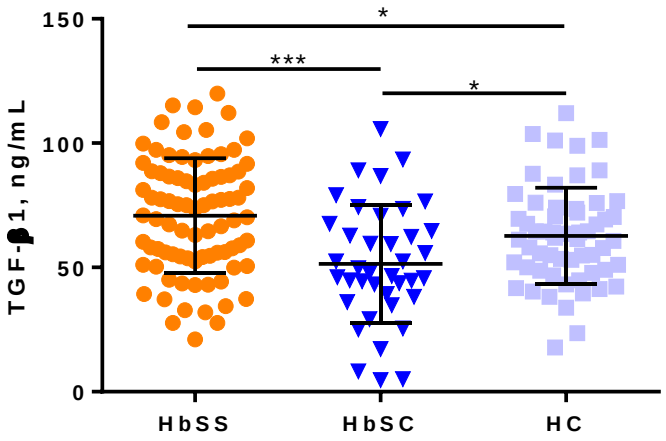


FIGURE 2

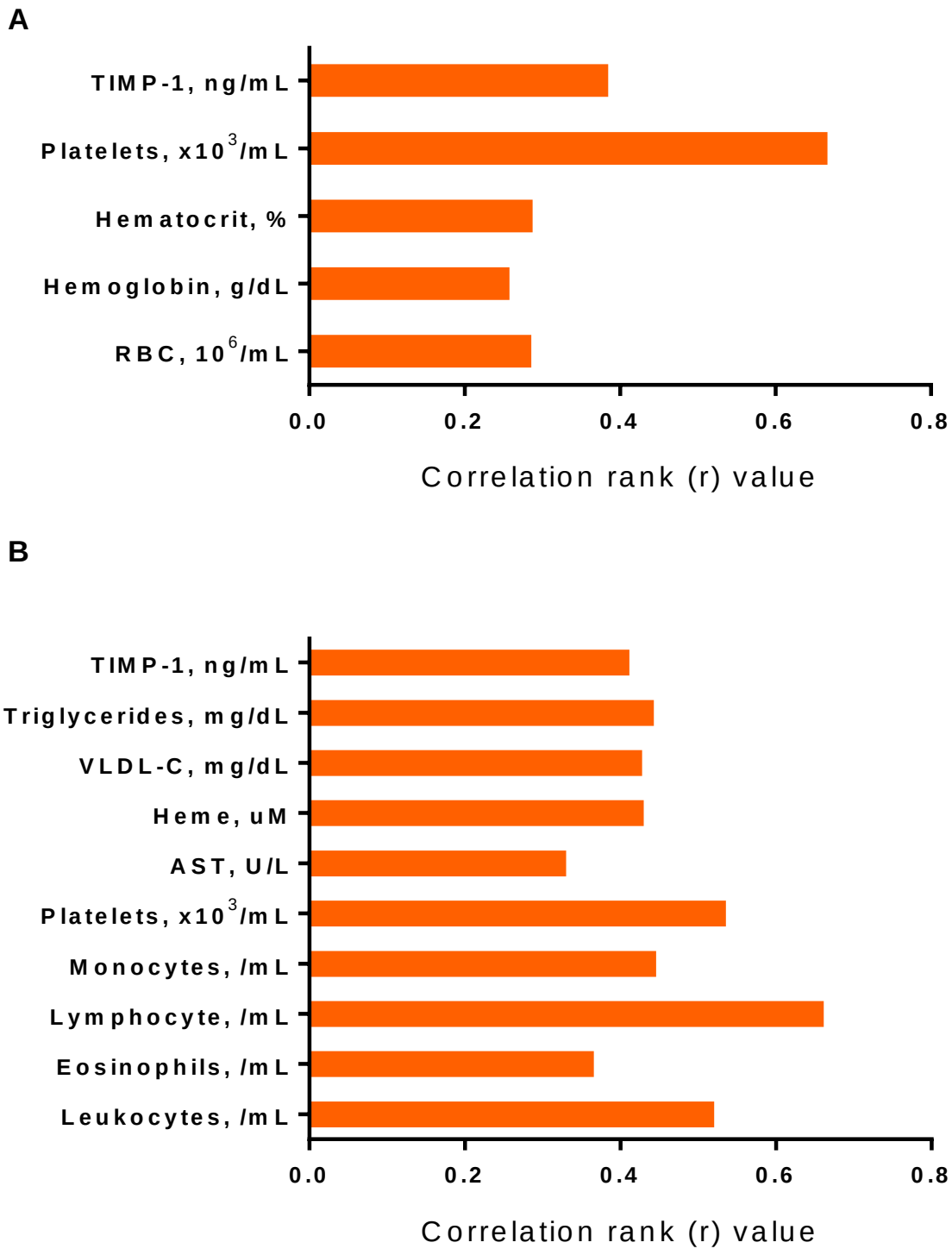

A
TIMP-1, ng/mL
Platelets, x10^3/mL
Hematocrit, %
Hemoglobin, g/dL
RBC, 10^6/mL
0.0
0.2
0.4
0.6
0.8
Correlation rank (r) value
B
TIMP-1, ng/mL
Triglycerides, mg/dL
VLDL-C, mg/dL
Heme, uM
AST, U/L
Platelets, x10^3/mL
Monocytes, /mL
Lymphocyte, /mL
Eosinophils, /mL
Leukocytes, /mL
0.0
0.2
0.4
0.6
0.8
Correlation rank (r) value

FIGURE 3

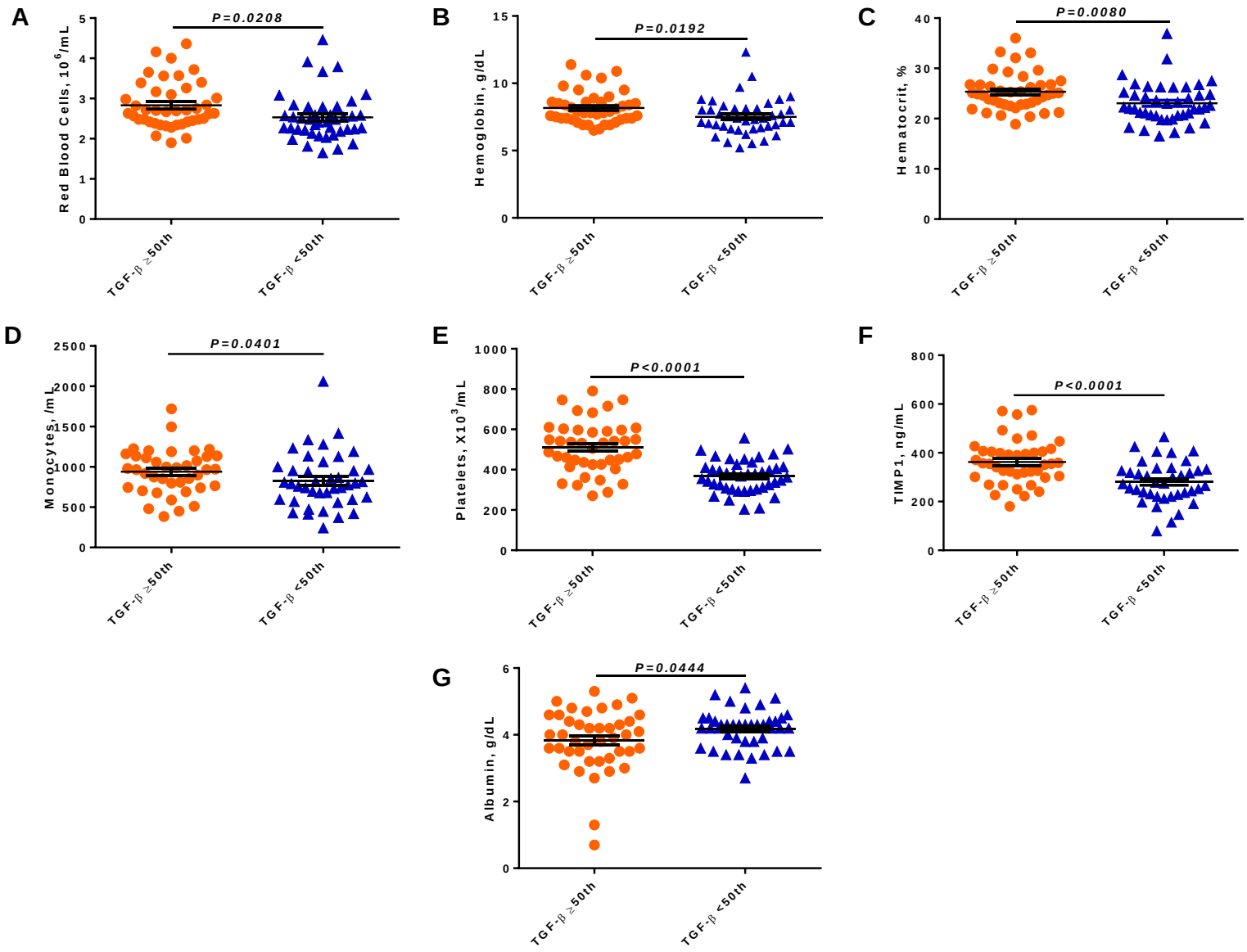

A
P=0.0208
Red Blood Cells, 10^6/mL
0
1
2
3
4
5
TGF-β ≥50th
TGF-β <50th
B
P=0.0192
Hemoglobin, g/dL
0
5
10
15
TGF-β ≥50th
TGF-β <50th
C
P=0.0080
Hematocrit, %
0
10
20
30
40
TGF-β ≥50th
TGF-β <50th
D
P=0.0401
Monocytes, /mL
0
500
1000
1500
2000
2500
TGF-β ≥50th
TGF-β <50th
E
P<0.0001
Platelets, X10^3/mL
0
200
400
600
800
1000
TGF-β ≥50th
TGF-β <50th
F
P<0.0001
TIMP1, ng/mL
0
200
400
600
800
TGF-β ≥50th
TGF-β <50th
G
P=0.0444
Albumin, g/dL
0
2
4
6
TGF-β ≥50th
TGF-β <50th

FIGURE 4

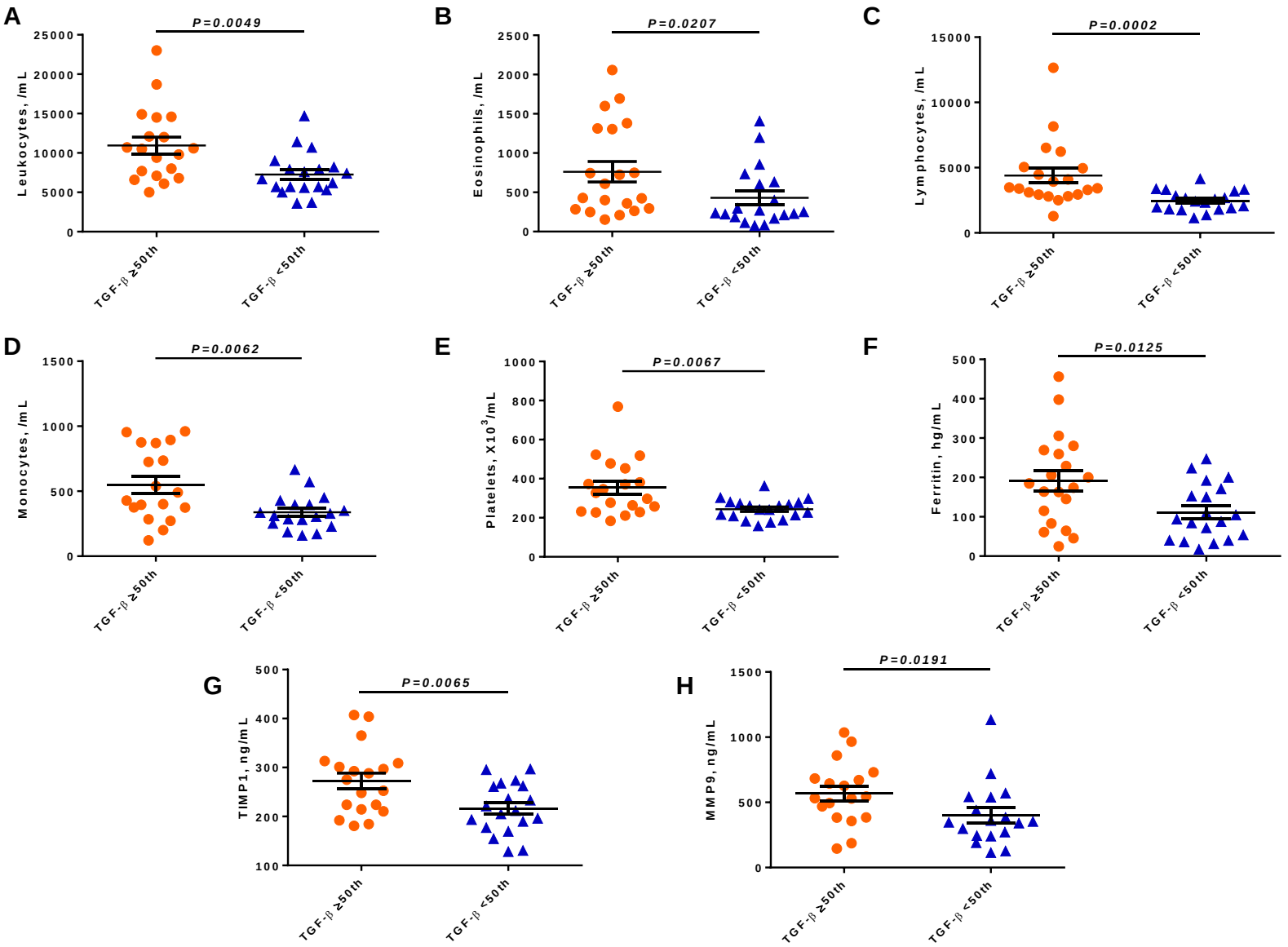
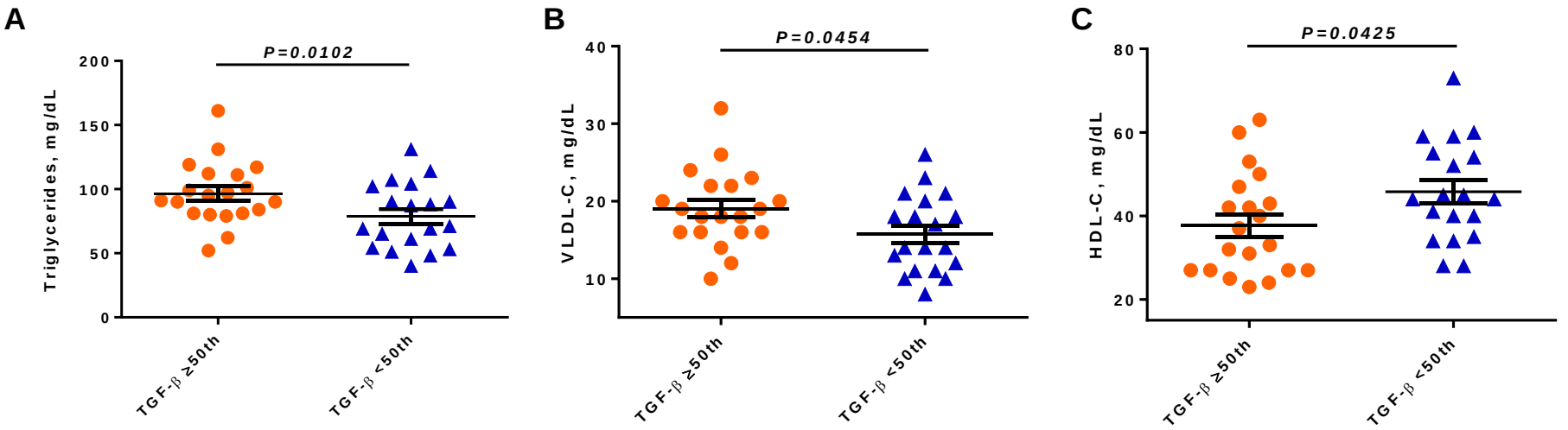

A
P=0.0049
Leukocytes, /mL
25000
20000
15000
10000
5000
0
TGF-β ≥50th
TGF-β <50th
B
P=0.0207
Eosinophils, /mL
2500
2000
1500
1000
500
0
TGF-β ≥50th
TGF-β <50th
C
P=0.0002
Lymphocytes, /mL
15000
10000
5000
0
TGF-β ≥50th
TGF-β <50th
D
P=0.0062
Monocytes, /mL
1500
1000
500
0
TGF-β ≥50th
TGF-β <50th
E
P=0.0067
Platelets, X10³/mL
1000
800
600
400
200
0
TGF-β ≥50th
TGF-β <50th
F
P=0.0125
Ferritin, hg/mL
500
400
300
200
100
0
TGF-β ≥50th
TGF-β <50th
G
P=0.0065
TIMP1, ng/mL
500
400
300
200
100
TGF-β ≥50th
TGF-β <50th
H
P=0.0191
MMP9, ng/mL
1500
1000
500
0
TGF-β ≥50th
TGF-β <50th

FIGURE 5


A
P=0.0102
Triglycerides, mg/dL
200
150
100
50
0
TGF-β ≥50th
TGF-β <50th
B
P=0.0454
VLDL-C, mg/dL
40
30
20
10
TGF-β ≥50th
TGF-β <50th
C
P=0.0425
HDL-C, mg/dL
80
60
40
20
TGF-β ≥50th
TGF-β <50th

FIGURES LEGENDS

Figure 1. TGF-β1 plasmatic levels in individuals with HbSS, HbSC and healthy controls (HC). * $p<0.05$; *** $p<0.0001$

Figure 2. Correlations between TGF-β1 levels and laboratory biomarkers in individuals with HbSS and HbSC. A) Positive correlations between TGF-β1 levels and RBC, Hb, Ht, platelet and TIMP-1 in individuals with HbSS. B) Positive correlations between TGF-β1 levels and leukocytes, eosinophils, lymphocytes, monocytes, platelets, AST, VLDL-C, triglycerides, heme, and TIMP-1 in HbSC individuals.

Figure 3. Associations between TGF-β1 levels and laboratory biomarkers as well as the glycoprotein TIMP-1 in individuals with HbSS. Individuals with TGF-β1 levels above the median (TGF-β1 ≥ 72.29) exhibited increased A) RBC counts, B) hemoglobin levels, C) hematocrit, D) monocyte counts, E) platelet counts and F) TIMP-1 levels, as well as G) decreased albumin levels.
All p values obtained by the Mann–Whitney U test, except for TIMP-1 and platelet counts, for which the independent t-test was used.

Figure 4. Associations between TGF-β1 levels and laboratory biomarkers as well as the glycoprotein TIMP-1 and MMP-9 among individuals with HbSC. Individuals with TGF-β1 levels above the median (TGF-β1 ≥ 47.80) exhibited increased A) leukocytes, B) eosinophils, C) lymphocytes, D) monocytes and E) platelets, as well as higher levels of F) ferritin, G) TIMP-1 and H) MMP-9. All p values obtained by the Mann–Whitney U test, except for leukocytes, monocytes, ferritin, and TIMP-1, for which the independent t-test was used.

Figure 5. Associations between TGF-β1 levels and lipid biomarkers in individuals with HbSC. Individuals with TGF-β1 levels above the median (TGF-β1 ≥ 47.80) exhibited A) increased triglycerides, B) increased VLDL-C and decreased HDL-C levels (all p values obtained by the independent t-test).

6.2 MANUSCRITO 2

Título: *TGFBR3* polymorphisms (rs1805110 and rs7526590) are associated with laboratory biomarkers and clinical manifestations in sickle cell anemia

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Situação: Publicado na *Disease Markers* – DOI: 10.1155/2020/8867986

Objetivos:

- Avaliar os marcadores hemolíticos, renais, hepáticos, lipídicos, inflamatórios, bem como marcadores relacionados à disfunção endotelial em indivíduos com DF;
- Investigar a frequência dos polimorfismos rs1805110, rs2038931, rs2765888, rs284157, rs284875 e rs7526590 no gene *TGFBR3* em indivíduos com DF;
- Investigar as associações entre polimorfismos no gene *TGFBR3* e os biomarcadores investigados, bem como as complicações clínicas apresentadas por estes indivíduos.

Principais resultados:

O alelo variante A do polimorfismo *rs1805110* esteve associado a concentrações elevadas de hemoglobina, hematócrito, LDL-C, ácido úrico e contagem de reticulócitos. Encontrou-se a associação ao PDW e alterações ósseas. O alelo variante T do polimorfismo *rs7526590* esteve associado aos níveis elevados de RDW, fosfatase alcalina, aspartato aminotransferase, bilirrubina indireta e lactato desidrogenase, índice elevado de PDW, níveis diminuídos de ferritina e ocorrência de úlceras de perna. Os resultados encontrados sugerem que o alelo variante A do polimorfismo *rs1805110* pode influenciar o estado inflamatório e hemolítico na DF, enquanto o alelo variante T do polimorfismo *rs7526590* pode influenciar as características descritas no sub-fenótipo hemolítico e suas manifestações clínicas.

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Research Article

TGFBR3 Polymorphisms (rs1805110 and rs7526590) Are Associated with Laboratory Biomarkers and Clinical Manifestations in Sickle Cell Anemia

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Individuals with sickle cell anemia (SCA) present chronic anemia, hemolysis, an exacerbated inflammatory response, and heterogeneous clinical complications, which may be modulated by the transforming growth factor beta (TGF-β) pathway. Thus, we aimed to investigate polymorphisms (*rs1805110* and *rs7526590*) of the transforming growth factor beta receptor III gene (*TGFBR3*) with regard to laboratory biomarkers and clinical manifestations in individuals with SCA. Hematological, biochemical, immunological, and genetic analyses were carried out, as well as serum endothelin-1 measurements. The minor allele (A) of the *TGFBR3 rs1805110* polymorphism was associated with increased hemoglobin, hematocrit, reticulocyte counts, total cholesterol, low-density lipoprotein, uric acid, and endothelin levels, as well as decreased platelet distribution width (PDW) and the occurrence of bone alterations. The minor allele (T) of *TGFBR3 rs7526590* was associated with increased red cell distribution width, PDW, alkaline phosphatase, aspartate aminotransferase, total and indirect bilirubin, and lactate dehydrogenase levels, as well as lower ferritin levels and the occurrence of leg ulcers. Our data suggest that the minor allele (A) of *TGFBR3 rs1805110* is associated with inflammation and bone alterations, while the minor allele (T) of *TGFBR3 rs7526590* is related to hemolysis and the occurrence of leg ulcers.

1. Introduction

Sickle cell anemia (SCA) is an inherited hematological disorder characterized by the presence of the beta allele S (β^S) in homozygosis. SCA, the most common and severe form of sickle cell disease (SCD), is associated with more prominent hemolytic anemia, vaso-occlusive events (VOE), and a broad spectrum of clinical complications [1, 2].

Clinical manifestations in SCA are closely related to two main pathophysiological mechanisms: hemolysis and vaso-occlusion. Hemolysis reduces the bioavailability of nitric oxide (NO), which alters the homeostasis of vascular functions,

triggering several clinical manifestations, such as pulmonary hypertension (PH), priapism, and stroke. Sickle erythrocyte adherence can also occur due to decreased NO bioavailability, leading to VOE, acute chest syndrome (ACS), and osteonecrosis [3–5].

In accordance with the heterogeneity and complexity of SCD, three subphenotypes have been established: viscosity vaso-occlusive, hemolysis-endothelial dysfunction, and dyslipidemic [3, 4]. The former subphenotype refers to individuals with high hemoglobin levels and an elevated frequency of clinical manifestations associated with the sickling of red blood cells (RBC), such as osteonecrosis, ACS, VOE, and pain crisis [3]. Differently, the hemolysis-endothelial dysfunction subphenotype characterizes individuals with more intense anemia and hemolysis who present clinical manifestations including stroke, leg ulcers, priapism, and PH [3, 6]. In the dyslipidemic subphenotype, individuals present an inflammatory profile decreased levels of low-density lipoproteins (LDL-C), high-density lipoprotein (HDL-C), and NO [4].

The influence of an individual's genetic background on the spectrum of clinical manifestations in SCD has prompted the search for novel biomarkers of disease severity. In this context, the transforming growth factor beta receptor III (*TGFBR3*) gene, also known as betaglycan, which is expressed in endothelial and hematopoietic cells, fibroblasts, and other cell types, is thought to be a candidate genetic factor that modulates SCA severity [7]. *TGFBR3* encodes a receptor of the transforming growth factor beta (TGF-β) family, TGF-β type III receptor (TβRIII), which presents affinity to all three TGF-β isoforms [8]. Polymorphisms in *TGFBR3* have been previously associated with clinical manifestations in SCD, such as pain crisis, ACS, infection, stroke, leg ulcers, priapism, osteonecrosis, and PH [7, 9–15]. A previous report suggested the association of *TGFBR3 rs7526590* with priapism in individuals with SCD [9]. Another polymorphism, *TGFBR3 rs1805110*, despite not being previously investigated in the context of SCD, was associated with Behcet's disease, an inflammatory disorder in the Chinese population characterized by blood vessel inflammation [16].

Carvalho et al. described lower TGF-β levels in individuals with SCD in crisis state in comparison to those in steady state or healthy volunteers [17]. The TGF-β pathway, which consists of TGF-β, activins, and bone morphogenetic proteins (BMP), has been implicated in a shortened life expectancy in individuals with SCD [18]. Moreover, inflammation, hematopoiesis, immune response, angiogenesis, and other cellular processes are known to be regulated by the TGF-β pathway [11]. Considering that these processes are also related to the pathogenesis of SCA, we endeavored to investigate polymorphisms *TGFBR3 rs1805110* and *rs7526590* with respect to associations between laboratory biomarkers and clinical manifestations in individuals with SCA.

2. Material and Methods

2.1. Subjects. The present cross-sectional study included 120 individuals with SCA, 54 (45%) females, all seen at the Bahia State Hematology and Hemotherapy Foundation (HEMOBA) from 2016 to 2017. All SCA patients were in steady state, defined as the absence of acute episodes as well as any significant medical support within the three months prior to inclusion. Median participant age was 15 years (IQR 12-17).

The research protocol was approved by the Institutional Research Board of the São Rafael Hospital (HSR protocol number 1400535) and was conducted in accordance with the Declaration of Helsinki (1964) and its subsequent revisions. All individuals or their legal guardians agreed to the biological sample collection procedures and provided signed terms of informed consent. Data pertaining to clinical manifestations was collected from patient medical records.

2.2. Hematological and Biochemical Parameters. Blood samples were collected by HEMOBA staff following a fasting period of no less than 12 hours. Hematological and biochemical parameter analysis was performed at the Clinical Analyses Laboratory of the College of Pharmaceutical Sciences, Federal University of Bahia (FACFAR-UFBA), while genetic and immunological analyses were performed at the Laboratory of Genetic Investigation and Translational Hematology at the Gonçalo Moniz Institute-FIOCRUZ (LIGHT-IGM/FIOCRUZ), both located in Salvador, Bahia-Brazil.

A Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) was used to quantify hematological parameters. Hemoglobin profiles were determined by high-performance liquid chromatography using an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA).

Biochemical parameters, including lipid profile (total cholesterol and high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides), renal profile (urea and creatinine), hepatic profile (alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl-transferase, alkaline phosphatase, uric acid, total protein, and fractions), total bilirubin and fractions, iron, and lactate dehydrogenase (LDH) were determined using an automated A25 chemistry analyzer (Biosystems S.A., Barcelona, Catalunya, Spain). C-reactive protein (C-RP) and alpha-1 antitrypsin (AAT) levels were measured using the IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). Ferritin levels were measured using the Access 2 Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA).

Serum endothelin-1 levels were measured using an Endothelin-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's protocol.

2.3. Genetic and Linkage Disequilibrium Analyses. Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Westphalia, Germany) following the manufacturer's recommendations. *TGFBR3* polymorphisms (rs1805110 and rs7526590) were detected, and genotyping was performed using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Table 1: Genotype frequencies of *TGFBR3* polymorphisms.

Individuals	Genotype frequency					
	rs1805110 (G>A)			rs7526590 (A>T)		
	G/G	G/A	A/A	A/A	A/T	T/T
Sickle cell anemia ($N = 120$)	85 (0.71)	33 (0.28)	2 (0.02)	81 (0.68)	37 (0.31)	2 (0.02)

Proportions of genotype frequency are indicated in parentheses.

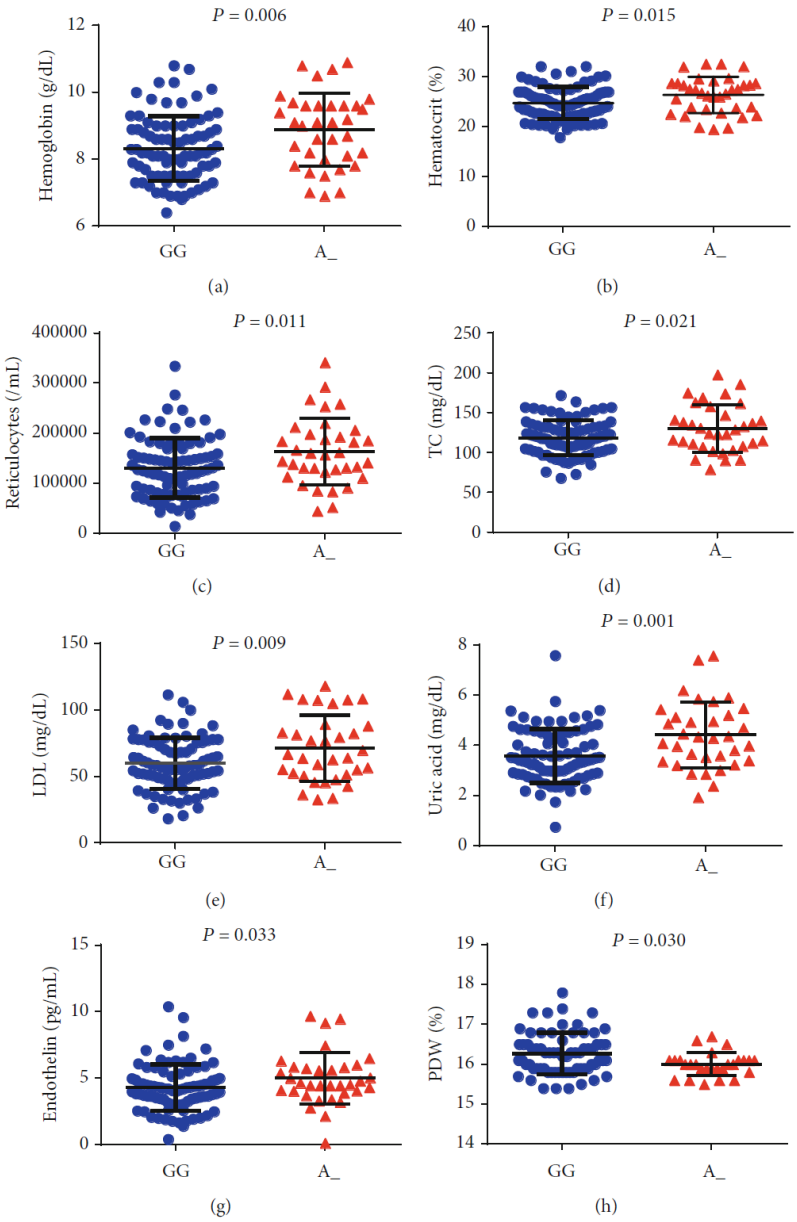

Figure 1: Association of *TGFBR3 rs1805110* polymorphism with laboratory biomarkers using the dominant genetic model. SCA individual carriers of the minor allele A (A/G+A/A) presented increased (a) hemoglobin, (b) hematocrit, (c) reticulocyte counts, (d) total cholesterol, (e) LDL-C, (f) uric acid, and (g) endothelin levels as well as (h) decreased PDW. All p values obtained by the Mann–Whitney U test, except for hemoglobin, hematocrit, and LDL-C, for which the independent t-test was used.

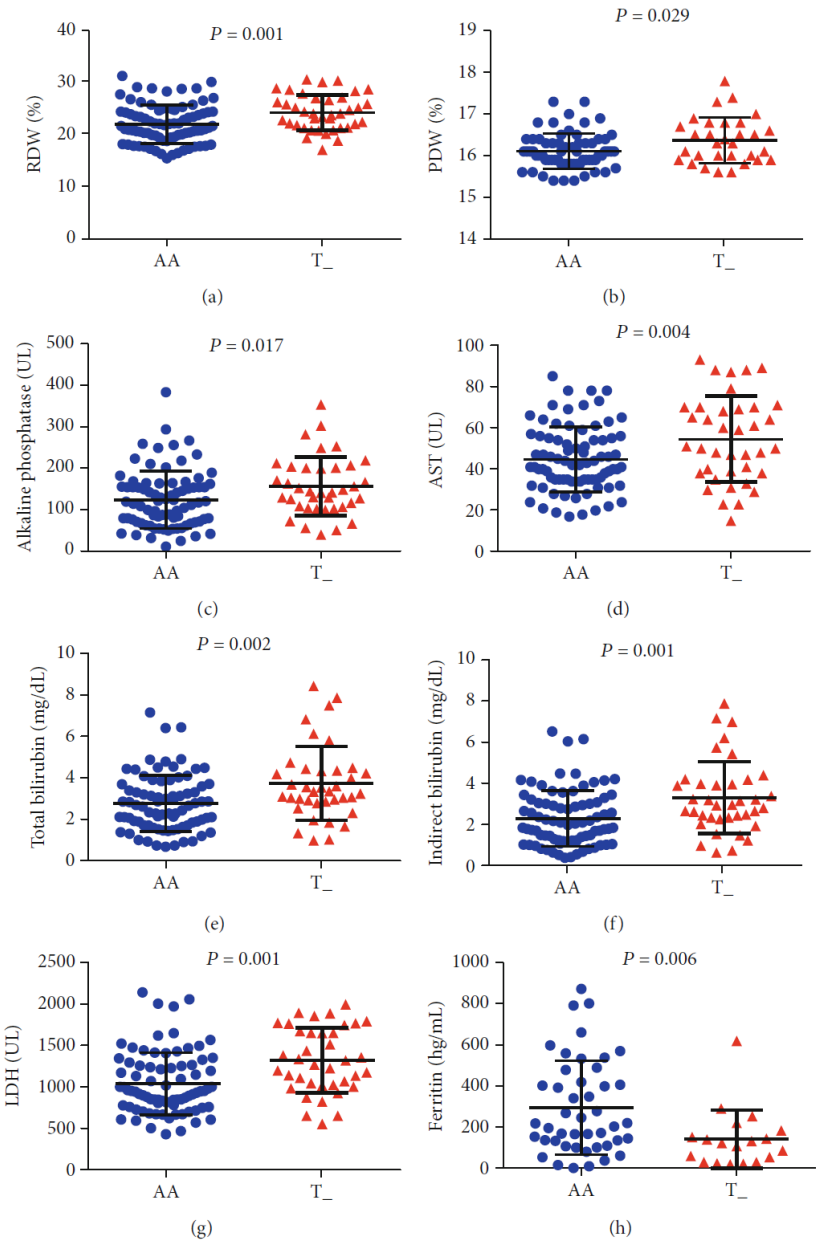

FIGURE 2: Association of *TGFBR3 rs7526590* polymorphism with laboratory biomarkers using the dominant genetic model. SCA individual carriers of the minor allele T (AT+TT) presented increased (a) RDW, (b) PDW, (c) alkaline phosphatase, (d) aspartate aminotransferase (AST), (e) total bilirubin, (f) indirect bilirubin, and (g) LDH, as well as (h) decreased ferritin levels. All p values obtained by the Mann–Whitney U test, except for RDW and AST, for which the independent t-test was used.

Linkage disequilibrium between these two SNPs was determined using the SNPStats web tool, considering D^2 and r values. [19]

2.4. Statistical Analysis. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) v. 20.0 software (IBM, Armonk, New York, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA). Significance was considered when $p < 0.05$. The Shapiro-Wilk test was used to determine the distribution of quantitative variables. The Mann–Whitney U test or independent t-test was used to compare two numerical variables depending on distribution. Hardy–Weinberg equilibrium (HWE) was assessed using the chi-squared test (χ^2 test). Associations between polymorphisms and clinical data were performed using Fisher's exact test or the χ^2 test with Yates correction.

3. Results

3.1. Individual Characteristics. S1 Table lists the hematological and biochemical laboratory parameters of SCA patients, with results expressed as means ± standard deviation and median (IQR).

3.2. Polymorphism Frequencies. With regard to the distribution of the frequencies of the *TGFBR3 rs1805110* genetic polymorphism, 71% (85/120) of the patients were homozygous for the ancestral allele (G/G), 28% (33/120) were heterozygous (G/A), and 2% (2/120) were homozygous for the minor allele (A/A) (Table 1). The analysis of *TGFBR3 rs7526590* revealed that 68% (81/120) were homozygous for the ancestral allele (A/A), 31% (37/120) were heterozygous (A/T), and 2% (2/120) were homozygous for the minor allele (T/T) (Table 1). Both polymorphisms were found to be in Hardy–Weinberg equilibrium.

3.3. Linkage Disequilibrium Analysis. Linkage disequilibrium (LD) calculations revealed that *rs1805110* and *rs7526590* were not in LD ($D^2 = 0.09$; $r = -0.0182$) and, consequently, were not inherited as a haplotype.

3.4. Associations between Laboratory Biomarkers and Polymorphisms TGFBR3 rs1805110 and rs7526590. The dominant genetic model was employed in all analyses to evaluate associations between minor alleles and laboratory biomarkers. With regard to *TGFBR3 rs1805110*, carriers of the minor allele (A/G+A/A) presented increased hemoglobin (Hb) ($p = 0.006$), hematocrit (Ht) ($p = 0.015$), reticulocyte counts ($p = 0.011$), total cholesterol ($p = 0.021$) LDL-C ($p = 0.009$), uric acid ($p = 0.001$), and endothelin levels ($p = 0.033$), as well as decreased PDW ($p = 0.030$) (Figure 1).

Regarding the *TGFBR3 rs7526590* polymorphism, carriers of the minor allele (AT+TT) presented increased RDW ($p = 0.001$), PDW ($p = 0.029$), alkaline phosphatase ($p = 0.017$), aspartate aminotransferase ($p = 0.004$), total bilirubin ($p = 0.002$), indirect bilirubin ($p = 0.001$), and LDH ($p = 0.001$), as well as decreased ferritin ($p = 0.006$) (Figure 2).

3.5. Associations between Clinical Manifestations and Polymorphisms TGFBR3 rs1805110 and rs7526590. The dominant genetic model was used to investigate associations between the minor alleles of the investigated polymorphisms and clinical manifestations.

The minor allele (A/G+A/A) of the *TGFBR3 rs1805110* polymorphism was found to be associated with a previous history of bone alterations ($p = 0.006$) (Figure 3) (Table 2).

The minor allele of *TGFBR3 rs7526590* was associated with a history of leg ulcers ($p = 0.037$) (Table 2). We also observed that individuals with a previous history of leg ulcers (LU+) presented higher AST ($p = 0.006$) and LDH levels ($p = 0.004$) (Figure 4) than those without leg ulcers (LU-). Importantly, all individuals with a history of leg ulcers who were carriers of the T allele presented increased AST levels ($p = 0.024$).

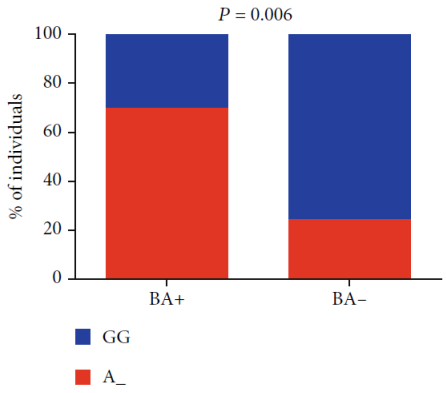

Figure 3: Association of *TGFBR3 rs1805110* with bone alteration occurrence (BA) among SCA individuals (p value was obtained by Fisher's exact test).

4. Discussion

It has been previously described that *TGFBR3* encodes TβRIII, which is able to bind to all three isoforms of TGF-β [8]. The TGF-β pathway is involved in the regulation of inflammation, hematopoiesis, immune response, angiogenesis, and other cellular processes, which are relevant in the context of SCD [11]. Thus, we chose to investigate associations between the *TGFBR3 rs1805110* and *rs7526590* polymorphisms with laboratory biomarkers and clinical manifestations in a group of steady-state SCA patients.

We identified that individuals who carried the minor allele (A/G+A/A) of the *TGFBR3 rs1805110* polymorphism presented increased Hb, Ht, reticulocyte counts, LDL-C, uric acid, and endothelin levels, as well as decreased PDW. In addition, individuals with the minor allele A presented milder anemia when compared with the GG genotype. Our results suggest that inflammation was more prominent in these individuals than in carriers of the GG genotype. LDL-C plays an important proinflammatory role in vascular disease [20]. LDL-C attaches to the walls of blood vessels and becomes oxidized by reactive oxygen species (ROS), resulting in oxidized LDL. In SCA, LDL-C is more susceptible to oxidation and is considered an important marker of oxidative stress and vasculopathy [21]. Additionally, uric acid can accelerate the oxidative process in mildly oxidized LDL-C, contributing to endothelial activation and oxidative stress [22]. A previous study demonstrated the role of uric acid in activating the inflammasome pathway in individuals with SCA, leading to a proinflammatory state. These authors also demonstrated the participation of this biomarker in inflammatory events associated with SCD [23].

Carriers of the minor allele (A/G+A/A) of the *TGFBR3 rs1805110* polymorphism also had higher endothelin levels, which suggests a greater propensity of presenting vasoconstriction events and endothelial dysfunction. TGF-β is one of the factors that induces endothelin-1 expression by way of the overexpression of Smad3 [24]. Endothelin is a mediator produced by endothelial and immune cells, as well as neurons. This molecule acts as a vasoconstrictor by activating

Table 2: Association of *TGFBR3 rs1805110* and rs7526590 polymorphism with clinical manifestation in SCA.

Clinical manifestation	Polymorphisms					
	rs1805110		*p* value	rs7526590		*p* value
	GG ($N = 85$)	AG+AA ($N = 35$)		AA ($N = 81$)	AT+TT ($N = 39$)	
Acute chest syndrome	19	12	0.259	22	9	0.797
Bone alterations	3	7	**0.006***	7	3	1.000*
Cholelithiasis	27	9	0.661	26	10	0.609
Infections	60	22	0.540	52	30	0.232
Leg ulcer	9	2	0.506	4	7	**0.037***
Pneumonia	42	24	0.086	47	19	0.444
Painful crises	48	25	0.186	53	20	0.197
Splenomegaly	34	21	0.072	38	17	0.883
Stroke	8	4	0.744*	9	3	0.749*
Vaso-occlusive events	28	14	0.598	28	14	0.951

Bold values indicate significance at $p < 0.05$. All *p* values obtained by the chi-squared test, except for those with asterisk (*), for which the Fisher exact test was used.

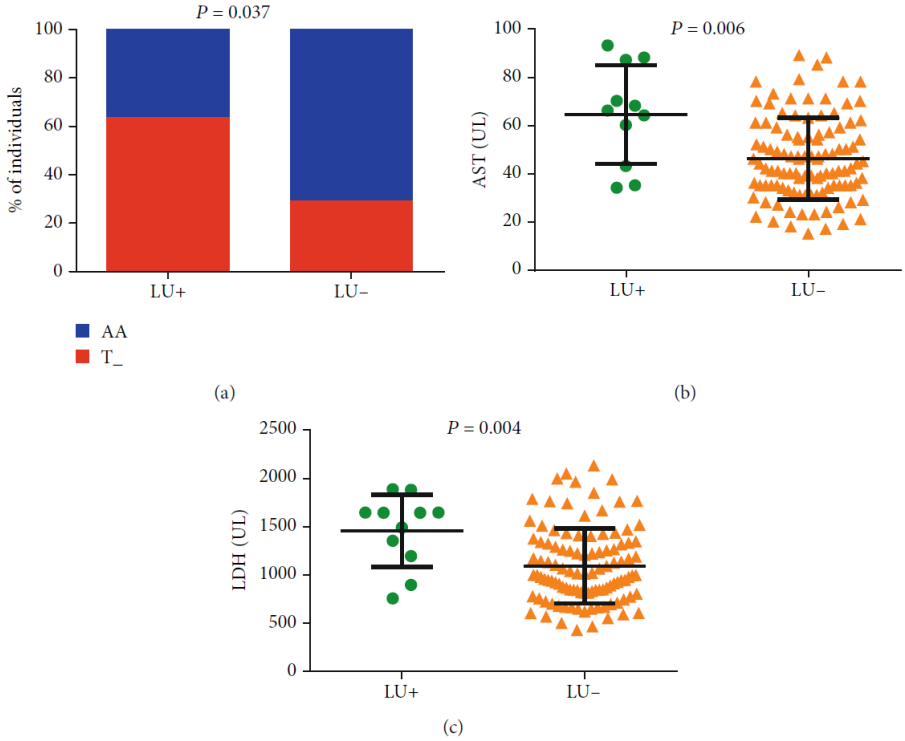

Figure 4: Association of TGFBR3 rs7526590 polymorphism of leg ulcer occurrence (LU). (a) SCA individual carriers of the allele T of TGFBR3 rs7526590 had high leg ulcer occurrence (*p* value was obtained with Fisher's exact test). SCA individuals with leg ulcers had high (b) AST and (c) LDH levels (*p* values were obtained by Mann–Whitney *U* test).

endothelial cells and promoting vascular inflammation [25, 26]. In addition, these individuals also presented decreased PDW, indicating better platelet uniformity than in individuals with the GG genotype [27]. An in vitro evaluation showed that TGF-β1 can inhibit erythropoiesis, consequently affecting thrombopoiesis, by blocking the proliferation of erythroid progenitors and accelerating differentiation in these cells [28].

The minor allele (A) of the *TGFBR3 rs1805110* polymorphism was also associated with the occurrence of bone alterations, such as osteonecrosis of the femoral head and osteomyelitis, both clinical complications commonly seen in individuals with SCD [29]. A previous study demonstrated that other polymorphisms in *TGFBR3* were associated with osteonecrosis in individuals with SCD [15]. Furthermore, the *TGFBR3 rs1805110* polymorphism was also found to be

associated with Behcet's disease in both Caucasians and the Chinese Han population [16, 30]. Behcet's disease is a multisystem inflammatory disease, which can lead to the development of osteonecrosis and bone infarction in some cases [30, 31].

Our association analysis further identified high PDW in SCA patients who carried the minor allele (T) of the *TGFBR3 rs7526590* polymorphism. A previous study found that individuals with SCD presented increased PDW, suggesting bone marrow hyperplasia with the release of subfunctional platelets [27]. In addition, another study demonstrated the involvement of TGF-β in hematopoiesis through the control of behavior, quiescence, and the renewal of hematopoietic stem cells [32].

High levels of alkaline phosphatase, an indicator of tissue injury, were identified in SCA individuals who carried the minor allele (T) of the *TGFBR3 rs7526590* polymorphism. Previous studies have also observed high alkaline phosphatase levels in individuals with SCA [33, 34], and the literature attributes these high levels to intrahepatic sinusoidal sickling capable of damaging liver tissue [34].

The SCA individuals who carried the minor allele (T) of the *TGFBR3 rs7526590* polymorphism also presented increased RDW and higher indirect markers of intravascular hemolysis, such as AST, IB, and LDH, as well as decreased ferritin levels, which is suggestive of an association with hemolysis. During intravascular hemolysis, RBCs release LDH and AST simultaneously with hemoglobin and arginase into the bloodstream [6, 35]. In extravascular hemolysis, macrophages in the spleen and liver remove senescent and damaged RBCs, leading to the release of the heme group of hemoglobin, which is converted into unconjugated bilirubin [5]. Individuals with SCA present the most severe form of SCD due to more pronounced hemolysis have more intensive anemia and frequently present complications arising from hemolysis [1, 36].

The minor allele (T) of the *TGFBR3 rs7526590* polymorphism was found to be associated with the occurrence of leg ulcers. A recent study demonstrated that this polymorphism was associated with priapism in individuals with SCD [9]. Leg ulcers and priapism were classified by Kato et al. [3] as part of the hemolytic subphenotype, which is reinforced by Taylor et al. [37] who reported that PH, leg ulcers, and priapism were more prevalent in individuals with SCD and hyperhemolysis syndrome. Our results indicate that SCA individuals with a prior history of leg ulcers present higher levels of hemolytic markers, AST, and LDH than individuals who did not report this condition. Indeed, several studies have associated high AST and LDH levels with leg ulcers and priapism in individuals with SCD [3, 6, 38, 39]. A further analysis of the SCA individuals with a previous history of leg ulcers identified that those who carried the minor allele of the *TGFBR3 rs7526590* polymorphism presented increased AST levels. This finding serves to reinforce the association of the minor allele in this polymorphism with a hemolytic subphenotype and the occurrence of hemolysis in SCA.

Our findings suggest that *rs1805110* seems to be related to inflammation, and *rs7526590* appeared to be linked to hemolysis and clinical manifestations, especially the occurrence of leg ulcers arising from a hemolytic process. Collectively, our results corroborate the role of hemolytic parameters in addition to clinical manifestations associated with *TGBR3* polymorphism, highlighting the relevance investigating novel biomarkers of disease severity in the clinical management of individuals with SCA. To the best of our knowledge, the present study is the first attempt to demonstrate associations between these polymorphisms and hematological and biochemical parameters in SCD.

5. Conclusion

The results presented herein suggest that the minor allele (A) of *TGFBR3* rs1805110 is associated with an inflammatory state and the occurrence of bone alterations in SCA, while the minor allele (T) of *TGFBR3* rs7526590 is associated with a hemolytic subphenotype and related clinical manifestations, such as leg ulcers. Thus, we suggest that gene *TGFBR3* plays an important role in the physiopathology of SCA. Further studies are essential to evaluate *TGFBR3* as a prognostic marker and identify possible therapeutic targets in individuals suffering from SCD. It is important to emphasize that although this study focused on pediatric individuals, disease complications tend to worsen with increasing age.

Data Availability

All relevant data used to support the findings of this study are included within the article and the supplementary information file.

Disclosure

The sponsors of this study, who played no role in gathering, analyzing, or interpreting the data presented herein, are public organizations whose role is to support science in general.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

See Supplementary Table 1 in the Supplementary Material for comprehensive data analysis. *(Supplementary Materials)*

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Supplemental Table 1. Laboratory profiles of individuals with sickle cell anemia.

Parameter	HbSS N=120 (Mean ± SD)
Hemoglobin pattern	
Fetal hemoglobin, %	9.05 ± 5.68
S hemoglobin, %	84.05 ± 9.22
Hematological markers	
RBC, $x10^6$/mL	2.74 ± 0.47
Hemoglobin, g/dL	8.48 ± 1.03
Hematocrit, %	25.18 ± 3.39
MCV, fL	92.64 ± 11.74
MCH, ρg	31.41 ± 4.01
MCHC, %	33.92 ± 1.04
Reticulocyte count, %	4.98 ± 1.92
Leukocyte count, /mL	11424.75 ± 3168.73
Neutrophil count, /mL	5522.78 ± 2425.85
Eosinophil count, /mL	398.96 ± 274.21
Lymphocyte count, /mL	4146.18 ± 1335.15
Monocyte count, /mL	1087.63 ± 543.49
Platelet count, $x10^3$/ mL	421.28 ± 135.02
MPV (fL)	7.91 ± 0.82
PDW (%)	16.19 ± 0.48
Biochemical markers	
Total Cholesterol, mg/dL	121.85 ± 24.89
HDL-C, mg/dL	35.85 ± 8.86
LDL-C, mg/dL	63.14 ± 21.53
VLDL-C, mg/dL	20.31 ± 7.09
Triglycerides, mg/dL	101.75 ± 35.27
Total bilirubin, mg/dL	3.00 ± 1.56
Direct bilirubin, mg/dL	0.41 ± 0.16
Indirect bilirubin, mg/dL	2.59 ± 1.54
LDH, U/L	1135.73 ± 399.98
ALT, U/L	19.27 ± 10.09
AST, U/L	47.81 ± 17.97
Total protein, g/dL	8.42 ± 0.87
Albumin, g/dL	4.79 ± 0.33
Globulin, g/dL	3.63 ± 0.74
Alkaline phosphatase, U/L	133.81 ± 71.35
Iron serum, mcg/dL	103.28 ± 41.39
Ferritin, ηg/mL	260.04 ± 215.27
Urea, mg/dL	17.22 ± 5.65
Creatinine, mg/dL	0.43 ± 0.14
C-reactive protein, mg/L	4.09 ± 2.58
Alpha 1 antitrypsin, mg/dL	81.15 ± 44.56
Uric acid, mg/dL	3.81 ± 1.20
Endothelin, pg/mL	4.47 ± 1.83

RBC: Red blood cells; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular 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; AST: Aspartate aminotransferase; ALT: alanine aminotransferase; LDH: Lactate dehydrogenase; MPV: mean platelet volume; PDW: platelet distribution width; N: number; SD: Standard Deviation.

6.3 MANUSCRITO 3

Título: Transforming growth factor beta receptor 3 haplotypes in sickle cell disease are associated with lipid profile and clinical manifestations

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Situação: Aceito para publicação na *Mediators of Inflammation* – ID: 3185015

Objetivos:

- Avaliar os marcadores hemolíticos, renais, hepáticos, lipídicos, inflamatórios, bem como marcadores relacionados à disfunção endotelial em indivíduos com DF;
- Investigar a frequência dos polimorfismos rs1805110, rs2038931, rs2765888, rs284157, rs284875 e rs7526590 no gene *TGFBR3* em indivíduos com DF;
- Identificar os haplótipos no gene *TGFBR3* e associá-los a biomarcadores hematológicos, bioquímicos, genéticos, imunológicos e a complicações clínicas apresentadas pelos indivíduos com DF.

Principais resultados:

Os indivíduos com DF e portadores do haplótipo GG apresentaram níveis mais elevados de colesterol total (TC), lipoproteína de baixa densidade (LDL-C), triglicérides, colesterol não HDL (não-HDL-C), proteínas totais e globulina que aqueles portadores do haplótipo não-GG. Além disso, o haplótipo GG esteve associado com a ocorrência de pneumonia. Os indivíduos com DF portadores do haplótipo não-GG apresentaram ocorrência maior de colelitíase. Indivíduos com DF e o haplótipo CGG apresentaram níveis elevados de plaquetócrito, TC, LDL-C e não-HDL-C. Esse mesmo haplótipo, ainda esteve associado à ocorrência de pneumonia.

Transforming growth factor beta receptor 3 haplotypes in sickle cell disease are associated with lipid profile and clinical manifestations

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Abstract

Individuals with sickle cell disease (SCD) present both chronic and acute inflammatory events. The TGF-β pathway is known to play a role in immune response, angiogenesis, inflammation, hematopoiesis, vascular inflammation and cell proliferation. Polymorphisms in the transforming growth factor beta receptor 3 (*TGFBR3*) gene have been linked to several inflammatory diseases. This study investigated associations between two *TGFBR3* haplotypes and classical laboratory parameters, as well as clinical manifestations, in SCD. We found that individuals with the GG haplotype presented higher levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), triglycerides, non-HDL cholesterol, total proteins and globulin than individuals with non-GG haplotypes. In addition, the GG haplotype was associated with a previous history of pneumonia. Individuals with the CGG haplotype presented increased plateletcrit, TC, LDL-C levels and non-HDL cholesterol. The CCG haplotype was also associated with a previous history of pneumonia. Our findings suggest that individuals with the GG and CGG haplotypes of *TGFBR3* present important alterations in lipid profile.

KEY WORDS: *TGFBR3* haplotypes, sickle cell disease, lipids and dyslipidemia.

Introduction

Sickle cell disease (SCD) is an autosomal genetic disorder marked by a chronic inflammatory status with acute episodes. The acute condition occurs as a result of vaso-occlusion, persistent cycles of red blood cell sickling and unsickling and hemolysis, leading to leukocyte and endothelial cell activation that induces the release of cytokines and adhesion molecules. By contrast, the chronic inflammatory status is the result of ischemic and reperfusion processes, which cause endothelial cell and vascular wall damage [1-3].

The clinical complications, which occur frequently in individuals with SCD, are classified as acute or chronic according to the age of affected individuals, yet are not restricted to any specific stage of life [4, 5]. SCD individuals also present clinical manifestations including vaso-occlusive (VO) and painful crises, pneumonia, cholelithiasis, stroke, priapism and chronic injury in a variety of organs [6]. One common acute complication in SCD is acute chest syndrome (ACS), characterized by cough, shortness of breath and signs of hypoxemia that are difficult to distinguish from acute pneumonia [7]. Pulmonary complications in SCA are mostly associated with vascular impairment and vasoconstriction, leading to VO [8]. Among the clinical manifestations associated with hemolysis, cholelithiasis, which is related to gallstone formation and gallbladder obstruction, tends to increase in frequency with age [9]. Cholelithiasis occurs due to the accelerated rate of chronic erythrocyte destruction in individuals with SCD. Heme is released by hemolysis and becomes metabolized into bilirubin, which can form insoluble calcium bilirubinate in the bile and precipitate as pigments that form gallstones [10].

The immunological aspects of SCD have been widely studied among individuals with sickle cell anemia (SCA), with high levels of cytokines detected, including interleukin (IL) 4, 6, 8, 10 and tumor necrosis factor alpha (TNF-a) [11]. In addition, transforming growth factor beta (TGF-β) and IL-17 were also found to be associated with vascular activation and inflammation based on a direct association with arginase levels [12]. Differences were found in the levels of cytokines (IL1β, IL6, TNF-a and TGF-β), lipid inflammatory mediators (LTB4 and PGE2) and modulators of vascular remodeling (MMP9 and TIMP1) between SCA individuals in steady- and crisis-state, permitting the characterization of these two groups using these parameters [13].

The TGF-β pathway is involved in several cellular processes, since signal transduction involves binding with transforming growth factor (TGF)-beta receptors (TGFβRI, TGFβRII or TGFβRIII), which activates mothers against decapentaplegic homolog (SMAD) proteins and other mediators. As TGF-β is known to activate several mediators, the TGF-β pathway is considered to play a role in immune response, angiogenesis, inflammation, hematopoiesis, vascular inflammation and cell proliferation [12, 14].

The transforming growth factor beta receptor 3 (*TGFBR3*) gene encodes a receptor of the transforming growth factor beta (TGF-β) family, the TGF-β type III receptor (TβRIII), which presents affinity with all three TGF-β isoforms [15]. Polymorphisms in gene *TGFBR3* have been linked to several diseases, such as Marfan syndrome, bladder cancer, Behçet's disease and SCD [14, 16-18]. In individuals with SCD, some polymorphisms have been associated with stroke, leg ulcers, priapism, pulmonary hypertension, osteonecrosis and acute chest syndrome, all severe clinical manifestations [19-25].

Considering the complex mechanisms underlying the pathogenesis of SCD, we sought to investigate associations between *TGFBR3* haplotypes and classical laboratory parameters, as well as clinical manifestations.

Materials and Methods

Subjects

One hundred seventy-five individuals with SCD (HbSS and HbSC genotypes) were seen at the Bahia Hemotherapy and Hematology Foundation between October 2016 and September 2017. The individuals, 83/175 (47.4%) of whom were female, had an average age of 14.46 ± 3.35 years and a median age of 14 years (interquartile range [IQR]: 12-17 years). All individuals with SCD were in steady-state, characterized by the absence of acute crisis in the three months prior to blood collection procedures. None of the patients were undergoing therapy with lipid-lowering agents, such as statins. All individuals or their legal guardians agreed to biological sample collection procedures and signed terms of informed consent. The present research protocol was approved by the Institutional Research Board of the São Rafael Hospital (HSR protocol number: 1400535) and was conducted in compliance with the Declaration of Helsinki (1964) and its subsequent amendments. Biochemical, hematological, genetic and immunological analyses were performed at the Clinical Analyses

Laboratory of the College of Pharmaceutical Sciences, Federal University of Bahia (LACTFAR-UFBA), and at the Laboratory of Genetic Investigation and Translational Hematology at the Gonçalo Moniz Institute-FIOCRUZ (LIGHT-IGM/FIOCRUZ).

Clinical manifestations

All legal guardians of the individuals with SCD were asked to complete a questionnaire containing information on clinical data regarding the occurrence of previous clinical manifestations. All information provided was confirmed by individual patient medical records.

Hematological and Biochemical Parameters

Blood samples were collected by HEMOBA staff following a fasting period of no less than 12 hours.

Hematological parameters were determined using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA) and hemoglobin profile was confirmed by high-performance liquid chromatography using an HPLC/Variant-II hemoglobin testing system (Bio-Rad, Hercules, California, USA).

An automated A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain) was used to determine biochemical parameters, including total bilirubin and fractions, lactate dehydrogenase (LDH), total protein and fractions, iron, hepatic metabolism and renal profile. Ferritin levels were measured using an Access 2 Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA). C-reactive protein (CRP) and alpha-1 antitrypsin (AAT) levels were measured using an IMMAGE® Immunochemistry System (Beckman Coulter Inc., Pasadena, California, USA).

Lipid profile

Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride levels were determined using an A25 chemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain), while LDL-C and VLDL-C levels were determined by the Friedewald equation [26]. TC/HDL-C, LDL-C/HDL-C and triglyceride/HDL-C ratio were calculated to evaluate cardiovascular risk [27-31]. In addition, non-HDL-C was calculated by TC – HDL-C [32].

Genotype analysis

A QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Westphalia, Germany) was used to extract genomic DNA from peripheral blood in accordance with the manufacturer's recommendations. Genotyping of *TGFBR3* polymorphisms (rs1805110, rs2038931, rs2765888, rs284157, rs284875 and rs7526590) was performed by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Linkage Disequilibrium Analysis

Haploview software (version 4.2) was used to calculate the linkage disequilibrium (LD) between each pairwise combination of SNPs and haplotype frequencies [33].

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) v. 20.0 software (IBM, Armonk, New York, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA) were used to perform all analyses, with p values <0.05 considered significant. χ^2 test and Fisher's exact test were used to evaluate associations between polymorphisms and clinical data. Hardy-Weinberg equilibrium (HWE) was assessed using the χ^2 test. The Shapiro-Wilk test was used to determine the distribution of quantitative variables, followed by the Mann-Whitney *U* test or independent t-test to compare two numerical variables according to distribution.

Results

Baseline characteristics of individuals with SCD

The baseline laboratory parameters of 175 individuals with SCD, expressed as means ± standard deviation, are shown in Supplementary Table 1. Baseline laboratory characteristics showed that SCD individuals presented anemia, hemolysis, leukocytosis, together with decreased levels of HDL-C.

Linkage disequilibrium and haplotype analysis

Genotype frequency testing revealed all six SNPs to be in HWE (Table 1).

Haploview software indicated that all six SNPs met qualifications for LD analysis, which was performed using the four-gamete rule and solid spine of LD methods. The four-gamete testing method created one block with two SNPs (rs284875 and rs2038931) showing complete LD with D'=1 and the logarithm of odds ratio (LOD)<2 (Figure 1a). The haplotype evaluation of this block revealed a higher frequency of the GG haplotype (f=0.731) than the others (GA=0.160 and AG=0.109) (Figure 1b). LD analysis using the solid spine of LD method created one block with three SNPs (rs2765888, rs284875 and rs2038931) (Figure 2a). Haplotype analysis of this block showed a higher frequency of the CGG haplotype (f=0.621) than the others identified (CGA=0.160, TGG=0.110, CAG=0.093 and TAG=0.016) (Figure 2b).

Association of *TGFBR3* rs2765888, rs284875 and rs2038931 polymorphisms with laboratory parameters and clinical manifestations in SCD

The dominant genetic model was employed in rs2765888 and rs2038931 polymorphisms, while the recessive genetic model was employed in rs284875 polymorphism to evaluate associations between alleles and laboratory biomarkers. With regard to *TGFBR3* rs2765888, individuals with CC genotype presented higher mean corpuscular hemoglobin concentration (MCHC) (p=0.039) and plateletcrit (p=0.027) than those with T_ genotypes (Table 2).

Regarding the *TGFBR3 rs284875* polymorphism, individuals with GG genotype presented decreased triglycerides/ HDL-C ratio (p=0.022) than those with A_ genotypes (Table 3).

When the *TGFBR3 rs2038931* polymorphism was evaluated individuals with GG genotype presented increased VLDL-C (p=0.007), triglycerides (p=0.006), TC/HDL-C ratio (p=0.035), triglycerides/ HDL-C ratio (p=0.006), LDL-C/HDL-C ratio (p=0.033) and total protein (p=0.030) than those with A_ genotypes (Table 4).

Regarding clinical manifestations, no significant association were found to rs2765888 and rs284875 polymorphisms, while the A_ genotypes of rs2038931 polymorphism was associated with a previous history of cholelithiasis (*P*=0.038) (Table 5).

Association of GG haplotype with laboratory parameters and clinical manifestations in SCD

A comparison of the hematological, biochemical and immunological parameters between the GG and non GG-haplotypes revealed that individuals with the GG haplotype presented higher total cholesterol (TC) (*P*=0.019), low density lipoprotein cholesterol (LDL-C) (*P*=0.034), triglycerides (*P*=0.040), non-HDL-C (*P*=0.022), total proteins (*P*=0.022) and globulin levels (*P*=0.046) than those with non-GG haplotypes, although statistically higher, serum lipid levels in the GG haplotype are within the normal clinical range (Figure 3) (Table 6). Regarding clinical manifestations, the GG haplotype was associated with a previous history of pneumonia (*P*=0.015) (Figure 4a), while non-GG haplotypes were associated with a previous history of cholelithiasis (*P*=0.021) (Figure 4b) (Table 5).

Association of CGG haplotype with laboratory parameters and clinical manifestations in SCD

A comparison of hematological, biochemical, and immunological laboratory parameters between CGG and non CGG-haplotypes showed that individuals with CGG haplotype presented increased plateletcrit (PCT) (*P*=0.046), TC (*P*=0.029), LDL-C (*P*=0.035) and non-HDL-C (*P*=0.030) levels, although statistically higher, serum lipid levels in the CGG haplotype are within the normal clinical range (Figure 5) (Table 7). In addition, the CCG haplotype was associated with a previous history of pneumonia (*P*=0.018) (Figure 4c) (Table 5).

Association of clinical manifestations with laboratory parameters in SCD

Once the GG and CCG haplotypes were associated with a previous history of pneumonia and the non-GG haplotype was associated with a previous history of cholelithiasis, the association between these clinical manifestations and laboratory parameters were investigated. A comparison of the hematological, biochemical and immunological parameters between the individuals with and without previous history of pneumonia revealed that individuals with previous history of pneumonia presented higher TC (*P*=0.004), LDL-C (*P*=0.025), non-HDL-C (*P*=0.012) and CRP (*P*<0.001), as well as, decreased creatinine levels (*P*=0.014) than those without previous history of pneumonia (Table 8).

With regard to cholelithiasis, a comparison of the hematological, biochemical and immunological parameters between the individuals with and without previous history of this clinical manifestation revealed that individuals with previous history of

cholelithiasis presented increased fetal hemoglobin (*P*<0.001), S hemoglobin (*P*=0.002), MCV (*P*<0.001), MCHC (*P*<0.001), total bilirubin (*P*=0.016) and indirect bilirubin (*P*=0.010), as well as, decreased red blood cell counts (*P*=0.003), TC (*P*=0.012), LDL-C (*P*=0.003), non-HDL-C (*P*=0.007), TC/HDL-C ratio (*P*<0.001), LDL-C/HDL-C ratio (*P*<0.001) and urea levels (*P*=0.002) than those without previous history of cholelithiasis (Table 9).

Discussion

Although polymorphisms in the *TGFBR3* gene, when evaluated individually, have been associated with several clinical manifestations in SCD [14, 20-24, 34, 35], Kim et al. (2010) found that, in asthmatic individuals, these polymorphisms are more informative when evaluated in the context of haplotype, versus on an individual basis [36]. Accordingly, this study evaluated associations between *TGFBR3* haplotypes and laboratory markers, as well as clinical manifestations, in individuals with SCD.

Laboratory parameters of SCD individuals presented classical markers of hemolysis, anemia, increased leukocyte counts, as well as decreased HDL-C levels. These findings are consistent with previous reports describing SCD individuals [37-40].

In our study, six polymorphisms were evaluated, the polymorphisms rs7526590, rs284875, rs2038931, rs7526590 and rs284157 are intronic variants. Intronic variants might affect alternative splicing of the mRNA, however until this moment there are no evidence of association between these polymorphisms and TGFBR3 function or TGFbeta signaling. We reinforce that all the selected SNPs were previously studied and associated with occurrence of clinical manifestation in SCD [20-24]. With regard to rs1805110 polymorphism, this is a missense variant that causes a change in the polypeptide chain where a S (Ser) > F (Phe). This polymorphism has been associated with Behçet's disease, susceptibility in HBV-related hepatocellular carcinoma, pulmonary emphysema and aneurysm, however, it was not previously investigated in SCD [41-44].

Our results showed that individuals with the GG haplotype of *TGFBR3* had higher levels of TC, LDL-C, triglycerides and non-HDL-C than those with non-GG haplotypes. In addition, individuals with the CGG haplotype of *TGFBR3* also presented lipid profile alterations, i.e. higher TC, LDL-C and non-HDL-C levels than those with non-CGG haplotypes. Importantly, even statistically higher, serum lipid

levels in the GG and CGG haplotypes were within the normal clinical range. The individual evaluation of polymorphisms showed that individuals with GG genotype of rs2038931 polymorphism presented increased VLDL-C, triglycerides, TC/HDL-C ratio, triglycerides/HDL-C ratio and LDL-C/HDL-C ratio than those with A_ genotypes. Moreover, the CC genotype of rs2765888 polymorphism was also associated with increased triglycerides/HDL-C ratio. Indeed, when the *TGFBR3* rs2765888, rs284875 and rs2038931 polymorphisms, which compose the haplotypes, were individually investigated, none of them seems to contribute more than the other polymorphism to the associations found by the haplotypes. Thus, the associations between the haplotypes and lipid parameters were equally modulated by the polymorphisms. Despite the rs2038931 polymorphism has been associated with lipid markers, they were different from those identified by haplotypes.

High total cholesterol, as well as LDL-C levels triggers the process of atherosclerosis by stimulating cholesterol accumulation and promotes an inflammatory response in the artery wall [45]. The pathophysiological mechanisms present in atherosclerosis are analogous to those identified in SCA vasculopathy, marked by enhanced oxidative stress, low NO bioavailability in addition to endothelial dysfunction, however, it is important to emphasize that in SCD there is no formation of atheroma plaques [4, 46]. Vasculopathy is responsible for several clinical manifestations in SCD, such as stroke, priapism, pulmonary hypertension and leg ulcers [4].

Moreover, high triglyceride and non-HDL-C levels have also been associated to atherosclerosis. In the lipolysis of triglycerides, triglyceride-rich remnants are released in vessel contributing to increase inflammation, coagulation and endothelial dysfunction [47]. Increased triglyceride levels were shown to be a potential risk factor for pulmonary hypertension, a frequent respiratory complication in individuals with SCD [29].

Non-HDL-C is a cholesterol carried by apolipoprotein B, including those carried by LDL-C and VLDL-C, and is considered a useful marker of atherosclerosis [48]. Previous studies performed in individuals with cardiovascular disease reported high non-HDL-C levels [49, 50], however no significant alterations in non-HDL-C levels were found in SCD individuals [32].

TC, LDL-C, triglycerides and non-HDL-C are markers of the lipid metabolism associated with inflammation related to vascular response [45, 46, 48]. The TGF-β

pathway has been associated with vascular inflammatory responses in several diseases. In many cases, cholesterol uptake and trafficking is also responsible for vessel wall modifications, which can contribute to inflammation [51, 52]. Previous study reported that TGF-β signaling regulates lipid metabolism through the induction of lipogenesis genes resulting in increased triglyceride synthesis and lipid accumulation. TGF-β signaling activates SMADs proteins, triggering the activation of these pathways. The inhibition of these pathways suppresses changes in gene expression associated with lipid metabolism [53]. Therefore, the identification of markers which may modulate the endothelial inflammatory response in SCD is relevant to monitor the clinical course and may contribute to understand the disease pathophysiology.

TGFBRIII, encoded by the *TGFBR3* gene, plays an important role in regulating and mediating the signal transduction of TGF-β [54]. In the literature, polymorphisms in *TGFBR3* have been associated with inflammatory diseases other than SCD, such as Marfan syndrome, bladder cancer and Behçet's disease [14, 16-18]. Marfan syndrome presents as clinical complications in the cardiovascular, skeletal, pulmonary, ocular and nervous system; its physiopathology includes alterations in extracellular matrix deposition in vessels, similar to findings reported in SCD [16]. Behçet's disease is characterized by multisystemic vasculitis with marked inflammatory lesions in the central nervous system, skin, joints, oro-genital mucosa and eyes; analogous to SCD, the inflammatory component is the main physiopathological feature of this disease [18].

Individuals with the GG haplotype presented high levels of total proteins and globulin, which is similar to previous reports in individuals with SCD compared to controls [55, 56]. This hyperproteinemia arises from hyperglobulinemia, which is known to occur in SCD individuals as a result of erythrocyte destruction during sickling [57]. Thus, it is expected that individuals with the GG haplotype would present more prominent hemolysis than those with non-GG haplotypes. High levels of total protein were also identified in individuals with GG genotype of rs2038931 polymorphism when compared to A_ genotypes.

With regard to clinical manifestations, the haplotypes have showed to be more informative than the polymorphisms individually analyzed, both the GG and CGG haplotypes of gene *TGFBR3* were associated with the occurrence of pneumonia. In a large cohort of SCD individuals, pneumonia was one of the leading causes of

hospitalization, second only to sickle cell crisis [58]. A previous study performed in individuals with SCA, the majority of whom presented *Streptococcus pneumoniae* infection, identified that a polymorphism in *TGFBR3* was associated with increased susceptibility to bacteremia [34]. *Streptococcus pneumoniae* infection is one of the main causes of pneumonia in individuals with SCD [34]. It is important to note that all individuals in this study were immunized against pneumococcal disease; therefore, it is possible that these children could have been undergoing a process of autosplenectomy, which consequently increases susceptibility to serious infections [59].

Other respiratory complications, such as asthma and bronchopulmonary dysplasia, have also been associated with *TGFBR*3 in the literature [36, 60]. Similarities exist in the pathogenesis of asthma, bronchopulmonary dysplasia and SCD, which is marked by inflammation and the activation of several cytokines, including TGF-β [36, 60].

We found that individuals with previous history of pneumonia presented increased TC, LDL-C, non-HDL-C, CRP levels and decreased creatinine. A previous study performed in individuals with SCD identified that occurrence of pneumonia is related to increased frequency of other respiratory complications, such as acute chest syndrome (ACS), arising from fat embolism and pathogenic infection [61]. In a cohort without SCD, high cholesterol levels were associated with death related to a miscellaneous of respiratory diseases in a large study involving adults [62].

Serum CRP levels are clinically used to differentiate pneumonia from other acute respiratory infections [63]. In SCD, community-acquired pneumonia triggers inflammatory response and lung injury [64]. Alterations in CRP levels and others immunological markers were found in SCA individuals with lung dysfunction deriving from ACS [65]. High creatinine levels were also described as severity marker of community-acquired pneumonia, however in our results the individuals with SCD and previous history of pneumonia presented lower creatinine levels [66].

We also found that non-GG haplotypes were associated with occurrence of cholelithiasis, one of the most frequent SCD complications that occurs in 26-58% of patients. In addition, individual with previous history of cholelithiasis presented more prominent hemolysis than those without history. This is often associated with increased bilirubin levels, and consequently with hemolysis, and has been described as part of the hemolysis-endothelial dysfunction subphenotype [4, 67, 68].

Cholelithiasis, a clinical manifestation of SCD related to chronic hemolysis, triggers bilirubin production, leading to the formation of gallstones [69]. The upregulation of the TGF-β pathway was previously detected in the gallbladders of individuals with cholelithiasis [70]. Moreover, an investigation of gene expression in patients undergoing cholecystectomy found significant expression of TGF-β receptor (TGFβR) I and TGFβRII during chronic cholelithiasis in comparison to acute cholelithiasis. However, no significant increase in TGFβRIII expression was found [71].

Altogether, our results suggest that *TGFBR3* haplotypes seem to be related to inflammation and the occurrence of pneumonia. Inflammation is a physiopathological mechanism present in SCD, highlighting the relevance investigating novel biomarkers of disease severity in the clinical management of individuals with SCD. To the best of our knowledge, the present study is the first attempt to demonstrate associations between *TGFBR3* haplotypes and hematological and biochemical parameters, as well as, clinical manifestations in SCD.

Conclusion

Collectively, the present findings suggest that individuals with the GG and CGG haplotypes of *TGFBR3* present significant lipid profile alterations and could be associated with the occurrence of pneumonia, while the non-GG haplotypes was associated with the occurrence of cholelithiasis. Further studies are essential to evaluate *TGFBR3* haplotypes as prognostic markers and identify possible therapeutic targets in SCD individuals.

Data Availability

All relevant data used to support the findings of this study are included within the article and the supplementary information file.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

See Supplementary Table 1 in the Supplementary Material for comprehensive data analysis.

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Table 1. Hardy-Weinberg equilibrium values for each *TGFBR3* polymorphism.

Polymorphism	Frequencies	χ^2	*P* value
rs1805110 (G>A)			
GG	129/175		
AG	44/175	0.680	0.409
AA	2/175		
rs2038931 (G>A)			
GG	120/175		
AG	54/175	3.831	0.050
AA	1/175		
rs2765888 (C>T)			
CC	133/175		
CT	40/175	0.277	0.598
TT	2/175		
rs284157 (C>T)			
CC	73/175		
CT	81/175	0.041	0.838
TT	21/175		
rs284875 (A>G)			
AA	4/175		
AG	30/175	2.289	0.130
GG	141/175		
rs7526590 (A>T)			
AA	118/175		
AT	54/175	1.300	0.254
T	3/175		

χ^2: Chi-square test

Table 2. Association of *TGFBR3* rs2765888 polymorphism with laboratory biomarkers using the dominant genetic model.

Parameter	***TGFBR3* rs2765888 polymorphism**		***P* value**
	CC **N=133** Median (IQR)	**T_** **N= 42** Median (IQR)	
Hemoglobin pattern			
Fetal hemoglobin, %	5.70 (1.70 – 10.78)	4.00 (1.27 – 8.60)	0.217
S hemoglobin, %	80.90 (54.70 – 90.25)	76.20 (52.80 – 85.58)	0.098
Hematological markers			
RBC, 10^6/mL	2.84 (2.49 – 3.96)	3.29 (2.72 – 3.95)	0.114
Hemoglobin, g/dL	9.00 (7.90 – 10.80)	9.60 (8.20 – 11.10)	0.219
Hematocrit, %	27.00 (23.40 – 32.20)	28.50 (25.33 – 33.00)	0.215
MCV, fL	88.85 (80.65 – 96.33)	85.50 (79.80 – 94.35)	0.361
MCH, ρg	30.35 (27.35 – 32.80)	28.65 (26.60 – 32.05)	0.146
MCHC, g/dL	33.90 (33.20 – 34.40)	33.70 (33.18 – 33.90)	**0.039***
RDW, %	20.80 (18.00 – 24.20)	20.60 (17.43 – 23.10)	0.515
Reticulocyte count, /mL	138650 (94770 – 171380)	140025 (88680 – 180015)	0.793
WBC, /mL	10600 (8075 – 13300)	10250 (7800 – 13150)	0.520*
Neutrophils, /mL	4687 (3384 – 6650)	5550 (3550 – 6518)	0.403
Eosinophils, /mL	315.00 (153.30 – 578.80)	351.00 (155.00 – 548.00)	0.984
Lymphocytes, /mL	3834 (2833 – 4601)	3164 (2348 – 4264)	0.050
Monocytes, /mL	900 (600 – 1313)	818 (500 – 1158)	0.122
Platelet count, $x10^3$/mL	389.00 (289.00 – 474.00)	332.50 (257.00 – 422.50)	0.071
Platelet Volume Average, fL	7.90 (7.40 – 8.60)	8.10 (7.45 – 8.60)	0.694*
Plateletcrit, %	0.31 (0.23 – 0.37)	0.27 (0.20 – 0.31)	**0.027**
Biochemical markers			
TC, mg/dL	124.00 (105.00 – 145.00)	121.00 (108.80 – 134.50)	0.633*
HDL-C, mg/dL	36.00 (31.00 – 42.00)	36.50 (32.75 – 43.00)	0.789*
LDL-C, mg/dL	64.00 (50.60 – 80.60)	59.00 (48.10 – 77.85)	0.638
VLDL-C, mg/dL	20.20 (14.80 – 25.40)	19.40 (16.50 – 23.20)	0.964
Triglycerides, mg/dL	101.00 (74.00 – 127.00)	97.00 (82.50 – 116.00)	0.994
Non-HDL-C, mg/dL	85.50 (70.00 – 104.80)	79.50 (69.75 – 97.75)	0.537
TC/HDL-C ratio	3.27 (2.75 – 4.14)	3.22 (2.66 – 4.16)	0.739
Triglycerides/ HDL-C ratio	2.57 (1.84 – 3.76)	2.61 (1.88 – 3.46)	0.661
LDL-C/HDL-C ratio	1.76 (1.29 – 2.32)	1.72 (1.18 – 2.29)	0.828
Total bilirubin, mg/dL	2.05 (1.26 – 3.13)	1.82 (1.21 – 3.42)	0.704
Direct bilirubin, mg/dL	0.35 (0.26 – 0.50)	0.35 (0.24 – 0.43)	0.662
Indirect bilirubin, mg/dL	1.63 (0.89 – 2.74)	1.41 (0.91 – 3.12)	0.924
LDH, U/L	868.00 (654.00 – 1239.00)	847.50 (610.80 – 1265.00)	0.593
ALT, U/L	15.00 (11.00 – 19.00)	12.00 (9.00 – 20.00)	0.213
AST, U/L	38.00 (26.00 – 51.25)	34.50 (22.75 – 56.75)	0.418
Total protein, g/dL	8.19 (7.72 – 8.84)	8.27 (7.93 – 8.90)	0.469
Albumin, g/dL	4.75 (4.57 – 4.95)	4.86 (4.65 – 5.02)	0.237*
Globulin, g/dL	3.48 (3.03 – 4.00)	3.42 (3.14 – 3.94)	0.912
Albumin /Globulin Ratio	1.38 (1.21 – 1.58)	1.37 (1.25 – 1.61)	0.679*
Iron, mcg/dL	92.00 (73.75 – 117.00)	93.00 (62.00 – 134.50)	0.762
Ferritin, ηg/ mL	147.50 (90.68 – 243.80)	121.20 (45.10 – 169.80)	0.221

Urea nitrogen, mg/dL	17.00 (13.96 – 21.00)	18.02 (14.31 – 21.82)	0.201
Creatinine, mg/dL	0.48 (0.38 – 0.58)	0.51 (0.39 – 0.68)	0.147
CRP, mg/L	2.61 (1.73 – 3.78)	2.39 (1.81 – 5.89)	0.255
AAT, mg/dL	69.30 (37.60 – 121.30)	72.00 (38.00 – 91.30)	0.886

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

Table 3. Association of *TGFBR3* rs284875 polymorphism with laboratory biomarkers using the recessive genetic model.

Parameter	***TGFBR3* rs284875 polymorphism**		***P* value**
	GG	**A_**	
	N=141 Median (IQR)	**N= 34** Median (IQR)	
Hemoglobin pattern			
Fetal hemoglobin, %	4.70 (1.30 – 10.30)	6.20 (2.30 – 9.80)	0.344
S hemoglobin, %	79.90 (52.00 – 88.70)	83.55 (56.35 – 90.13)	0.239
Hematological markers			
RBC, 10^6/mL	2.98 (2.59 – 3.98)	2.63 (2.40 – 3.68)	0.090
Hemoglobin, g/dL	9.10 (8.10 – 11.10)	8.35 (7.77 – 10.35)	0.183
Hematocrit, %	27.50 (24.00 – 32.60)	25.70 (22.60 – 31.05)	0.141
MCV, fL	87.10 (80.30 – 94.80)	90.35 (82.03 – 98.30)	0.151*
MCH, ρg	29.55 (27.20 – 32.40)	31.15 (27.60 – 34.10)	0.152*
MCHC, g/dL	33.70 (33.20 – 34.20)	34.00 (33.28 – 34.53)	0.193
RDW, %	20.70 (17.90 – 23.50)	21.70 (17.78 – 25.45)	0.350
Reticulocyte count, /mL	138650 (94770 – 179340)	136010 (87075 – 184758)	0.605
WBC, /mL	10750 (8000 – 13225)	10100 (7875 – 13525)	0.489
Neutrophils, /mL	5060 (3461 – 6600)	4348 (3386 – 6677)	0.413
Eosinophils, /mL	309.50 (153.80 – 546.00)	396.50 (162.50 – 613.50)	0.671
Lymphocytes, /mL	3629 (2723 – 4508)	3795 (2375 – 4656)	0.825
Monocytes, /mL	900 (600 – 1296)	810 (560 – 1140)	0.686
Platelet count, $x10^3$/mL	376.00 (278.00 – 467.00)	388.50 (273.30 – 457.00)	0.909*
Platelet Volume Average, fL	8.10 (7.40 – 8.60)	7.80 (7.37 – 8.42)	0.256*
Plateletcrit, %	0.29 (0.23 – 0.36)	0.29 (0.19 – 0.36)	0.704
Biochemical markers			
TC, mg/dL	123.00 (108.00 – 140.00)	118.00 (97.00 – 140.50)	0.296
HDL-C, mg/dL	37.00 (32.00 – 43.00)	33.00 (29.00 – 41.00)	0.180*
LDL-C, mg/dL	64.60 (51.55 – 80.35)	55.20 (44.30 – 75.30)	0.120
VLDL-C, mg/dL	19.40 (14.60 – 23.60)	21.00 (16.00 – 27.60)	0.091
Triglycerides, mg/dL	97.00 (73.00 – 118.00)	105.00 (80.00 – 138.00)	0.098
Non-HDL-C, mg/dL	85.00 (72.00 – 103.00)	81.00 (64.50 – 107.00)	0.489
TC/HDL-C ratio	3.24 (2.76 – 4.15)	3.29 (2.73 – 4.50)	0.629
Triglycerides/ HDL-C ratio	2.48 (1.82 – 3.50)	2.93 (2.31 – 4.30)	**0.022**
LDL-C/HDL-C ratio	1.75 (1.33 – 2.34)	1.78 (1.21 – 2.20)	0.877
Total bilirubin, mg/dL	2.04 (1.24 – 3.23)	2.00 (1.40 – 3.06)	0.786
Direct bilirubin, mg/dL	0.35 (0.24 – 0.43)	0.39 (0.29 – 0.54)	0.072
Indirect bilirubin, mg/dL	1.58 (0.89 – 2.90)	1.48 (1.01 – 2.78)	0.886
LDH, U/L	856.00 (630.00 – 1217.00)	954.00 (614.50 – 1354.00)	0.614
ALT, U/L	14.00 (10.00 – 19.00)	15.00 (9.25 – 19.50)	0.841
AST, U/L	37.00 (26.00 – 50.00)	41.00 (25.50 – 57.00)	0.899
Total protein, g/dL	8.22 (7.85 – 8.85)	8.09 (7.51 – 8.89)	0.427
Albumin, g/dL	4.76 (4.60 – 4.98)	4.88 (4.50 – 4.99)	0.858
Globulin, g/dL	3.47 (3.11 – 3.96)	3.25 (2.98 – 4.11)	0.437
Albumin /Globulin Ratio	1.36 (1.22 – 1.58)	1.42 (1.22 – 1.61)	0.442*
Iron, mcg/dL	92.00 (72.00 – 120.00)	92.00 (72.50 – 126.00)	0.921
Ferritin, ηg/ mL	145.70 (91.63 – 214.90)	239.60 (50.93 – 524.70)	0.272

Urea nitrogen, mg/dL	17.00 (14.00 – 21.00)	17.99 (14.11 – 21.07)	0.996
Creatinine, mg/dL	0.48 (0.38 – 0.60)	0.50 (0.34 – 0.64)	0.962
CRP, mg/L	2.39 (1.71 – 3.57)	2.66 (1.87 – 4.82)	0.240
AAT, mg/dL	71.30 (37.10 – 113.00)	69.00 (45.20 – 131.30)	0.444

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

Table 4. Association of *TGFBR3* rs2038931 polymorphism with laboratory biomarkers using the dominant genetic model.

Parameter	***TGFBR3* rs2038931 polymorphism**		***P* value**
	GG	**A_**	
	N=120 Median (IQR)	**N= 55** Median (IQR)	
Hemoglobin pattern			
Fetal hemoglobin, %	4.80 (1.70 – 8.75)	5.50 (1.30 – 12.63)	0.486
S hemoglobin, %	80.30 (54.50 – 89.90)	79.95 (52.15 – 87.60)	0.802
Hematological markers			
RBC, 10^6/mL	2.96 (2.51 – 3.97)	2.95 (2.56 – 3.79)	0.978
Hemoglobin, g/dL	9.00 (7.90 – 10.88)	9.20 (8.10 – 10.80)	0.510
Hematocrit, %	27.00 (23.30 – 32.65)	27.50 (24.30 – 32.10)	0.524
MCV, fL	87.30 (80.10 – 94.30)	91.20 (80.80 – 97.30)	0.177*
MCH, ρg	29.60 (27.15 – 32.65)	30.90 (27.60 – 32.60)	0.270*
MCHC, g/dL	33.80 (33.20 – 34.40)	33.70 (33.20 – 34.30)	0.393*
RDW, %	21.30 (18.00 – 24.40)	20.00 (17.60 – 23.00)	0.074
Reticulocyte count, /mL	131330 (88245 – 174570)	144690 (98880 – 180540)	0.070*
WBC, /mL	10700 (8150 – 13025)	10450 (7550 – 13625)	0.349
Neutrophils, /mL	5078 (3478 – 6600)	4402 (3356 – 6675)	0.270
Eosinophils, /mL	300.00 (160.00 – 544.00)	326.00 (142.00 – 590.00)	0.996
Lymphocytes, /mL	3702 (2783 – 4601)	3493 (2666 – 4454)	0.339
Monocytes, /mL	894 (600 – 1297)	800 (552 – 1288)	0.499
Platelet count, x10^3/mL	383.00 (278.00 – 457.00)	372.00 (274.00 – 467.00)	0.951
Platelet Volume Average, fL	7.90 (7.50 – 8.52)	8.10 (7.30 – 8.70)	0.278*
Plateletcrit, %	0.29 (0.23 – 0.36)	0.29 (0.22 – 0.36)	0.877
Biochemical markers			
TC, mg/dL	126.50 (109.30 – 141.80)	116.00 (103.00 – 137.50)	0.116
HDL-C, mg/dL	36.00 (32.00 – 42.00)	37.00 (32.00 – 45.00)	0.121
LDL-C, mg/dL	64.40 (50.60 – 81.40)	58.60 (49.90 – 78.60)	0.236
VLDL-C, mg/dL	20.50 (16.15 – 25.65)	17.40 (12.35 – 23.20)	**0.007**
Triglycerides, mg/dL	103.00 (81.00 – 129.00)	88.00 (62.00 – 116.00)	**0.006**
Non-HDL-C, mg/dL	90.00 (71.00 – 106.00)	79.00 (67.00 – 98.50)	0.068
TC/HDL-C ratio	3.33 (2.77 – 4.35)	3.10 (2.67 – 3.57)	**0.035**
Triglycerides/ HDL-C ratio	2.69 (1.97 – 3.73)	2.32 (1.53 – 3.10)	**0.006**
LDL-C/HDL-C ratio	1.81 (1.34 – 2.43)	1.58 (1.15 – 2.00)	**0.033**
Total bilirubin, mg/dL	2.07 (1.31 – 3.06)	1.76 (1.15 – 3.52)	0.654
Direct bilirubin, mg/dL	0.36 (0.24 – 0.49)	0.35 (0.28 – 0.43)	0.966
Indirect bilirubin, mg/dL	1.60 (0.98 – 2.63)	1.29 (0.85 – 3.12)	0.695
LDH, U/L	907.50 (630.50 – 1318.00)	841.00 (609.00 – 1085.00)	0.227
ALT, U/L	14.50 (10.75 – 20.00)	15.00 (10.00 – 19.00)	0.784
AST, U/L	37.00 (26.00 – 54.00)	38.00 (24.00 – 49.00)	0.627
Total protein, g/dL	8.27 (7.93 – 8.87)	8.00 (7.50 – 8.66)	**0.030***
Albumin, g/dL	4.83 (4.60 – 5.00)	4.71 (4.53 – 4.90)	0.066*
Globulin, g/dL	3.50 (3.11 – 4.00)	3.30 (2.94 – 3.93)	0.078
Albumin /Globulin Ratio	1.37 (1.21 – 1.51)	1.40 (1.23 – 1.64)	0.381*
Iron, mcg/dL	92.00 (72.00 – 117.00)	92.00 (72.50 – 128.30)	0.483
Ferritin, ηg/ mL	134.00 (71.55 – 189.10)	190.30 (89.33 – 263.00)	0.082

Urea nitrogen, mg/dL	17.22 (14.30 – 20.94)	15.00 (13.40 – 22.00)	0.367*
Creatinine, mg/dL	0.49 (0.36 – 0.62)	0.48 (0.38 – 0.59)	0.815
CRP, mg/L	2.40 (1.71 – 3.79)	2.58 (1.77 – 3.51)	0.866
AAT, mg/dL	68.20 (38.08 – 117.80)	73.80 (37.30 – 120.00)	0.859

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

Table 5. Association of *TGFBR3* rs2765888, rs284875 and rs2038931 polymorphism and *TGFBR3* haplotypes with clinical manifestations in SCD.

Clinical manifestation	Polymorphisms								Haplotypes						
	rs2765888			rs284875			rs2038931								
	CC N=133	T_ N=42	*P* value	GG N=141	A_ N=34	*P* value	GG N=120	A_ N=55	*P* value	GG N=93	nonGG N=82	*P* value	CGG N=63	nonCGG N=112	*P* value
Acute chest syndrome	28	11	0.485	32	7	0.791	25	14	0.495	19	20	0.529	12	27	0.440
Cholelithiasis	37	6	0.075	33	10	0.465	24	19	**0.038**	16	27	**0.015**	13	30	0.364
Infections	85	28	0.744	89	24	0.413	79	34	0.606	61	52	0.763	39	74	0.580
Leg ulcer	12	6	0.327	12	6	0.115	11	7	0.471	8	10	0.434	3	15	0.117*
Pneumonia	71	19	0.357	74	16	0.570	66	24	0.162	56	34	**0.013**	40	50	**0.016**
Painful crises	88	31	0.354	96	23	0.960	84	35	0.402	67	52	0.222	47	72	0.160
Stroke	10	4	0.745*	12	2	0.999*	9	5	0.767*	7	7	0.805	4	10	0.772*
Vaso-occlusive events	34	17	0.063	39	12	0.379	35	16	0.991	27	24	0.972	16	35	0.413

Bold values indicate significance at p<0.05. All p values obtained by the Chi square, except for those with asterisk (*), for which the Fisher's exact test was used.

Table 6. Association of *TGFBR3* GG haplotype with laboratory biomarkers

Parameter	*TGFBR3* GG haplotype		*P* value
	GG	**NonGG**	
	N=93 Median (IQR)	**N= 82** Median (IQR)	
Hemoglobin pattern			
Fetal hemoglobin, %	80.10 (52.08 – 89.88)	80.20 (54.50 – 88.60)	0.688
S hemoglobin, %	4.50 (1.40 – 8.60)	6.20 (1.70 – 11.600	0.142
Hematological markers			
RBC, 10^6/mL	2.98 (2.57 – 3.98)	2.90 (2.49 – 3.71)	0.345
Hemoglobin, g/dL	9.10 (8.00 – 11.10)	9.10 (8.10 – 10.73)	0.800
Hematocrit, %	27.00 (23.90 – 33.00)	27.35 (23.48 – 32.10)	0.765
MCV, fL	86.20 (80.15 – 93.85)	90.70 (80.78 – 97.50)	0.063*
MCH, ρg	29.10 (27.03 – 32.33)	30.95 (27.55 – 32.85)	0.778*
MCHC, g/dL	33.70 (33.20 – 34.20)	33.80 (33.20 – 34.40)	0.819
RDW, %	21.30 (18.10 – 24.40)	20.25 (17.70 – 23.70)	0.217
Reticulocyte count, /mL	133140 (90480 – 173360)	142055 (94085 – 181290)	0.478*
WBC, /mL	10800 (8700 – 13000)	10100 (7650 – 13550)	0.135*
Neutrophils, /mL	5282 (3800 – 6600)	4348 (3350 – 6713)	0.091
Eosinophils, /mL	300.00 (176.50 – 557.80)	326.00 (143.50 – 572.00)	0.862
Lymphocytes, /mL	3629 (2842 – 4600)	3700 (2626 – 4514)	0.591
Monocytes, /mL	900.00 (600.00 – 1300.00)	805.00 (563.50 – 1265.00)	0.322
Platelet count, x10^3/mL	379.00 (281.00 – 474.00)	383.00 (271.80 – 462.30)	0.655*
Platelet Volume Average, fL	8.00 (7.50 – 8.60)	7.95 (7.30 – 8.60)	0.875*
Plateletcrit, %	0.29 (0.23 – 0.36)	0.28 (0.21 – 0.36)	0.375
Biochemical markers			
TC, mg/dL	127.00 (111.50 – 145.00)	116.50 (100.30 – 138.00)	**0.019**
HDL-C, mg/dL	36.00 (32.00 – 42.75)	36.00 (31.00 – 43.75)	0.798*
LDL-C, mg/dL	68.40 (52.50 – 82.60)	57.70 (48.10 – 77.45)	**0.034**
VLDL-C, mg/dL	20.60 (16.20 – 25.40)	17.80 (13.80 – 23.80)	0.050
Triglycerides, mg/dL	103.50 (81.00 – 127.50)	89.50 (69.25 – 118.80)	**0.040**
Non-HDL-C, mg/dL	90.50 (74.00 – 106.80)	79.00 (66.25 – 99.75)	**0.024**
TC/HDL-C ratio	3.34 (2.83 – 4.26)	3.20 (2.67 – 3.88)	0.133
Triglycerides/ HDL-C ratio	2.71 (1.93 – 3.73)	2.47 (1.70 – 3.30)	0.092
LDL-C/HDL-C ratio	1.80 (1.38 – 2.43)	1.64 (1.20 – 2.11)	0.135
Total bilirubin, mg/dL	2.07 (1.26 – 3.18)	1.86 (1.22 – 3.14)	0.573
Direct bilirubin, mg/dL	0.25 (0.23 – 0.45)	0.35 (0.28 – 0.50)	0.378
Indirect bilirubin, mg/dL	1.67 (0.98 – 2.90)	1.34 (0.87 – 2.75)	0.339
LDH, U/L	917.00 (644.50 – 1304.00)	852.00 (614.00 – 1137.00)	0.276
ALT, U/L	14.50 (11.00 – 20.00)	14.00 (10.00 – 19.00)	0.326
AST, U/L	37.50 (26.00 – 54.75)	37.00 (24.25 – 50.75)	0.535
Total protein, g/dL	8.33 (8.01 – 8.88)	8.09 (7.53 – 8.82)	**0.022***
Albumin, g/dL	4.79 (4.60 – 5.05)	4.74 (4.53 – 4.94)	0.158
Globulin, g/dL	3.52 (3.19 – 4.00)	3.27 (2.98 – 3.94)	**0.046**
Albumin /Globulin Ratio	1.35 (1.20 – 1.50)	1.41 (1.22 – 1.62)	0.147*
Iron, mcg/dL	92.00 (74.00 – 117.00)	92.00 (70.25 – 127.00)	0.795
Ferritin, ηg/ mL	144.60 (98.60 – 200.60)	177.30 (72.70 – 300.40)	0.440
Urea nitrogen, mg/dL	17.19 (14.81 – 21.00)	15.74 (13.50 – 21.14)	0.169

Creatinine, mg/dL	0.48 (0.38 – 0.61)	0.48 (0.38 – 0.60)	0.999
CRP, mg/L	2.41 (1.67 – 3.78)	2.57 (1.80 – 3.53)	0.730
AAT, mg/dL	71.30 (37.35 – 118.50)	69.05 (38.23 – 118.30)	0.984

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

Table 7. Association of *TGFBR3* CGG haplotype with laboratory biomarkers

Parameter	***TGFBR3* CGG haplotype**		***P* value**
	CGG	**nonCGG**	
	N=63 Median (IQR)	**N= 112** Median (IQR)	
Hemoglobin pattern			
Fetal hemoglobin, %	4.50 (1.70 – 8.15)	5.85 (1.52 – 11.70)	0.331
S hemoglobin, %	80.70 (52.00 – 90.53)	79.70 (55.35 – 88.20)	0.676
Hematological markers			
RBC, 10^6/mL	2.98 (2.54 – 3.99)	2.95 (2.53 – 3.78)	0.681
Hemoglobin, g/dL	9.00 (7.85 – 11.35)	9.15 (8.10 – 10.80)	0.958
Hematocrit, %	27.00 (23.70 – 33.50)	27.40 (23.58 – 32.10)	0.977
MCV, fL	86.20 (80.05 – 93.78)	89.75 (80.73 – 97.03)	0.230
MCH, ρg	29.35 (27.13 – 32.38)	30.25 (27.40 – 32.68)	0.242*
MCHC, g/dL	33.90 (33.20 – 34.45)	33.70 (33.20 – 34.28)	0.304*
RDW, %	21.60 (18.10 – 24.50)	20.40 (17.70 – 23.70)	0.177
Reticulocyte count, /mL	131100 (106860 – 170440)	139385 (91350 – 180125)	0.948
WBC, /mL	10800 (9150 – 13000)	10300 (7800 – 13400)	0.152*
Neutrophils, /mL	4876 (3750 – 6650)	4810 (3392 – 6600)	0.452
Eosinophils, /mL	300.00 (182.00 – 520.00)	335.00 (146.50 – 552.50)	0.969
Lymphocytes, /mL	3840 (2943 – 4640)	3500 (2574 – 4440)	0.090
Monocytes, /mL	944.00 (597.00 – 1346.00)	840.50 (575.30 – 1253.00)	0.209
Platelet count, x10^3/mL	389.00 (310.50 – 484.50)	371.50 (267.30 – 454.80)	0.171*
Platelet Volume Average, fL	7.90 (7.50 – 8.50)	8.00 (7.30 – 8.60)	0.989*
Plateletcrit, %	0.32 (0.24 – 0.40)	0.28 (0.21 – 0.35)	**0.046**
Biochemical markers			
TC, mg/dL	128.00 (112.00 – 147.00)	119.00 (103.50 – 138.30)	**0.029**
HDL-C, mg/dL	36.00 (31.75 – 42.00)	36.00 (32.00 – 43.00)	0.645*
LDL-C, mg/dL	68.40 (55.40 – 82.80)	58.50 (48.40 – 78.00)	**0.035**
VLDL-C, mg/dL	20.80 (15.45 – 26.40)	19.10 (14.45 – 23.50)	0.176
Triglycerides, mg/dL	105.00 (77.50 – 132.50)	94.00 (72.50 – 117.00)	0.141
Non-HDL-C, mg/dL	91.00 (76.00 – 108.00)	79.00 (68.00 – 101.50)	**0.030**
TC/HDL-C ratio	3.36 (2.86 – 4.32)	3.22 (2.67 – 3.96)	0.134
Triglycerides/ HDL-C ratio	2.89 (1.87 – 3.96)	2.51 (1.82 – 3.45)	0.086
LDL-C/HDL-C ratio	1.83 (1.41 – 2.43)	1.68 (1.20 – 2.20)	0.135
Total bilirubin, mg/dL	2.08 (1.26 – 3.14)	1.92 (1.25 – 3.20)	0.657
Direct bilirubin, mg/dL	0.34 (0.23 – 0.45)	0.36 (0.26 – 0.49)	0.373
Indirect bilirubin, mg/dL	1.60 (0.96 – 2.66)	1.47 (0.88 – 2.79)	0.664
LDH, U/L	886.00 (651.00 – 1261.00)	855.00 (614.50 – 1224.00)	0.558
ALT, U/L	15.00 (11.00 – 18.00)	14.00 (10.00 – 20.00)	0.675
AST, U/L	36.50 (26.00 – 49.50)	38.00 (24.00 – 54.00)	0.855
Total protein, g/dL	8.33 (8.02 – 8.89)	8.09 (7.66 – 8.82)	0.055*
Albumin, g/dL	4.77 (4.60 – 5.05)	4.79 (4.59 – 4.98)	0.625*
Globulin, g/dL	3.60 (3.28 – 4.03)	3.32 (3.00 – 3.93)	**0.022**
Albumin /Globulin Ratio	1.31 (1.20 – 1.50)	1.40 (1.26 – 1.61)	0.059*
Iron, mcg/dL	88.00 (74.25 – 105.00)	92.00 (67.50 – 127.50)	0.363
Ferritin, ηg/ mL	142.00 (98.30 – 219.20)	155.70 (73.25 – 254.30)	0.931
Urea nitrogen, mg/dL	17.00 (14.70 – 20.49)	17.10 (14.00 – 21.89)	0.915

Creatinine, mg/dL	0.48 (0.38 – 0.59)	0.49 (0.38 – 0.61)	0.605
CRP, mg/L	2.43 (1.59 – 3.78)	2.37 (1.80 – 3.53)	0.780
AAT, mg/dL	65.80 (34.75 – 121.00)	72.55 (39.20 – 116.00)	0.566

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

Table 8. Association of occurrence of pneumonia with laboratory biomarkers

Parameter	Pneumonia - N= 85 Median (IQR)	Pneumonia + N= 90 Median (IQR)	*P* value
Hemoglobin pattern			
Fetal hemoglobin, %	4.90 (1.30 – 8.30)	6.00 (1.77 – 11.90)	0.208
S hemoglobin, %	76.20 (51.70 – 89.50)	80.95 (56.75 – 89.20)	0.347
Hematological markers			
RBC, 10^6/mL	2.86 (2.53 – 4.10)	2.98 (2.53 – 3.73)	0.499
Hemoglobin, g/dL	9.00 (8.05 – 11.35)	9.10 (8.00 – 10.65)	0.685
Hematocrit, %	27.00 (23.45 – 33.35)	27.45 (23.93 – 31.95)	0.760
MCV, fL	85.60 (80.30 – 93.50)	89.80 (81.40 – 97.80)	0.194
MCH, ρg	29.30 (27.20 – 32.45)	30.50 (27.40 – 33.10)	0.365*
MCHC, g/dL	33.90 (33.45 – 34.40)	33.60 (33.10 – 34.30)	0.226*
RDW, %	20.40 (17.40 – 23.75)	21.20 (18.00 – 24.38)	0.103
Reticulocyte count, /mL	127880 (86020 – 171955)	145305 (105170 – 183728)	0.100*
WBC, /mL	10500 (7350 – 13050)	10600 (8300 – 13500)	0124*
Neutrophils, /mL	4621 (3290 – 6468)	5060 (3600 – 7400)	0.172
Eosinophils, /mL	349.00 (130.00 – 595.50)	300.00 (179.00 – 553.00)	0.781
Lymphocytes, /mL	3549 (2400 – 4490)	3790 (2954 – 4537)	0.152
Monocytes, /mL	804.00 (600.00 – 1259.00)	915.50 (573.80 – 1300.00)	0.412
Platelet count, $x10^3$/mL	357.00 (247.00 – 432.50)	395.00 (315.50 – 491.00)	0.051
Platelet Volume Average, fL	8.10 (7.35 – 8.70)	7.90 (7.50 – 8.40)	0.245*
Plateletcrit, %	0.28 (0.20 – 0.35)	0.30 (0.24 – 0.40)	0.062
Biochemical markers			
TC, mg/dL	117.00 (102.00 – 138.00)	128.50 (110.50 – 150.80)	**0.004***
HDL-C, mg/dL	37.00 (31.50 – 42.50)	35.00 (32.00 – 43.00)	0.979
LDL-C, mg/dL	58.80 (49.80 – 78.00)	68.40 (50.90 – 87.35)	**0.025***
VLDL-C, mg/dL	18.50 (14.15 – 23.30)	20.60 (15.75 – 25.95)	0.084
Triglycerides, mg/dL	92.50 (70.75 – 116.50)	103.00 (79.50 – 131.30)	0.073
Non-HDL-C, mg/dL	78.00 (67.50 – 100.00)	91.00 (73.00 – 111.00)	**0.012**
TC/HDL-C ratio	3.13 (2.62 – 3.94)	3.28 (2.87 – 4.37)	0.052
Triglycerides/ HDL-C ratio	2.48 (1.81 – 3.35)	2.71 (1.88 – 4.00)	0.073
LDL-C/HDL-C ratio	1.67 (1.16 – 2.20)	1.77 (1.40 – 2.43)	0.184
Total bilirubin, mg/dL	2.02 (1.16 – 3.06)	2.02 (1.29 – 3.24)	0.716
Direct bilirubin, mg/dL	0.34 (0.25 – 0.46)	0.37 (0.26 – 0.49)	0.318
Indirect bilirubin, mg/dL	1.53 (0.88 – 2.74)	1.60 (0.99 – 2.91)	0.565
LDH, U/L	866.50 (605.00 – 1214.00)	858.50 (665.00 – 1324.00)	0.326
ALT, U/L	12.50 (10.00 – 18.75)	15.00 (11.00 – 20.00)	0.161
AST, U/L	38.00 (25.25 – 50.75)	36.00 (26.00 – 53.50)	0.968
Total protein, g/dL	8.30 (7.70 – 8.85)	8.16 (7.85 – 8.85)	0.957*
Albumin, g/dL	4.85 (4.60 – 5.02)	4.77 (4.57 – 4.98)	0.123*
Globulin, g/dL	3.42 (3.00 – 4.01)	3.47 (3.13 – 3.94)	0.560
Albumin /Globulin Ratio	1.40 (1.22 – 1.60)	1.35 (1.21 – 1.50)	0.267*
Iron, mcg/dL	92.00 (70.75 – 127.50)	92.00 (72.50 – 108.50)	0.231
Ferritin, ηg/ mL	151.50 (89.75 – 238.20)	139.30 (71.70 – 213.40)	0.466
Urea nitrogen, mg/dL	16.64 (14.00 – 21.22)	17.50 (14.39 – 21.00)	0.776
Creatinine, mg/dL	0.50 (0.42 – 0.66)	0.46 (0.36 – 0.57)	**0.014**

CRP, mg/L	2.12 (1.63 – 2.98)	3.21 (1.96 – 4.79)	**<0.001**
AAT, mg/dL	70.25 (39.23 – 114.80)	72.00 (37.10 – 120.00)	0.869

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

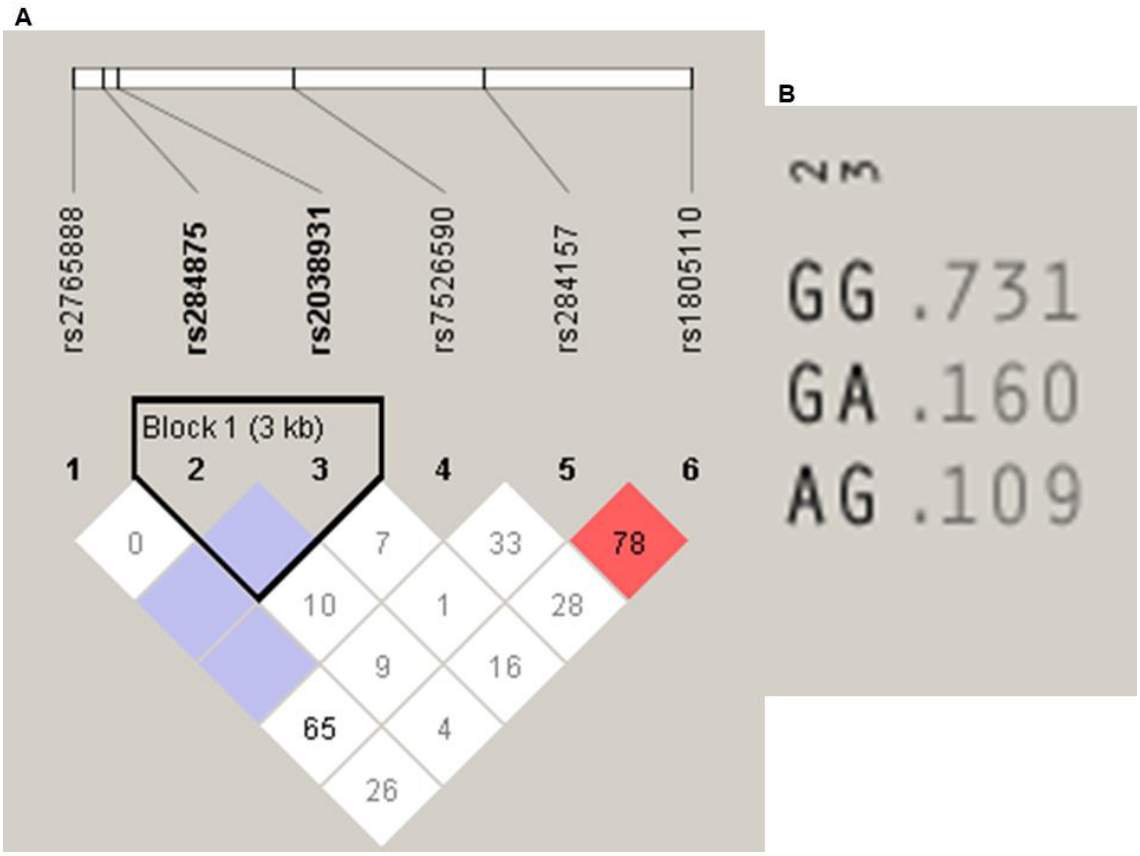
Table 9. Association of occurrence of cholelithiasis with laboratory biomarkers

Parameter	Cholelithiasis - N= 132 Median (IQR)	Cholelithiasis + N= 43 Median (IQR)	*P* value
Hemoglobin pattern			
Fetal hemoglobin, %	3.50 (1.20 – 7.50)	7.50 (3.60 – 13.75)	**<0.001**
S hemoglobin, %	78.70 (51.93 – 89.35)	85.40 (78.35 – 90.45)	**0.002**
Hematological markers			
RBC, 10^6/mL	3.11 (2.59 – 3.97)	2.63 (2.33 – 3.34)	**0.003**
Hemoglobin, g/dL	9.25 (8.10 – 11.03)	8.60 (7.60 – 10.50)	0.130
Hematocrit, %	27.50 (24.00 – 32.55)	26.00 (22.70 – 31.80)	0.176
MCV, fL	85.60 (79.80 – 93.30)	94.30 (88.40 – 104.80)	**<0.001***
MCH, ρg	28.80 (26.80 – 31.80)	32.30 (30.10 – 35.53)	**<0.001***
MCHC, g/dL	33.80 (33.23 – 34.40)	33.70 (33.10 – 34.10)	0.458*
RDW, %	20.80 (17.73 – 24.15)	20.80 (18.10 – 24.00)	0.999
Reticulocyte count, /mL	142285 (99920 – 142285)	123200 (79580 – 157800)	0.085
WBC, /mL	10800 (8100 – 13300)	9900 (7400 – 13200)	0.277*
Neutrophils, /mL	5026 (3600 – 6600)	4200 (3080 – 6600)	0.096
Eosinophils, /mL	345.00 (163.00 – 597.00)	288.00 (100.00 – 444.00)	0.097
Lymphocytes, /mL	3658 (2800 – 4532)	3800 (2652 – 4700)	0.626
Monocytes, /mL	915.50 (600.00 – 1300.00)	784.00 (500.00 – 1260.00)	0.224
Platelet count, x10^3/mL	390.00 (289.30 – 476.00)	352.00 (238.00 – 462.00)	0.149
Platelet Volume Average, fL	8.10 (7.50 – 8.60)	7.90 (7.20 – 8.60)	0.173*
Plateletcrit, %	0.30 (0.23 – 0.36)	0.28 (0.20 – 0.32)	0.104
Biochemical markers			
TC, mg/dL	126.00 (110.00 – 145.00)	110.00 (97.00 – 132.00)	**0.012***
HDL-C, mg/dL	36.00 (32.00 – 43.00)	36.00 (32.00 – 44.00)	0.796*
LDL-C, mg/dL	64.70 (52.15 – 83.15)	53.80 (45.60 – 75.60)	**0.003**
VLDL-C, mg/dL	20.20 (15.35 – 23.90)	17.50 (14.45 – 25.20)	0.399
Triglycerides, mg/dL	101.00 (77.00 – 121.00)	88.00 (72.50 – 125.00)	0.355
Non-HDL-C, mg/dL	90.00 (72.00 – 106.00)	74.00 (65.00 – 91.00)	**0.007**
TC/HDL-C ratio	3.38 (2.78 – 4.24)	2.97 (2.67 – 3.23)	**<0.001**
Triglycerides/ HDL-C ratio	2.68 (1.87 – 3.60)	2.17 (1.76 – 3.16)	0.110
LDL-C/HDL-C ratio	1.83 (1.38 – 2.43)	1.41 (1.16 – 1.76)	**<0.001**
Total bilirubin, mg/dL	1.89 (1.18 – 3.00)	2.79 (1.54 – 3.91)	**0.016**
Direct bilirubin, mg/dL	0.35 (0.27 – 0.49)	0.36 (0.24 – 0.44)	0.880
Indirect bilirubin, mg/dL	1.44 (0.88 – 2.61)	2.17 (1.21 – 3.50)	**0.010**
LDH, U/L	879.00 (618.00 – 1269.00)	855.00 (673.00 – 1153.00)	0.873
ALT, U/L	14.00 (10.25 – 19.00)	15.00 (10.00 – 19.00)	0.779
AST, U/L	39.00 (26.00 – 54.00)	35.00 (27.00 – 52.00)	0.512
Total protein, g/dL	8.21 (7.85 – 8.85)	8.44 (7.76 – 9.00)	0.215*
Albumin, g/dL	4.79 (4.60 – 4.97)	4.79 (4.57 – 5.00)	0.868
Globulin, g/dL	3.43 (3.10 – 3.90)	3.50 (3.03 – 4.11)	0.200*
Albumin /Globulin Ratio	1.39 (1.25 – 1.55)	1.30 (1.12 – 1.62)	0.380*
Iron, mcg/dL	92.00 (74.00 – 120.80)	94.00 (67.00 – 124.00)	0.960
Ferritin, ηg/ mL	154.40 (72.70 – 248.00)	148.90 (89.33 – 410.80)	0.577
Urea nitrogen, mg/dL	18.00 (14.53 – 21.62)	15.00 (12.76 – 17.22)	**0.002***
Creatinine, mg/dL	0.48 (0.38 – 0.63)	0.48 (0.36 – 0.57)	0.380

CRP, mg/L	2.37 (1.71 – 3.71)	2.75 (1.87 – 4.71)	0.128
AAT, mg/dL	68.20 (37.10 – 120.00)	73.20 (39.65 – 100.20)	0.873

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; IQR: Interquartile range. Bold values indicate significance at $p<0.05$. All p values obtained by the Mann–Whitney U test, except for those with asterisk (*), for which the independent t-test was used.

FIGURE 1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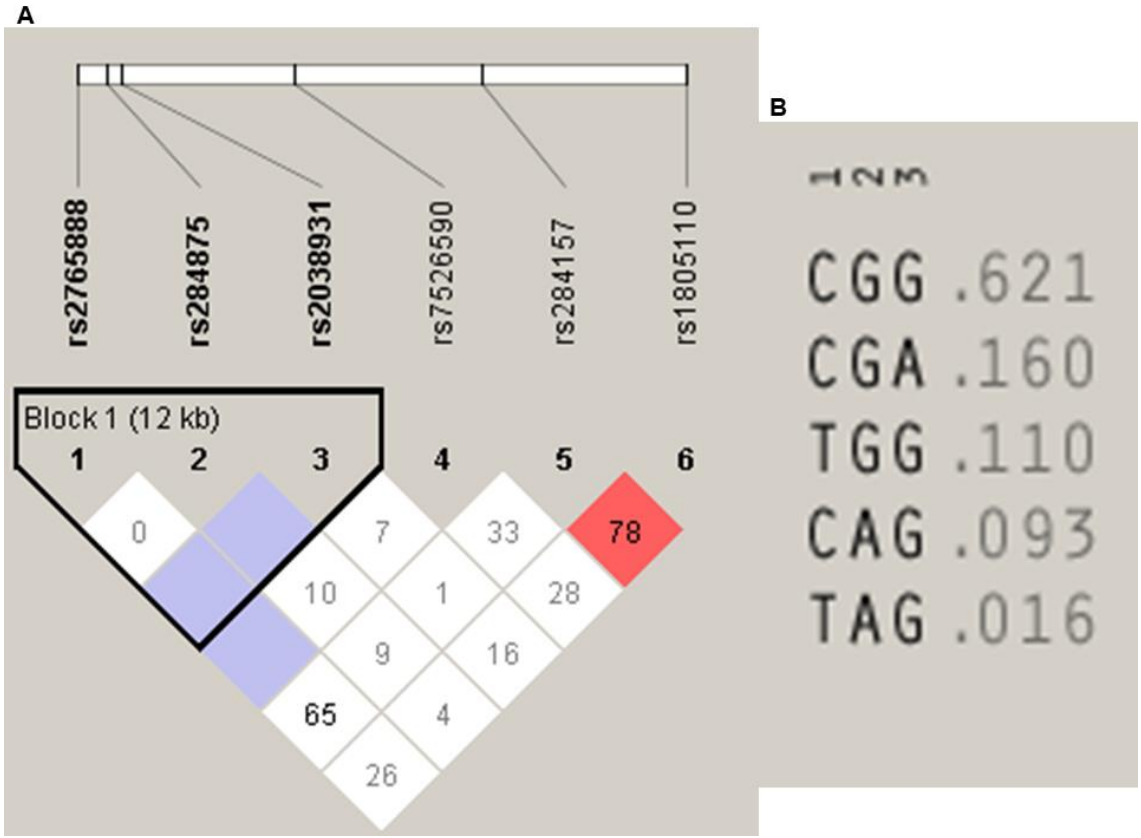
rs2765888
rs284875
rs2038931
rs7526590
rs284157
rs1805110
Block 1 (3 kb)
1
2
3
4
5
6
0
7
33
78
10
1
28
9
16
65
4
26
B
2 3
GG .731
GA .160
AG .109

FIGURE 2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rs284875
rs2038931
rs7526590
rs284157
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Block 1 (12 kb)
1
2
3
4
5
6
0
7
33
78
10
1
28
9
16
65
4
26
B
1 2 3
CGG .621
CGA .160
TGG .110
CAG .093
TAG .016

FIGURE 3

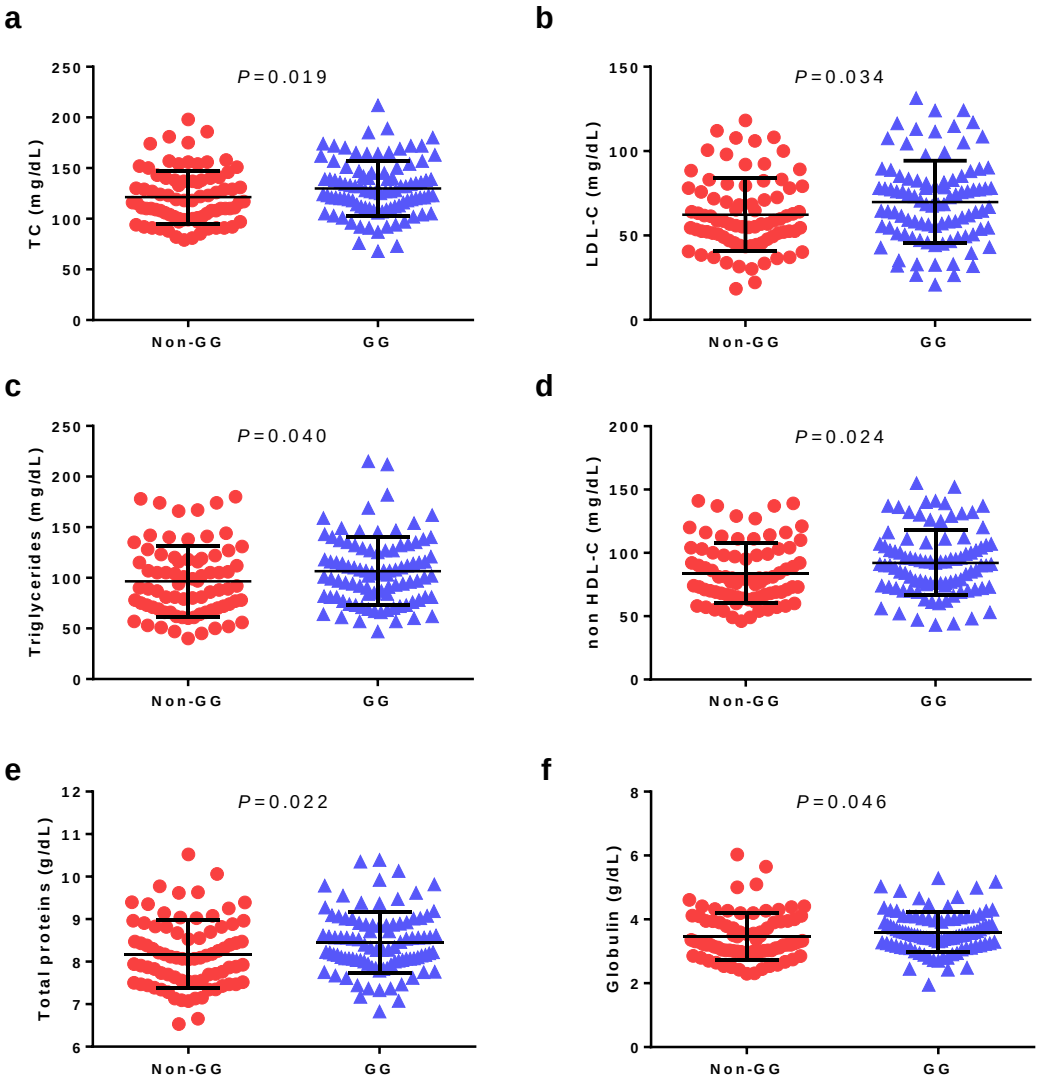
a
P=0.019
TC (mg/dL)
250
200
150
100
50
0
Non-GG
GG
b
P=0.034
LDL-C (mg/dL)
150
100
50
0
Non-GG
GG
c
P=0.040
Triglycerides (mg/dL)
250
200
150
100
50
0
Non-GG
GG
d
P=0.024
non HDL-C (mg/dL)
200
150
100
50
0
Non-GG
GG
e
P=0.022
Total proteins (g/dL)
12
11
10
9
8
7
6
Non-GG
GG
f
P=0.046
Globulin (g/dL)
8
6
4
2
0
Non-GG
GG

FIGURE 4

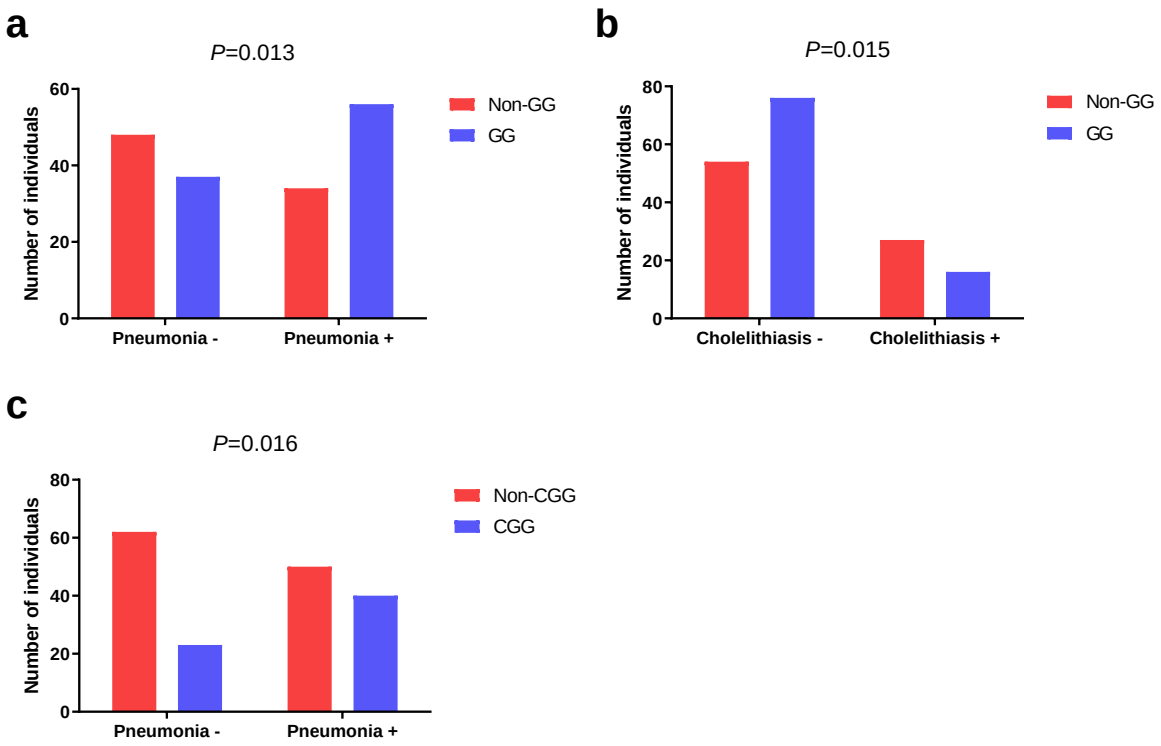
a
P=0.013
Number of individuals
60
40
20
0
Pneumonia -
Pneumonia +
Non-GG
GG
b
P=0.015
Number of individuals
80
60
40
20
0
Cholelithiasis -
Cholelithiasis +
Non-GG
GG
c
P=0.016
Number of individuals
80
60
40
20
0
Pneumonia -
Pneumonia +
Non-CGG
CGG

FIGURE 5

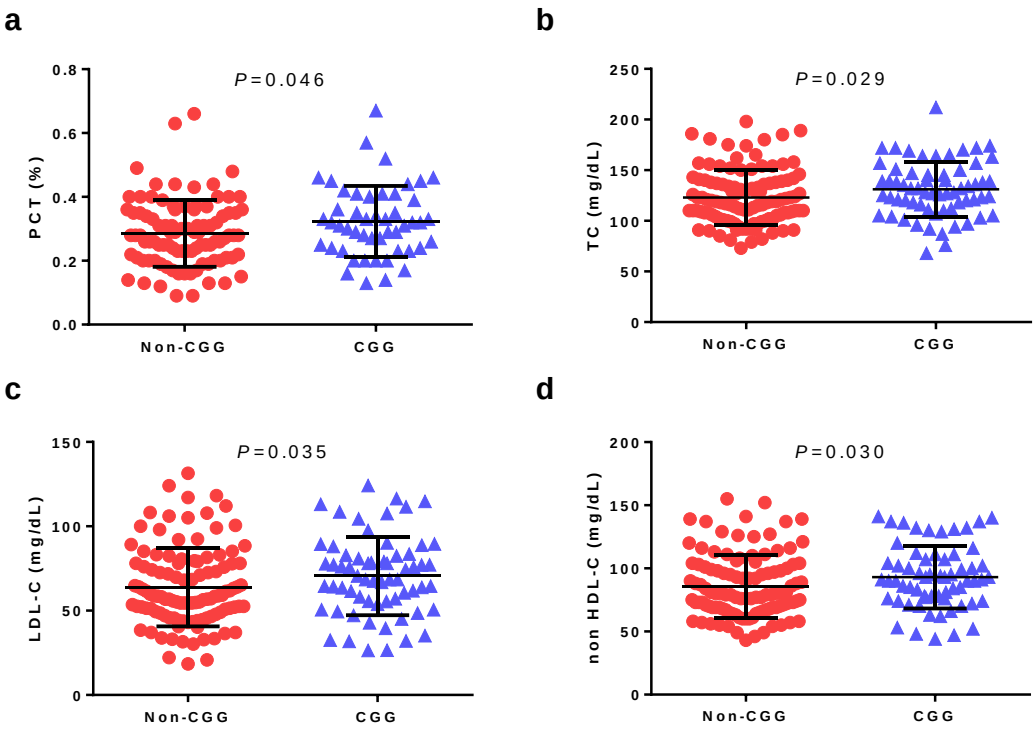
a
P=0.046
PCT (%)
0.8
0.6
0.4
0.2
0.0
Non-CGG
CGG
b
P=0.029
TC (mg/dL)
250
200
150
100
50
0
Non-CGG
CGG
c
P=0.035
LDL-C (mg/dL)
150
100
50
0
Non-CGG
CGG
d
P=0.030
non HDL-C (mg/dL)
200
150
100
50
0
Non-CGG
CGG

FIGURE LEGENDS

Figure 1. Linkage disequilibrium analysis of rs2765888 (C>T), rs284875 (A>G), rs2038931 (G>A), rs7526590 (A>T), rs284157 (C>T) and rs1805110 (G>A) using the four-gamete rule method. (a) According to HaploView 4.2 color schemes, white corresponds to D' <1 and LOD <2, shades of pink/red indicate D' < 1 and LOD ≥ 2 and light blue indicates complete LD with D' = 1 and LOD <2. (b) Identified haplotypes and associated frequencies. LOD: Log of odds ratio, a measure of confidence of the value of D'.

Figure 2. Linkage disequilibrium analysis of rs2765888 (C>T), rs284875 (A>G), rs2038931 (G>A), rs7526590 (A>T), rs284157 (C>T) and rs1805110 (G>A) using the solid spine of LD method. (a) According to HaploView 4.2 color schemes, white corresponds to D' <1 and LOD <2, shades of pink/red indicate D' < 1 and LOD ≥ 2 and light blue indicates complete LD with D' = 1 and LOD <2. (b) Identified haplotypes and associated frequencies. LOD: Log of odds ratio, a measure of confidence in the value of D'.

Figure 3. Associations between the GG haplotype and laboratory parameters in individuals with SCD. Carriers of the GG haplotype presented increased (a) total cholesterol (TC) levels, (b) low density lipoproteins cholesterol (LDL-C) levels, (c) triglycerides and (d) non-low-density lipoprotein cholesterol (non-HDL-C) (all p values obtained by the Mann-Whitney *U* test). Carriers of the GG haplotype also had presented increased (e) total protein levels (p value obtained with t-testing) and (f) increased globulin levels (p value obtained by the Mann-Whitney *U* test).

Figure 4. Associations between the GG and CGG haplotypes and clinical manifestations in individuals with SCD. GG haplotype was associated with (a) a previous history of pneumonia, while non-GG haplotypes were associated with (b) a previous history of cholelithiasis. The CCG haplotype was associated with (c) a previous history of pneumonia (all p values obtained by the Chi square test).

Figure 5. Associations between the CGG haplotype and laboratory parameters in individuals with SCD. Carriers of the CGG haplotype presented increased levels of (a) plateletcrit (PCT), (b) total cholesterol (TC), (c) low-density lipoprotein cholesterol

(LDL-C), and (d) non-high-density lipoprotein cholesterol (non-HDL-C) (p values obtained by the Mann-Whitney U test).

Supplementary Table 1. Baseline characteristics of individuals with SCD.

Parameter	N	Mean ± SD	Reference value*
Hemoglobin pattern			
Fetal hemoglobin, %	175	6.80 ± 5.98	≤ 2.00
S hemoglobin, %	175	73.70 ± 17.20	-
Hematological markers			
RBC, 10^6/mL	175	3.20 ± 0.84	4.00 – 5.40
Hemoglobin, g/dL	175	9.41 ± 1.72	11.30 – 16.30
Hematocrit, %	175	27.98 ± 5.30	35.00 – 49.00
MCV, fL	175	89.00 ± 11.61	80.00 – 99.00
MCH, ρg	175	30.10 ± 4.04	27.00 – 32.00
MCHC, g/dL	175	33.81 ± 0.93	31.50 – 35.50
RDW, %	175	21.19 ± 4.07	11.00 – 14.00
Reticulocyte Count, %	175	4.47 ± 1.90	0.50 – 1.50
WBC, /mL	175	10640.52 ± 3275.07	3700 – 10000
Neutrophils, /mL	175	5304.01 ± 2363.04	2500 – 7500
Eosinophils, /mL	175	390.87 ± 276.50	400 – 600
Lymphocytes, /mL	175	3717.92 ± 1379.72	720 – 4800
Monocytes, /mL	175	976.87 ± 519.15	120 – 1000
Platelet count, x10^3/mL	175	381.62 ± 139.14	150 – 450
Platelet Volume Average, fL	175	8.02 ± 0.89	6.50 – 12.00
Plateletcrit, %	175	0.29 ± 0.10	0.22 – 0.24
Biochemical markers			
TC, mg/dL	175	125.95 ± 27.04	< 200.00
HDL-C, mg/dL	175	37.12 ± 9.36	> 40.00
LDL-C, mg/dL	175	66.38 ± 23.46	< 130.00
VLDL-C, mg/dL	175	20.37 ± 6.87	< 40.00
Triglycerides, mg/dL	175	102.01 ± 34.24	< 130.00
Non-HDL-C, mg/dL	175	88.33 ± 25.06	< 145.00
TC/HDL-C ratio	175	3.56 ± 1.13	-
Triglycerides/ HDL-C ratio	175	2.97 ±1.60	-
LDL-C/HDL-C ratio	175	1.88 ± 0.82	-
Total bilirubin, mg/dL	175	2.46 ± 1.56	≤ 1.20
Direct bilirubin, mg/dL	175	0.37 ± 0.16	≤ 0.40
Indirect bilirubin, mg/dL	175	2.09 ± 1.51	≤ 0.90
LDH, U/L	175	967.33 ± 422.52	≤ 480
ALT, U/L	175	16.81 ± 9.52	Male: < 45.00 Female: < 37.00
AST, U/L	175	40.80 ± 18.77	< 42.00
Total protein, g/dL	175	8.33 ± 0.80	6.00 – 8.00
Albumin, g/dL	175	4.80 ± 0.33	3.50 – 5.50
Globulin, g/dL	175	3.53 ± 0.68	2.30 – 3.50
Albumin /Globulin Ratio	175	1.39 ± 0.26	1.00 – 2.50
Iron, mcg/dL	175	99.38 ± 39.10	Male: 65 – 170 Female: 50 – 170
Ferritin, ηg/ mL	175	216.45 ± 187.64	Male: 23.90 – 336.20 Female: 11.00 – 306.80

Urea nitrogen, mg/dL	175	17.61 ± 5.54	15.00 – 45.00
Creatinine, mg/dL	175	0.49 ± 0.16	0.40 – 1.30
CRP, mg/L	175	3.49 ± 2.42	< 6.00
AAT, mg/dL	175	77.71 ± 46.16	80.00 – 200.00

RBC: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red Cell Distribution Width; LDH: Lactate dehydrogenase; WBC: White blood cells; TC: total cholesterol; 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; CRP: C reactive protein; AAT: Alpha 1-antitrypsin; SD: standard deviation. *References values for adolescents.

7 DISCUSSÃO

Considerando que a via do TGF-β está associada a inflamação, hematopoese, resposta imune, angiogênese e outros processo celulares, e que estes processos têm sido relacionados à patogênese da DF, o presente estudo buscou investigar a associação dos níveis plasmáticos de TGF-β1, dos polimorfismos e haplótipos no gene *TGFBR3* com biomarcadores hematológicos, bioquímicos e imunológicos em indivíduos com DF, bem como com as complicações clínicas.

A DF é caracterizada por uma tríade de eventos: hemólise, vaso-oclusão e inflamação. Os mecanismos são indissociáveis e os resultados apresentados no presente estudo mostram que os níveis de TGF-β1, assim como os polimorfismos e haplótipos no gene *TGFBR3* estiveram associados a esses três fenômenos.

Primeiro, os resultados demonstraram que os níveis de TGF-β1, o alelo variante (T) do polimorfismo rs7526590 e o haplótipo GG estiveram associados a hemólise.

A HbS forma polímeros em tensões baixas de oxigênio, o que promove o aumento da rigidez celular e a distorção da membrana do eritrócito que adquire o formato de foice. Esse processo desencadeia a falência energética celular, estresse, desidratação e hemólise prematura (SUNDD et al., 2018). A hemólise é ocasionada pela ruptura do eritrócito, que libera Hb, heme e arginase no espaço vascular, promovendo o aumento do estresse oxidativo, redução dos níveis de NO e respostas inflamatórias (SMITH et al.,2015; PIEL et al., 2017; GUARDA et al., 2017; SANTIAGO et al., 2018). Esse contexto oxidativo e inflamatório contribui para a ativação de hemácias, leucócitos, plaquetas e células endoteliais, fazendo com que o ciclo inflamatório se mantenha com mais produção de ROS e citocinas pró-inflamatórias (VILAS BOAS et al., 2010; DUTRA et al., 2014).

No presente estudo identificou-se correlações positivas entre os níveis de TGF-β1 e os níveis de heme e aspartato aminotransferase (AST) em indivíduos com HbSC demonstrando que os níveis de TGF-β1 estão ligados a hemólise. Esse é um achado novo, contudo, genes envolvidos na via de sinalização do TGF-β/BMP têm sido associados ao subfenótipo hemolítico e as manifestações clínicas decorrentes do processo hemolítico, como por exemplo as úlceras de perna (NOLAN et al., 2006).

A avaliação do polimorfismo rs7526590 do gene *TGFBR3* revelou que o alelo variante (T) esteve associado a concentrações elevadas de RDW e marcadores bioquímicos de hemólise, como a AST, bilirrubina indireta e LDH e a concentrações diminuídas de ferritina, sugerindo uma provável influência desse polimorfismo na hemólise. Quando a hemólise intravascular ocorre, os eritrócitos liberam LDH, AST, Hb e arginase no sangue (KATO et al., 2006; NSIAH et al., 2011). Na hemólise extra vascular, macrófagos do baço e fígado removem eritrócitos senescentes que são lisados, sendo que o grupo heme da Hb é convertido em bilirrubina não conjugada (GUARDA et al., 2017). Os indivíduos com AF apresentam a forma mais grave de DF, já que a hemólise é acentuada, a anemia é mais grave e as complicações resultantes da hemólise ocorrem com frequência (STEINBERG, 2001; REES et al., 2012).

O alelo variante (T) do polimorfismo rs7526590 no gene *TGFBR3* esteve associado à ocorrência de úlceras de perna. Esse mesmo polimorfismo já foi previamente associado ao priapismo em indivíduos com DF (ELLIOTT et al., 2007). As úlceras de perna e os episódios de priapismo são classificados por Kato e colaboradores (2007) como parte do sub-fenótipo hemolítico. Taylor e colaboradores (2008) mostraram que indivíduos com DF e hiper-hemólise apresentavam prevalência elevada de úlcera de perna, priapismo e hipertensão pulmonar. No presente estudo, os indivíduos com histórico prévio de úlceras de perna apresentaram concentrações elevadas dos marcadores hemolíticos, AST e LDH, sendo que estes marcadores já foram associados, em estudos anteriores, com a ocorrência de úlceras de perna e priapismo na DF (KATO et al., 2006; KATO et al., 2007; NOLAN et al., 2005; MINNITI et al., 2010). Esses dados reforçam a associação do alelo variante (T) do polimorfismo rs7526590 com o sub-fenótipo hemolítico e os marcadores laboratoriais de hemólise na DF.

Quando se avaliou os haplótipos no gene *TGFBR3* foi identificado que indivíduos com DF portadores do haplótipo GG apresentavam concentrações elevadas de proteínas totais e globulina. Estudos anteriores descreveram concentrações elevadas de proteína total e globulina em indivíduos com DF quando comparados aos controles (PANDEY et al., 2012; OZGUNES et al., 2015). O estudo de Matthew e colaboradores (2012) descreveu que a hiperproteinemia apresentada pelos indivíduos com DF é resultado da hiperglobulinemia que acontece em resposta

à destruição de eritrócitos durante a falcização. Assim, indivíduos com haplótipo GG apresentaram hemólise mais proeminente que indivíduos com haplótipo não-GG.

Descreveu-se, também, a associação do haplótipo não-GG à ocorrência de colelitíase. A colelitíase é uma das complicações mais frequentes na DF, geralmente é encontrada em 26-58% dos pacientes, sendo frequentemente associada ao aumento nas concentrações de bilirrubina e, consequentemente, à hemólise, que é uma característica importante associada ao sub-fenótipo hemolítico descrito na doença (EBERT et al., 2010; BOND et al., 1987; KATO et al., 2007). A colelitíase é uma manifestação clínica da DF e está relacionada à hemólise crônica que desencadeia a produção de bilirrubina e, portanto, a formação de cálculos biliares (MARTINS et al., 2017). A via do TGF-β foi previamente associada à colelitíase, quando foram avaliadas vesículas biliares de indivíduos com colelitíase e constatou-se que essa via estava super expressa (KONINGER et al., 2005). Além disso, a investigação da expressão gênica em pacientes submetidos à colecistectomia encontrou expressão significativa do TGFβRI e TGFβRII durante a colelitíase crônica quando comparada à colelitíase aguda, embora não tenha sido encontrado o aumento significativo na expressão de TGFβRIII (WALAWALKAR et al., 2016).

Durante a hemólise intravascular, o eritrócito libera o seu conteúdo para o plasma, mais especificamente Hb livre, heme e arginase. A Hb livre degrada o NO disponível levando a produção de nitratos, meta-hemoglobina e nitrosil-hemoglobina, enquanto a arginase 1 consome a L-arginina que é o substrato da NO sintase endotelial (eNOS) para a produção de NO (GUARDA et al., 2017; KATO et al., 2018). A diminuição da biodisponibilidade de NO é a característica principal da disfunção endotelial, reduzindo a vasodilatação, enquanto causa ativação, proliferação endotelial e o remodelamento vascular (GUARDA et al., 2017). O presente estudo evidenciou a associação dos níveis de TGF-β1 e o do polimorfismo rs1805110 no gene *TGFBR3* a vasculopatia e disfunção endotelial na DF.

Identificou-se que os níveis de TGF-β1 estavam aumentados em indivíduos HbSS quando comparados aos indivíduos controles sadios (CS) e HbSC (SS>CS>SC). Um estudo realizado em indivíduos adultos com DF também identificou que os indivíduos com AF, forma mais grave da DF, apresentaram níveis elevados de TGF-β1 quando comparados aos indivíduos com HbSC e HbSβ-talassemia (TORRES et al., 2016). Keikhaei e colaboradores (2013) também observaram níveis elevados de TGF-β em indivíduos com DF em estado estável

quando comparados a indivíduos sadios. Carvalho e colaboradores (2018) demonstraram que os níveis de TGF-β estavam aumentados em indivíduos com AF em estado estável quando comparados aqueles em crise, mostrando que o TGF-β pode ser preditor do estado estável da DF.

De acordo com os resultados obtidos no presente estudo, acredita-se que os níveis elevados de TGF-β podem estar associados ao remodelamento endotelial e vasculopatia, assim como foi descrito por Nolan e colaboradores (2006). O endotélio tem a propriedade de controlar a liberação de fatores responsáveis pelo relaxamento e contração para regular o tônus vascular. A vasculopatia, em associação com a disfunção endotelial, é o principal evento crônico descrito na DF, uma vez que está presente na patogênese do AVC, doença renal e pulmonar e hipertensão (REES e GIBSON, 2012).

Reforçando o papel do TGF-β na angiogênese, vasculopatia e disfunção endotelial, identificou-se que o TGF-β foi positivamente correlacionado com TIMP-1 e, na análise de associação, os indivíduos com níveis elevados de TGF-β apresentaram níveis aumentados de TIMP-1. Além disso, os indivíduos HbSC também apresentaram níveis elevados de MMP-9. O TIMP-1 é uma proteína que pode modular o crescimento celular, apoptose, angiogênese e inibir a atividade da MMP-9 (KWAK et al., 2006). O TGF-β1 é capaz de estimular a expressão de MMP-2, MMP-9 e TIMP-1, além de aumentar os níveis de TIMP-1, o que pode impactar diretamente na angiogênese (PANTSULAIA et al., 2004). Na síndrome metabólica, a elevação da MMP-9 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 (WAKEFIELD et al., 2008; APLIN et al., 2009; ARDI et al., 2009; GONÇALVES et al., 2009).

Quando se avaliou os polimorfismos em *TGFBR3* identificou-se que os indivíduos com o alelo variante (A) do polimorfismo rs1805110 apresentaram concentrações elevadas de endotelina, sugerindo que eles podem estar sujeitos a vasoconstricção e disfunção endotelial. O TGF-β é um dos fatores que induz a expressão da endotelina-1, assim como a trombina, mediadores inflamatórios e a hipóxia, que atua como vasoconstrictor, ativando células endoteliais e promovendo a inflamação vascular (RODRIGUEZ-PASCUAL et al., 2004; VILAS-BOAS et al., 2016, LUTZ et al., 2018).

Além da hemólise, a vaso-oclusão também é importante clinicamente, pois contribui com vários episódios dolorosos. No presente estudo, os níveis de TGF-β1 e os polimorfismos rs1805110 e rs7526590 estiveram associados a marcadores relacionados aos fenômenos vaso-oclusivos da DF.

Na fisiopatologia da DF, a falcização além de desencadear o processo hemolítico também promove o fenômeno vaso-oclusivo. Os eventos vaso-oclusivos ocasionam a isquemia tecidual devido a interação entre os eritrócitos falcizados, leucócitos, plaquetas e o endotélio vascular que geram a oclusão microvascular (PIEL et al., 2017; GUARDA et al., 2017; KATO et al., 2018; SUNDD et al., 2018).

Os indivíduos com níveis elevados de TGF-β1 apresentaram contagem elevada de plaquetas. Estudos anteriores demonstraram que as plaquetas produzem mais TGF-β quando esta produção é comparada a outros tipos celulares; além disso, depois de ativadas, as plaquetas liberam rapidamente o TGF-β (ASSOIAN et al., 1983; AHAMED et al., 2008; SEIZER e MAY, 2013). O estudo de Torres e colaboradores (2016) mostrou que as contagens de plaquetas foram positivamente correlacionadas com os níveis de TGF-β em indivíduos HbSS e HbSC (TORRES et al., 2016). Esses dados corroboram com o resultado da análise de correlação apresentada no presente estudo, que demonstrou a correlação positiva entre plaquetas e TGF-β1.

Quando os polimorfismos em *TGFBR3* foram avaliados, identificou-se que os indivíduos com alelo variante A do polimorfismo rs1805110 apresentaram valores diminuídos do índice de anisocitose plaquetária, do inglês *platelet distribution width* (PDW). O presente estudo foi o primeiro a associar esse polimorfismo a parâmetros hematológicos na DF. A avaliação *in vitro* mostrou que o TGF-β1 é capaz de modular a eritropoiese, uma vez que pode inibir a eritropoiese bloqueando a proliferação e diferenciação dos progenitores eritróides (ZERMATI et al., 2000). Além disso, o alelo variante A do polimorfismo rs1805110 esteve associado à ocorrência de alterações ósseas. Alterações ósseas, como osteonecrose de cabeça do fêmur e osteomielite são complicações comuns desenvolvidas por indivíduos com DF (ADESINA et al., 2017). Um estudo identificou outros polimorfismos no gene *TGFBR3* associados à osteonecrose em indivíduos com DF (BALDWIN et al., 2005). Além disso, o polimorfismo rs1805110 já foi associado à doença de Behcet's, uma doença inflamatória caracterizada por vasculite sistêmica, com lesões inflamatórias acentuadas no sistema nervoso central, pele, articulações, mucosas oro-genital e

olhos; de forma análoga à DF, o componente inflamatório é a principal característica fisiopatológica dessa doença que pode levar ao desenvolvimento de osteonecrose e infarto ósseo (BARRY et al., 2015; CHEN et al., 2012; CHANG et al., 2001).

Na análise do polimorfismo *rs7526590*, o alelo variante T esteve associado a valores elevados de PDW, o que já foi associado a hiperplasia da medula óssea, com liberação de plaquetas subfuncionais na DF (AMIN et al., 2004). O alelo variante T do polimorfismo rs7526590 também esteve associado a concentrações elevadas de fosfatase alcalina, que podem ser explicadas pela falcização intra-hepática que gera lesões no tecido do hepático (TRIPATHI e TRIPATHI, 2016).

O terceiro mecanismo fisiopatológico da DF corresponde à resposta inflamatória, onde observamos grande ativação das células e produção elevada de moléculas inflamatórias. Os dados do presente estudo sugerem que tanto os níveis de TGF-β1 quanto o alelo variante A do polimorfismo rs1805110 e os haplótipos GG e CGG no gene *TGFBR3* estiveram associados a marcadores inflamatórios.

A fisiopatologia da DF é complexa, mas sabe-se que a inflamação desempenha papel importante e tem sido associada à ativação de eritrócitos, reticulócitos, leucócitos, plaquetas e células endoteliais, com a expressão de moléculas secundárias, expressas nesse microambiente inflamatório e na superfície dessas células (CARVALHO et al., 2017).

As citocinas pró-inflamatórias IL-1β, TNF-α, IL-6, IL-8, IL-17 e TGF-β são produzidas por leucócitos, células vasculares endoteliais e plaquetas, durante a hemólise, oclusão microvascular ou infecção, e todas elas implicam no desenvolvimento de complicações clínicas na DF (KEIKHAEI et al., 2013). Alguns estudos têm demonstrado que as citocinas TGF-β1, IL-17 e IL-18 participam da inflamação e ativação vascular, apresentando ainda papel importante na vaso-oclusão (GUARDA et al., 2017; VILAS BOAS et al., 2016; CERQUEIRA et al., 2011).

Na literatura, polimorfismos no gene *TGFBR3* têm sido associados a doenças que apresentam forte componente inflamatório, assim como o identificado na DF, como, por exemplo, a síndrome de Marfan, câncer de bexiga e doença de Behçet (STEINBERG e SEBASTIANI, 2012; GROENEVELD et al., 2018; LIU et al., 2013; BARRY et al., 2015). A síndrome de Marfan causa complicações clínicas nos sistemas cardiovascular, esquelético, pulmonar, ocular e nervoso e sua fisiopatologia inclui alterações na deposição da matriz extracelular nos vasos, semelhante aos achados na DF (GROENEVELD et al., 2018).

A análise de associação mostrou que os indivíduos HbSC e níveis elevados de TGF-β1 apresentavam contagem elevada de leucócitos. Além disso, identificou-se que os níveis de TGF-β1 estavam positivamente correlacionados a contagem de leucócitos, eosinófilos, linfócitos e monócitos indicando a presença de uma resposta inflamatória. O estudo de Torres e colaboradores (2016) encontrou resultados similares de correlação entre os níveis de TGF-β1 e a contagem total de leucócitos em indivíduos com HbSβ–talassemia. O TGF-β é uma citocina que está envolvida na quimiotaxia de neutrófilos e monócitos, com impacto no recrutamento de leucócitos para os locais de inflamação (FRANGOGIANNIS, 2017). O estado inflamatório crônico que caracteriza os pacientes com DF é marcado pelo aumento da expressão de moléculas de adesão e produção de fatores quimiotáticos e de citocinas inflamatórias que promovem o recrutamento dos leucócitos (KATO et al., 2018; GUARDA et al., 2017; SUNDD et al., 2019).

Quando avaliamos o perfil lipídico, os indivíduos HbSC com níveis elevados de TGF-β1 apresentavam concentrações diminuídas de HDL-C. Os níveis de TGF-β1 ainda foram positivamente correlacionados com VLDL-C e triglicérides. As concentrações diminuídas de HDL-C estão associadas ao estado inflamatório encontrado em indivíduos com DF, visto que ele apresenta ações anti-inflamatórias e vaso protetoras bem documentadas pela literatura (ALELUIA et al., 2017, SEIXAS et al., 2010). A HDL-C é uma lipoproteína composta por várias partículas, cuja função mais relevante é o transporte reverso de colesterol que consiste na retirada do excesso de colesterol dos tecidos periféricos, transportando-o para o fígado para excreção pela via biliar (SEIXAS et al., 2010; ALELUIA et al., 2017). Outra função importante da HDL-C 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 anti-inflamatórias, antioxidante, anticoagulante e pró-fibrinolítica. O HDL-C exerce papel importante também no endotélio vascular, interferindo na interação entre monócitos e moléculas de adesão, como a molécula de adesão celular vascular (VCAM), molécula de adesão intercelular (ICAM) e E-selectina expressas pelo endotélio (NOFER et al., 2002). O HDL-C é um marcador que está associado ao sub-fenótipo dislipidêmico que abrange o estado inflamatório da DF que ocorre devido a alterações nos lipídeos (ALELUIA et al., 2017). Triglicérides e VLDL-C são moléculas capazes de induzir a

ocorrência de eventos inflamatórios na aterosclerose, apresentando assim propriedades pró-inflamatórias (WELTY et al., 2013).

Na avaliação dos polimorfismos e haplótipos em *TGFBR3* identificou-se que o alelo variante (A) do polimorfismo rs1805110, assim como os haplótipos GG e CGG estiveram associados a concentrações elevadas de LDL-C. Os indivíduos portadores dos haplótipos GG e CGG ainda apresentaram concentrações elevadas de colesterol total quando comparados a indivíduos portadores dos haplótipos não-GG e não-CGG, respectivamente. Concentrações elevadas de colesterol total e LDL-C desencadeiam o processo da aterosclerose, promovendo o acúmulo do colesterol na parede da artéria e desencadeando a resposta inflamatória (TALL et al., 2015). Os mecanismos fisiopatológicos presentes na aterosclerose são análogos aos que acontecem na vasculopatia da DF, marcados pelo estresse oxidativo, baixa disponibilidade de NO e disfunção endotelial; contudo, é importante enfatizar que na DF não há a formação de placas de ateroma (KATO et al., 2007; LIBBY et al., 2011).

Os portadores do haplótipo GG ainda apresentaram níveis elevados de triglicérides quando comparados a indivíduos com o haplótipo não-GG. Além disso, os indivíduos portadores dos haplótipos GG e CGG apresentaram níveis elevados de colesterol não-HDL (não-HDL-C) quando comparados aos portadores dos haplótipos não-GG e não-CGG. Níveis elevados de triglicerídeos e não-HDL-C também foram associados à aterosclerose. Na lipólise de triglicerídeos, remanescentes ricos em triglicerídeos são liberados no vaso, contribuindo para aumentar a inflamação, coagulação e disfunção endotelial (HANDELSMAN et al., 2017). Na DF, níveis elevados de triglicerídeos mostraram ser um fator de risco potencial para hipertensão pulmonar, uma complicação respiratória frequente em indivíduos com DF (ZORCA et al., 2010).

A fração não-HDL-C é um colesterol transportado pela apolipoproteína B, incluindo os que são transportados pelo LDL-C e VLDL-C, sendo considerado um marcador da aterosclerose (ZHANG et al., 2016). Estudos anteriores realizados em indivíduos com doença cardiovascular relataram níveis elevados de não-HDL-C (LIU et al., 2005; BOEKHOLDT et al., 2012). Na DF, não foram encontradas alterações significativas nos níveis de não-HDL-C (EPHRAIM et al., 2016).

Os marcadores de metabolismo lipídico mais especificamente, T-CHOL, LDL-C, triglicerídeos e não HDL-C, são associados à inflamação e relacionados à resposta vascular (TALL et al., 2015; LIBBY et al., 2011; ZHANG et al., 2016). A via

do TGF-β está diretamente associada a resposta inflamatória vascular em várias doenças. Na maioria dessas doenças, a captação e o tráfego de colesterol também são responsáveis por modificações na parede do vaso que contribuem para a inflamação (SALTER et al., 2016; THOMPSON et al., 2018). Diversos estudos demonstram que os pacientes com AF apresentam alterações no perfil lipídico e que estas estão relacionadas a parâmetros hematológicos, crises vaso-oclusivas, via do TGF-β, hipertensão pulmonar, entre outras complicações (SEIXAS et al., 2010; ZORCA et al., 2010; AKINLADE et al., 2014; FIGUEIREDO et al., 2016).

A avaliação do polimorfismo rs1805110 no gene *TGFBR3* mostrou que o alelo variante A esteve associado a concentrações elevadas de ácido úrico. O ácido úrico pode acelerar o processo oxidativo do LDL-C levemente oxidado contribuindo para a ativação endotelial e o estresse oxidativo (PATTERSON et al., 2003). O ácido úrico também demonstrou ter papel na ativação da via do inflamassoma em indivíduos com AF, desencadeando o estado pró-inflamatório. Os mesmos autores demonstraram a participação desse marcador em eventos inflamatórios associados a DF (CERQUEIRA et al., 2011).

Além de estarem associados a todos os processos fisiopatológicos da DF, os haplótipos GG e CGG demonstraram ainda estar associados a ocorrência de infecção, mais especificamente pneumonia nos indivíduos com DF. Em uma grande coorte de indivíduos com DF, a pneumonia foi a segunda principal causa de hospitalizações (BROUSSEAU et al., 2010; DA GUARDA et al., 2020). Estudo anterior realizado em indivíduos com AF, dos quais a maioria teve infecção por *Streptococcus pneumoniae*, identificou que o polimorfismo no gene *TGFBR3* esteve associado ao aumento da suscetibilidade à bacteremia (ADEWOYE et al., 2006). Outros membros da família do TGF-β também podem estar envolvidos nas complicações infecciosas da DF. Além do gene do *TGFBR3*, já foi descrita a associação entre polimorfismos nos genes *BMP6*, *BMPR1A*, *SMAD6* e *SMAD3* e episódios infecciosos e bacteremia na AF (ADEWOYE et al., 2006). A infecção pelo *Streptococcus pneumoniae* é uma das principais causas de pneumonia em indivíduos com DF (ADEWOYE et al., 2006). É importante ressaltar que todos os indivíduos que participaram do presente estudo eram imunizados contra doenças pneumocócicas, assim acredita-se que essas crianças podem estar passando ou já terem passado pelo processo de auto esplenectomia, o que aumenta, consequentemente, a suscetibilidade a infecções graves por bactérias encapsuladas

(BRAGA et al., 2007). A família de moléculas do TGF-β é multifuncional e exerce diversos papéis na fisiopatologia da DF, sendo que as propriedades imunorregulatórias estão associadas a processos infecciosos (BRITTAIN e PARISEA, 2007).

Outras complicações respiratórias, como asma e displasia bronco pulmonar, também estiveram associadas à *TGFBR3* (KIM et al., 2010, POZARSKA et al., 2016). A patogênese da asma e da displasia bronco pulmonar é semelhante à DF, pois apresenta estado inflamatório marcado pela ativação de várias citocinas, incluindo o TGF-β (KIM et al., 2010, POZARSKA et al., 2016).

Os resultados do presente estudo permitem inferir que os níveis de TGF-β, os polimorfismos rs1805110 e rs7526590 e os haplótipos no gene *TGFBR3* estão associados a hemólise, disfunção endotelial, vasculopatia, vaso-oclusão, inflamação, processos infecciosos que fazem parte da fisiopatologia da DF, assim como as manifestações clínicas que acometem os indivíduos com DF. A associação entre os níveis de TGF-β e os polimorfismos em *TGFBR3* com os marcadores laboratoriais reforçam a importância em identificar associações entre biomarcadores inflamatórios e biomarcadores clássicos investigados através do laboratório clínico. Os parâmetros laboratoriais muitas vezes são os mais acessíveis tanto para o paciente quanto para o clínico. A identificação de marcadores que podem modular a resposta fisiopatológica da DF contribui para o entendimento da fisiopatologia da doença e se mostra relevante no monitoramento do curso clínico e pode também auxiliar para o manejo clínico dos pacientes. É importante ressaltar que a casuística do presente estudo é composta por indivíduos em idade pediátrica o que torna importante a identificação precoce de marcadores associados ao quadro clínico mais grave da doença para melhor manejo clínico do paciente, uma vez que a história natural da doença tende a ser mais grave e mais crônica à medida que os pacientes se tornam adultos.

8 CONCLUSÕES

- Os níveis de TGF-β estiveram mais elevados nos indivíduos com AF que em indivíduos controles e HbSC, sugerindo a participação mais efetiva dessa via na fisiopatologia da AF;
- Os níveis de TGF-β estiveram associados ao TIMP-1 em ambos os genótipos de DF, mostrando que essa molécula pode desempenhar papel importante no remodelamento vascular e contribuir para a vasculopatia devido à deposição da matriz extracelular;
- Os níveis de TGF-β estiveram associados a marcadores de hemólise, leucócitos, plaquetas e marcadores do metabolismo lipídico, sugerindo a modulação inflamatória na DF;
- O alelo variante (A) do polimorfismo rs1805110 no gene *TGFBR3* esteve associado ao estado inflamatório encontrado na AF e a ocorrência de alterações ósseas, sugerindo a possível influência na gênese clínica desse evento na DF;
- O alelo variante (T) do polimorfismo rs7526590 no gene *TGFBR3* esteve associado ao sub-fenótipo hemolítico e a ocorrência de úlcera de pernas em indivíduos com DF, sugerindo a possível influência na gênese clínica desse evento na DF;
- Indivíduos com os haplótipos GG ou CGG no gene *TGFBR3* apresentaram alterações importantes no perfil lipídico, sinalizando que estes podem estar associados ao estado pró-inflamatório encontrado nos indivíduos com DF;
- Os haplótipos GG ou CGG no gene *TGFBR3* estiveram associados a manifestação clínica presente na DF, mais especificamente a pneumonia, enquanto o haplótipo não-GG esteve associado a colelitíase.

Coletivamente, os dados do presente estudo sugerem que os níveis de TGF-β1 e polimorfismos no gene *TGFBR3* estão associados a fisiopatologia e manifestações clínicas da DF. Além disso, TGF-β1 e polimorfismos no gene *TGFBR3* também podem modular o desenvolvimento, progressão e auxiliar no monitoramento clínico da DF, uma vez que associações importantes com parâmetros laboratoriais e episódios clínicos foram identificadas reforçando o ciclo fisiopatológico da DF baseado na hemólise, vaso-oclusão, inflamação e disfunção

endotelial. Dessa forma, acreditamos que os dados obtidos neste estudo podem auxiliar o manejo clínico e laboratorial dos pacientes com DF.

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APÊNDICES

A – MANUSCRITOS EM COLABORAÇÃO

(Artigos produzidos em colaboração durante o período do doutorado e que não entraram no corpo da tese).

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Letter to the Editor

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

ARTICLE INFO

Editor: Mohandas Narla

To the Editor:

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

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

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

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

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was defined as an absence of any blood transfusion and acute events for a period of four months prior to blood sampling. In addition, exclusion criteria for the study included infectious diseases or inflammatory episodes at the time of blood sampling. This study received approval from the Institutional Research Board of the Oswaldo Cruz Research Foundation and is in compliance with the Declaration of Helsinki 1964 and its subsequent amendments. Since all individuals were under 18 years, their legal guardians signed terms of informed consent.

Hematological parameters were obtained using a Coulter Counter T-890 system hematologic analyzer (Coulter Corporation, Miami, FL, USA) and hemoglobin profiles were analyzed by high-performance liquid chromatography (Bio-Rad, Hercules, California, USA). Biochemical parameters were determined using an A25 immunochemistry analyzer (Biosystems S.A, Barcelona, Catalunya, Spain). Serum hemopexin and haptoglobin levels were investigated by enzyme-linked immunoassay (ELISA, Kamiya Biomedical, Seattle, WA, USA) following the manufacturer's instructions, as well as the plasma concentrations of total heme using the QuantiChrom Heme Assay Kit (Bioassay Systems, Hayward, CA, USA). Statistical analyses were performed using the Statistical Package for the Social Sciences v. 20.0 software (IBM, Armonk, NY, USA). *P* values were obtained using the Kruskal-Wallis test. Comparisons between SS, SC and AA were obtained using Dunn's multiple comparisons test. All data and statistical analyses are presented in Table 1. Spearman's rank correlation (r) was carried out to determine correlations between pairs of variables.

Evaluating the haptoglobin and hemopexin levels, we found that SS and SC patients have lower haptoglobin and hemopexin levels (SS < SC < AA) than AA controls (Table 1). Similarly, SS and SC patients have lower hemoglobin (SS < SC < AA) and increased reticulocyte counts and serum LDH (SS > SC > AA) than AA controls. Total and indirect bilirubin followed a similar pattern (SS > SC > AA). These data suggest more hemolysis in the following pattern: SS > SC > AA.

CRP levels and white blood cell counts are evidence for more inflammation in SCD (SS > SC > AA). Heme levels and platelet counts were higher in SS patients, but similar in SC and AA patients. Finally, Total, HDL, and LDL-cholesterol levels were lower in patients with more hemolysis (SS < SC < AA). Hypocholesterolemia is associated with various forms of anemia [12]. The rapid turnover of red blood cells in SCD patients might reduce plasma cholesterol levels by increasing the demand for cholesterol in newly synthesized reticulocyte membranes.

The correlations between hemopexin, haptoglobin, heme and other markers of hemolysis within SCD patients (SS + SC) are presented in Fig. 1. Hemopexin was negatively correlated to LDH ($r = -0.509$, $p < 0.001$, Fig. 1A) and plasma heme ($r = -0.592$, $p < 0.001$, Fig. 1D). Total plasma heme was positively correlated to total bilirubin ($r = 0.582$, $p < 0.001$, Fig. 1B) and indirect bilirubin ($r = 0.602$, $p < 0.001$, Fig. 1C). Haptoglobin was not correlated to total plasma heme ($r = 0.155$, $p = 0.293$, Fig. 1E) or LDH ($r = -0.0292$, $p = 0.7905$, Fig. 1F). Also haptoglobin and hemopexin were not significantly correlated ($r = 0.0890$, $p = 0.3989$, Fig. 1G). Although both haptoglobin and hemopexin were depleted in SCD patients relative to normal controls, hemopexin was superior to haptoglobin as a marker of hemolysis within the SCD patient subgroup.

Our data demonstrate that serum haptoglobin and hemopexin are depleted in children with SS and SC disease and should be considered as clinical indicators of hemolysis. Recent data in hyperhemolytic sickle mice suggest that haptoglobin and hemopexin supplementation might be beneficial to decrease vascular inflammation and vaso-occlusion in SCD [13].

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Competing interests

JDB and GMV receive research funding from CSL Behring for work on haptoglobin and hemopexin supplementation in sickle cell disease.

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Research Article

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

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

1. Introduction

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

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ORIGINAL ARTICLE

Hemoglobin Variant Profiles among Brazilian Quilombola Communities

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ABSTRACT

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

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Africans; hemoglobinopathy; hemoglobin (Hb) variant; Quilombola

Introduction

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

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

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

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

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Supplemental data for this article can be accessed here.

PLOS ONE

RESEARCH ARTICLE

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

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Abstract

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

Introduction

Sickle cell disease (SCD) consists of a group of hemoglobinopathies in which individuals inherit hemoglobin variants derived from single point mutations, that causes morphological abnormalities in the red blood cells (RBC) [1]. Sickle cell anemia (SCA) is characterized by the homozygosity for hemoglobin S (HbS) and is the most frequent and severe form of the disease.

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

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

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

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Research Article

Effect of N(Epsilon)-(carboxymethyl)lysine on Laboratory Parameters and Its Association with β^S Haplotype in Children with Sickle Cell Anemia

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The present study aimed to investigate the association of N^{ϵ}-carboxymethyllysine (CML) with laboratory parameters and β^S haplotypes in pediatric sickle cell anemia (SCA) patients with or without hydroxyurea (HU) therapy. We included 55 children with SCA (SCA_{total}), where 27 were on HU treatment (SCA-HU^+) and 28 without HU treatment (SCA-HU^-). Laboratory characteristics were determined using electronic methods while CML was measured using competitive ELISA. β^S haplotypes were determined by RFLP-PCR. Significant increases in MCV and MCH and significant decreases in leukocytes, eosinophils, basophils, atypical lymphocytes, lymphocytes, and monocytes were found in SCA-HU^+ compared to SCA-HU^-. SCA-HU^+ presented significant reduction in aspartate transaminase and lactate dehydrogenase and increase in creatinine levels compared to SCA-HU^-. CML levels were significantly higher in both SCA-HU^+ and SCA-HU^- compared to the healthy control. In addition, a negative correlation was found between CML and alanine transaminase in SCA-HU^+ and SCA_{total} ($p < 0.01$). A significant association was found between CML levels and β^S haplotypes. The results suggest that CML has a role to play in SCA complications, independent of HU therapy.

1. Introduction

Sickle cell anemia (SCA) is a monogenic hematological disorder caused by substitution GAG>GTG at the 6th position of the beta globin gene (*HBB*) located in chromosome 11 [1]. SCA patients present a wide variability in clinical manifestations regarding the functions of vital organs as well as frequency and severity of vasoocclusive crises [2, 3]. This can be explained by factors such as fetal hemoglobin (HbF) levels, coexistence of alpha (α) thalassemia, haplotypes associated with the β^S globin gene, oxidative stress, features intrinsic to the red blood cell (RBC), and extracellular environment [4, 5]. Higher HbF levels were associated with improved survival, decreased rates of painful crises, acute chest syndrome, and osteonecrosis [6–9]. Furthermore, Senegal (SEN), Cameroon (CAM), Bantu or Central African

PLOS ONE

RESEARCH ARTICLE

Hydroxyurea alters hematological, biochemical and inflammatory biomarkers in Brazilian children with SCA: Investigating associations with βS haplotype and α-thalassemia

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Abstract

This study investigated the effects of hydroxyurea (HU) on hematological, biochemical and inflammatory parameters in children with sickle cell anemia (SCA) in association with β^S haplotype and α-thalassemia. We included 22 children with SCA who were followed for an average of 14.5 months. Laboratory parameters were assessed by electronic methods, and molecular analysis was investigated by PCR-RFLP and allele-specific PCR. Results showed significant increases in hemoglobin, HbF, hematocrit, MCV, MCH, glucose, HDL-C and albumin levels, as well as significant decreases in MCHC and AST levels, WBC, neutrophils, eosinophils, lymphocytes and reticulocytes, in children during HU therapy. HbF levels were positively correlated with hemoglobin, hematocrit, MCV and total protein, yet negatively correlated with MCHC, RDW, AAT and AST during HU therapy ($p<0.05$). Children who carried the Central African Republic haplotype, in response to HU therapy, presented significant increases in hemoglobin, hematocrit, triglycerides and uric acid levels, as well as significant decreases in MCHC, AST and direct bilirubin levels, WBC, neutrophils, eosinophils, lymphocytes and reticulocytes. Those with the Benin haplotype presented increases in HbF and albumin levels, and a reduction in platelet counts ($p<0.05$). Children with α-thalassemia presented decreased ALT during HU use, while those without this deletion presented increases in hemoglobin, hematocrit, MCV, MCH, HDL-C and albumin, as well as decreases in MCHC, neutrophils, lymphocytes, reticulocytes and AST ($p<0.05$). Hence, regardless of its use in association with β^S haplotypes or α-thalassemia, HU seems to be linked to alterations in hemolytic, inflammatory, hepatic, lipid and glycemic profiles.

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ORIGINAL ARTICLE

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

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Abstract

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

KEYWORDS

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

1 | INTRODUCTION

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

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REVIEW ARTICLE

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

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

Introduction

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

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

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Research Article

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

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

1. Introduction

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

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

frontiers in Immunology

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

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

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

INTRODUCTION

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

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

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ORIGINAL RESEARCH
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Evaluation of Cardiometabolic Parameters among Obese Women Using Oral Contraceptives

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

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

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

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

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

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

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BMC Hematology

RESEARCH ARTICLE Open Access

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

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Abstract

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

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

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

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

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

Background

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

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

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

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

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

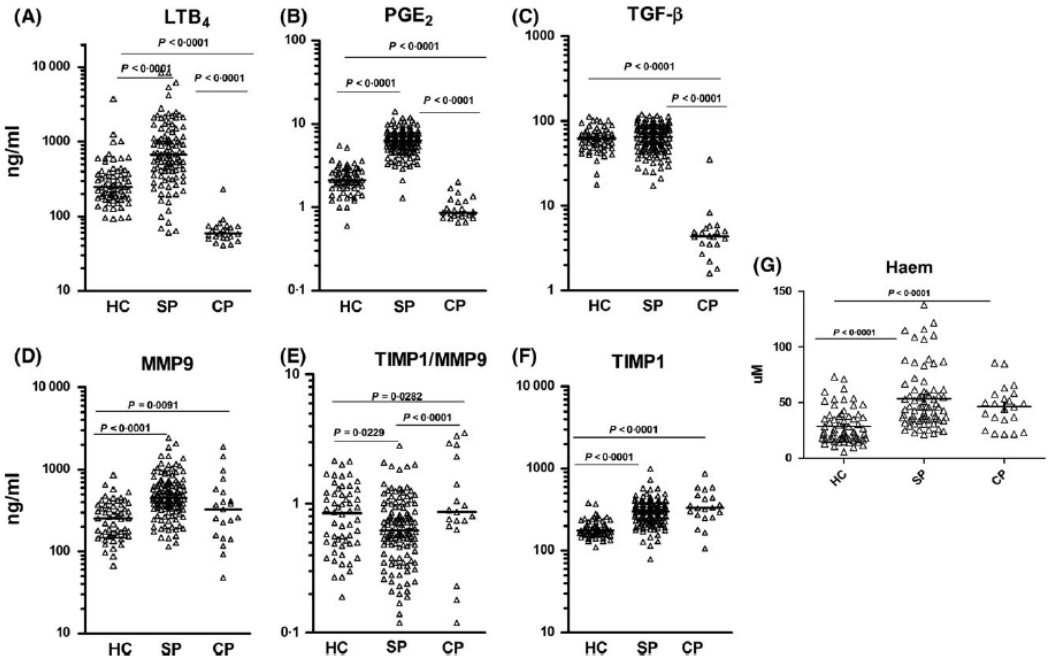

Fig 1. Inflammatory mediator and tissue remodelling marker concentrations in sickle cell anaemia patients and controls. Evaluation of LTB_4 (A), PGE_2 (B), TGF-β (C), MMP9 (D), TIMP1/MMP9 ratio (E), TIMP1 (F) and haem (G) levels among sickle cell anaemia patients in steady state and crisis, as well as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

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were used to compare two numerical variables according to distribution. Additionally, the 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.

Characteristics of the SCA patient groups and the healthy control group are described in Tables S1 and S2. As expected, the crisis state SCA patients had the lowest haematological and chemical marker concentrations. Our results show that LTB_4, PGE_2 and TGF-β were increased in steady state SCA patients. In addition, higher concentrations of haem, TIMP1, and MMP9 were increased in both patient groups (steady and crisis state) (Fig 1A–G).

The inflammatory cytokines IL1β, IL6, IL10 and TNF-α were increased in SCA patients in crisis state, and IL12 and IL8 were increased in both crisis and steady state SCA patients (Fig 2A–G).

The ROC curve showed a predictive power and demonstrates that LTB_4, TGF-β and PGE_2 serve as markers of steady state SCA, whereas TNF-α, IL1β and IL10 serve as markers of crisis state SCA (Figure S1).

Inflammation is a complex mechanism modulated by several cell types as well as many different molecules. In this context, the inflammatory response in SCA is driven by a considerable amount of stimuli, such as monocytes, neutrophils, platelets, irreversibly sickled erythrocytes, cytokines, lipid mediators and even haem released during haemolysis (Taylor *et al*, 2008; Lanaro *et al*, 2009; Monteiro *et al*, 2011; Zhang *et al*, 2016). We found increased levels of LTB4, PGE2 and TGF-β among steady state patients. These molecules have been previously associated with neutrophil chemotaxis and vascular endothelial cells activation, and were also correlated with leucocyte counts (Setty & Stuart, 2002; Monteiro *et al*, 2011; Torres *et al*, 2016). Cytokines are molecules responsible for the signalling mechanisms during immune response and we identified higher IL1β, IL6, IL10 and TNF-α levels in crisis state SCA. These cytokines are produced during acute inflammatory conditions, such as the crisis state, and were associated with vascular dysfunction and leucocytosis. It is believed that neutrophils, followed by monocytes and endothelial cells, may serve as a source of these mediators (Brittain & Parise, 2007; Lanaro *et al*, 2009). The levels of free haem, IL12, IL8, TIMP1 and MMP9 remained elevated on both states. SCA patients exhibit a chronic haemolytic feature and systemic free haem levels and reactive oxygen species are released during haemoglobin (Hb) catabolism following intravascular haemolysis (Taylor *et al*, 2008). Both IL8 and free haem were associated with acute chest syndrome in SCA (Adisa *et al*, 2013).

Systemic levels of MMPs and TIMPs, as well as their ratio, have been associated with normal and pathological events, including tissue remodelling, metastasis, angiogenesis, multiple sclerosis, obesity, metabolic syndrome and atherosclerosis (Belo *et al*, 2009). Our data suggest continuous production in both SCA groups, which may represent active matrix remodelling and maintenance of tissue destruction and degradation in these patients.

The ROC curve analysis allowed us to identify markers associated with steady state and crisis state SCA patients. The markers had high sensitivity, specificity and accuracy, showing high predictive values for these biomarkers in the follow-up of SCA patients.

Our data highlight the molecular differences in the chronic inflammatory response exhibited by both states of the disease. LTB_4, PGE_2 and TGF-β may be useful predictors of steady state SCA while IL1β, IL6 and TNF-α may be useful predictors of crisis state SCA. The high levels of free haem, MMP9 and TIMP1 in both SCA states suggest a possible role of free haem in the chronic haemolysis and vasculopathy.

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Authorship

MOSC was involved in the study design, collected the samples, performed the experiments at IGM/FIOCRUZ and at College of Pharmaceutical Sciences (UFBA), performed statistical analyses and wrote the paper. TAS, BAVC, NFL, JHOR and CGB helped with the sample collection and performed the enzyme immunoassays. CCG, CVBF, LMF, RPS provided technical support, discussed the results and co-wrote the paper. LCR, IML and VML assist the patients enrolled in the study. MBN, VMB and MSG were involved in the design and coordination of the study, provided academic support, co-wrote and critically revised the manuscript. All authors read and approved the final manuscript.

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Research Article

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

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

1. Introduction

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

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REVIEW

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

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ABSTRACT

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

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

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

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

1. Sickle cell anemia

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

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

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

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

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RESEARCH Open Access

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

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Abstract

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

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

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

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

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

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frontiers in Microbiology

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

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

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

INTRODUCTION

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

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

To the Editor:

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

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

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

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

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

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

TABLE 1 Laboratory characteristics of SCA patients taking and not taking hydroxyurea (HU)

Laboratory value	All patients *N* = 141 Median (25th-75th)	No HU use* *N* = 99 Median (25th-75th)	HU * *N* = 42 Median (25th-75th)	*P value**
RBC, $\times 10^{12}$/L	2.4 (2.2-2.8)	2.6 (2.3-2.8)	2.2 (2.0-2.6)	**.004**
Hemoglobin, g/dL	7.7 (7.2-8.7)	7.6 (7.2-8.5)	8.0 (7.3-9.2)	.206
Hematocrit, %	22.0 (20.0-25.0)	21.8 (20.0-24.4)	22.4 (19.6-25.2)	.757
MCV, fL	89.2 (83.6-96.3)	86.2 (81.4-90.9)	97.2 (90.7-103.4)	**<.001**
Total bilirubin, mg/dL	2.4 (1.6-3.5)	2.6 (1.7-3.8)	2.1 (1.5-3.0)	.084
Direct bilirubin, mg/dL	0.4 (0.3-0.6)	0.4 (0.3-0.6)	0.4 (0.3-0.5)	.501
Indirect bilirubin, mg/dL	2.0 (1.2-3.0)	2.2 (1.2-3.2)	1.6 (1.1-2.5)	.072
LDH, U/L	999.0 (738.5-1586.5)	1094.0 (791.0-1710.0)	897.0 (677.2-1480.5)	.101
HbF, %	9.9 (5.7-14.6)	9.3 (5.2-14.1)	11.1 (9.0-15.4)	**.013**
HbF, g/dL	0.76 (0.43-1.10)	0.71 (0.40-1.09)	0.85 (0.69-1.20)	**.010**
HbS, %	85.4 (81.1-90.0)	86.7 (81.2-91.5)	83.6 (80.3-86.6)	**.007**
WBC, $\times 10^9$/L	12700.0 (9700.0-15600.0)	13900.0 (11,200.0-16100.0)	9700.0 (8050.0-12800.0)	**<.001**
Neutrophils, $\times 10^9$/L	5396.0 (4005.0-7153.0)	5760.0 (4081.0-7182.0)	4839.0 (3684.5-6705.7)	.102
Eosinophil count, $\times 10^9$/L	642.0 (249.0-1541.0)	696.0 (282.0-1807.0)	394.5 (146.0-738.0)	**.001**
Lymphocyte count, $\times 10^9$/L	4998.0 (3918.0-6726.5)	5560.0 (4350.0-7224.0)	4156.5 (3329.2-5085.0)	**<.001**
Monocyte count, $\times 10^9$/L	338.0 (200.0-611.5)	360.0 (220.0-688.0)	279.5 (156.7-517.5)	**.045**

RBC, red blood cell; MCV, mean cell volume; LDH, lactate dehydrogenase; WBC, white blood cell. *P*-value obtained using Mann-Whitney and **P*-value using t test. Bold values indicate significance at $P < .05$ between patients not using and using HU.

associated with decreased HbF. In our patients, *BCL11A* rs6732518 C > T and rs766432 A > C had minor allele frequencies of 0.59 and 0.27. Homozygotes for the minor C allele of *BCL11A* rs766432 had a higher RBC count, hematocrit, hemoglobin concentration and HbF level, suggesting that in the presence of this allele patients treated with HU had less hemolysis that could contribute to clinical improvement. Our results suggest that SCA patients homozygous for the minor allele of *BCL11A* rs766432 and who use HU had greater increases in HbF expression and corresponding improvement in hematological parameters.

The results of a study with polymorphisms in *BCL11A* and *OR51B5/6*, and *HBS1L-MYB* in 622 Brazilian sickle cell disease patients including patients with HbSC disease and SCA who were not taking HU were similar to the present study.[5] A study of Tanzanian SCA found SNPs in *HBS1L-MYB* and *BCL11A* associated with HbF.[6] The multivariate model shows that *HBS1L-MYB*, sex, HU treatment, and HbS gene haplotype modulated HbF expression. This was a cross-sectional study and a determination of the change in HbF in response to HU according to SNP genotype requires prospective studies.

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MMA performed the interview, collected the samples, analyzed the data and wrote the manuscript. RPS, CCG analyzed the data and co-wrote the manuscript. TCCF, FIN, RSQ, attended the patients. CVBF, SCMAY, RMO, JRDF reviewed the article. BAVC, CGB, JNM, MHS, and MSG analyzed the data, provided academic support and revised the article critically.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study received approval from the institutional review board of the Instituto Gonçalo Moniz at the Fundação Oswaldo Cruz (IGM-FIOCRUZ – Bahia - Brazil) (CAAE 08452913.9.0000.0040) and T32HL007501 from the National Heart Lung and Blood Institute, NIH (JNM).

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Heme changes HIF-α, eNOS and nitrite production in HUVECs after simvastatin, HU, and ascorbic acid therapies

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ABSTRACT

The sickle cell disease (SCD) is a hemolytic genetic anemia characterized by free heme and hemoglobin release into intravascular spaces, with endothelial activation. Heme is a proinflammatory molecule able to directly activate vascular endothelium, thus, endothelial dysfunction and vascular disease are major chronic events described in SCD. The aim of this study was to evaluate the production of endothelial nitric oxide synthase (eNOS), nitrite and hypoxia inducible factor alpha (HIF-α) in HUVECs (human umbilical vein endothelial cells) activated by heme in response to simvastatin, hydroxyurea (HU), and ascorbic acid therapies. eNOS and HIF-α production were evaluated by ELISA and nitrite was measured by the Griess technique. The production of HIF-α increased when the cells were stimulated by heme ($p < 0.01$), while treatment with HU and simvastatin reduced the production ($p < 0.01$), and treatment with ascorbic acid increased HIF-1a production by the cells ($p < 0.01$). Heme increased eNOS production, ($p < 0.01$) but showed a heterogeneous pattern, and the lowest concentrations of all the treatments reduced the enzyme production ($p < 0.01$). The nitrite production by HUVECs was enhanced by stimulation with heme ($p < 0.001$) and was reduced by treatment with HU ($p < 0.001$), ascorbic acid ($p < 0.001$) and simvastatin ($p < 0.01$). In summary, our results suggest that the hemolytic vascular microenvironment in SCD requires different therapeutic approaches to promote clinical improvement, and that a combination of therapies may be a viable strategy for treating patients.

Introduction

Vascular complications are frequent in SCD and one of the main pathophysiological features is chronic endothelial activation. Endothelial cells of SCD patients express adhesion molecules and chemotactic factors when stimulated by the inflammatory stimuli commonly from cytokines, lipid mediators, reticulocytes, normal and irreversibly sickled red blood cells, platelets and even heme (Hebbel et al., 2004; Kumar et al., 1996; Belcher et al., 2000). Endothelial cells are capable of expressing procoagulants, anticoagulants, vasoconstrictors, vasodilators factors, adhesion molecules and cytokines. For that reason they are one of the main regulators of hemostasis (Wakefield et al., 2008).

Heme has many proinflammatory properties including: leukocyte activation and migration, increased expression of adhesion molecules, and cytokine and acute-phase protein induction. It can also activate the endothelial vascular cells (Figueiredo et al., 2007; Graca-Souza et al., 2002; Wagener et al., 1997). After challenge with heme, an increased expression in intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin (Wagener et al., 1997), increased tissue factor expression (Setty et al., 2008), increased mobilization of Weibel–Palade body and von Willebrand factor production (Belcher et al., 2014) were observed in vascular endothelial cells.

The hypoxia-inducible factor (HIF-1 alpha (α), HIF-1 beta (β), and HIF-2α) is known for its influence on vascular genes' transcription. Expression and activity of HIF-1α that forms heterodimers with HIF-1β beta are finely regulated by cellular oxygen concentration, vascularization, and angiogenesis. In a hypoxia environment, the HIF-1α activates gene transcription of erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor (VEGF) and its receptors, and other genes whose proteins increase oxygen bioavailability or ameliorate metabolic adaptation to hypoxia. Nitric oxide synthases (NOSs) are an enzyme family (hemeproteins, as p450 cytochrome) responsible for NO synthesis from L-arginine and molecular oxygen (Cokic et al., 2007). NO is generated by the conversion of L-arginine into L-citruline by NOS, that requires nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, and also the co-factors tetrahydrobiopterin

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Sickle red cells as danger signals on proinflammatory gene expression, leukotriene B4 and interleukin-1 beta production in peripheral blood mononuclear cell

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ABSTRACT

This study tested the hypothesis that sickle red blood cell (SS-RBC) induce Toll-like receptors (TLR) and Nod-like receptor family, pyrin domain containing 3 (NLRP3)- inflammasome expression in peripheral blood mononuclear cells (PBMC). TLR and NLRP3 inflammasome could contribute to the maintenance of the inflammatory status in sickle cell anemia (SCA) patients, since SS-RBC act as danger signals activating these pathways. In this study, first, we evaluated *TLR (2, 4, 5* and *9), NLRP3, Caspase-1, interleukin (IL)-1β* and *IL-18* expression in PBMC freshly isolated from SCA patients (SS-PBMC) in comparison with PBMC from healthy individuals (AA-PBMC). In the second moment, we investigated whether SS-RBC could interfere with the expression of these molecules in PBMC from healthy donor, in the absence or presence of hydroxyurea (HU) in vitro. TLRs and NLRP3 inflammasome expression were investigated by qPCR. IL-1β, Leukotriene-B4 (LTB_4) and nitrite production were measured in PBMC (from healthy donor) culture supernatants. *TLR2, TLR4, TLR5, NLRP3* and *IL-1β* were highly expressed in SS-PBMC when compared to AA-PBMC. Additionally, SS-RBC induced *TLR9, NLRP3, Caspase-1, IL-1β* and *IL-18* expression and induced IL-1β, LTB_4 and nitrite production in PBMC cultures. HU did not prevent *TLR* and *NLRP3* inflammasome expression, but increased *TLR2* and *IL-18* expression and reduced nitrite production. In conclusion, our data suggest that TLR and inflammasome complexes may be key inducers of inflammation in SCA patients, probably through SS-RBC; also, HU does not prevent NLRP3 inflammasome- and TLR-dependent inflammation, indicating the need to develop new therapeutic strategies to SCA patients that act with different mechanisms of those observed for HU.

1. Introduction

Sickle cell anemia (SCA) is a genetic disorder characterized by the production of abnormal red blood cells, homozygosity of hemoglobin S (HbS) and oxygen transport dysfunction. Clinically, SCA patients can be in a steady state, with subclinical manifestations, or in crisis, showing systemic inflammation, with vaso-occlusive phenomena and painful episodes, as well as susceptibility to infections and hemolysis [1–3]. SCA has been characterized as a chronic inflammatory state, with abnormal activation and elevated number of peripheral blood mononuclear cells (PBMCs),

Abbreviations: AA-PBMC, peripheral blood mononuclear cells freshly isolated from healthy individuals; AA-RBC, red blood cell from healthy individuals; ASC, apoptosis-associated speck-Like protein containing card; DAMPs, damage-associated molecular pattern molecules (DAMPs); HbF, fetal hemoglobin; HMGB1, high-mobility group protein B1; HU, hydroxyurea; LT, leukotriene; LTB4, leukotriene B4; NLR, Nod-like receptors; NLRP3, Nod-like receptor family, pyrin domain containing 3; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; RBC, red blood cell; SCA, sickle cell anemia; SCD, sickle cell disease; SS-PBMC, peripheral blood mononuclear cells freshly isolated from sickle cell anemia patients; SS-RBC, red blood cells from sickle cell anemia patients; TLR, Toll-like receptors.

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

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

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

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Introduction

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

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

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

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

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

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ABSTRACT

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

1. Introduction

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

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

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

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RESEARCH ARTICLE

WILEY Pediatric Blood & Cancer

Transcranial Doppler in hemoglobin SC disease

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Abstract

Background: Stroke is a severe clinical disorder in sickle cell disease (SCD), and few studies have evaluated transcranial Doppler (TCD) flow velocities in hemoglobin SC disease (HbSC). The guidelines for stroke risk are based on evaluations in sickle cell anemia (SCA) or HbS/β thalassemia.

Procedure: In this study, we compare cerebral blood flow in patients with SCD stratified by genotypes. A total of 1,664 pediatric patients with SCD underwent TCD velocity screening, and the time-averaged maximum mean velocity (TAMM) was determined in the middle cerebral artery (MCA), anterior cerebral artery (ACA), and distal intracranial internal carotid artery (ICA).

Results: Abnormal velocities were not identified in the ACA; therefore, we only use ICA and MCA velocities. TAMM from the left and right in the ICA and MCA was 134.3 ± 32.0 and 134.4 ± 32.6 cm/s in patients with SCA, and 105.2 ± 20.6 and 104.7 ± 20.0 cm/s in the patients with HbSC, respectively. Mean TAMM between right and left ICA/MCA was 134.5 ± 30.5 cm/s in the SCA group, and 104.9 ± 19.3 cm/s in the HbSC group. Notably, our data show that TCD velocities were significantly lower among the patients with HbSC compared to SCA. TAMM was negatively correlated with hemoglobin and hematocrit in both genotypes.

Conclusion: These results suggest that a different cut-off value for abnormal TCD velocities could be considered for patients with HbSC. Additional studies are warranted to determine the actual risk of stroke in HbSC genotype associated with this possible TCD risk value.

KEYWORDS
hemoglobinopathies, neurology and sickle cell, sickle cell disease, transcranial Doppler ultrasound

1 | INTRODUCTION

Stroke is a common clinical manifestation in sickle cell disease (SCD) in children of 1 year or older.[1–5] However, there are differences in stroke incidence among the SCD genotypes, with a rate of 0.61/100 patients/year for sickle cell anemia (SCA) patients, 0.17/100 patients/year for hemoglobin SC disease (HbSC), 0.11/100 patients/year for HbS/β^+ thalassemia, and 0.11/100 patients/year for HbS/β^0 thalassemia.[3]

The transcranial Doppler (TCD) monitors the cerebral mean blood flow velocities of patients with SCD allowing the identification of those with an increased risk to developing stroke.[6–10] The stratification of stroke risk can be determined by measuring the average maximum velocity or the time-averaged maximum mean velocity (TAMM) in the distal intracranial internal carotid artery (ICA), anterior cerebral artery (ACA), and middle cerebral artery (MCA). Values ≥200 cm/s are considered of high risk, whereas values <170 cm/s are considered of low risk; speeds ≥170 cm/s and <200 cm/s are considered conditional.[6] After the first episode of stroke in SCA patients, there is a 46–90% risk of stroke recurrence without prophylactic red cell transfusion therapy.[11,12] Despite the high incidence of stroke in patients with HbSC compared with the pediatric population without SCD,[3,4] few studies have evaluated flow velocities by TCD in this patient subset, using TCD values for measuring risk stratification that are obtained from SCA or HbS/β thalassemia patients.[3,6,11] Therefore, theoretically, these values may not extrapolate well to patients with HbSC. The aim

Abbreviations: ACA, anterior cerebral artery; HbSC, hemoglobin SC disease; ICA, Intracranial internal carotid artery; MCA, Middle cerebral artery; SCA, sickle cell anemia; SCD, sickle cell disease; SD, standard deviation; STOP, Stroke Prevention in Sickle Cell Anemia; TAMM, time-averaged maximum mean velocity; TCD, transcranial Doppler

B – TERMOS DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PARA MENORES DE 18 ANOS

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

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

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

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

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

Os registros da participação do menor no estudo serão mantidos confidencialmente, sendo do conhecimento apenas da equipe participante do projeto e do médico que o acompanha. Os dados individuais dos exames e informações do prontuário serão do

conhecimento somente dos pesquisadores envolvidos na pesquisa. Desta forma, a sua identidade será mantida em segredo e nenhum outro grupo terá acesso às informações coletadas, tais como seguradoras, empregadores ou superiores, de acordo com a resolução Res. CNS 340/2004, item V.1.e.

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

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

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

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

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

Se tiver qualquer dúvida, você pode procurar a Dra. Marilda de Souza Gonçalves na FIOCRUZ (telefone: 3176-2226).

C – QUESTIONÁRIO

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

QUESTIONÁRIO PARA PACIENTES E CONTROLES

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

Endereço: _____

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

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

01. Qual a sua cor? {cor} ( ) Branca[0] ( ) Negra[1] ( ) Parda[2] ( ) Amarela[3] ( ) Indígena[4]
02. Você estuda? {EST} ( ) NÃO [0] ( ) SIM [1]
03. Nível de escolaridade: {NESC} ( ) Alfabetiz.[0] ( ) Até 4 FM[1] ( ) Até 8 FM[2] ( ) Até 3 MD[3]
04. Número de irmãos:{NIRM} ( ) 0 [0] ( ) 1 [1] ( ) 2 [2] ( ) 3 [3] ( ) 4 ou + [4]
05. Familiares com DF? {FDFALC} ( ) Nenhum[0] ( ) Pai [1] ( ) Mãe [2] ( ) Irmão [3]
06. Idade primeira menstruação: {IPM} ( ) Não menst.[0] ( ) 09-11[1] ( ) 12-14 [2] ( ) 15-17 [3]
07. Já engravidou? {ENGRA} ( ) NÃO [0] ( ) SIM [1]
08. Está grávida? {GRA} ( ) NÃO [0] ( ) SIM [1]
09. Usa anticoncepcional? {ANTICO} ( ) NÃO [0] ( ) SIM [1]
10. Menstruação é regular? {MREG} ( ) NÃO [0] ( ) SIM [1]
11. Idade do 1° diagnóstico de Doença Falciforme: {ID} ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2] ( ) 10 - 14anos [3] ( ) 15 - 17anos [4]
12. Eletroforese de Hb {EHB} ( ) AA[0] ( ) SS[1] ( ) SC[2] ( ) SB+[3] ( ) SB_0[4] ( ) SD[5]
13. Haplótipo {HAPL} ( ) Sen[0] ( ) Car[1] ( ) Ben[2] ( ) Cam[3] ( ) Sau-Ara [4] ( ) Atip[5] ( ) I[6] ( ) II[7] ( ) III[8]
14. Talassemia {TAL} ( ) Negativo[0] ( ) Hetero 3.7[1] ( ) Homo 3.7[2] ( ) Hetero 4.2[3] ( ) Homo 4.2[4]

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

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

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

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

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

Qual especialidade? {ESPEC} ( ) Cardiologia [0] ( ) Oftalmologia [1] ( ) Neurologia [2]
( ) Infectologia [3] ( ) Pneumologia [4] ( ) Cirurgia [5]
( ) Angiologia [6] ( ) Nefrologia [7] ( ) Clínica da Dor [
( ) Outras [9]

16. Já teve pneumonia? {PNEU} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas vezes? {QPNEU} ( ) 1[0] ( ) 2-3[1] ( ) 4-6[2] ( ) 7 ou + [3
Se SIM, teve febre? {FEBRE} ( ) NÃO [0] ( ) SIM [1]
Anormalidade no RX? {ARX} ( ) NÃO [0] ( ) SIM [1]
Quando internado, usou medicação? {MPNEU} ( ) NÃO [0] ( ) SIM [1]
Quais? {DESCMPNEU} _____

17. Teve ou tem esplenomegalia? {ESPLE} ( ) NÃO [0] ( ) SIM [1]
Em que período? {PERIOESPLE} ( ) <6m[0] ( ) 6m-1ano [1] ( ) 2-3a [2] ( ) 4-5a [3] ( ) >6a [4]
Teve crise de seqüestro esplênico? {SEQESPLE} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas vezes? {QSEQESPLE} _____

18. Faz uso profilático de Penicilina? {PROP} ( ) NÃO[0] ( ) SIM [1]
Se SIM, qual? {QPEN} ( ) Penicilina V oral [0] ( ) Penicilina benzatina [1]
Se Sim, há quanto tempo? {QTPEN} ( ) até 1 ano [0] ( ) + de 1 ano a 3 anos [1]
( ) + 3 anos a 5 anos [2] ( ) + 5 anos a 7 anos [3]
( ) + de 7 anos [4]

19. Já teve AVC? {AVC} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas vezes? {QAVC} ( ) 1 [0] ( ) 2 [1] ( ) 3 [2] ( ) 4 ou + [3]
Se SIM, seqüelas do AVC? {SEQAVC} ( ) NÃO [0] ( ) SIM [1]
Já fez ressonância magnética? {RESSOMAG} ( ) NÃO [0] ( ) SIM [1]
Alguma alteração? {ALTRESSOMAG} ( ) NÃO [0] ( ) SIM [1]

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

21. Apresenta asma? {ASMA} ( ) NÃO [0] ( ) SIM [1]
Se SIM, quantas crises nos últimos 06 meses? {QASMA} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+
Faz uso regular de nebulização? {NEBU} ( ) [0] NÃO ( ) SIM [1]

22. Tem crises de dor? {CRISDOR} ( ) [0] NÃO ( ) SIM [1]
Se SIM, quantas crises nos últimos 06 meses? {QCRISDOR} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+
Quando foi a última crise? {ULTCRISDOR} ( ) <1mês [0] ( ) 1-3m [1] ( ) 4m ou+[2]
Usa medicação para a dor? {MDOR} ( ) NÃO [0] ( ) SIM [1]
Prescrita por um médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]
Assistido por especialista em dor? {ESPECDOR} ( ) NÃO [0] ( ) SIM [1]
Faz tratamento com hidroxiuréia? {HIDROXI} ( ) NÃO [0] ( ) SIM [1]

23. Faz uso de alguma medicação? {MEDIC} ( ) NÃO [0] ( ) SIM [1]
Se SIM, qual? {DESCMEDIC} _____
Com que freqüência? {FREQMEDIC} ( ) Diário [0] ( ) Dias alternados[1] ( ) Semanal[2]
( ) Quinzenal[3] ( ) Mensal [4] ( ) Bimestral[5] ( ) Semestral [6]

24. Vaso-Ocusão: {VO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QVO} _____
Fez uso de alguma medicação? {MVO} ( ) NÃO [0] ( ) SIM [1]
25. Retinopatia: {RETIN} ( ) NÃO [1] ( ) SIM [2]
Se SIM, fez uso de alguma medicação? {MRETIN} ( ) NÃO [0] ( ) SIM [1]
Faz consultas periódicas com oftalmo? {CONSOFTAL} ( ) NÃO [0] ( ) SIM [1]
26. Infecções: {INFEC} ( ) NÃO[0] ( ) SIM [1]
Quais? {DESCINFEC} ( ) Rinite [0] ( ) Sinusite [1] ( ) Otite [2]
( ) Faringite [3] ( ) Amigdalite [4] ( ) Outros [5]
Fez uso de alguma medicação? {MINFEC} ( ) SIM [0] ( ) NÃO [1]
27. Priapismo: {PRIAP} ( ) NÃO [0] ( ) SIM [1]
N° de vezes: {QPRIAP} ( ) Até 4 [0] ( ) 05-09 [1] ( ) 10 ou +[2]
Fez uso de alguma medicação? {MPRIAP} ( ) NÃO [0] ( ) SIM [1]
28. Úlcera maleolar: {ULCMALEO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QULCMALEO} _____
Idade da primeira úlcera: {IDULC} ( ) Até 4 anos [0] ( ) 5-9 [1] ( ) 10 ou + [2]
Tratou a úlcera? {TRATULC} ( ) NÃO [0] ( ) SIM [1]
Qual tratamento? {QUALTRAT} _____
29. Síndrome toráxica aguda: {SDTOR} ( ) NÃO [0] ( ) SIM [1]
Quantas vezes? {QSDTOR} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]
30. Alterações ósseas: {ALTOSSEA} ( ) NÃO [0] ( ) SIM [1]
Quais? {DESCALTOSSEA} _____
31. Insuficiência Renal Aguda: {INSRENAG} ( ) NÃO [0] ( ) SIM [1]
Quantas vezes? {QINSRENAG} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]
32. Insuficiência Renal Crônica: {INSRENCRO} ( ) NÃO [0] ( ) SIM[1]
Idade diagnóstico: {IDINSRENCRO} ( ) Até 5 anos [0] ( ) 06-11[1] ( ) 12 ou + [2]
33. Alterações cardíacas: {INSCARD} ( ) NÃO [0] ( ) SIM[1]
Qual alteração? {QUALALTCA} _____
Idade diagnóstico: {IDINSCARD} ( ) Até 5 anos [0] ( ) 06-11 [1] ( ) 12 ou + [2]
Fez eletrocardiograma? {ELETRO} ( ) NÃO [0] ( ) SIM [1]
Fez ecocardiograma? {ECOCARD} ( ) NÃO [0] ( ) SIM [1]
34. Seqüestro hepático: {SEQHEP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QSEQHEP} _____
35. Insuficiência respiratória: {INSRESP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QINSRESP} _____
36. Distúrbio do sono? {DISTSONO} ( ) NÃO [0] ( ) SIM [1]
37. Litíase biliar: {LITIBILI} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QLITIBILI} _____
38. Cirurgia: {CIRURG} ( ) NÃO [0] ( ) SIM [1]
Quais? {QUALCIRURG} _____
39. Se SIM, fez uso de profilaxia antibiótica? {PROFANTIB} ( ) NÃO [0] ( ) SIM [1]
40. Completou o calendário vacinal? {CALVAC} ( ) NÃO [0] ( ) SIM [1]
Fez uso das seguintes vacinas? {USOVAC} ( ) 7 valente [0] ( ) 23 valente [1]
( ) Meningo [2] ( ) Haemophilus [3]
41. Faz uso de hemoderivados? {HEMODER} ( ) NÃO [0] ( ) SIM [1]

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

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

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

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

Tipo de profissão: {QTRAB} _____

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

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

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

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

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

Se SIM, que freqüência? {FREQBEBE} _____

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

Se SIM, que freqüência? {FREQFUMA} _____

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

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

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

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

49. Quantos amigos vc têm aproximadamente? {AMIGO}

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

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

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

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

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

D – APROVAÇÃO DO COMITÊ DE ÉTICA

HOSPITAL SÃO
RAFAEL/MONTE TABOR-BA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

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

Pesquisador: Marilda Gonçalves

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

Versão: 1

CAAE: 52280015.1.0000.0048

Instituição Proponente: Hospital São Rafael/Monte Tabor-BA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.400.535

Apresentação do Projeto:

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

Endereço: Av. São Rafael 2152, 6º andar
Bairro: São Marcos **CEP:** 41.256-900
UF: BA **Município:** SALVADOR
Telefone: (71)3281-6484 **Fax:** (71)3281-6855 **E-mail:** cep@hsr.com.br

HOSPITAL SÃO
RAFAEL/MONTE TABOR-BA

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SALVADOR, 02 de Fevereiro de 2016

Assinado por:
Regina Maria Pereira Oliveira
(Coordenador)