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CENTRO DE PESQUISAS GONÇALO MONIZ

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

TESE DE DOUTORADO

Estudo de Marcadores Genéticos Associados a Inflamação em pacientes com Anemia Falciforme

Cyntia Cajado de Souza

Salvador - Bahia - Brasil

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Tese apresentada ao Colegiado do Curso de Pós-graduação em Biotecnologia em Medicina e Saúde Investigativa, como pré-requisito obrigatório para obtenção do grau de Doutor.

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“O que vale na vida não é o ponto de partida e sim a caminhada.

Caminhando e semeando, no fim terás o que colher.”

Cora Coralina

Dedico este trabalho

a meus pais Alcides e Zélia que foram e sempre serão meus alicerces;

aos meus filhos Lucas e Pedro , meus amores , que transformaram meu viver;

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CAJADO, Cyntia de Souza. Estudo de marcadores genéticos associados a inflamação em pacientes com anemia falciforme. 154 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Instituto de Pesquisas Gonçalo Moniz, Salvador, 2013.

RESUMO

A anemia falciforme (HbSS) é uma doença genética com prevalência mundial elevada, caracterizada pela heterogeneidade clinica apresentando manifestações agudas e crônicas de caráter multifatorial, sendo caracterizada pela presença do estado inflamatório sistêmico. As alterações no sistema imune têm sido relacionadas a predisposição a infecções em pacientes HbSS, com produção exacerbada de anticorpos, alterações na função de leucócitos e na imunidade celular, entre outros. Estão inclusos nesta tese o conjunto de três manuscritos com o objetivo de investigar marcadores genéticos relacionados a inflamação em pacientes com HbSS, com ênfase para os polimorfismo e níveis séricos das citocinas *TNFα* -308G>A e *IL-8* 251A>T, para a provável influência dos polimorfismos gênicos *TNFα*-308G>A, *FcγRIIA*H/R131, *MPO*-463G>A, *TLR4* 896A>G, e *TLR9*- 1237T>C na gravidade de infecções e complicações inflamatórias na HbSS, e ainda a expressão gênica dos receptores TLR2, TLR4, TLR5 e TLR9 em células dendríticas de pacientes HbSS. Nossos resultados mostraram que os níveis séricos elevados das citocinas TNFα e IL-8, bem como a presença do alelo mutante dos polimorfismos de TNFα e da IL-8 estudados estão associados com a gravidade clínica da doença. O estudo dos polimorfismos *TLR9* -1237T>C e *TLR4* 896A>G mostraram associação com a ocorrência de infecção respiratória e acidente vascular encefálico (AVE), respectivamente, e o polimorfismo *MPO*-463G>A esteve associado com a ocorrência de infecções.
Além disso, a presença concomitante do polimorfismo *FcγRIIA* H/R131 com o *MPO*-463G>A, com o *TLR4* 896A>G, ou com o *TNF-α* influenciou a ocorrência de internações hospitalares, de sequestro esplênico, de AVE e do número de crises vaso-oclusivas. O estudo de novos marcadores de inflamação na anemia falciforme pode ajudar no entendimento da complexidade da fisiopatologia desta doença. Nossos dados enfatizam a identificação de novos biomarcadores genéticos e sua associação marcadores clássicos podem ser uma ferramenta importante para elucidar a diversidade fenotípica da HbSS.

Palavras chaves: 1. Anemia Falciforme. 2. Inflamação. 3. Marcadores Genéticos. 4. Receptores semelhantes a toll.

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ABSTRACT

Sickle cell anemia (HbSS) is a genetic disease with elevated worldwide distribution characterized by clinical heterogeneity and acute and chronic complication presenting systemic inflammatory state. The immune system changes are related to infection predisposition in HbSS, exacerbated antibody production, changes in leucocytes function and innate immunity. It was included in this thesis a set of three manuscripts that aim to investigate genetic markers related to inflammation in HbSS emphasizing polymorphisms and cytokine serum levels of *TNFα* -308G>A and *IL-8* 251A>T, to the influence of genetic polymorphisms of *TNFα*-308G>A, *FcγRIIA*H/R131, *MPO*-463G>A, *TLR4* 896A>G and *TLR9*- 1237T>C in infection and inflammatory complication in HbSS and the gene expression of dendritic cell TLR2, TLR4, TLR5 and TLR9 receptors. Our results shows high serum levels and the presence of TNFα and IL-8 mutant allele were related to disease clinical severity. The study of *TLR9* -1237T>C and *TLR4* 896A>G polymorphisms were associated to respiratory infection and encephalic vascular accident (EVA), respectively, and the polymorphism *MPO*-463G>A was associated to infection. The double presence of polymorphism *FcγRIIA* H/R131 with *MPO*-463G>A or with *TLR4* 896A>G or with *TNF-α* influence the presence of hospitalization, spleen sequestration, EVA and vasooclusive crisis. The new sickle cell anemia inflammation markers study may help to highlight the disease physiopathology complexity. Our finds emphasizes new genetic and immunological biomarkers identification and its association with classical markers may be important to elucidate phenotypic HbSS diversity.

Key world: : 1. Sickle cell. 2. Inflammation. 3. Genetic biomarkers. 4. Toll like receptor.

LISTA DE ABREVIATURAS

APC	Célula Apresentadora de Antígeno
AVE	Acidente Vascular Encefálico
CD	Grupo ou cluster de diferenciação (moléculas da superfície celular que servem como marcadores, do inglês *cluster of differentiation*
CD1c	Cluster de diferenciação 1c
CD11	Cluster de diferenciação 11
CD123	Cluster de diferenciação 123
DC	Célula dendrítica, do inglês *dendritic cell*
DNA	Ácido desoxirribonucléico
Fc	Fragmento cristalizável, porção efetora da imunoglobulina reconhecida por receptores específicos
FcγR	Receptor gama do fragmento cristalizável de IgG, classificados como I, II e III
GM-CSF	Fator estimulante de colônias de Granulócito e Macrófago
HbC	Hemoglobina C
HbF	Hemoglobina F
HbS	Hemoglobina S
HbSC	Doença SC
HbSS	Anemia Falciforme
HLA-DR	Complexo Principal de Histocompatibilidade de Classe II
IgG	Imunoglobulinas da classe G
INF-γ	Interferon gama

IL-1	Interleucina 1
IL-1β	Interleucina 1 beta
IL-4	Interleucina 4
Il-6	Interleucina 6
IL-8	Interleucina 8
IL-10	Interleucina 10
IL-12	Interleucina 12
LPS	Lipopolissacarídeo
mDC	Célula Dendritica mielóide
MPO	Mieloperoxidase
NK	Célula *Natural Killer*
PAMP	Padrão Molecular Associado a Patógeno
PCR	Reação em cadeia da polimerase
PCR-RFLP	Reação em cadeia da polimerase do polimorfismo de tamanho de fragmento de restrição
PCR-RT	Reação em cadeia da polimerase com transcriptase reversa
pDC	Célula dendritica plasmocitóide
PMN	Neutrófilo Polimorfonuclear
PRR	Receptores de Reconhecimento de Padrão
RNA	Ácido Ribonucléico
SIRS	Síndrome da Resposta Inflamatória Sistêmica
STA	Síndrome Torácica Aguda
Th-1	Linfócito T auxiliador (helper) CD4 positivo Th1

Th-2	Linfócito T auxiliador (helper) CD4 positivo Th2
TLR	Receptores semelhantes a toll, do inglês *toll like receptor*
TNF-α	Fator de Necrose Tumoral alfa

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1. Introdução

As hemoglobinopatias são alterações hereditárias na molécula da hemoglobina (Hb) associadas a mutações nos genes da globina. Os principais grupos de hemoglobinopatias são as relacionadas a mudanças estruturais, com ocorrência de Hb variantes, tais como as hemoglobina S (HbS) e hemoglobina C (HbC); e aquelas caracterizadas pela redução ou ausência de síntese de cadeias de globina, comumente denominadas talassemias, sendo as principais as associadas a defeitos de síntese das cadeias beta (β) e alfa (α) (WEATHERALL & CLEGG, 2001).

A hemoglobina S é uma hemoglobina variante, caracterizada pela presença da mutação pontual G**A**G>G**T**G localizada no sexto códon do gene da globina β (beta) (*HBB*), com substituição do ácido glutâmico por valina na sexta posição da cadeia polipeptídica β ($\beta^{S\ 6Glu\rightarrow Val}$). A presença do resíduo de valina na posição 6 da cadeia polipeptídica β caracteriza a HbS e promove a interação hidrofóbica entre os tetrâmeros de globina, principalmente em condições de baixas de oxigênio ou hipóxia, com polimerização e formação de filamentos de HbS no interior da hemácia, fenômeno que altera a sua forma bicôncava original tornando-as falcizadas e rígidas (STEINBERG & RODGERS, 2001). Entretanto, quando os níveis de oxigênio são restabelecidos ocorre o retorno da hemácia a sua forma bicôncava original, sendo que eventos sucessivos de falcização podem alterar a estrutura da membrana eritrocitária, com exposição da fosfatidilserina, tornando-os irreversivelmente falcizados (FRENETTE & ATWEH, 2007; STEINBERG, 2009).

Alterações hereditárias na molécula de hemoglobina possuem distribuição mundial elevada, sendo frequentes entre os países africanos, do Mediterrâneo, Ásia, China e Américas, com prevalência elevada da doença falciforme (DF) nos países africanos. Desta forma, as hemoglobinopatias estão presentes em todos os continentes, em decorrência, principalmente, das migrações populacionais e afetam cerca de 7% da população mundial (WEATHERALL, 2008). A cada ano nascem cerca de 300.000 a 500.000 crianças com DF no mundo, sendo considerado um problema crescente de saúde pública e social (PATEL *et. al.,* 2005*;* STEINBERG & RODGERS, 2001; MCCAVIT, 2012).

O Brasil possui uma população com miscigenação racial elevada, sendo que a frequência do alelo βS varia de acordo com a região estudada (PARRA *et al.*, 2003). De acordo com os dados do programa nacional de triagem neonatal do

Ministério da Saúde, o estado da Bahia possui a incidência de 1:650 para DF e a frequência do traço falciforme (HbAS) de 1:17 entre os nascidos vivos. Segundo os dados de triagem neonatal, a incidência de DF no Rio de Janeiro é 1:1200 e a frequência do traço é de 1:21; em Minas Gerais a incidência de DF é de 1:1400 e a frequência do traço falciforme é de 1:23. Com base nesses dados estima-se o nascimento de 3.500 crianças com DF e 200.000 portadores do traço falciforme/ ano no Brasil (JESUS, 2010).

Adorno e cols. (2005) realizaram a investigação de hemoglobinas variantes em uma maternidade pública de Salvador-Bahia e encontraram a frequência de 9,8% para os heterozigotos HbAS e a prevalência de 0,9% para a HbSC e de 0,2% para a HbSS. Amorim e cols. (2010) descreveram com base nos dados obtidos na triagem neonatal do estado da Bahia a incidência de 1:601 para a DF e de 1:1.435 para a AF.

1.1- A Anemia falciforme: heterogeneidade fenotípica

A anemia falciforme (AF) é caracterizada pela homozigose da HbS (HbSS), sendo a forma mais grave do grupo de doença falciforme. Os indivíduos com AF apresentam anemia hemolítica grave, com quadro clínico heterogêneo. Um dos fatores determinantes da gravidade da doença é a concentração de HbS, uma vez que os pacientes apresentam hemácias com cerca de 80% ou mais de HbS (FERWERDA *et al.*, 2007; STEINBERG, 2009).

Os pacientes com AF podem apresentar gravidade clínica elevada com retardo no crescimento e desenvolvimento, bem como alterações em vários órgãos, hemólise, fenômenos vaso-oclusivos e hospitalizações frequentes, sendo que a intensidade e gravidades destas características clínicas variam entre pessoas. A heterogeneidade clínica tem sido também relacionada a fatores ambientais, tais como o nível socioeconômico e estado nutricional, além de fatores genéticos relacionados aos pacientes (EMBURY, 1995; WEATHERALL, 2008; REES *et al.*, 2010).

Entre as manifestações clínicas mais prevalentes na AF podemos citar as crises vaso-oclusivas, o acidente vascular encefálico (AVE); a síndrome torácica aguda (STA); úlceras de perna; sequestro esplênico; alterações pulmonares e

oftalmológicas; priapismo; retardo no crescimento e desenvolvimento, bem como susceptibilidade elevada a infecções, além de alterações em diversos órgãos (STUART & NAGEL, 2004; STEINBERG, 2008).

As manifestações clínicas podem estar relacionadas a idade do indivíduo, como por exemplo, o sequestro esplênico, a dactilite, a STA e as infecções bacterianas, uma vez que estas são mais comuns na infância (REDDING-LALLINGER & KNOLL, 2006); por outro lado, o priapismo, a retinopatia, a hipertensão pulmonar, a vasculopatia progressiva, a falência renal e a necrose óssea são mais frequentes na idade adulta; embora, as crises de dor e alguns tipos de infecção acometam desde a infância agravando-se na idade adulta (KATO, 2007; GLADWIN & VICHINSKY, 2008).

Apesar da AF estar relacionada a origem genética, este motivo parece não ser suficiente para explicar a heterogeneidade fenotípica da doença, que tem sido atribuída a efeitos epistáticos entre diferentes genes, que modulam a expressão clínica diferenciada já descrita na doença (STEINBERG, 2009).

Entre os biomarcadores clássicos associados com a modulação clínica da AF encontram-se os níveis de hemoglobina fetal (HbF), o tipo de haplótipos ligados ao grupo de genes da globina β^S e presença de talassemia alfa (tal-α) (STEINBERG, 2009).

Os níveis elevados de HbF no paciente com AF têm sido associados a efeitos benéficos na clínica do paciente (PLATT *et. al*., 1994), com redução dos episódios dolorosos (PLATT *et. al*., 1991). Os haplótipos ligados ao grupo de genes da globina beta também são considerados marcadores do fenótipo dos pacientes com AF; por exemplo, o haplótipo Ben está associado a níveis intermediários de HbF e ao curso clínico também intermediário da doença; o haplótipo CAR, que está associado a níveis diminuídos de HbF e quadro clínico grave e, o Sen e Saudi Arabia, que estão associados a níveis elevados de HbF e curso clínico menos grave da doença (NAGEL & RANNEY, 1990; NAGEL & STEINBERG, 2001; STEINBERG & SEBASTIANI, 2012).

A presença da talassemia alfa (tal-α) diminui a concentração de hemoglobina (Hb) no interior do eritrócito, diminuindo também a polimerização da HbS, com o aumento da concentração de Hb e a diminuição da hemólise. O efeito clínico da tal-α é variável, mas geralmente benéfico, diminuindo a ocorrência de

vários sintomas clínicos, como o AVE (BERNAUDIN, 2009). Porém, a tal-α parece não influenciar a ocorrência de crises de dor (PLATT *et. al.*, 1991) e tem sido também associada ao aumento de osteonecrose (KATO *et al.*, 2007).

Porém, os biomarcadores acima citados, não podem ser considerados como as principais causas da diversidade clínica dos pacientes com AF, sendo que outros fatores genéticos têm sido relacionados à patogênese da doença justificando seu caráter poligênico (STEINBERG, 2009).

A fisiopatologia da AF é multifatorial, sendo caracterizada pela ocorrência de mecanismos diversos, com presença de inflamação, vasculopatia, danos associados a isquemia/reperfusão, lesão a órgãos e neuropatia, sendo que cada processo contribui individualmente para a instalação da dor, causa comum de hospitalização dos pacientes (KOHLI *et al*, 2010).

Nesse contexto, muitos fatores parecem contribuir para a presença do estado inflamatório sistêmico presente na AF, tais como a quantidade elevada de HbS no interior das hemácias, que favorece a polimerização intracelular e a falcização da hemácia; o aumento da viscosidade sanguínea; a adesão de hemácias falcizadas, leucócitos e plaquetas ao endotélio vascular pela expressão de moléculas de adesão; alterações na concentração de Hb e de HbF; o aumento do número de leucócitos, ativação de monócitos, síntese de proteínas de fase aguda, citocinas e quimiocinas (CONRAN *et al.*, 2004; STUART & NAGEL, 2004; REES *et al.*, 2010); ativação de fatores da coagulação e de plaquetas (TOMER *et al*. 2001; SOLOVEY *et al*., 2004); alteração na biodisponibilidade do óxido nítrico (NO) (BELHASSEN *et. al*., 2000). Desta forma, a complexidade de fenômenos decorrentes da fisiopatologia da AF, bem como, a diversidade de manifestações clinicas resultam na manutenção de um estado pró-inflamatório crônico (CHIES & NARDI, 2001; KUTLAR, 2005).

As infecções são comuns entre os pacientes com AF, sendo caracterizadas como uma das causas mais importante de morbidade e mortalidade na idade pediátrica (COSTA, 2001; DI NUZZO & FONSECA, 2004). Apesar do uso profilático de antibióticos, as infecções ainda são causa de hospitalização em crianças, principalmente associada a crises vaso-oclusivas (QUINN *et al.*, 2004). Em crianças menores de 5 (cinco) anos de idade as infecções por *Streptococcus pneumoniae* apresentam prevalência de 7 em 100 (QUINN *et al*., 2004), sendo

também frequentes as infecções causadas por *Staphylococcus aureus* e *Salmonella species* (NORRIS *et al*., 2003). As alterações no sistema imune têm sido relacionadas a predisposição a infecções em pacientes com AF, com produção exacerbada de anticorpos, deficiências na via alternativa do complemento, alterações na opsonização, alterações na função de leucócitos e na imunidade celular (COSTA, 2001; OHENE-FREMPONG & STEINBERG, 2001, SALAWU *et al*, 2009).

1.*2*- ***Imunidade inata na anemia falciforme***

A principal função da resposta inflamatória é a de combater a infecção e o dano tecidual (MEDZHITOV, 2008). A resposta imune inata reconhece os patógenos ou a lesão celular pela ação de células fagocíticas e síntese de diferentes moléculas, induzida por infecção bacteriana ou por dano tecidual e apresenta importante papel na inflamação aguda (MEDZHITOV, 2010).

A resposta imune inata corresponde à primeira linha de defesa contra patógenos e contra estímulos gerados pela resposta inflamatória. Os componentes celulares da imunidade inata são representados, principalmente, pelos neutrófilos polimorfos nucleares e pelos fagócitos mononucleares, como os monócitos, macrófagos, células dendríticas e células *Natural Killer* (NK) (NEWTON & DIXIT, 2012). Ressaltamos que as células dendríticas caracterizam-se pela presença de projeções longas na membrana e, quando estimuladas, executam tanto a pinocitose quanto a fagocitose de partículas (MEDZHITOV, 2010). Entre as moléculas que podem ser sintetizadas pelas células dendríticas encontram-se as citocinas, quimiocinas e proteínas do complemento e de fase aguda (MEDZHITOV, 2010).

Quando os agentes infecciosos ultrapassam as barreiras epiteliais e alcançam os tecidos subjacentes eles entram em contato com macrófagos, fibroblastos ou células dendríticas. Entretanto, a interação dessas células com os agentes infecciosos ocorre por intermédio dos Receptores de Reconhecimento de Padrões (PRRs), que por sua vez reconhecem os Padrões Moleculares Associados a Patógenos (PAMPs), desencadeando a cascata de sinalização que promove o recrutamento de leucócitos para aquela região (NEWTON & DIXIT, 2012). Os

PRRs são encontrados em diferentes populações celulares e podem estar presentes tanto na membrana plasmática ou endossomal, como no citoplasma. Os receptores TLR (Receptores semelhantes a Toll) são PRRs de membrana que têm sido bastante estudados nos últimos anos em especial quanto a sua participação na inflamação e nas doenças autoimunes (AKIRA, 2006).

Os aspectos imunológicos na AF têm sido bastante estudados, sendo que níveis elevados de citocinas Th2 (IL-4, IL-6 e IL-10) e de citocinas pró–inflamatórias, como a IL-8 e fator de necrose tumoral alfa (TNF-α) plasmática já foram descritos em pacientes com AF (ETIENNE-JULAN *et al*.,2004; REDDING-LALLINGER & KNOLL, 2006). As alterações em citocinas pró-inflamatórias e anti-inflamatórias estão associadas ao aumento da expressão de moléculas de adesão em leucócitos e do numero de reticulócitos e eritrócitos circulantes, e a ativação de plaquetas e do endotélio vascular nos individuos com AF (MALAVE, *et al.,* 1993; TAYLOR *et al.*, 1997).

Ainda quanto a imunidade inata, a inflamação presente na AF sofre influência de mediadores inflamatórios mesmo durante o estado estável da doença (ASSIS *et al*., 2005). O aumento na expressão de moléculas de adesão, já referido anteriormente, está associado a diferentes graus de necrose tecidual e promove o recrutamento de granulócitos, monócitos, eosinófilos, plaquetas, citocinas, proteínas de fase aguda e proteínas do sistema complemento para o endotélio vascular, elevando o *status* inflamatório (ASSIS *et al*., 2005).

É importante ressaltar a participação da endotelina-1 na inflamação da AF, sendo que esta promove o aumento da secreção de citocinas inflamatórias, tais como IL-1, IL-6, IL-8, TNF-α, as quais aumentam a secreção de superóxidos pelos neutrófilos (TURHAN, *et al*., 2002). Assim, essas citocinas têm a habilidade de regular a síntese de outras citocinas, que é o que ocorre, por exemplo, com a IL-1 que induz a produção de TNF-α e vice versa. Desta forma, as citocinas apresentam ação moduladora na expressão de seus próprios receptores (ABBAS, 2012). O impacto desse estado inflamatório crônico, subclinico e multifatorial no funcionamento do sistema inflamatório ainda não esta definido, mas a estreita relação entre a imunidade inata e a patogenia é bastante evidente.

1.2.1 – Células dendríticas (DCs) e Receptores semelhantes a Toll (TLRs): Importância e polimorfismos gênicos

As células dendríticas (DC) são células apresentadoras de antígenos (APCs) em diferentes tecidos, sendo consideradas a principal classe deste tipo de células. As células dendríticas são eficientes na ativação de linfócitos T e na capacidade de desencadear resposta imune (STEINMAN *et al*., 2003). Além disso, as DCs também estão relacionadas com a integração entre a imunidade inata e a adaptativa. As principais funções das DCs são a captura, o processamento e a apresentação de antígeno, seguido pela migração celular, ativação dos linfócitos T, secreção de citocinas e ativação da imunidade adaptativa (HARDIN, 2005).

Existem duas categorias de CDs no sangue periférico, as CDs de linhagem mielóide (mCDs; HLA-DR/CD11+/CD1c+) e as de linhagem plasmocítóide (pCDs; HLA-DR/CD123+/CD11-) (FACCHETTI *et al*., 2003). As mCDs podem ser consideradas como CDs clássicas, uma vez que são responsáveis pela ativação dos linfócitos T e são encontradas em quantidade elevada nos tecidos linfoides, no cutâneo e nas mucosas, onde residem na sua forma imatura. As pCDs também estão presentes nos tecidos linfoides, porém em quantidade menor que as mCDs, sendo que sua função está diretamente ligada as infecções virais (FACCHETTI *et al*., 2003).

Antes do contato com antígenos as CDs apresentam fenótipo imaturo com expressão diminuída de moléculas co-estimulatórias. Após o estímulo antigênico as DCs sofrem alterações fenotípicas, morfológicas e funcionais que as tornam apta a apresentação de antígenos, tornando-se maduras (STEINMAN, 2003). O processo de maturação é contínuo e se inicia nos tecidos periféricos e se completa nos órgãos linfoides com a interação com as células T, sendo estimulada por produtos bacterianos, como o lipopolissacarídeo (LPS) e por citocinas pró-inflamatórias. Durante a inflamação, as CDs são recrutadas e possuem a sua capacidade fagocítica ativada, migrando em direção aos linfonodos e baço tornando-se maduras e APCs potentes (PULENDRAN, 2005). A diferenciação de monócitos em CDs imaturas, *in vitro,* ocorre quando o meio de cultura é enriquecido com a adição do fator de crescimento de ganulócito e monócito (GM-CSF) e da interleucina-4 (IL-4) (KADOWAKI *et al*., 2001).

Os pacientes com AF em estado estável apresentam a expressão diferenciada de CDs com fenótipo CD1 positivo (CD1+), sendo que as moléculas CD1+ estão presentes na CDs de 75% desses pacientes, enquanto que indivíduos saudáveis expressam cerca de 30% de células CD1+ (SLOMA *et al*., 2004).

Os TLRs são PRRs que agem como sensores de produtos microbianos e outros patógenos (PATEL *et al.*, 2005) e são expressos constitutivamente ou por indução na superfície das APCs, incluindo macrófagos, células dendríticas (CDs) e linfócitos B (IWASAKI & MEDZHITOV, 2004).

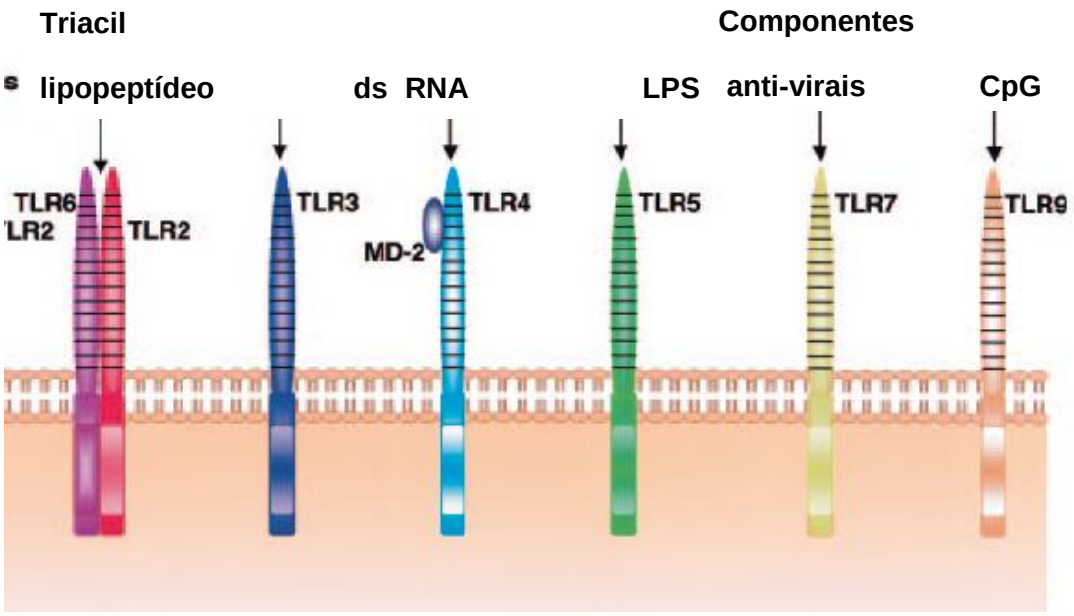

Figura 1: Sumário das principais famílias de TLR e seus ligantes (adaptado de Akira, 2003).

Até o presente momento já foram descritos 10 tipos diferentes de TLRs humanos, os quais podem ser expressos em diferentes tecidos. (O'MAHONY *et al.*, 2008). Os TLRs do tipo TLR1, TLR2, TLR4, TLR5, TLR6 e TLR11 são expressos na superfície das células apresentadoras de antígeno, enquanto os TLR3, TLR7, TLR8, e TLR9 possuem localização intracelular. (AKIRA, 2006). Os monócitos expressam constitutivamente os TLR3, TLR6, TLR7 e TLR10 (KOKKINOPOULOS *et al.*, 2005). Algumas citocinas pró-inflamatórias contribuem para a regulação da expressão dos TLRs em monócitos e neutrófilos, mas o mecanismo regulatório

dessa expressão ainda é desconhecido. O reconhecimento bacteriano ocorre por meio do TLR2, TLR4, TLR5 e TLR9, sendo que o TLR2 está associado ao reconhecimento de bactérias gram-positivas; o TLR4 é considerado fundamental para a detecção de LPS de bactérias gram-negativas; o TLR5 é ativado por flagelina e o TLR9 reconhece sequências de DNA bacteriano, de fungo ou de vírus (AKIRA, 2004).

Diversos trabalhos têm descrito a associação entre polimorfismos no gene dos *TLRs* e a ocorrência de infecções (SCHRÖDER & SCHUMANN, 2005, MILLER *et al., 2005*). O polimorfismo 896A>G no gene *TLR4* tem sido relacionado a ocorrência de infecções por bactérias gram negativas (AGNESE *et a.l*, 2002) e com a gravidade da Síndrome da Resposta Inflamatória Sistêmica (SIRS) (CHILD *et al*., 2003). O polimorfismo -1237T>C na região promotora do gene *TLR9* parece estar relacionado com a doença de Crohn (TOROK *et al*., 2008), e com a ocorrência de surdez pós meningite bacteriana (VAN WELL *et al.*, 2012).

1.2.2 – Citocinas na anemia falciforme

As citocinas são glicoproteínas de peso molecular baixo (~20kDa), secretadas por células da imunidade inata e da imunidade adaptativa. Essas moléculas possuem atividade pleiotropica e participam tanto dos processos inflamatórios quanto na resposta imune (JANEWAY *et al*., 2005). Além de promover o crescimento, a sobrevivência e a diferenciação de células B e T, a síntese de diferentes citocinas está associada a resposta do organismo contra infecções (JANEWAY *et al*., 2005). Desta forma, o padrão de citocinas produzido, a duração e a localização de sua produção são importantes na geração de resposta imune adequada. Esses fatores são regulados pelo controle da expressão de genes associados a sua síntese (HOLLOWAY *et al.*, 2001).

A modulação da síntese de citocinas é realizada pela ligação da citocina ao seu receptor especifico na célula alvo, desencadeando uma rede de sinalização que se inicia com a transdução de sinais intracelulares na célula efetora da resposta. A ligação de citocinas aos seus receptores específicos dará inicio à transdução de sinais e de vias secundárias dentro da célula alvo que poderão

resultar na ativação de genes responsáveis pela divisão celular, crescimento, diferenciação, migração ou apoptose (JANEWAY *et al*., 2010). Assim, a partir da maturação de CDs, os linfócitos podem assumir os padrões de citocinas Th1 (estimulada pela liberação de IL-12 e INF-γ) ou Th2 (pela secreção de IL-4) (STEINMAN, 2003).

Como consequência da anemia hemolítica crônica, da vaso-oclusão e da susceptibilidade elevada a infecções, os indivíduos com AF possuem produção exacerbada e recorrente de citocinas e de proteínas de fase aguda. As citocinas liberadas contribuem para a ativação do endotélio vascular e expressão de moléculas de adesão, ativação plaquetária, produção de endotelina-1 e desregulação da apoptose de células endoteliais (MAKIS *et al*., 2000). Entre as citocinas que contribuem para esse mecanismo na patogênese da AF, podem ser citados o TNF-α, a IL-1 e a IL-8 ((MAKIS *et al*. 2000).

O TNF-α possui papel imunomodulador importante, participando da resposta do hospedeiro em condições inflamatórias agudas e crônicas (HAJEER & HUTCHINSON, 2001). Essa citocina promove o aumento da expressão de moléculas de adesão, elevando a circulação de células nos sítios de infecção, estimulando macrófagos e neutrófilos a exercerem fagocitose, bem como a liberação de outras citocinas e mediadores inflamatórios. A expressão de TNF-α nos estados infecciosos está associada a maioria dos sintomas clínicos presentes, tais como a febre e reações inflamatórias locais. Em contrapartida, quando produzido em excesso ou liberado de forma sistêmica em grandes quantidades, pode levar a complicações fatais, tais como colapso circulatório e falência múltipla de órgãos (JANEWAY *et al.,* 2010). Apesar da expressão do *TNF-α* possuir regulação multifatorial, tais como fatores transcricionais e alterações na forma membranar do TNF- α, que resulta na liberação da forma solúvel e expressão de receptores, sendo que os níveis elevados desta citocina parecem influenciar na susceptibilidade ou gravidade de condições inflamatórias (HAJEER-HUTCHINSON, 2001). O polimorfismo -308G>A na região promotora do gene *TNF-α* tem sido associado a expressão elevada do TNF- α (HAJEER & HUTCHINSON, 2001), a susceptibilidade a sepse pós-traumática (O'KEEFE *et al*., 2002), bem como a ocorrência de AVE em pacientes Com AF (HOPPE *et al*., 2004).

A IL-8 é uma quimiocina secretada por monócitos, macrófagos, fibroblastos e células endoteliais que participa do recrutamento de neutrófilos, basófilos e de células T para os locais da infecção, e que está envolvida tanto na resposta inflamatória local como na crônica (HULL *et al*., 2001). A IL-8 parece atuar de duas formas principais na resposta inflamatória: primeiro, participando do rolamento de leucócitos nas células endoteliais nos sítios inflamatórios, estabelecendo mudança conformacional nas integrinas de leucócitos, de maneira a permitir a passagem de leucócitos pelas paredes dos vasos e entre as células endoteliais. Segundo, a IL-8 promove um gradiente quimiotático que induz a migração e eleva a concentração de leucócitos nos locais da infecção (JANEWAY *et al.*, 2010; THARP *et al.*, 2006).

O papel da IL-8 na AF parece estar relacionado com a ativação de neutrófilos nos sítios inflamatórios e na luz dos vasos sanguíneos, promovendo o aumento da aderência de hemácias ao endotélio vascular pela ativação de receptores de membrana, com exacerbação e propagação da inflamação (ETIENNE-JULAN *et al.,* 2004). Além disso, a expressão de IL-8 e de moléculas de adesão associadas ao endotélio é regulada pela IL-1 e pelo TNF-α, que apresentam níveis elevados em indivíduos com AF (MAKIS *et al.*, 2000; LANARO *et al*., 2009). Alguns estudos demonstraram a presença de níveis elevados de IL-8 em pacientes com AF em crise vaso-oclusiva, quando comparados a indivíduos assintomáticos e saudáveis, independente do fator indutor da crise (GONÇALVES *et al*., 2001, LANARO *et al*. 2009).

1.3 – FCGR2A (FcγRIIA): receptores de IgG - polimorfismo gênico e interação clinica.

Os receptores gama do fragmento cristalizável de IgG (FCGR) são glicoproteínas de membrana que atuam como mediadores importantes das respostas imune humoral e celular devido a sua capacidade de interagir com diferentes tipos celulares por meio da sua ligação com os domínios Fc (fragmento cristalizável) da imunoglobulina G (IgG) (BOURNAZOS *et al.*, 2009).

Os receptores FCGR (FcγR) são expressos em diferentes tipos celulares, principalmente os de origem hematopoiética, que medeiam várias respostas

biológicas, tais como fagocitose, endocitose, captura e clareamento de imunocomplexos, citotoxicidade, geração de espécies reativas de oxigênio, liberação de enzimas lisossomais, e liberação de mediadores inflamatórios, a depender do tipo celular, do tipo de receptor Fcγ e da natureza do complexo de IgG ativado (BRUHNS *et al*., 2009).

Três classes principais de receptores FcγR, são identificadas no homem e descritas como FcγRI (CD64), FcγRII (CD32) e FcγRIII (CD16), ressaltando que a mesma célula pode expressar mais de um receptor, o que contribui para a diversidade de respostas biológicas (JANEWAY, 2010; BOURNAZOS *et al.*, 2009).

O receptor para IgG humano mais amplamente distribuído entre as células do sistema imune é o FcγRII (CD32). Essa glicoproteína de 40 kDa, pode ser codificada por três genes (*A, B* e *C*), sendo que seis RNAs mensageiros (RNAm) diferentes são transcritos (JANEWAY, 2010). O receptor FcγRIIA é expresso em células polimorfonucleares, monócitos, macrófagos, em alguns tipos de células endoteliais e nas plaquetas; o FcγRIIB nas células B, monócitos e macrófagos; enquanto o FcγRIIC é restrito a células natural killer (NK) (BOURNAZOS *et al.*, 2009).

O FcγRII liga-se a imunocomplexos de IgG1 e IgG3, sendo que sua ligação a IgG2 depende do polimorfismo no gene *FcγRIIA*, que altera a capacidade de ligação do FcγRII, apresentando duas variantes alotípicas: o *FcγRIIA-R131* (arginina 131) e o *FcgRIIA-H131* (histidina 131). Esse polimorfismo é o mais estudado e aparentemente o de interesse e implicações clínicas maiores, uma vez que a variação alélica representada pela mutação de um único aminoácido na posição 131 (arginina ou histidina) é crítica para a ligação da IgG2. O alótipo *FcgRIIA-H131* é o único capaz de ligar IgG2 eficientemente, com reperpurssão na imunidade mediada por IgG2 (WARMERDAM *et al*., 1991). Assim, a variante H131 apresenta papel importante em condições onde há resposta imune com elevação de anticorpos IgG2, conferindo ativação de leucócitos e o aumento da capacidade de clearence de complexos de imunoglobulinas circulantes (BOURNAZOS *et al.*, 2009). Ressalta-se que esta subclasse de IgG é essencial para a defesa contra bactérias encapsuladas, tais como *Neisseria meningitidis* e *Haemophilus influenzae* (KIMBERLY *et al*., 1995; NORRIS *et al*., 1996).

O estudo do polimorfismo *FcγRIIA H/R131* tem contribuído para esclarecer a importância desse receptor nas alterações da imunidade, em doenças hematológicas e em doenças autoimunes (JIANG *et al*., 1996), como o lúpus eritematoso sistêmico (YUAN *et al*., 2009), síndrome coronariana aguda (RAAZ *et al*., 2009) e a proteção contra malária (SINHA *et al*., 2008).

2- Justificativa

Os pacientes com AF apresentam quadro clínico heterogêneo, com internações hospitalares frequentes, devido principalmente a ocorrência de infecções e/ou de crises vaso-oclusivas, que podem levar a falência de órgãos vitais, reduzindo a qualidade e expectativa de vida. Além disso, portadores de AF apresentam estado pró-inflamatório crônico que parece estar relacionado a susceptibilidade elevada a infecções bacterianas e alterações na imunidade individual (STEINBERG, 2008).

As alterações no sistema imune têm sido relacionadas a predisposição a infecções apresentada pelos pacientes com AF, tais como produção exacerbada de anticorpos, deficiências na via alternativa do complemento, alterações na opsonização, alterações na função de leucócitos e na imunidade celular (OHENE-FREMPONG & STEINBERG, 2001, SALAWU et al., 2009), desencadeando o estado pró-inflamatório constante (KUTLAR, 2005; REDDING-LALLINGER & KNOLL, 2006).

Desta forma, no presente estudo foram avaliados aspectos relativos a imunidade inata, visando contribuir para o aumento do conhecimento do sistema imune na patogênese da AF. Para tanto, o presente estudo investigou a presença de polimorfismos nos genes do *TLR4*, *TLR9*, da *MPO*, do *TNF-α*, da *IL-8* e do receptor *FcγRIIA*, associando-os a presença de talassemia $\alpha_2^{-3.7Kb}$, aos níveis séricos das citocinas IL-8, TNF-α, IL-1β e IL-10 em indivíduos com AF em estado estável e em crise e aos dados encontrados no quadro clinico do paciente, bem como a participação desses elementos nos fenômenos vaso-oclusivos e infecciosos presentes na AF. Também foi investigada a expressão de *TLR2*, *TLR-4*, *TLR-5* e *TLR-9* em células dendríticas derivadas de monócitos de indivíduos com AF.

Assim, a avaliação do estado inflamatório crônico e agudo em pacientes pediátricos com AF em estado estável e em crise foi realizada pela investigação dos genes de moléculas do sistema imune e sua associação aos aspectos clínicos e laboratoriais desses indivíduos, visando identificar os mecanismos associados a resposta imune na AF e levantar dados que poderão ser contribuir futuramente no manejo terapêutico e na prevenção da gravidade da doença desses indivíduos.

3- Objetivos

3.1- Objetivo geral

Investigar ,em pacientes com AF, a presença de polimorfismos nos genes *IL-8* e *TNF-α*, dos receptores *TLR4*, *TLR9*, *FcγRIIA* e da *MPO* e do marcador clássico talassemia-α_2-$^{3.7Kb}$, associando-os aos níveis séricos das citocinas IL-1β, IL-8, IL-10 e TNF-α e a expressão de moléculas co-estimulatórias, como os TLR2, TLR4, TLR5 e TLR9 em células dendríticas; a aspectos clínicos e laboratoriais e moleculares apresentados por esses indivíduos, de maneira a contribuir com informações relacionadas a mecanismos associados aos fenômenos vaso-oclusivos e estados infecciosos em pacientes em idade pediátrica.

3.2- Objetivos específicos

- **Objetivo 1**: Investigar a presença da talassemia-$\alpha_2^{3.7Kb}$ e dos polimorfismos gênicos -251T>A, -308G>A presentes na região promotora dos genes da *IL-8*, e *TNF-α,* respectivamente;
- **Objetivo 2**: Investigar a presença dos polimorfismos gênicos -1237T>C e -463G>A na região promotora dos genes do *TLR9* e da *MPO*, bem como dos polimorfismos 896A>G no gene do *TLR4*, *e* R131H no gene do *FcγRIIA*, respectivamente;
- **Objetivo 3**: Quantificar os níveis séricos das citocinas TNF-alfa e IL-8 em indivíduos com AF em estado estável e internados por crises vaso-oclusivas ou infecção;
- **Objetivo 4**: Investigar a expressão dos receptores *TLR2*, *TLR4, TLR5* e *TLR9* em células dendríticas derivadas de monócitos;
- **Objetivo 5**: Correlacionar os dados obtidos com o perfil clínico dos pacientes pediátricos com AF incluídos no estudo.

4- Materiais e Métodos

4.1- Casuística

Foi realizado um estudo de corte transversal com a casuística de 346 pacientes com AF em idade pediátrica, compondo três grupos distintos, de acordo com o período de inclusão no estudo, como mostra a descrição dos desenhos experimentais I, II e III (Figuras 1, 2 e 3, respectivamente). O primeiro grupo de estudo foi composto por 210 indivíduos AF com idade média de 9,3±4,5 anos, cuja coleta de amostras foi realizada no período de 2003 a 2007, sendo denominado G1. O segundo grupo foi composto por 129 pacientes Com AF, com idade média de 12,6±8,8 anos, cuja coleta de amostras foi realizada no período de 2008 a 2010, sendo denominado G2. Esses dois grupos foram compostos por indivíduos acompanhados regularmentm,e no ambulatório de hematologia da Fundação de Hematologia e Hemoterapia da Secretária de Saúde do Estado da Bahia-HEMOBA/SESAB.

Os grupos G1 e G2 foram compostos por pacientes com AF em idade pediátrica em estado estável que concordaram em participar do estudo e cujos pais ou responsáveis assinaram o termo de consentimento livre e esclarecido (TCLE). Foram incluídos no estudo indivíduos que não fizeram uso de hemoderivados ou que apresentaram alguma comorbidade nos 3 meses que precederam a coleta de amostras para o estudo. Foram excluídos desse estudo pacientes que não assinaram o TCLE ou aqueles com história de infecção ou episódios de crise vaso-oclusiva nos três meses que precederam a coleta de amostras e/ou análises de hemoglobinas não confirmatórias do perfil HbSS. A coleta de sangue foi realizada durante a consulta ambulatorial e os dados clínicos foram obtidos pela busca retrospectiva aos prontuários médicos desses pacientes.

Pareado ao grupo G1 foram estudados 200 indivíduos saudáveis, em idade pediátrica e com perfil de hemoglobinas normal (AA), sem histórico de anemia, ou condição inflamatória ou doença hematológica, triados durante o atendimento regular no laboratório de análises clínicas da Faculdade de Farmácia da Universidade Federal da Bahia (FACFAR-UFBA).

O terceiro grupo (G3) foi incluído no estudo prospectivo que incluiu 8 pacientes Com AF internados no Hospital da Criança das Obras Sociais Irmã Dulce (HC/OSID), no período de janeiro de 2011 a agosto de 2011. Nesse grupo foram incluídos pacientes menores de 18 anos, internados por vaso-oclusão e/ou

infecção, cujos responsáveis concordaram com a participação do paciente no estudo após a explicação e assinatura do TCLE, mas que não fizeram uso de hemocomponentes nos últimos 3 meses. Os critérios de exclusão foram a recusa do menor em coletar o sangue, a falta de anuência do responsável e a não assinatura do TCLE e a realização de terapia transfusional nos últimos 3 meses. O projeto foi submetido ao comitê de Ética em Pesquisa em Seres Humanos do HC/OSID, sob número 103/03; e ao comitê de Ética em Pesquisa em Seres Humanos do Centro de Pesquisa Gonçalo Moniz da Fundação Oswaldo Cruz (CEP/CPqGM-FIOCRUZ).

Em todos os grupos estudados as amostras de sangue foram coletadas e encaminhadas ao CPqGM-FIOCRUZ (CPqGM/FIOCRUZ) para a realização das análises de biologia molecular, de cultura de células e os ensaios imunoenzimáticos (ELISA- *Enzyme-linked Immunosorbent Assay*); e uma alíquota da amostra coletada foi encaminhada para o Laboratório de Pesquisa em Anemias da FAC-FAR-UFBA para análise do perfil hematológico e de hemoglobinas. Os dados clínicos foram obtidos por entrevista com o paciente e/ou responsável pelo menor e a consulta aos prontuários médicos dos indivíduos acompanhados na HEMOBA e no HC/OSID. As amostras coletadas para os ensaios de cultura de células foram imediatamente processadas.

Todos os experimentos realizados seguiram as normas de Biossegurança de acordo com a Lei no. 11.105 de 24 de março de 2005, regulamentada pelo decreto no. 5.591 de 22 de novembro de 2005, seguindo as normas técnicas existentes no manual de Procedimentos para a manipulação de microrganismos patogênicos e/ou recombinantes na Fiocruz (Comissão Técnica de Biossegurança da FIOCRUZ – Ministério da Saúde, 2005).

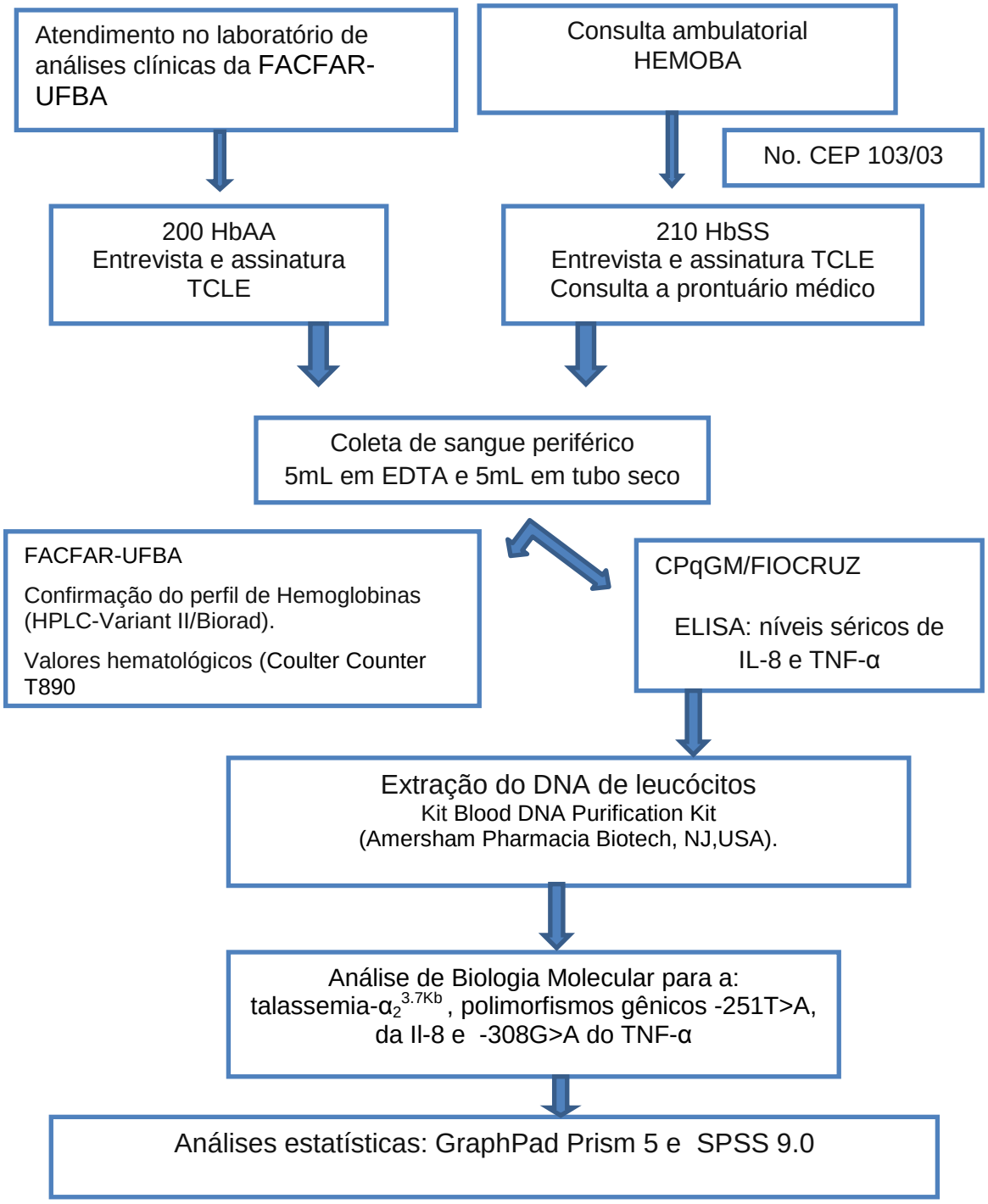

Figura 2: Representação do desenho experimental I, referente ao estudo de corte transversal realizados entre 2003 a 2007 (G1).

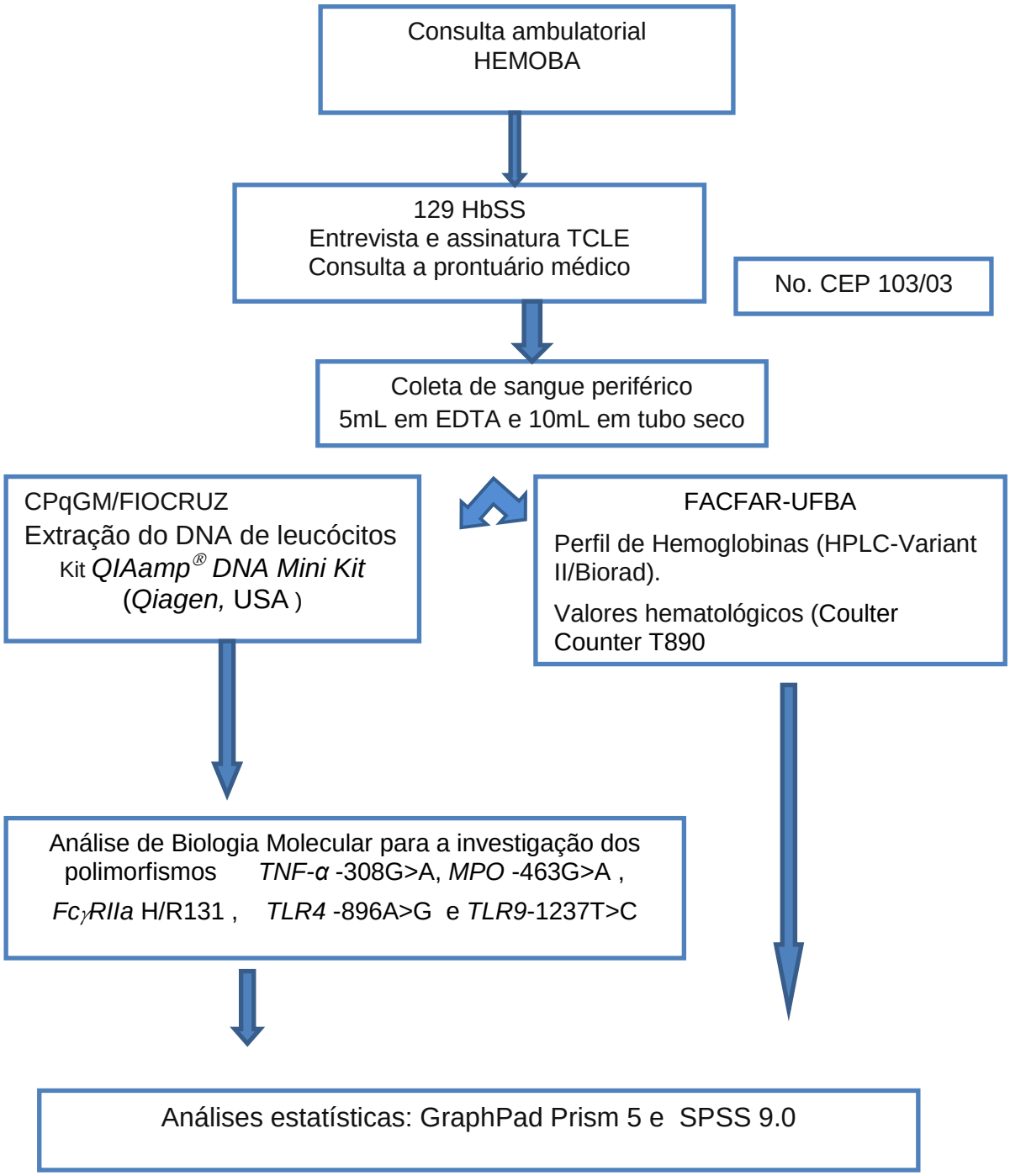

Figura 3: Representação esquemática do Desenho experimental II, referente ao estudo de corte transversal realizados entre 2008 a 2010 (G2).

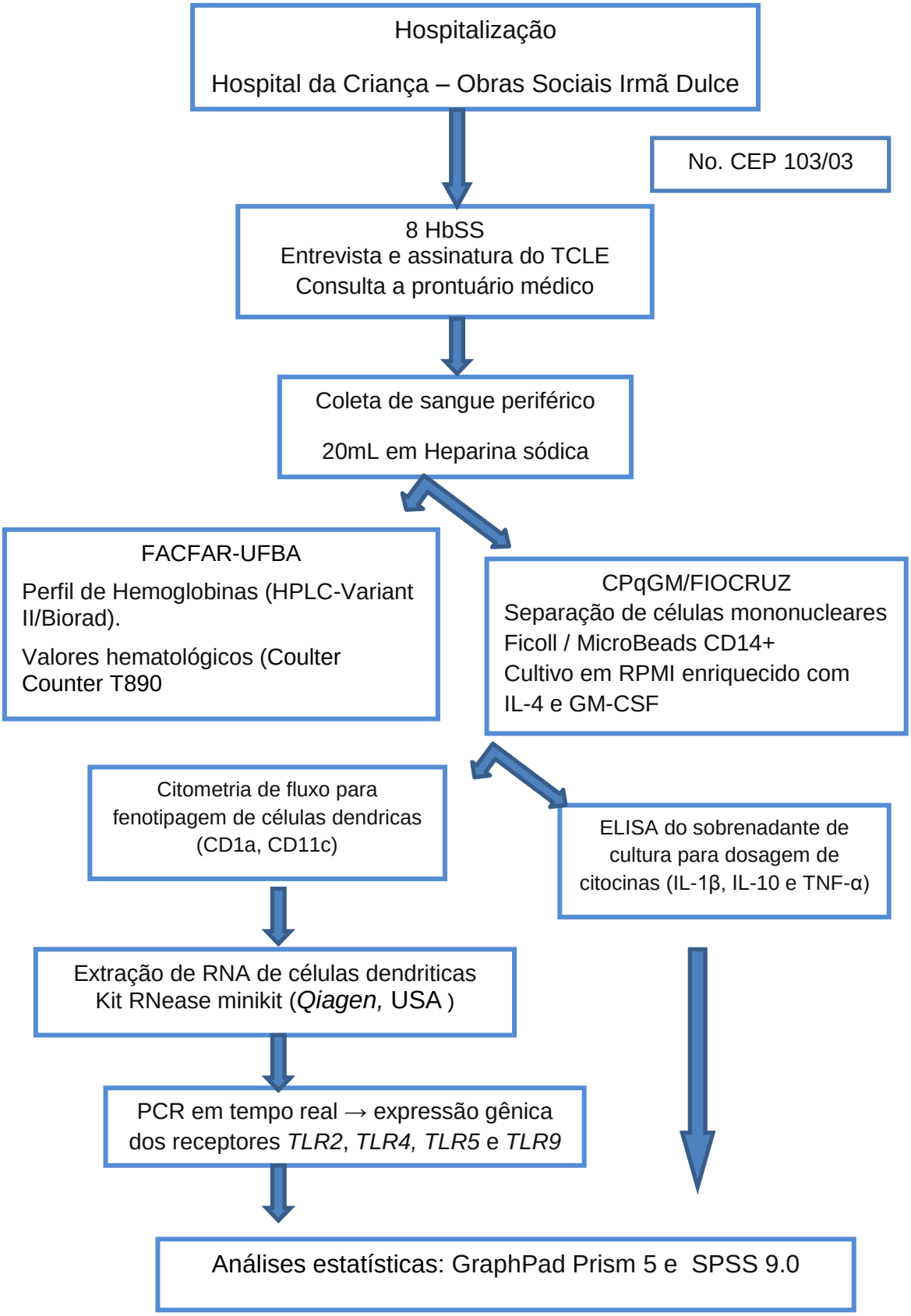

Figura 4: Representação esquemática do Desenho experimental III, referente ao estudo prospectivo com Com AF pediátricos internados no HC/OSID (G3).

4.2- **Métodologia utilizada nos experimentos denominados G1 e G2**

4.2.1- **Coleta das amostras de sangue**

Para os grupos G1 e G2 foram coletados 5mL de sangue venoso em EDTA (ácido etileno de aminotetracético di-sódico), na concentração de 1,5mg/mL (DACIE & LEWIS, 2006), que foram utilizados para a determinação das análises hematológicas, do perfil de hemoglobinas e extração do DNA genômico. Também foram coletados 10mL de sangue venoso, em tubo sem aditivo, utilizados para obtenção de soro destinado a dosagem de citocinas. O DNA genômico e o soro foram armazenados a -20 ^{o}C até a sua utilização.

4.2.2- **Análises hematológicas e do perfil de hemoglobinas**

Todos os indivíduos incluídos no estudo tiveram o perfil de hemoglobinas confirmado pela técnica de cromatografia líquida de alto desempenho (HPLC) em equipamento automatizado (*Variant II- Bio-Rad*). Os valores hematológicos e índices hematimétricos foram avaliados em contador eletrônico de células (*Coulter Count T* – 890, *Beckman Coulter*) e a análise morfológica das hemácias pela observação microscópica de esfregaços sanguíneos corados pelo método de Wright (DACIE & LEWIS, 1984).

4.2.3- **Análise de biologia molecular**

4.2.3.1- **Extração do DNA Genômico**

O DNA genômico foi isolado de leucócitos a partir de 200μL de sangue periférico, utilizando-se o método direto *QIAamp® DNA Mini Kit (Qiagen),* conforme protocolo do fabricante. O DNA foi armazenado a -20 ^{o}C até o momento das análises.

4.2.3.2- **Determinação dos haplótipos ligados ao grupo de genes da globina beta S e determinação da talassemia $\alpha 2^{3.7\ Kb}$**

Os haplótipos ligados ao grupo de genes da globina beta S foram investigados pela técnica de reação da polimerase em cadeia (PCR) utilizando oligonuclotídeos sintéticos (*primers*) específicos, seguida por digestão dos fragmentos obtidos com enzimas de restrição (RFLP). Para determinação dos

haplótipos βS foram investigados 5 sítios de restrição: 5'γG (*XmnI*),5γAγG (*Hind III*) , 3'γAγG (*HindIII*), 5'ψβ (*HIncII*) e 3'ψβ (*HIncII*) (SUTTON *et al*., 1989).

A talassemia $\alpha 2^{3.7\ Kb}$ foi investigada pela técnica da reação de PCR alelo-específico, utilizando *primers* contendo as sequências normal (A + C) e mutante (A + B) para o gene α2 (Tabela 1), de acordo com protocolo previamente estabelecido (DODE *et al.*, 1993). As reações realizadas com *primers* A + B e A + C amplificaram fragmentos de 1.700 pb (1,7 kb), para a presença da deleção e dos genes normais, respectivamente.

4.2.3.3- **Estudo de polimorfismos nos genes de citocinas, nos genes de receptores do tipo *TLRs* e *FcγRIIA* e da *mieloperoxidase* (*MPO*).**

Os polimorfismos nos genes da *IL-8*, do *TNF-α* e nos receptores *TLR4, TLR9, FcγRIIA* e da *MPO* foram investigados pela reação de PCR e digestão posterior dos fragmentos amplificados com enzimas de restrição (RFLP). Os respectivos *primer*s ou oligonuclotídeos sintéticos, temperatura de anelamento e enzimas de restrição estão enumerados na Tabela 1. O padrão de amplificação, ou seja, o tamanho do fragmento gerado e o tamanho dos fragmentos dos respectivos genótipos encontrados após digestão estão representados na tabela 2.

Os produtos obtidos nas reações de PCR/RFLP do *IL-8*, *TNF-α, FcγRIIa e MPO* foram analisados em gel de agarose a 1% e os produtos da digestão com as enzimas de restrição em gel de poliacrilamida a 7%, em tampão TBE 1X (Tris–Borato - 0,04M; EDTA - 0,001M), corados pelo brometo de etídio (0,002%) e visualizados sob luz ultra-violeta.

Os fragmentos obtidos das reações de PCR/RFLP para *TLR4* e *TLR9* foram visualizados em gel de agarose de alta resolução a 3% (*Metaphor*®/Cambrex), também em tampão TBE 1X (Tris–Borato - 0,04M; EDTA - 0,001M), corados pelo brometo de etídio (0,002%) e visualizados sob luz ultra-violeta.

Para cada reação foram incluídos controles negativos e positivos visando testar a presença de contaminantes e a qualidade da amostra, respectivamente.

Tabela 1: Lista dos *primers* e condições da reação PCR/RFLP dos polimorfismos gênicos estudados.

Polimorfismo gênico	Sequencia dos Primers (5'→3')	Temperatura de anelamento/ $MgCl_2$(°C/mM)	Enzima de restrição
MPO -463G>A	F- CGG-TAT-AGG-CAC-ACA-ATG-GTG-AG R-GCA-ATG-GTT-CAA-GCG-ATT-CTT-C	58/2.5	*AciI*
FcγRIIa H/R131	F- GGA-AAA-TCC-CAG-AAA-TTC-TCG-C R-CAA-CAG-CCT-GAC-TAC-CTA-TTA-CGC-GGG	55/1.5	*BstUI*
TNF-alpha -308G>A	F- AGG-CAA-TAG-GTT-TTG-AGG-GCC-AT R- TCC-TCC-CTG-CTC-CGA	59/3.5	*NcoI*
TLR4 -896A>G	F-GAT-TAG-CAT-ACT-TAG-ACT-ACT-ACC-TCC-ATG R-GAT-CAA-CTT-CTG-AAA-AAG-CAT-TCC-CAC	55/3.0	*NcoI*
TLR9 -1237T>C	F-ATG-GGA-GCA-GAG-ACA-TAA-TGG-A R-CTG-CTT-GCA-GTT-GAC-TGT-GT	61/3.0	*BstNI*
Talassemia **α2** $^{3.7Kb}$	**A:** CCC-TCC-CCC-TCG-CCA-AGT-CCA-CCC-C **B:** GGG-GGG-AGG-CCC-AAG-GGG-CAA-GAA **C:** GGG-AGG-CCC-ATC-GGG-CAG-GAG-GAA-C	58/2.5	-

Tabela 2: Tamanho dos fragmentos gerados após digestão do produto de PCR com enzima de restrição específica.

Polimorfismo gênico	Selvagem	Homozigoto mutante	Heterozigoto	Referencia
IL-8 -251A>T	174pb	154 e 20pb	174, 154 e 20 pb	HEINZMANN *et al*., 2004
TNF-α -308G>A	87pb e 20pb	107pb	107, 87e 20 pb	SEITZER *et al*., 1997
MPO -463G>A	169, 120 e 61bp	289 e 61bp	289, 169, 120, e 61bp	NIKPOOR *et al*, 2001.
FcγRIIa H/R131	343 e 23 bp	322 e 44pb	343, 322, 44 e 23 pb	ISRAELSSON *et al*., 2008.
TLR9 -1237(T>C)	108pb e 27pb	135pb	108pb, 60pb, 48pb e 23pb	HAMANN et al., 2004
TLR4 -896A>G	249pb	23pb	223pb e 23pb	GAZOULI et al., 2005; LORENZ et al., 2002

4.2.4- Determinação dos Níveis de Citocinas

Os níveis séricos de IL-8 e TNF-α foram determinados pela técnica de ELISA, utilizando anticorpos de detecção e de captura (*BD OptEIA - Biosciences)*. Para as dosagens foi utilizado protocolo único, alterando apenas as concentrações de anticorpos para sensibilização das placas e as concentrações do padrão para

determinação da curva padrão. Para as dosagens das citocinas IL-8 e TNF-α foram utilizados anticorpos monoclonais de captura para IL-8 e para TNF-α na concentração de 2µg/mL e anticorpo de detecção monoclonal biotinilado anti-IL-8 humano e anti-TNF-α na concentração de 1µg/mL. A curva padrão teve 8 pontos em diluição seriada a partir de 1:2 para cada citocina estudada, de acordo com a recomendação do fabricante. As amostras foram aplicadas em duplicata, seguindo o protocolo descrito abaixo, conforme orientação do fabricante:

- **Sensibilização da placa**: A placa de 96 poços foi sensibilizada com 100uL/poço do anticorpo monoclonal de captura humano diluído em solução Na_2HO_4 0,1M pH9,0 na concentração de 2ug/mL, seguido por incubação por 12 horas à 4^OC;
- **Bloqueio**: Após remover a solução com anticorpo de captura foram adicionados 200µL/poço do tampão de bloqueio (tampão salina fosfatado acrescido de albumina bovina sérica a 1% - PBS/BSA 1%). Incubação por 2 horas à temperatura ambiente;
- **Lavagem**: Após cada etapa de incubação foi realizada a lavagem com PBS/Tween 0,05% (tampão salina fosfatado acrescido de albumina bovina sérica a 1% e Tween 20 0,05%), sendo cada placa lavada 4 vezes;
- **Padrões, controles e amostras**: Foram distribuídos 100µL/poço do padrão IL-8 e TNF-α humano recombinante na diluição 1:2 até 1:256 e 100µL/poço do soro dos pacientes em duplicata. Também foram incluídos o branco da reação, controle positivo e negativo (PBS/BSA1%Tween0,05%), seguidos de incubação por 12 horas à 4^OC.
- **Detecção**: Foram adicionados à placa, 100µL/poço do anticorpo monoclonal biotinilado anti-IL-8 humano diluído em PBS/BSA1%Tween0,05% na concentração de 1ug/mL. Após esta etapa foi realizada incubação durante 1 hora à temperatura ambiente;
- **Conjugado**: Nesta etapa foram adicionados 100µL/poço do conjugado HRP-strepatavidina (SAv-HRP), diluído em PBS/BSA1%Tween0,5% na concentração de 1:2000 e incubado por 30 minutos;
- **Substrato**: O substrato TMB (3,3',5,5'-tetrametilbenzidina) em tampão citrato fosfato, DMSO e H_2O_2 foi preparado 20 minutos antes do uso, com a adição de 100µL/poço;

- **Solução de parada**: A reação de cor foi parada pela adição de 50uL/poço de H_2SO_4 8N após 5 minutos de incubação. A leitura da reação foi realizada em espectofotômetro a 450nm.

4.3- Métodos utilizados para a realização dos experimentos com grupo de pacientes internados.

4.3.1- **Coleta das amostras de sangue**

Para o grupo G3 foram coletados 12 a 15mL de sangue venoso em heparina sódica, para a realização imediata de cultura de células.

4.3.2- **Separação de células mononucleares em sangue periférico em gradiente de Ficoll seguida de separação magnética por *beads***

Foi utilizado volume de 15 a 20mL de sangue venoso heparinizado coletado dos pacientes Com AF e destinados a obtenção de células mononucleares por gradiente de Ficoll (Histopaque 1077, Sigma Aldrich Co, LLA). O sangue foi diluído em volume igual de solução fisiológica 0,9%, distribuído lentamente em tubos de fundo cônico contendo 3mL de Ficoll para cada 10mL de sangue diluído. Em seguida, a solução foi submetida a centrifugação a 1500rpm durante 30 minutos. As células mononucleares formaram um anel no gradiente de Ficoll e foram coletadas e lavadas três vezes em solução fisiológica 0,9%, centrifugados 1500 rpm durante 5 minutos, decrescendo gradualmente a velocidade de centrifugação a cada lavagem até 1000rpm. Após as lavagens, as células foram ressuspensas em meio RPMI 1640 (Sigma-Aldrich Co, LLA). Após as lavagens procedeu-se a contagem das células suspensas em RPMI, utilizando câmara de *Neubawer* e coloração pelo Azul de Trypan para avaliação da viabilidade celular.

A suspensão de células contendo aproximadamente 10^8 células por mL de solução foi submetida ao processo de separação magnéticas de monócitos pelo kit CD14 *Microbeads human-Macs* (*Miltenyi-Biotec*, USA). Seguindo o protocolo recomendado pelo fabricante, após a marcação das células com as microesferas cobertas por CD14, as amostras foram passadas em coluna magnética. A concentração final de células foi ajustada para 10^5 células/mL e plaqueadas em

meio RPMI enriquecido com 10% soro bovino fetal (Sigma-Aldrich Co, USA),com fator de crescimento GM-CSF (50 ng/mL) (PeProTech, USA), IL-4 (20 ng/mL) (PeProTech, USA), penicilina (100µg/mL) (Gibco, USA), sendo incubadas em média 2 a 3 poços de 1mL por indivíduo. As culturas foram mantidas durante 6 dias a 37^{o}C em estufa de CO_2. A troca do meio foi realizada a cada 3 dias, sendo que no sexto dia de cultura foram coletadas amostras contendo cerca de 10^5 células/mL para a caracterização imunofenotípica. Outras amostras foram mantidas em cultura e estimuladas com 1µg/mL LPS (lipopolissacarídeo) por 24 horas, quando então foram congeladas à -70ºC em meio RPMI para posterior extração de RNA e transcrição do cDNA para realização de qRT- PCR (PCR em tempo real). Os sobrenadantes de cultura das células cultivadas por 6 e por 7 dias também foram aliquotados (500uL) e congelados à -70ºC, sendo destinados a dosagem de citocinas.

4.3.3- **Citometria de Fluxo – IMUNOFENOTIPAGEM**

Para realização de imunofenotipagem foram utilizadas concentrações celulares que variaram de 2 a 6 x10^5 células. As subpopulações celulares foram caracterizadas pela técnica de imunofluorescência por meio de citometria de fluxo (FACSAria - BD Biosciences, USA), técnica que determina e quantifica a expressão de moléculas de superfície ou intracelulares, mediante a utilização de anticorpos marcados com substâncias fluorescentes ou fluorocromos. Para caracterização fenotípica foram utilizados os marcadores de células dendriticas provenientes de monócito CD14 (cluster de diferenciação), CD1a e CD11c. Os anticorpos monoclonais utilizados foram o anti-CD14 e o anti-CD1a marcados com isocianato de fluoresceina (FITC), o anti-CD11c marcado com aloficocianina (APC) (BD Biosciences, USA). A aquisição e análise das amostras foram realizadas no FACSAria, utilizando o programa CELLQUEST.

As células foram distribuídas na concentração de 2 x 10^5 células / mL em tubos de polipropileno (BD Biosciences, USA) e centrifugadas a 4ºC a 1800 rpm durante 5 minutos. Os sobrenadantes foram desprezados e os precipitados celulares foram homogeneizados em agitador de tubos. Em seguida, as células foram incubadas durante 30 minutos ao abrigo da luz, com 2,5 µL dos anticorpos monoclonais anti-CD14, anti-CD1a e anti-CD11c. Após o período de incubação, as

células foram lavadas três vezes em tampão PBS/BSA1% e centrifugadas a 1800 rpm por 5 minutos. Em seguida, as células foram ressuspensas em 400 µL de solução de paraformaldeido a 2% e 0,02% de azida sódica e mantidas a 4ºC até o momento da aquisição. As amostras foram adquiridas em citômetro de fluxo FACSAria (BD Biosciences, USA), utilizando controle negativo contendo só células, e controle positivo IgG especifico (BD Biosciences, USA). para cada fluorescência. As culturas apresentaram em média mais que 72% de céulas (Figura 4).

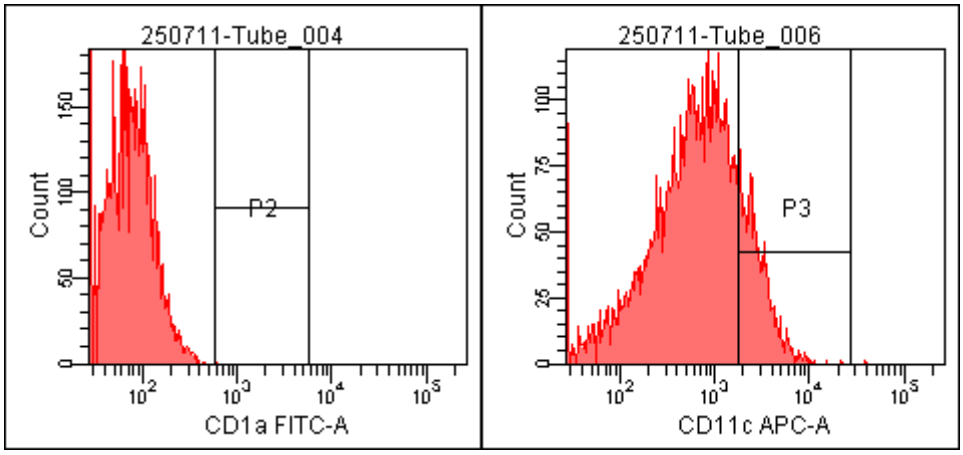

Figura 5: Imunofenotipágem de células dendríticas proveniente de monócitos marcadas com CD1a e CD11c.

4.3.4- Extração do RNA de células dendríticas, quantificação de RNA e Tratamento com DNase

A extração de RNA das células dendriticas foi realizada a partir de 10^7 células dendriticas/mL, utilizando-se o método direto *RNeasy® Mini Kit* (*Qiagen*, USA), conforme protocolo descrito pelo fabricante.

O RNA obtido foi quantificado por meio de leitura no espectofotômetro (*Nanodrop,* Thermo Scientific, USA) nos comprimentos de onda (λ) de 260 RNA e 280 nm (proteína). O grau de pureza da amostra foi avaliado pela relação das leituras 260/280 nm, sendo considerados ideais os valores entre 1,8 e 2,0. Os resultados foram obtidos em ng/µL onde o valor de densidade ótica (DO) igual a 1, no comprimento de onda de 260 nm correspondeu a 40 µg de RNA (Sambrook 1989). Uma alíquota do RNA foi submetida a eletroforese em gel de agarose a 1%

para visualização da integridade das amostras, observando as subunidades do RNA ribossômico, 18S e 28S, e também possíveis contaminações com DNA. Para evitar possíveis contaminações com DNA, o RNA foi tratado com rDNase (*Invitrogen*, USA), de acordo com a recomendação do fabricante. As amostras foram consideradas adequadas quando as bandas de 28S e 18S mostraram-se íntegras, sem nenhum traço de DNA genômico.

4.3.5 - Transcrição reversa em DNA complementar (cDNA)

A transcrição reversa de 5µg de RNA total para cDNA foi realizada após controle da qualidade e pureza do RNA e do tratamento do RNA total com DNase, utilizando a enzima transcriptase reversa Super-scriptII™ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Para este protocolo foi adicionado ao RNA 0,2uM dos primers específicos para *TLR2*, *TLR4*, *TLR5* e *TLR9* (descritos na tabela 3), 0,4 mM de dNTP, (desorribonuleosídeo trifosfatado do tipo dATP, dCTP, dGTP e dTTP) e água DEPC (água Milli-Q tratada com dietilpirocarbonato) para um volume final de 50µL. Esta mistura foi aquecida a 65ºC por 5 minutos em termociclador (Eppendorf, Germany). Em seguida foram adicionados ao tubo de reação 4µL de tampão de transcrição 5x, 2µL de DTT (Dithiothreitol) 0,1M e 1µL de inibidor de RNase (40U/µL) (RNAse OUT™, Invitrogen, Carlsbad, CA, USA). As amostras foram então colocadas em termociclador a 42ºC por 2 minutos. Por último foi adicionado 1µL da enzima transcriptase reversa (200U/µL), e esta solução foi colocada novamente em termociclador a 42ºC por 50 minutos seguidos de 70ºC por 15 minutos. Ao final da transcrição, o cDNA foi mantido a -20ºC.

4.3.6- Reação de PCR quantitativo em tempo real (qRT-PCR)

As reações de PCR em tempo real (RT-PCR) foram realizadas em duplicata, em placas ópticas de 96 poços (*Applied Biosystems,* USA) no equipamento ABI 7500, utilizando *software* SDS 2.0 (*Applied Biosystems* USA). Os primers para essas reações estão descritos na tabela 3. Para análise de fluorescência foi utilizado o fluoróforo SYBR-Green PCR. A reação foi realizada com desnaturação inicial a 95ºC por 10 minutos, seguida por 40 ciclos de 15 segundos a 95ºC e 1

minuto a 60 ºC, de acordo com o manual de instruções do fabricante *Applied Biosystems*.

O cálculo da quantificação relativa foi realizado pelo método de delta delta Ct (*cycle threshold)*, utilizando como gene de referência (endógeno) o *hypoxanthine-guanine phosphoribosyltransferase* HPRT e calibrado com controles normais (TAVARES *et al*., 2010). Foram considerados os valores de quantificação relativa maior ou igual a 1,0 (QR≥1,0) para os genes regulados positivamente e de QR<1,0 para genes regulados negativamente em relação ao calibrador. A fórmula utilizada para o cálculo de QR foi:

$QR = 2^{-\Delta\Delta Ct}$, onde,

ΔCt= Ct alvo – Ct referência, e $\Delta\Delta Ct = \Delta Ct$ amostra - ΔCt Controle.

As reações de PCR seguiram o seguinte protocolo: foram utilizados 12,5uL de *SYBR Green (Invitrogen)*, 0,5μL de *primer*s direto e reverso na concentração inicial de 10μM; 2,0μL de cDNA de cada amostra (concentração de 50ng de DNA) e água livre de DNA e RNA para ajustar o volume final e 25uL.

Tabela 3. Sequencia de *primers* utilizados nas reações de PCR em tempo real.

Gene	Sequencia dos Primers (5'→3')	Definição do Locus (GeneBank)
TLR9	F AAC CTC CCC AAG AGC CTA CAG	017442
	R CAG CAC TTA AAG AAG GCC AGG TA	
TLR5	R AGC CCC GGA ACT TTG TGA CT	003268
	F TGT ATG CAC TGT CAC TCT GAC TCT GT	
TLR4	R CAA CAA TCA CCT TTC GGC TTT T	024169
	F GGC CAT TGC TGC CAA CAT	
TLR2	F TTG TGA CCG CAA TGG TAT CTG	
	R GCC CTG AGG GAA TGG AGT TT	

4.3.7- Padronização da Concentração de uso dos *primer*s

A concentração de uso de cada par de sequências de *primer*s foi inicialmente padronizada, utilizando amostras de cDNA de indivíduos saudáveis, onde foram testadas as concentrações de 0,1μM, 0,2μM, 0,3μM, 0,4μM, 0,5μM e 0,6μM para cada par de sequência de *primer*s. Após as reações de qRT-PCR, os resultados obtidos foram comparadas aos valores de Ct de cada par de *primer*s em cada concentração e foi escolhida a concentração com base no valor de Ct, de forma que a concentração de *primer* não fosse um fator limitante da reação. A **Tabela 3** mostra as sequência e características dos *primer*s que foram utilizados nas reações de qRT-PCR. Para a padronização foi utilizada a concentração de 0,2μM para todos os sítios estudados.

4.3.8-Cálculo da Eficiência dos primers

A avaliação da eficiência indica a cinética real de amplificação da sequência de *primer* testada, bem como sua reprodutibilidade e exatidão. Para o cálculo do valor da eficiência de cada par de *primer*s (*F* e *R*), utilizou-se um *pool* de cDNA de indivíduos saudáveis, em diluições seriadas (1:10 a 1:1250), e foram realizados ensaios de qRT-PCR, com 0,2μM de cada *primer* de interesse, já padronizado quanto a sua concentração de uso. Após a reação de qRT-PCR, as médias dos valores de Ct das triplicatas de cada diluição foram utilizadas no cálculo da eficiência (E). Para cada sequência de *primer* construiu-se um gráfico com base no valor logaritmo das diluições testadas (log ng RNA; eixo X), e pelos valores da média de Ct (eixo Y). O valor da inclinação da reta (slope) foi utilizado para determinar a eficiência dos *primer*s para cada gene de interesse, com base na fórmula:

$$E = (10^{-1/slope}) - 1$$

Através das curvas de eficiência também se determina a diluição de cDNA das amostras que são utilizadas nas reações de qRT-PCR para determinar a expressão de cada gene (Tabela3). O gráfico da eficiência, a amplificação das amostras e a curva de dissociação estão mostrados na Figura **4.** Os experimentos de quantificação da expressão gênica foram iniciados após confirmar se os

experimentos preencheram todos os critérios de validação para qRT-PCR (eficiência e especificidade) e determinar a diluição cDNA de 1:10 a ser usada para cada gene de interesse.

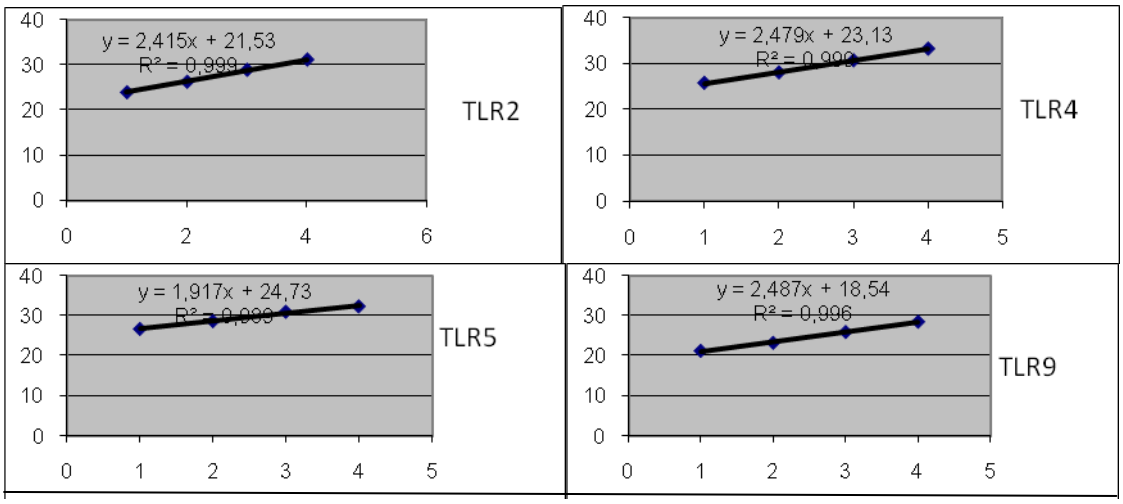

Figura 6: Representação do cálculo de eficiência dos *primers* para expressão dos genes *TLR2*, *TLR4*, *TLR5* e *TLR9*.

4.3.9- Determinação dos Níveis de Citocinas no sobrenadante de cultura

As dosagens das citocinas IL1-β, TNF-α e IL-10 foram realizadas nas amostras de plasma dos pacientes Com AF internados e no sobrenadante de cultura de células dendríticas utilizando a técnica de ELISA com anticorpos de detecção e de captura (*BD OptEIA - Biosciences, USA)*. Para cada dosagem foi utilizado protocolo único, alterando apenas as concentrações de anticorpos para sensibilização das placas e as concentrações do padrão para realização da curva padrão, conforme orientação do fabricante (*BD OptEIA – Biosciences, USA)* .

4.4- **Análise estatisitica**

As análises estatísticas foram realizadas utilizando-se os programas *Graphpad Prism* versão 3.0 e SPSS 9.0 *for Windows*. As distribuições das variáveis quantitativas foram determinadas pelo teste de Kolmogorov–Smirnov. As análises de correlação bivariada foram realizadas utilizando o teste de Spearman.

A análise de variáveis qualitativas ou categóricas foi realizada pelo teste não paramétrico do Qui-quadrado (χ^2), devidamente corrigido pelos testes de Mantel-Haenszel e Yates. Na presença de valores inferiores a 4 nas tabelas 2 x 2, as análises foram realizadas pelo teste exato de Fisher. Para a análise de duas variáveis numéricas com distribuição normal, utilizamos o teste T independente na comparação de dois grupos de valores dentro de uma mesma variável e o teste T pareado quando foram analisados valores de variáveis numéricas, com distribuição normal, obtidos em diferentes momentos de uma mesma variável. No caso de distribuição não normal, utilizamos o teste de Mann Whitney. Os valores de $p<0,05$ (5%) foram considerados significantes para as análises realizadas.

5.Artigos

5.1- Artigo 1

TNF-alpha and IL-8: Serum levels and gene polymorphisms (-308G>A and -251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia.

C. Cajado, B.A.V. Cerqueira, F.D. Couto, J.P. Moura-Neto, W. Vilas-Boas, M.J. Dorea, I.M. Lyra, C.G. Barbosa, M.G. Reis, M.S. Goncalves. **Cytokine, 56:312–317, 2011.**

Resumo: A anemia falciforme é uma doença caracterizada pela heterogeidade clinica. Neste trabalho nos investigamos a associação entre a presença do o polimorfismo -308G>A no gene do *TNF-alfa* e -251A>T no gene da *IL-8* com a historia clinica de crianças portadoras de anemia falciforme em estado estável. Também foram estudados biomarcadores clássicos como os haplótipos ligados ao gene da globina beta S e a presença da talassemia α2 com deleção de 3.7-kb por PCR/RFLP. As citocinas foram quantificadas por ELISA. A esplenomegalia foi mais prevalente em crianças com idade até 5 anos (p=0,032). O alelo A do polimorfismo *TNF-alfa* -308G>A e a presença de talassemia-$\alpha_2^{3.7kb}$ foram associados com o risco para ocorrência de sequestro esplênico (p=0.001; p =0.046), enquanto o alelo T do polimorfismo IL-8 _251A>T foi considerado fator protetor para esplenomegalia (p=0.032). O alelo A do polimorfismo *TNF-alfa* -308G>A foi associado aos níveis séricos elevados de TNF-alfa (p=0.021), concentrações de hemoglobina fetale os haplótipos foram associados aos níveis séricos de Il-8. As análises de regressão mostraram associação estatisticamente significativa entre os polimorfismos no gene *TNF-alfa* , dos haplótipos e talassemia-$\alpha_2^{3.7kb}$ com a ocorrência de sequestro esplênico. Nosso estudo enfatiza que a identificação de fatores genéticos novos e biomarcadores imunológicos em associação com os marcadores clássicos são estratégias importantes para elucidar as causas de fenótipos diferentes apresentados pelos pacientes com anemia falciforme.

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TNF-alpha and IL-8: Serum levels and gene polymorphisms (−308G>A and −251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia

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ABSTRACT

Sickle cell anemia (SCA) is a disorder characterized by a heterogeneous clinical outcome. In the present study, we investigated the associations between *Tumor Necrosis Factor-alpha* (*TNF-alpha*) −308G>A and *Interleukin 8* (*IL-8*) −251A>T gene polymorphisms, medical history and classical biomarkers in children with steady-state SCA. In total, 210 SCA patients aged 2–21 years and 200 healthy controls were studied. Gene polymorphisms, *beta*^{S}*-globin* haplotypes and a 3.7-kb deletion in *alpha*2-thalassemia (α_2-thal$^{3.7\ kb}$) were investigated by PCR/RFLP analysis, and cytokine levels were determined by ELISA. Splenomegaly ($p = .032$) was more prevalent among children younger than 5 years of age. The A allele of the *TNF-alpha* −308G>A gene polymorphism and the presence of α_2-thal$^{3.7\ kb}$ were associated with an increase risk of splenic sequestration events ($p = .001$; $p = .046$), while the T allele of the *IL-8* −251A>T gene polymorphism was considered to be a protective factor for splenomegaly events ($p = .032$). Moreover, the A allele of the *TNF-alpha* −308G>A gene polymorphism was associated with high TNF-alpha levels ($p = .021$), and the hemoglobin F and hemoglobin S haplotypes were correlated with serum levels of IL-8. The logistic regression analysis showed significant effects of the *TNF-alpha* and *IL-8* gene polymorphisms, *beta*^{S}*-globin* gene haplotypes and α_2-thal$^{3.7\ kb}$ on the occurrence of splenic sequestration events. Our study emphasizes that the identification of new genetic and immunological biomarkers and their associations with classical markers is an important strategy to elucidate the underlying causes of different SCA phenotypes and their effects on patient outcome.

1. Introduction

Sickle cell anemia (SCA) is an inherited recessive autosomal disorder characterized by clinical heterogeneity that may be influenced by environmental factors, ethnicity, social and economic factors and genetic markers secondary to epigenetic phenomena. These genetic factors include associations between SCA and *beta*^{S}*-globin* haplotypes, the presence of a 3.7-kb deletion in *alpha*2-thalassemia (α_2-thal$^{3.7\ kb}$) and fetal hemoglobin concentration, which is a well-known prognostic marker [1].

Clinical manifestations of SCA are based on vaso-occlusive episodes that impair blood flow as a consequence of intravascular sickling in capillaries, hemolysis, cellular activation, leukocytosis and the breakdown of homeostasis [2–4]. The major clinical features include pain, stroke, priapism, acute chest syndrome, osteonecrosis and renal failure [5].

The *beta*^{S}*-globin* gene haplotypes have been shown to correlate with the clinical features of SCA patients; the CAR haplotype may be associated with more severe symptoms, while the SEN haplotype correlates with a better prognosis [6–8]. The concurrent α_2-thal$^{3.7\ kb}$ is correlated with protection against the hemolysis-associated phenotypes of leg ulcers and priapism [9] and is associated with increased risk for the viscosity-vaso-occlusive phenotypes of acute pain and osteonecrosis [10,11]. Despite a common genetic background, the phenotypic expression in SCA patients varies widely, from mild clinical symptoms with survival into 60–70 years of age to very severe clinical symptoms with multi-organ damage and early mortality [6,12].

TNF-alpha and IL-8 are pro-inflammatory molecules involved in endothelial cell and leukocyte activation, macrophage stimulation, affinity of leukocyte surface molecules and endothelial receptors

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and leukocyte chemotaxis and recruitment [13–16]. Sickle cell anemia patients have increased serum levels of circulating TNF-alpha and IL-8 at steady state and during crisis events [17,18]; these inflammatory molecules also possibly contribute to the complex mechanisms involved in vascular occlusion events. Thus, aberrations in cell activation and interaction, the pro-inflammatory and oxidant profiles, genetic background and environmental factors possibly result in recurrent vascular events [19,20].

Changes in the cytokine balance in SCA patients are an important risk factor for the occurrence of clinical events [21]. Moreover, inter-patient variations in cytokine levels could be attributed to gene polymorphisms, notably the A alleles of -308 G>A and −251 A>T, which are positioned in the promoter regions of the *TNF* and *IL-8* genes, respectively, and have been associated with higher *TNF-alpha* and *IL-8* transcript levels [22,23].

Based on these observations, the present study investigated polymorphisms in the *TNF-alpha* and *IL-8* genes and their association with the respective cytokine serum levels, medical history and classical biomarkers presented by SCA patients.

2. Materials and methods

2.1. Subjects

A cross-sectional study comprising 210 SCA children (123 male and 87 female; 9.3 ± 4.5 years) selected from the hematology outpatient clinic of the Hematology and Hemotherapy Foundation of Bahia State (HEMOBA) was performed. The samples were collected during the period from 2003 to 2007. Clinical data were collected from the patients' medical records, and demographic data were obtained by interviews with patients and their parents or guardians. Only pediatric SCA patients were eligible. All patients were at the steady state of the disease, which was characterized as a period of three months without any acute events and no blood transfusions for 120 days prior to blood sampling. Exclusion criteria included the presence of infectious diseases, hemoglobin profiles not compatible with SCA, previous blood transfusions (less than four months before the study) and inflammatory episodes during the study.

The study was approved by the *Gonçalo Moniz* Research Center of the *Oswaldo Cruz* Foundation (FIOCRUZ) Ethics Committee, and all parents or guardians provided written informed consent followed by the children's agreement, in accordance with the Declaration of Helsinki of 1975, as revised in 2000. Clinical information was collected from the patients' charts and their physicians.

The control group consisted of 200 individuals who attended the clinical laboratory of the Pharmacy College of the Federal University of Bahia (UFBA), and these individuals were age- and sex-matched with the SCA patients group. The control individuals had normal hemoglobin profiles and lacked a history of anemia, inflammatory conditions and hematological diseases.

2.2. Hematological and hemoglobin analyses

Hematological analyses were performed using an electronic cell counter (Coulter Counter T890, Brea, CA, USA). The hemoglobin profile was analyzed by high-performance liquid chromatography (HPLC) (Bio-Rad Variant, CA, USA).

2.3. Beta^S-globin gene haplotypes and a 3.7-kb deletion in alpha-2-thalassemia

DNA was isolated from blood leukocytes using the GFX™ Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, NJ, USA). The *beta^S-globin* gene haplotypes and α_2-thal$^{3.7\ kb}$ were investigated with PCR and restriction fragment length polymorphism (RFLP) techniques as previously described [24,25].

2.4. Typing of single nucleotide polymorphisms (SNPs) and measurement of serum cytokine levels

The *TNF-alpha* −308G>A and *IL-8* −251A>T gene polymorphisms were investigated with PCR and RFLP techniques as previously described [26,27].

Serum TNF-alpha and IL-8 levels were measured with an enzyme-linked immunosorbent assay (ELISA) (*BD Biosciences Pharmingen, USA*), according to the manufacturer's instructions, with cut-off levels of ⩽7.8 pg/mL and ⩽15.0 pg/mL for TNF-alpha and IL-8, respectively.

2.5. Statistical analysis

The baseline characteristics are presented as the means and proportions of the selected variables. The distributions of quantitative variables were determined using the Kolmogorov–Smirnov test. Bivariate correlation analysis was performed to determine correlations between pairs of variables using Spearman's rho correlation. Parametric ANOVA analyses confirmed by Bonferroni post hoc tests and the nonparametric Kruskal–Wallis tests were used to compare the means among two or more groups of interval variables that were normally distributed and not normally distributed, respectively. Interactions between specific categories of clinical variables were tested for significance using a χ^2 test corrected by Yates or Fisher's exact test, and the expected frequency in the cell tables was taken into account.

The logistic regression was applied to test several models compounded by variables associated with splenic sequestration episodes. The independent variables were *TNF-alpha* −308G>A, *IL-8* −251A>T, Gender, α_2-thal$^{3.7\ kb}$ and the *beta^S-globin* haplotypes.

The data analysis was performed using EPI Info 6.04 (CDC, Atlanta, Georgia), Statistical Data Analysis (STATA) SE 10 (StataCorp, Texas, USA) and GraphPad Prism 5.0. A *p*-value of less than .05 was considered statistically significant.

3. Results

Our study included a total of 210 SCA patients aged 2–21 years, 36.6% of which were female. Clinical features are described in Table 1. Vaso-occlusive pain episodes occurred in patients of all ages, and splenomegaly was more prevalent among children younger than 5 years of age (Fig. 1).

3.1. Alpha2-thalassemia 3.7-kb deletion and beta^S-globin gene haplotypes

Frequencies of α_2-*thal$^{3.7kb}$* and the *beta^S-globin* gene haplotypes in the SCA group are described in Table 2.

Table 1
Clinical features of the sickle cell anemia patients.

Clinical profile	Frequency	Percent
Vaso-occlusive events	180/210	85.7
Pneumonia	75/210	35.7
URTI	40/210	19.0
Splenic sequestration	23/210	10.9
Splenomegaly	19/210	9.0
Stroke	11/210	5.2
Urinary infection	8/210	3.8
Osteonecrosis	3/210	1.4
Leg ulcer	1/210	0.5

URTI: upper respiratory tract infection.

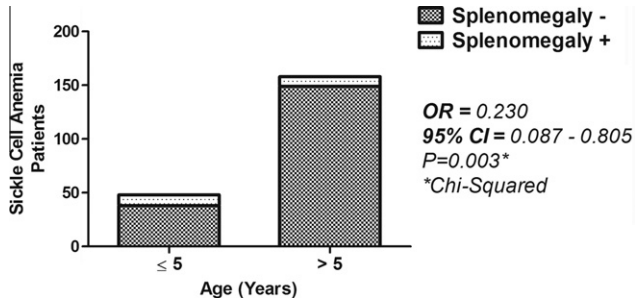

Fig. 1. Splenomegaly incidence among SCA patient of different ages. ***P**** **ANOVA.**

3.2. Analysis of the IL-8 −251A>T and TNF-alpha −308G>A gene polymorphisms

The *IL*-8 −251A>T and *TNF-alpha* −308G>A gene polymorphism frequencies of the 210 patients and the 200 individuals in the reference group were analyzed. The genotype frequencies were in Hardy–Weinberg equilibrium (Table 3).

3.3. Associations between the TNF-alpha −308G>A and IL-8 −251A>T gene polymorphisms, beta^S-globin gene haplotypes and alpha$_2$-thalassemia 3.7-kb deletion and clinical events in children with sickle cell anemia

The A allele of the *TNF-alpha* gene polymorphism and the presence of α_2-*thal*$^{3.7\ kb}$ were associated with splenic sequestration episodes (Table 4). The T allele of the *IL-8* gene polymorphism was characterized as a protection factor for splenomegaly (*OD*: 0.326; 95% *CI:* 0.114–0.937; $P = 0.032$).

3.4. Serum levels of IL-8 and TNF-alpha

The mean serum IL-8 level was 10.90 ± 13.13 pg/mL with a minimum of 1.67 and a maximum of 109.05 pg/mL. The mean serum TNF-alpha level was 29.71 ± 19.49 pg/mL with a minimum of 1.30 and a maximum of 128.41 pg/mL. The presence of the AG and AA genotypes of the −308G>A *TNF*-alpha gene polymorphism was associated with the highest serum levels of TNF ($p = .021$) (Fig. 2). The presence of the AT and TT genotypes of the −251A>T *IL-8* gene polymorphism was not associated with serum levels of IL-8.

3.5. Hemoglobin profiles and IL-8 serum levels

Fig. 3 shows the positive correlation between IL-8 and HbS and the negative correlation between this cytokine and HbF.

Table 2
Frequencies of classical genetic prognosis markers.

	Frequency	Percent
α_2-*thal*$^{3.7\ kb}$		
Heterozygous	37/174	21.3
Homozygous	2/174	1.1
beta^S-globin haplotypes		
CAR/CAR	41/210	19.5
CAR/BEN	98/210	46.7
CAR/atypical	5/210	2.4
CAR/CAM	2/210	0.9
BEN/BEN	52/210	24.8
BEN/atypical	7/210	3.3
BEN/CAM	5/210	2.4

3.6. Multivariate associations of independent markers, such as the TNF-alpha −308G>A and IL-8 −251A>T gene polymorphisms, beta^S-globin gene haplotypes, and alpha-2 thalassemia 3.7-kb deletion, and splenic sequestration in children with sickle cell anemia

Models show the possible interactions between independent variables and their influences on dependent variables, such as splenic sequestration, and were adjusted for age and gender.

Presence of the *TNF-alpha* gene polymorphism and α_2-*thal*$^{3.7\ kb}$ are independently related to the risk of splenic sequestration events. In the first model, patients with these genetic modifications have a decreased risk of this clinical phenotype. Moreover, we observed in the third model that patients with *TNF-alpha* gene polymorphisms, α_2-*thal*$^{3.7\ kb}$ and *IL-8* gene polymorphisms had a decreased risk of splenic sequestration. However, males with the CAR haplotype in the second and fourth models had an increased risk of splenic sequestration events (Table 5).

4. Discussion

The presentation and clinical course of sickle cell anemia show substantial viability between patients, from sporadic pain crises to organ damage, resulting in frequent hospitalization and early death [28].

The data presented herein demonstrate that vaso-occlusive pain episodes are found among patients of different ages, which confirms that these clinical events occur in all age groups. Moreover, the occurrence of splenomegaly was more prevalent among children younger than 5 years of age in the SCA patients studied. This result agrees with previous reports that describe children from the United States of America and emphasizes the finding that spleens from children with SCA progress through several changes and that dysfunction begins very early in infancy [2,6,29–31].

Several genetic association analyses have been performed to link single nucleotide polymorphisms or deletions with particular complications of sickle cell anemia [32–35]. α_2-thal$^{3.7\ kb}$ is frequently present in SCA patients and correlates with clinical profiles because its occurrence is related to an increase in hemoglobin concentration, a decrease in hemoglobin S polymerization and a reduction in hemolysis. The clinical effects of α_2-thal$^{3.7\ kb}$ are variable but are usually beneficial for patients, such as reductions in the occurrence of stroke [36], gall stones [37], leg ulcers [38] and priapism [39], which are based on the decrease in hemolysis; however, pain frequency is not reduced because there is an increase in blood viscosity [30]. Our results show that splenic sequestration is partly attributable to the presence of α_2-thal$^{3.7\ kb}$. The high hematocrit and increased blood viscosity generated by α_2-thal$^{3.7\ kb}$ could promote morphological sickling and lead to a lack of deformability, both of which are important etiological factors for splenic sequestration [30,40].

The association between the A allele of the *TNF-alpha* gene and splenic sequestration was observed; patients with the mutant genotype have a 4.6-fold increased risk for the development of this clinical manifestation. Other studies that correlated the A allele of the *TNF-alpha* gene and clinical events are controversial. Hoppe et al. [32] first identified a protective role of the A allele of the *TNF-alpha* gene using a logistic regression model with many independent variables related to large vessel stroke. Hoppe et al. [33] confirmed the role of the A allele of the *TNF-alpha* gene as a protective factor for large vessel stroke. However, Vicari et al. [35] did not find an association between the mutant allele of the *TNF* gene and stroke.

TNF-alpha, which is mainly produced by macrophages and T cells, is a potent cytokine with a wide range of pro-inflammatory activities, including the activation of endothelial cells; stimulation

Table 3
Frequencies of *IL-8* −251A>T and *TNF-alpha* −308G>A gene polymorphisms in children with steady-state sickle cell anemia compared with the healthy control group.

Cytokine genotype		Genotype frequency *N* (%)		
		SCA	Healthy control	
IL-8 −251A>T	AA	31 (14.8)	28 (14.0)	*P* = 0.68[a] *OD*:0.89 95% CI: 0.49–1.61
	AT	108 (51.4)	98 (49.0)	
	TT	71 (33.8)	74 (37.0)	
Total		210	200	
TNF-alpha −308G>A	GG	162 (77.1)	146 (73.0)	*P* = 0.28[a] *OD*: 0.78 95% CI: 0.49–1.25
	GA	46 (21.9)	50 (25)	
	AA	2 (1.0)	4 (2.0)	
Total		210	200	

The genotypic and allelic distributions of all polymorphisms were in Hardy–Weinberg equilibrium.
[a] χ^2.

Table 4
Associations of clinical variables with *TNF-alpha* −308G>A and *IL8* −251A>T gene polymorphisms, CAR/CAR and CAR/BEN *beta*^{S}*-globin* gene haplotypes and the 3.7-kb deletion of alpha-2 thalassemia among children with steady-state sickle cell anemia.

	Vaso-occlusive events	Pneumonia	Splenic sequestration	Stroke	URTI	Urinary infection
IL-8 −251A>T						
AA	26/31	11/31	4/31	1/31	7/31	1/31
AT + TT	154/179	64/173	19/179	10/179	33/179	7/179
	OR = 1.18,[b]	OR = 1.06,[a]	OR = 0.80,[b]	OR = 1.77,[b]	OR = 0.77,[b]	OR = 1.22,[b]
	95% CI:0.41–3.37	95% CI:0.48–2.37	95% CI:0.25–2.53	95% CI:0.21–14.38	95% CI:0.38–1.95	95% CI:0.14–10.28
	P = .75	*P* = .87	*P* = .70	*P* = .59	*P* = 0.58	*P* = 0.85
TNF −308G>A						
GG	141/162	60/156	11/162	10/162	30/162	7/162
GA + AA	40/48	15/48	12/48	1/48	10/48	1/48
	OR = 0.78,[a]	OR = 0.73,[a]	OR = 4.60,[a]	OR = 0.34,[b]	OR = 1.16,[a]	OR = 0.47,[b]
	95% CI:0.32–1.88	95% CI:0.37–1.46	95% CI:1.88–11.27	95% CI:0.04–2.60	95% CI:0.52–2.60	95% CI:0.05–4.00
	P = .58	*P* = .38	*P* = .001	*P* = .29	*P*=.70	*P* = .49
Haplotypes						
CAR/CAR	38/41	14/39	4/41	5/41	5/41	1/41
CAR/BEN	80/97	34/95	12/97	3/93	16/97	3/97
	OR = 0.37,[a]	OR = 0.99,[a]	OR = 1.30,[b]	OR = 0.23, [a]	OR = 1.42,[b]	OR = 1.28,[b]
	95% CI:0.10–1.34	95% CI:0.46–2.16	95% CI:0.39–4.31	95% CI:0.52–1.01	95% CI:0.48–4.18	95% CI:0.13–12.65
	P = .13	*P* = .099	*P* = .66	*P* = .052	*P* = 0.52	*p* = 0.83
Alpha-2-Thalassemia 3.7 kb						
Wild type	110/134	49/129	12/134	7/134	29/134	5/134
Heterozygous/Homozygous	35/38	18/37	8/38	3/38	7/38	1/38
	OR = 2.55,[a]	OR = 1.55,[a]	OR:2.71,[a]	OR = 1.55,[b]	OR:0.82,[a]	OR = 0.70,[b]
	95% CI:0.72–8.96	95% CI:0.74–3.23	95% CI:1.02–7.22	95% CI:0.38–6.32	95% CI:0.33–2.05	95% CI:0.08–6.15
	P=.14	*P*=.24	*P*=.046	*P*=.53	*P*=.67	*P* = 0.75

OR: odds ratio; URTI: upper respiratory tract infection.
[a] χ^2 Yates corrected.
[b] Fisher's exact test.

of inflammation; induction of the coagulation cascade, fevers and the synthesis acute phase proteins; activation of neutrophils; and stimulation of neutrophil adhesion [15]. These characteristics make serum TNF levels an important risk factor in SCA. Lanaro et al. [17] observed an increase in the circulating levels and an increase in mRNA expression of TNF-alpha in SCA patients at steady state, which is characteristic of a pro-inflammatory state. Moreover, Pathare et al. [21] observed an increase in the circulating concentration of TNF-alpha during crisis events. In our study, we observed an association between the A allele of *TNF-alpha* −308G>A and an increase in the serum levels of TNF-alpha in SCA patients. Another study observed that an increase in the serum levels of TNF-alpha was associated with the A allele of *TNF-alpha* −308G>A [41]. Using a reporter gene assay, Wilson et al. [22] suggested that the A allele of *TNF-alpha* −308G>A affects transcrip-

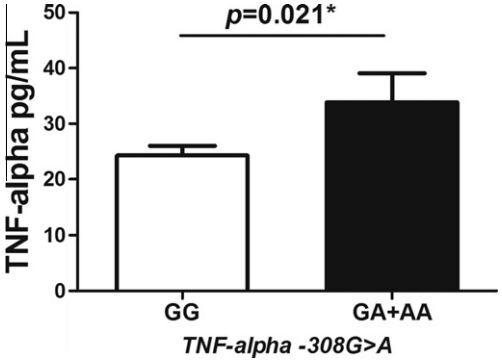

Fig. 2. *TNF-alpha* −308G > A gene polymorphism and serum cytokine levels in children with steady-state sickle cell anemia. *Spearman's Correlation.

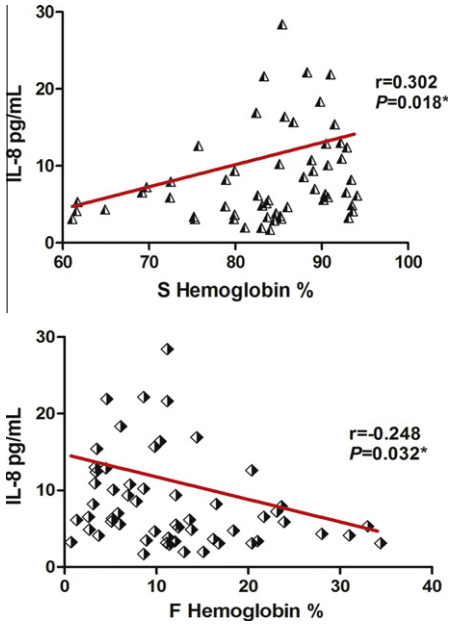

Fig. 3. Hemoglobin profile and serum IL-8 levels in children with steady-state sickle cell anemia.

tional activity and results in an increase in the expression of the *TNF* gene.

However, this is the first study that assessed the impact of the −251A>T *IL-8* gene polymorphism on the clinical phenotypes of SCA patients; the splenomegaly protection of the T allele of −251A>T *IL-8* gene could be related to its transcriptional activity [23].These aspects indicate that the A allele of the *TNF-alpha* gene and the T allele of the −251A>T *IL-8* gene are important clinical predictors of SCA.

The data presented in our study demonstrate a positive correlation between IL-8 and S hemoglobin and an inverse correlation with F hemoglobin. These results support a previous report in which high IL-8 levels and other pro-inflammatory markers were associated with SCA in patients during vascular occlusion episodes (regardless of the crisis-inducing factor) and in steady-state patients [17]. High serum levels of IL-8 are a marker of poor prognosis based on their association with an increase in S hemoglobin and decrease in F hemoglobin in red blood cells due to an increase in intravascular hemolysis and increases in oxidative damage, cellular activation, vascular occlusion and consequently inflammation, which characterize hemolysis, vascular occlusion and inflammation as cyclical events in SCA.

Logistic regression results were obtained by multivariate association of classical biomarkers, such as CAR *beta^S-globin* haplotypes, the presence of α_2-thal$^{3.7\ kb}$, the A allele of the *TNF-alpha* gene and the T allele of the *IL-8* gene, with splenic sequestration events. The models show that the risk of occurrence of spleen sequestration varies and depends on genetic abnormalities present in each patient and gender. The most interesting observation in the second and fourth models was that the inclusion of the CAR haplotype, a classical factor of poor prognosis [8], consequently increased the risk of spleen sequestration.

5. Conclusions

The results presented here indicate the importance of the A allele of the *TNF* gene and the T allele of the −251A>T *IL-8* in the clinical events of SCA and further highlights the contribution of genetic modifications to the risk of clinical phenotypes. Our study emphasizes that the identification of new genetic and immunological biomarkers and their association with classical markers is an important strategy for the elucidation of different SCA phenotypes and their effects on patient outcome. Further studies should be performed to investigate the mechanisms by which these gene polymorphisms affect clinical manifestations and the contribution of these associations to the expression of cytokines and adhesion molecules.

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Table 5
Coefficients, standard errors, *P* values, and confidence intervals for SCA patients with splenic sequestration.

Variable	Coefficient	Standard error	*P*-value	Odds ratio	Lower 95% confidence interval	Upper 95% confidence interval
Model 1						
TNF	1.1425	0.5091	**0.0248***	3.1347	1.1557	8.5028
TALA	0.9955	0.5192	0.0552	2.7061	0.9781	7.4870
Model 2						
TNF	1.4075	0.6715	**0.0361***	4.0859	1.0958	15.2356
TALA	1.6353	0.6976	**0.0191***	5.1308	1.3072	20.1381
HAPLO	0.2365	0.7759	0.7575	1.2668	0.2823	5.6840
GENDER	0.8709	0.7611	0.2525	2.3890	0.5375	10.6186
Model 3						
TNF	1.1440	0.5091	**0.0246***	3.1393	1.1573	8.5156
TALA	1.0020	0.5198	0.0539	2.7237	0.9834	7.5436
IL-8	−0.2022	0.6922	0.7702	0.8169	0.2104	3.1724
Model 4						
TNF	1.4072	0.6717	**0.0362***	4.0844	1.0949	15.2367
TALA	1.6339	0.7015	**0.0199***	5.1238	1.2955	20.2653
HAPLO	0.2380	0.7701	0.7573	1.2686	0.2804	5.7390
IL-8	0.0161	0.8679	0.9852	1.0162	0.1854	5.5690
GENDER	0.8765	0.7657	0.2545	2.3928	0.5335	10.7317

Models: TNF: *TNF-alpha* −308G>A; TALA: alpha-2-thalassemia with 3.7-kb deletion; HAPLO: CAR *betaS-globin gene haplotype*; IL-8: *IL-8* −251A>T; GENDER: male.
* *p* values in bold show significant variables that are contributing to the dependent variable occurrences in the model.

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5.2- Artigo 2

Polymorphisms of *Toll-like receptor*, *Myeloperoxidase* and *Fc gamma receptor IIa* genes in children with sickle cell anemia.

C. Cajado, B.A.V. Cerqueira, W. Vilas-Boas, M.O.S. Carvalho, M. Salvino, A. Zanette, I.M. Lyra, M.S. Goncalves.

Artigo Submetido ao periódico revista *Cytokine*, 2013.

Resumo:

Infecções graves e episódios de vaso-oclusão (VOE) têm sido associados com a morbidade e mortalidade na anemia falciforme. Alguns polimorfismos em genes de receptores do sistema imune têm sido associados a ocorrência de infecções e a gravidade de doenças. Neste trabalho, investigou-se a provável influência dos polimorfismos gênicos *TNFα*-308G>A, *FcγRIIA*H/R131, *MPO*-463G>A, *TLR4* 896A>G, e *TLR9*- 1237T>C na gravidade de infecções e complicações inflamatórias em pacientes HbSS. Foram investigados 129 indivíduos com AF em estado estável, com idade média de 12,6±8,8 no período de 2008-2010. Houve associação entre o polimorfismo *TLR9* -1237T>C e a ocorrência de infecção respiratória (p=0,021), *TLR4* 896A>G e a ocorrência de acidente vascular encefálico (AVE), e entre o polimorfismo *MPO*-463G>A e a ocorrência de infecções. O polimorfismo *FcγRIIA H/R131* esteve associado à frequencia elevada de internações hospitalares e a ocorrência de sequestro esplênico. A presença concomitante do polimorfismo *FcγRIIA* H/R131 com o *TLR4* 896A>G ou com o *MPO*-463G>A influenciou a ocorência de internações hospitalares, de sequestro esplênico e do número de crises vaso-oclusivas (p<0,05). Estudos de fatores imunológicos podem contribuir para a elucidação de mecanismos fisiopatológicos relacionados com a gravidade clinica de pacientes com AF.

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Abstract: Sickle cell anemia (HbSS) is an inherited recessive autosomal disorder characterized by clinical heterogeneity of symptoms and vascular occlusion events that represent a complex interaction of factors involving blood and endothelial cell activation and cell-to-cell interactions with several soluble inflammatory mediators. A wide spectrum of epigenetic events has been associated with the interactions of several genes. The Toll-like receptor 4 +896 A>G, Toll-like receptor 9 -1237 T>C, Myeloperoxidase -463 G>A and Fc gamma receptor IIA 131 H>R gene polymorphisms and their effects on hematological markers and clinical profiles were investigated in steady-state HbSS children. A total of 125 steady-state HbSS patients were studied. The mean patient age was 12.41±8.80 years. All patients were from Northeast Brazil and attended the outpatient clinic of the Fundação de Hematologia e Hemoterapia da Bahia. Hematological and hemoglobin analyses were performed using an electronic cell counter and HPLC respectively. Gene polymorphisms were investigated by PCR and RFLP. The mutant allele of the Fc gamma receptor IIA gene was a risk factor for splenic sequestration (OD: 3.115; 95% CI: 1.119-8.675; P= 0.025). The G allele of the Toll-like receptor 4 +896 polymorphism was associated with the occurrence of cerebrovascular accidents (OD: 11.05; 95% CI: 1.57-77.3; P= 0.039). The C allele of the Toll-like receptor 9 -1237 polymorphism was associated with upper respiratory tract infection (URTI) (OD: 2.75; 95% CI: 1.139-6.632; P= 0.021), and the A allele of the Myeloperoxidase -463 polymorphism was a protective factor against infection (OD: 0.45; 95% CI: 0.218-0.928; P= 0.029). The study of genes involved in innate and effector immune responses and their association with and clinical profiles may help elucidate the complex signaling cascades of the mechanisms involved in the pathogenesis of sickle cell anemia.

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Salvador, Bahia, Brazil, March 1st , 2013

To: Sir Gordon W. Duff

University of Sheffield, Sheffield, United Kingdom

Editor, CYTOKINE

From: Authorship of the manuscript entitled "**Polymorphisms of *Toll-like receptor*, *Myeloperoxidase* and *Fc gamma receptor IIA* genes in sickle cell anemia children**"

Subject: Submission letter

Dear Sir Duff,

We are sending the manuscript entitled "**Polymorphisms of *Toll-like receptor*, *Myeloperoxidase* and *Fc gamma receptor IIA* genes in sickle cell anemia children**" to be appreciated as a possible publication for the editorial office. The manuscript will not be submitted for publication elsewhere while under consideration for the CYTOKINE. We consider that this manuscript has no conflict of interest, once that it do not have financial relation with industry, and all subjects involved at the work had the participation allowed by them or by a responsible family member that signed a informed consent. Moreover, this manuscript is original work and that it is not under submission to any other journals. The protocol was approved by the Institutional Ethical Committee from Centro de Pesquisa Gonçalo Moniz / FIOCRUZ and all procedures presented in the manuscript are in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as

revised in 2000. The manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts and receive the Certificate Verification Key: 4100-9641-EEE1-EBA2-F05C.

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Sincerely Yours,

_____

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***Highlights (for review)**

Highlights of the manuscript entitled:

- *FcγRIIA* is associated to clinical complication in HbSS;
- The HbSS presents synergistic effect *of FcγRIIA and TLR4*, *MPO, TNF-α* SNPs;
- The immunogenetic modulation of HbSS involve a complex net of molecules interaction;
- The epistatic gene interaction has a pivotal role in HbSS immunomodulation.

Polymorphisms of *Toll-like receptor*, *Myeloperoxidase* and *Fc gamma receptor IIA* genes in children with sickle cell anemia

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Abstract

Sickle cell anemia (HbSS) is an inherited recessive autosomal disorder characterized by clinical heterogeneity of symptoms and vascular occlusion events that represent a complex interaction of factors involving blood and endothelial cell activation and cell-to-cell interactions with several soluble inflammatory mediators. A wide spectrum of epigenetic events has been associated with the interactions of several genes. The *Toll-like receptor 4* +896 A>G, *Toll-like receptor 9* -1237 T>C, *Myeloperoxidase* -463 G>A and *Fc gamma receptor IIA* 131 H>R gene polymorphisms and their effects on hematological markers and clinical profiles were investigated in steady-state HbSS children. A total of 125 steady-state HbSS patients were studied. The mean patient age was 12.41±8.80 years. All patients were from Northeast Brazil and attended the outpatient clinic of the Fundação de Hematologia e Hemoterapia da Bahia. Hematological and hemoglobin analyses were performed using an electronic cell counter and HPLC respectively. Gene polymorphisms were investigated by PCR and RFLP. The mutant allele of the *Fc gamma receptor IIA* gene was a risk factor for splenic sequestration (*OD*: 3.115; *95% CI:* 1.119–8.675; *P*= 0.025). The G allele of the *Toll-like receptor 4* +896 polymorphism was associated with the occurrence of cerebrovascular accidents (*OD*: 11.05; *95% CI:* 1.57–77.3; *P*= 0.039). The C allele of the *Toll-like receptor 9* -1237 polymorphism was associated with upper respiratory tract infection (URTI) (*OD*: 2.75; *95% CI:* 1.139–6.632; *P*= 0.021), and the A allele of the *Myeloperoxidase* -463 polymorphism was a protective factor against infection (*OD*: 0.45; *95% CI:* 0.218–0.928; *P*= 0.029). The study of genes involved in innate and effector immune responses and their association with and clinical profiles may help elucidate the complex signaling cascades of the mechanisms involved in the pathogenesis of sickle cell anemia.

Key word: Sickle cell anemia; Toll-like receptors 4; Toll-like receptors 9; Myeloperoxidase; Fc gamma receptor IIA.

1. Introduction

Sickle cell anemia (HbSS) is an autosomal recessive disorder characterized by the presence of the variant hemoglobin S (HbS), which is responsible for the erythrocyte shape change to a sickle shape in hypoxic environments. HbSS patients have a chronic hematological disease, characterized by microvascular occlusion, hemolytic anemia and jaundice. The pathophysiology of HbSS involves a cascade of complex events [1]: cell-cell junction interactions and/or cell-matrix interactions; the adhesion of red blood cells (RBCs), leukocytes, and platelets to their ligands, receptors, and vascular endothelium; the expression of several soluble proinflammatory molecules [2]; activation of the coagulation cascade [3]; increased nitric oxide (NO) scavenger levels; and the development of vascular dysfunction as well as chronic vasculopathy. All of these events have been implicated in the pathogenesis of the clinical complications presented by HbSS patients [4]. The clinical evolution of sickle cell disease (SCD) shows a high diversity, and the painful sickle cell crisis is a hallmark clinical presentation of the disease that is considered a parameter of disease severity [5]. Several clinical events have been related to the morbimortality increase among SCD patients, including cerebrovascular accidents (CVA) and acute chest syndrome (ACS), which are considered causes of premature death.

There are some very well-characterized genetic markers of SCD that are considered to modulate the HbSS phenotype, such as the β^S-globin gene haplotypes, alpha-thalassemia and HbF levels that have been associated with the quantitative trait loci (QTLs). This suggests epigenetic involvement in

the disease heterogeneity of SCD, which is now considered a complex multigenic disorder [6, 7]. The variable clinical expression of HbSS is difficult to explain. Several reports have described the role of the immune system as a key element in the disease pathogenesis, and it is now characterized as a chronic inflammatory condition [8, 9].

Severe recurrent infections and vaso-occlusive episodes (VOE) have been frequently associated with the high rate of morbidity and mortality of HbSS patients, and the host genetic background has been related to patient susceptibility and the severity of these clinical events [10]. Additionally, the immune response plays a vital role in the protection against infectious agents. Differences in susceptibility to several diseases, such as infection, cancer, and asthma, have been associated with the presence of specific gene polymorphisms. Toll-like receptors (TLRs) are essential components of the innate immune system. They recognize microbial products and induce a rapid and efficient immune response against invading microorganisms. Several TLR variants have been described and associated with altered responsiveness [11]. TLR9 has been shown to be responsible for mediating dendritic cell maturation and the production of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-12 (IL-12), by macrophages following exposure to unmethylated CpG-rich bacterial DNA [12]. Frequent single-nucleotide polymorphisms (SNPs) have been described for the *TLR4* and *TLR9* genes. For the *TLR4* +896 A>G polymorphism, the Asp299Gly was shown to reduce reactivity to inhaled lipopolysaccharide and has been associated with an increased risk of septic shock [13] and increased severity of systemic inflammatory response

syndrome (SIRS) [14]. The *TLR9* -1237T>C SNP is located on chromosome 3p21.3 and has been associated with asthma susceptibility [15] and Crohn's disease [16]. The A allele of the *TNF-alpha* -308 G>A polymorphism has been associated with increased clinical severity in SCD patients [17].

The FcγRII binds IgG in its oligomeric form or when it is bound to cells and is found on the surface of monocytes, macrophages, neutrophils, platelets, basophils, eosinophils, and other cells. The *FcγRIIA* gene contains a polymorphism at position 131 that results in an amino acid change (arginine: CGT or histidine: CAT) that alters the ability of the receptor to bind certain IgG subclasses [18]. The identification of the *FcγRIIA* H/R131 genotype has assumed a growing importance in host defense disorders, immunohematological diseases and systemic autoimmune disorders [18].

Myeloperoxidase (MPO) is a lysosomal enzyme that is highly expressed in neutrophils and monocytes and plays an important role in the host defense system, providing microbicidal activity against a number of organisms [19]. A polymorphism in the *MPO* gene promoter region (-463 G >A) has been shown to reduce *MPO* gene transcription and may modulate the incidence or severity of lung cancer [19, 20]. MPO deficiency has been associated with an increased occurrence of severe and chronic inflammatory processes [19].

To study the potential genetic modifiers of HbSS, gene polymorphisms, including *TNF-α* -308G>A, *FcγRIIa* H/R131, *MPO* -463G>A, *TLR4* +896A>G, and *TLR9* -1237T>C were selected and their relationships to the immune response were evaluated to determine if they had greater significance in SCD patients and to identify individuals with defects in the host defense

system. The coinheritance of susceptibility genes may influence the prevalence and severity of infectious and inflammatory complications.

2. Materials and methods

2.1. *Patients*

A total of 129 HbSS patients were investigated in the present study, and they were selected from the hematology outpatient clinic of the Hematology and Hemotherapy Foundation in Bahia State (HEMOBA). Only pediatric HbSS patients were eligible for inclusion in the study. Samples were collected from 2008 to 2010. All HbSS patients were at the steady-state of the disease, and clinical data were collected from the patients' medical records.

All patients and/or the patients' parents provided written informed consent and the children's agreement. The protocol was approved by the Gonçalo Moniz Research Center of the Oswaldo Cruz Foundation (FIOCRUZ) Ethics Research Board. The hemoglobin profile was confirmed by high performance liquid chromatography (HPLC) (Bio-Rad Variant, CA, USA). Hematological data were obtained using an electronic cell counter (Coulter Counter T890, Brea, CA, USA). All clinical information was extracted and organized into a database prior to the genotypic analysis of genomic DNA, and unique patient identifiers were removed.

2.2 *Polymorphism assay using genomic DNA*

Genomic DNA (50 ng/ml) was isolated from peripheral blood leukocytes using a *QIAamp® DNA Mini Kit* (*Qiagen,* Valencia, CA, USA). The

polymorphism analysis for *TNF-alpha* -308G>A [21], *MPO* -463G>A [22], *FcγRIIa* H/R131 [18], *TLR4* +896A>G [23], and *TLR9* -1237T>C [24] was performed according to protocols based on previously reported assays using PCR-RFLP. The primer pairs, annealing temperatures, and detection methods used in the PCR-based assays are described in Table 1.

2.3 *Statistical analysis*

Results are presented as frequencies and proportions of the selected variables. Interactions between specific categories of clinical variables were tested for significance using a $\chi 2$ test with a Yates' correction for parametric analyses. Fisher's exact test was used in the analysis of categorical data with sample sizes smaller than 5. The data analysis was performed using EPI Info 6.04 (CDC, Atlanta, Georgia), SPSS 17.0, and GraphPad Prism 5.0. P-value less than 0.05 were considered statistically significant.

3. Results

A total of 129 HbSS patients aged 12.6±8.8 years old were included in the present study, and 49.6% (64/129) were female. The clinical histories of the HbSS patients showed that 83.7% (108/129) of patients had been hospitalized, 62.8% (81/129) had an infection, 54.3% (70/129) had pneumonia, 20.9% (27/129) had a respiratory infection, 3.9% (5/129) had a urinary tract infection, 13.9% (18/129) had splenic sequestration, 7% (9/129) had a CVA, 86.8% (112/129) had VOE, and 6.2% (8/129) had a leg ulcer.

3.1 *Analysis of the TLR4 +896A>G, TLR9 -1237T>C, FcγRIIa* H/R131, *TNF-alpha and MPO -463G>A gene polymorphisms*

No differences in the hematological parameters of HbF, reticulocytes, leukocytes, and platelets were observed between the *TLR4*, *TLR9*, *FcγRIIa* *TNF-alpha* and *MPO* gene polymorphisms.

Genotypic frequencies of the gene polymorphisms are shown in Table 2 and the *TLR4* and *MPO* were not in Hardy-Weinberg equilibrium. The *MPO* polymorphism was associated with infection, and the *TLR4* and *TLR9* gene polymorphisms were associated with CVA and respiratory infection, respectively ($p<0.05$) (Figure 1).

3.2 *Association between FcγRIIA H/R131 and clinical events in children with HbSS*

The *FcγRIIA* mutation was associated with hospitalization events compared with individuals who did not have the mutation and were not hospitalized (Figure 2A, $p=0.028$) and was associated with splenic sequestration (Figure 2B, $p=0.024$). The risk of developing more than five pain crises events was lower for the variant allele of *FcγRIIA* ($p=0.013$).

3.3 *HbSS children who were combined carriers of FcγRIIA and MPO, TLR4 or TNF-α alleles had an increased incidence of clinical complications.*

Table 3 shows the association of combined carriers with two different polymorphic alleles and the risk of developing the clinical complications of HbSS patients. The *FcγRIIa* 131 H/R and *TLR4* + 896A>G polymorphisms, the *MPO* -463G>A and *TNF-α* -308G>A gene polymorphisms were observed to exert a combined influence. When the *FcγRIIA* H allele was associated to

TLR4 genotypes with the presence of the A allele, HbSS patients had a decreased incidence of hospitalization and painful crisis occurrence, but an increased risk for splenic sequestration. When the interaction between the *FcγRIIA* H allele and *MPO* genotypes with the presence of the G allele was analyzed, the HbSS patients had a decreased incidence of CVA and painful crisis, but an increased risk for splenic sequestration. However, when the association of *FcγRIIA* genotypes with the presence of the mutant allele R and *MPO* genotypes with the presence of the mutant allele A was analyzed, the HbSS patients had a decreased incidence of hospitalization. Additionally, when the interaction between *FcγRIIA* H allele and *TNF-α* genotype with the presence of the G allele was analyzed, a decreased risk of hospitalization was observed.

4. Discussion

Phenotypic heterogeneity is characteristic of HbSS patients and many of its complications, including CVA, ACS, premature death and the three major manifestations, painful vaso-occlusive crises, hemolytic anemia and infections [26, 27]. In this study, we found that the SNPS distribution on *TLR4* and *MPO* genes were not in Hardy-Weinberg equilibrium and it can be reflex of a genetic drift, which is a mechanism that modifies microevolutionary randomly allele frequencies over time. This mechanism results in the loss of genetic variation and allele's fixation at different loci. Alleles fixed by drift can be neutral, deleterious or advantageous and it could justify some of our results described here.

Two polymorphisms (-1237T>C and -1486T>C) within the *TLR9* promoter have been recently described, and one (-1237T>C) is potentially associated with an increased risk for asthma [28]. Asthma in SCD is known to be associated with increased morbidity and an elevated rate of clinical complications, such ACS, CVA, VOE, and early mortality [29]. In this study, we did not discuss asthma occurrence. However, we found that pneumonia was the most frequent infection in this group and that the mutant *TLR9* -1237 T>C allele was associated with respiratory infection. This result shows the extent of respiratory complications in HbSS patients. Previous studies have described the influence of *TLR4* +896A>G on gram-negative infections and septic shock susceptibility [13, 30]; other studies have shown a protective effect of this SNP on coronary atherosclerosis [31] and the risk of cerebral ischemia [32]. Our study demonstrated that HbSS patients with the mutant allele had more CVA episodes than wild type allele carriers. These results have never been described, but we emphasize the low number of patients with a CVA occurrence in this group. Because of the divergent results in previous report regarding the association of this SNP with ischemia [13, 30, 31], we could not conclusively determine how the *TLR4* +896A>G polymorphism affects the risk of CVA.

Myeloperoxidase has emerged as a potential participant in the promotion and/or propagation of atherosclerosis. Additionally, recent studies have strongly suggested an important role of MPO as a marker of endothelial dysfunction through the limitation of NO bioavailability [33, 34]. The *MPO* -463G>A polymorphism may be a significant genetic modulator that increases the susceptibility of HbSS patients to infection [35, 36]. In our study, we found

that the mutant allele was a protective factor against infection, which is in agreement with a previous study [36].

Few studies have evaluated the *FcγRIIA* H/R131 polymorphism and HbSS, although one study described the influence of the polymorphism on the incidence of infection with encapsulated organisms in children with HbSS [37]. Our study showed that the *FcγRII*A-*H131* allele conferred protection for hospitalization and crisis occurrence. When we analyzed this polymorphism in association with the *TLR4* or *MPO* gene polymorphism, we demonstrated a relationship with the risk of splenic sequestration and protection against hospitalization and crisis in this patient group. The combination of the *FcγRIIA* and *TNF-α* gene polymorphisms also influenced the occurrence of hospitalization. A number of chronic inflammatory diseases have been associated with genetic variants of the FcγRIIA receptor, including autoimmune pathologies, such as systemic erythematosus lupus [38], and vascular inflammatory disorders, such as acute coronary syndrome [39]. Previous studies have shown that the *FcγRIIA*R131H gene polymorphism is functional. Because of the abundant expression of FcγRIIA on endothelial cells, this polymorphism may also be associated with endothelial dysfunction [40]. We demonstrated that carriers of the H allele had a reduced rate of hospitalization, CVA and painful crisis when carried in association with another gene polymorphism and that the genotypes with the allele R had a reduced rate of hospitalization. The FcγRIIA appears to play an important role in inflammatory disease and in the function of the vascular endothelium.

5. Conclusion

These data suggest that the *FcγRIIA*H131R gene polymorphism is associated with clinical complications in HbSS patients, as previously observed in other diseases. Association of the *FcγRIIA* receptor gene polymorphism with the *TLR4*, *MPO* and *TNF-α* SNPs may modulate the clinical picture of HbSS, and these genes may have a synergistic effect. The hypothesis of an immunogenetic modulation of risk in HbSS remains difficult to confirm because of the extensive heterogeneity and possible epistatic gene interaction. Future studies should evaluate the interactions of related genes and the potential roles of these alleles and other candidate genes to define a genetic risk profile for HbSS patients.

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Table(s)

Table 1. Primers, conditions for PCR amplification and restriction enzymes used for the gene polymorphism assay.

Gene Polymorphism	Primer sequence (5'→3')	Annealing temperature/ $MgCl_2$(°C/mM)	Restriction enzyme
MPO -463G>A	[a] F- CGG-TAT-AGG-CAC-ACA-ATG-GTG-AG [b] R-GCA-ATG-GTT-CAA-GCG-ATT-CTT-C	58/2.5	*AciI*
FcγRIIa H/R131	F- GGA-AAA-TCC-CAG-AAA-TTC-TCG-C R-CAA-CAG-CCT-GAC-TAC-CTA-TTA-CGC-GGG	55/1.5	*BstUI*
TNF-α -308G>A	F- AGG-CAA-TAG-GTT-TTG-AGG-GCC-AT R- TCC-TCC-CTG-CTC-CGA	59/3.5	*NcoI*
TLR4 +896A>G	F-GAT-TAG-CAT-ACT-TAG-ACT-ACT-ACC-TCC-ATG R-GAT-CAA-CTT-CTG-AAA-AAG-CAT-TCC-CAC	55/3.0	*NcoI*
TLR9 -1237T>C	F-ATG-GGA-GCA-GAG-ACA-TAA-TGG-A R-CTG-CTT-GCA-GTT-GAC-TGT-GT	61/3.0	*BstNI*

[a] F=Forward and [b] R=Reverse

Table(s)

Table 2. Genotypic and allelic frequencies.

Genotypic frequencies				Allelic frequencies	H-W Equilibrium [a]	
Gene Polymorphisms	Wide type Homozygous n(%)	Heterozygous n(%)	Mutant Homozygous n(%)	Mutant	χ^2	p value
MPO -463G>A	68 (52.7)	43 (33.1)	18 (13.9)	0.58	5.9892	0.014
FcγRIIA H/R131	96 (74.4)	28 (21.7)	5 (3.9)	0.294	2.3819	0.122
TNF-α -308G>A	101 (78.3)	25 (19.4)	3 (2.3)	0.24	0.8976	0.343
TLR4 +896A>G	124 (96.1)	4 (3.1)	1 (0.8)	0.023	13.000	<0.001
TLR9 -1237T>C	90 (69.8)	35 (27.1)	4 (3.1)	0.33	0.0697	0.792

[a] H-W= Hardy-Weinberg equilibrium

Table(s)

Table 3- Combination of *FcγRIIA* polymorphisms and its interaction with different genes related to immune defense and clinical complications

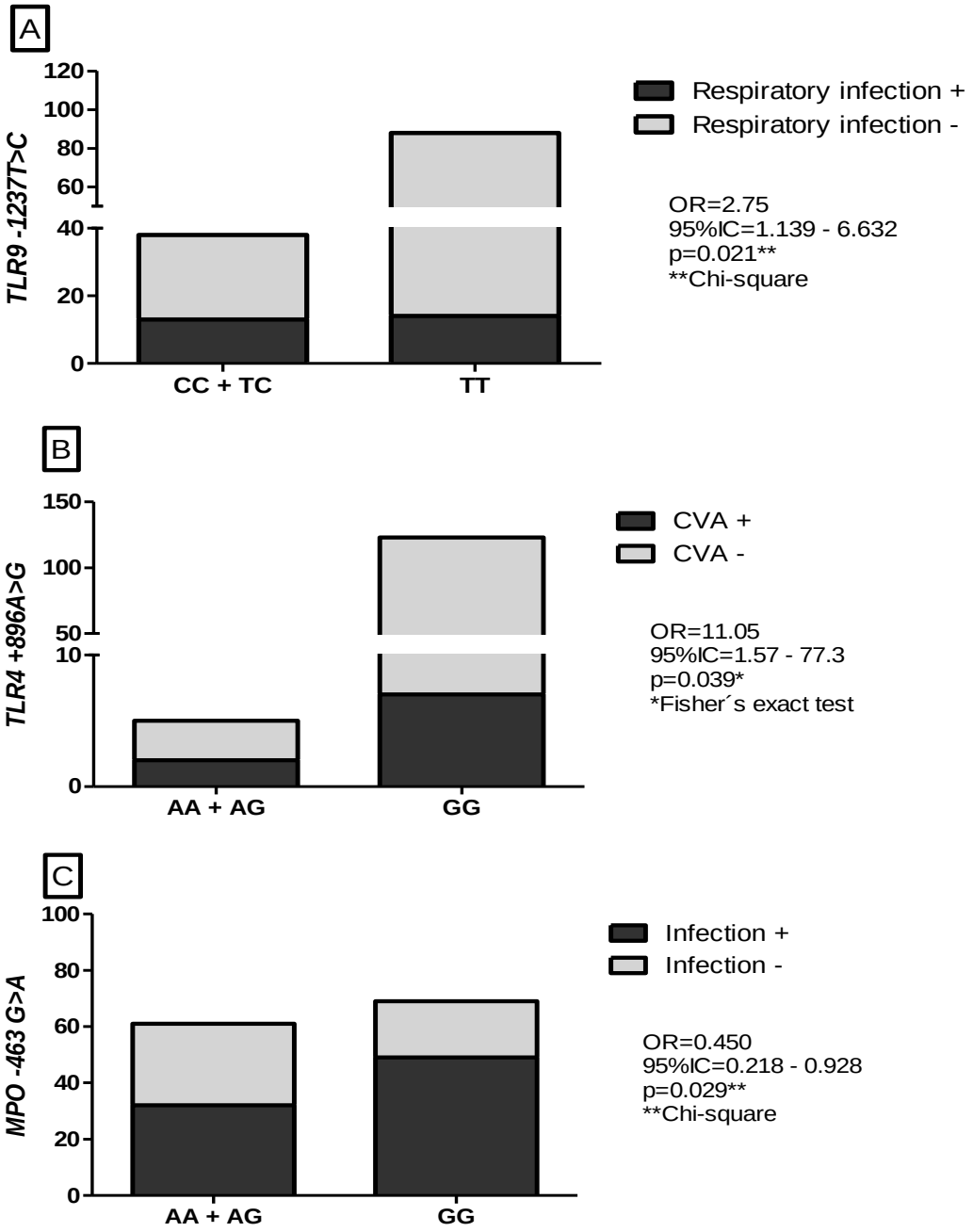
Combinations of gene polymorphisms	Genotypes	Hospitalization N (%)	Splenic sequestration N (%)	CVA N (%)	>5 Painful crisis N (%)
FcγRIIA + *MPO*	HH + GG/GA vs remaining	25/33 (75.5) vs 81/92 (88)	9/33 (27.2) vs 9/92 (9.8)	2/33 (6) vs 33/92 (35.8)	4/33 (12.1) vs 30/92 (32.6)
	p value, OR (95% CI)	0.05[a],0.34 (0.12 – 1.0)	0.043[a], OR=3.19 (1.03 – 9.97)	<0.001[b],0.11 (0.04 – 0.63)	0.022[b], 0.27 (0.08 - 0.78)
FcγRIIA + *MPO*	RR/HR + GA/AA vs remaining	11/19 (57.9) vs 95/110 (86.3)	3/19 (18.7) vs 15/110 (13.6)	2/19 (10.5) vs 7/110 (6.3)	1/19 (5.2) vs 33/110 (30)
	p value, OR (95% CI)	0.007[b], 0.21 (0.07-0.62)	0.99[a]	0.82[a]	0.88 [a]
FcγRIIA + *TNF-α*	HH + GG+GA vs remaining	5/5 (14.3) vs 100/122 (81.9)	1/5 (2.8) vs 15/122 (12.3)	3/5 (25.7) vs 6/122 (4.9)	4/5 (11.4) vs 31/122 (25.4)
	p value, OR (95% CI)	<0.001[b], 0.037 (0.01 – 0.10)	0.17[b]	0.64[b]	0.12[b]
FcγRIIA + *TLR4*	HH + AA+AG vs remaining	24/33 (72.7) vs 81/92 (88)	9/33 (27.2) vs 9/92 (9.8)	2/33 (6) vs 7/92 (7.6)[b]	4/33 (12.1) vs 28/92 (30.4)
	p value, OR (95% CI)	0.02[a], OR=0.3 (0.11 - 0.79)	0.04[a], OR=3.15 (1.10 – 9.05)	1.0[b]	0.04[b], OR=0.29 (0.09-0.90)

[a] Chi-square, Yates corrected [b] Fisher's exact test

Figure(s)

Figure 1. Associations between *TLR* and *MPO* gene polymorphisms and clinical complications in HbSS patients. A: *TLR9* and respiratory infection; B: *TLR4* and Cerebrovascular Accident (CVA); C: *MPO* and infection.

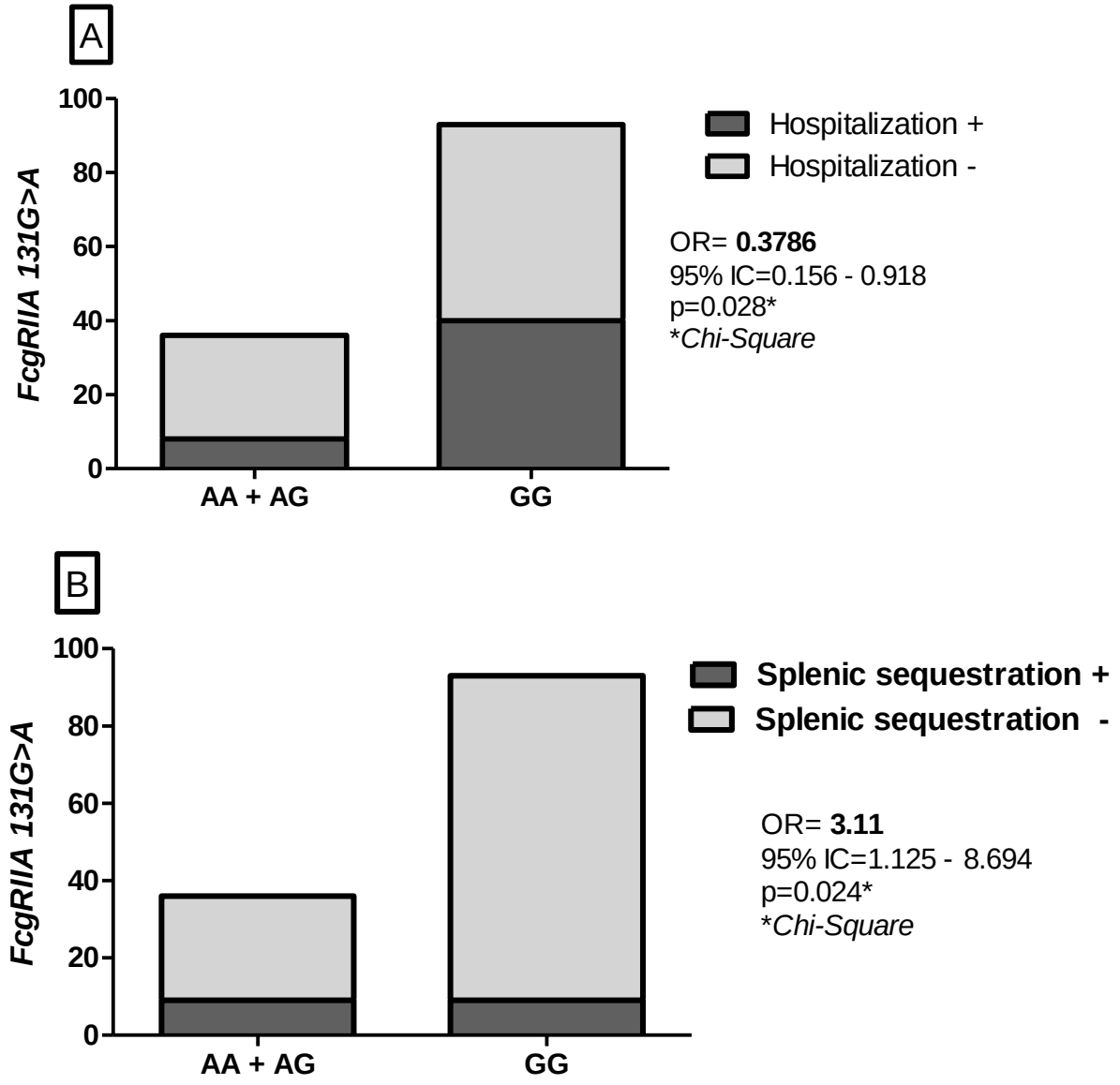
Figure(s)


A
TLR9 -1237T>C
120
100
80
60
40
20
0
CC + TC
TT
Respiratory infection +
Respiratory infection -
OR=2.75
95%IC=1.139 - 6.632
p=0.021**
**Chi-square
B
TLR4 +896A>G
150
100
50
10
0
AA + AG
GG
CVA +
CVA -
OR=11.05
95%IC=1.57 - 77.3
p=0.039*
*Fisher´s exact test
C
MPO -463 G>A
100
80
60
40
20
0
AA + AG
GG
Infection +
Infection -
OR=0.450
95%IC=0.218 - 0.928
p=0.029**
**Chi-square

Figure(s)

Figure 2. Association between *FcγRIIA H/R131* genotypes and hospitalization. (A) and splenic sequestration (B) in HbSS children.

Figure(s)


A
100
80
60
40
20
0
FcgRIIA 131G>A
AA + AG
GG
Hospitalization +
Hospitalization -
OR= 0.3786
95% IC=0.156 - 0.918
p=0.028*
*Chi-Square
B
100
80
60
40
20
0
FcgRIIA 131G>A
AA + AG
GG
Splenic sequestration +
Splenic sequestration -
OR= 3.11
95% IC=1.125 - 8.694
p=0.024*
*Chi-Square

5.3- Artigo 3

Toll like receptors have mRNA differentiated expression in dendritic cells in crisis-state sickle cell anemia patients, suggesting a pivotal role of these molecules and cell type in the maintenance of inflammatory response.

Cajado, C; Futukami K.F, Machado, N; Leite, I; Siqueira, I; Lyra IM; Adorno, EV; Goncalves, MS

As alterações vasculares apresentam um papel importante no estado inflamatório crônico da anemia falciforme com a participação de diferentes tipos celulares, incluindo macrófagos e células dendriticas (DCs). Aqui nós avaliamos a expressão do mRNA dos TLRs em células dendriticas pré e pós o estímulo com LPS e também a quantificação de citocinas IL-1β, TNF-α e IL-10 no plasma e sobrenadante de cultura de DCs pré e pós desafio com LPS. As células dendríticas imaturas (imDC) derivadas de monócitos foram obtidas de monócitos de sangue periférico de sete pacientes internados portadores de HbSS. A quantificação relative de mRNA de cada transcrito foi realizada por PCR em tempo real e os níveis de citocinas foram detectados por ELISA. Nós observamos que diferentes pacientes apresentaram expressão específica de TLRs e diferentes níveis de IL-1β em pacientes HbSS com e sem história clinica de pneumonia (p=0.0253) e com concentração elevada de hemoglobina (p=0.0288). a expressão diferenciada de TLR4 foi associada coma ocorrência de ulcera de perna (p=0.0016); de TLR2 com a contagem de plaquetas (0.0102) e de reticulócitos (p=0.0007); e o TLR5 com os níveis de TNF-α (p=0.0496) e IL-10 (p=0.0374). Nossos resultados mostram que essas moléculas e os tipos celulares possíveis apresentam um importante papel na manutenção do estado inflamatório descrito nesta doença.

Palavras chave: Anemia falciforme; inflamação; células dendriticas; receptores sememlhantes a toll.

Toll like receptors have mRNA differentiated expression in dendritic cells in crisis-state sickle cell anemia patients, suggesting a pivotal role of these molecules and cell type in the maintenance of inflammatory response.

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Abstract

The vascular alterations play an important role in the SCA chronic inflammatory state with the participation of different cell types, including macrophages and dendritic cells (DCs). Here, we evaluated the mRNA expression of *TLRs* and in DCs cells pre and post LPS stimulus and also measured IL-1β, TNF-α and lL-10 cytokines in plasma and supernatant of DCs pre and post LPS challenge. In vitro generation of immature DCs (imDC) monocyte-derived was obtained of monocytes from peripheral blood mononuclear cells (PBMC) of seven hospitalized HbSS patients. Relative mRNA abundance of each transcript was investigated by quantitative real time PCR and cytokines levels were measured by ELISA, We found that different HbSS patients showed specific DCs TLR expression and a differential levels of IL-1β among HbSS patients with and without pneumonia history (p=0.0253) and with hemoglobin concentration (p=0.0288). Differential expression of TLR4 and HbSS patients with leg ulcers history (p=0.0016); TLR2 with platelets (0.0102) and reticulocytes (p=0.0007) and TLR5 with TNF-α (p=0.0496) and lL-10 (p=0.0374) levels. Ours results show that these molecules and cell type possibly have an important role in maintain the inflammatory state described in this disease.

Key word: Sickle cell anemia; inflammation; dendritic cells; toll like receptors

1. INTRODUCTION

Sickle cell anemia (SCA) is a genetic disease characterized by an inflammatory state secondary to vaso-occlusive events (VOE) and/or infections (1). The HbS concentration is the main determinant of disease severity by co-inheritance of genetic factors that modulate the intracellular HbS or fetal hemoglobin concentration, such as the protective effects of co-inherited α-thalassemia or hereditary persistence of fetal hemoglobin (2). The SCA steady-state patients have chronic inflammatory surroundings, characterized by elevated leukocyte counts (1), activated leukocytes (3), platelets (4) and elevated cytokines levels (5). The VOE in SCA is transient and episodic (6) and together with the hemolysis generate radical of oxygen and nitrogen species, contribute to tissue damage, ischemia and reperfusion and vascular dysfunction (7).

Vascular alterations play an important role in the SCA chronic inflammatory state with the participation of different cell types such as macrophages, fibroblasts, mast cells and dendritic cells, as well as circulating leukocytes, including monocytes and neutrophils, in a very complex net of mechanisms, promoting tissues damage, with recognition of pathogen or cell damage products, with intracellular or surface-expressed pattern recognition receptors (PRRs) (8). These receptors have been related to either directly or indirectly bind pathogen-associated molecular patterns (PAMPs). For the other side, it was postulate by Matzinger (2002) that oxidative reactions products bring proteins, lipids, carbohydrates and DNA changes and are consider as danger or damage-associated molecular patterns (DAMPs) (8, 9).

TLRs are integral glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain (10). Ligand

binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. To date, 10 TLRs have been identified in humans and recognize distinct PAMPs derived from several microbial pathogens, including viruses, bacteria, fungi and protozoa. TLRs can be divided into subfamilies primarily recognizing related PAMPs; TLR1, TLR2, TLR4 and TLR6 recognize oxidized-lipids (ox-L), whereas TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids (9, 10).

Cells types expressing TLRs are antigens presenting cells (APCs), including macrophages, dendritic cells (DCs) and B lymphocytes, which expresses either inducible or constitutively way in the course of infection, oxidative events and (11). DCs play a pivotal role in antigen (Ag) presentation in different tissues and increase rapidly in numbers when recruited and efficiently captured by antigens due to their high phagocytic ability. Subsequently to antigen capture, DCs become activated and mature by pathogens or cell damage products with migrations into draining lymph nodes or the spleen to become mature and powerful antigen-presenting cells which are capable of activating naive T cells. The transition from immature to mature state is accompanied by the production of several cytokines and chemokine by the DCs that regulate their ability to interact with naïve T cells to direct T cell differentiation (12).

Two major DCs subsets can be detected in the peripheral blood and have distinct but overlapping functions. Myeloid DCs (mDCs) express HLA DR, CD11c, and CD1c and are the main producers of interleukin-12 (IL-12), while plasmacytoid DCs (pDCs) express HLA DR, CD123 and blood dendritic cell antigen 2 (BDCA2) and are the main producers of interferon-α (IFN-α) (13). However, there is a particular CD1-positive phenotype expression of CD1a, b, and c molecules at DCs, while the

classical phenotype described in the general population in which only 15% of the individuals express the CD1 molecules at the surface of their monocytes (14). The expression of CD1 molecules seems to be related to surveillance for pathogens to survey different endosomal Previous studies hypothesized that the absence of CD1 coexpression on monocytes of the vast majority of SCA patients could be responsible for the emergence of some of the infections occurring in these patients and because of this SCA monocytes have the ability to differentiate in efficient dendritic cells and if the differential susceptibility to infections of SCA patients is due to their atypical CD1 expression on monocytes (14).

Meanwhile, the elevated concentration of cytokines of SCA in monocyte activation in SS patient's plasma can contribute for their activated status and it may be hypothesized that CD1 expression on dendritic cells from SCA patients is a consequence of the elevated level of endothelin in the plasma of SCA (15).

The role of DCs in malaria affected population and its' association with hemoglobinopathies have been described and these founds shows that the activation of mDCs and pDCs during acute malaria may be faster or more profound in children with α-thalassemia than in children with normal hemoglobin (16). The *Plasmodium falciparum* glycosylphos- phatidylinositol (GPI) can bind to TLR2 and TLR4 expressed on mDCs and monocytes (16).

Considering that the role of immune response in SCA is poorly understood and change in the immune system has been associated to the morbidity of this disease, studies involving these aspects are required to elucidate the mechanisms and possible target molecules to be evaluated in further therapeutic approaches.

Here, we evaluated the mRNA expression of *TLRs* in CDs cells and we found that these molecules and cell type possibly have an important role in maintain the inflammatory state described in this disease.

2. MATERIAL AND METHODS

Patients

A total of 7 HbSS patients, four man and three women, were recruited from the "Hospital da Criança das Obras Sociais Irmã Dulce (HOSID)", which were hospitalized by pain crisis or infection events. The study was approved by the "Centro de Pesquisa Gonçalo Moniz- Fundação Oswaldo Cruz (CPqGM-FIOCRUZ)" and HOSID human subject research boards and the informed consent was signed by officials responsible. The work was developed in accord to the Helsinki Declaration of 1975, and its revision. Venous blood (10-20mL) was taken into heparin vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) and processed for each analysis.

In vitro generation of dendritic cells

In vitro generation of immature DCs (imDC) monocyte-derived was obtained of monocytes from peripheral blood mononuclear cells (PBMC) of hospitalized patients from the (HCOSID) after blood passage over a Ficoll Hypaque gradient (Sigma-Aldrich, Piscataway, USA). PBMC were washed three times, and the $CD14^+$ cell population was enriched by positive selection using magnetic cell sorting (Mini Macs, Miltenyi Biotec, Auburn, CA, USA). Monocytes were suspended in a concentration of $5x10^5$ cells/mL in RPMI-1640 medium Gibco (Grand Island, NY, USA) supplemented with 2 mM/L glutamine, 100 U/mL penicillin, 100µg/mL streptomycin Gibco (Grand Island, NY, USA), and 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Piscataway, USA), plus 100 UI/mL IL-4 (PeproTech, Rocky Hill, NJ, USA)

and 50 ng/mL Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA). Cells were plated in a 12-well tissue culture plates (Costar, Corning, NY, USA) and incubated at 37°C, under 5% CO2 for 7 days. Supernatants were collected on day 3 and 5 for replacement of IL-4 and GM-CSF supplemented medium. After 7 days, to characterize the DCs population, cells were stained with anti-CD1a (PharMingen, San Diego, CA, USA) and fluorescence was analyzed by FACS (FACSAria cell sorter, Becton Dickinson, San Jose, CA, USA). Cultures contained more than 80% CD1a-positive cells were harvested, washed twice with saline, and used in different phenotypic and functional experiments (Figure 1).

On day 7 of culture, DCs were harvested and cultured at 2 x10^5/mL in a 24-well tissue-culture plate in RPMI-1640 medium plus 10% heating activated FBS and medium was supplemented with 25 ng/mL lypopolissacaride (LPS) (Sigma-Aldrich, Saint Louis,USA) complete medium (CM) and supernatants and cells were collected after 48 h.

Quantitative real time PCR

Total RNA was extracted from freshly isolated mDCs with RNeasy Mini Kit (Qiagen, Texas, USA). First strand cDNA was synthetized using 5µg of total RNA using oligo (dT) (Invitrogen Life Technologies, Austin, USA) and Superscript II reverse transcriptase (Invitrogen Life Technologies, Burlingont, Canada). The PCR was performed with the primers 5'-TTG TGA CCG CAA TGG TAT CTG-3' and 5'-GCC CTG AGG GAA TGG AGT TT-3' for TLR-2, 5'-CAA CAA TCA CCT TTC GGC TTT T-3' and 5'-GGC CAT TGC TGC CAA CAT -3' for TLR-4 (gene bank 024169, 5'-AGC CCC GGA ACT TTG TGA CT-3' and 5'-TGT ATG CAC TGT CAC TCT GAC TCT GT-3' for TLR-5 (gene bank 003268) and 5'-AAC CTC CCC AAG AGC CTA CAG-3', 5'-CAG CAC TTA AAG AAG GCC AGG TA-3' for TLR-9 (gene bank 017442). PCR products were separated by electrophoresis in 2% agarose gels and were visualized by ethidium bromide staining.

Quantitative real time PCR (RT-PCR) reactions were performed twice for each sample in optical plates and analyzed by the ABI PRISM 7500 Sequence system (*Applied Biosystems, California,* USA) and software SDS 2.0 (*Applied Biosystems, California,* USA). The SYBR-Green with fluorescent particles was used for develop the RT-PCR reactions. Relative mRNA abundance of each transcript was normalized, calculated, where Ct represents the threshold cycle for each transcript. Relative mRNA abundance of each transcript was normalized with the constitutively expressed housekeeping gene, the *Hypoxanthinephophoribosyltransferase* (*HPRT*) and calculated as $\mathbf{2^{-\Delta\Delta Ct}}$**,** where Ct represents the threshold cycle for each transcript. This comparative CT method, also known as the ΔΔCT method, ensures that the target and endogenous control have similar or relatively equivalent PCR efficiencies. We used the healthy control as the calibrator, with the $\mathbf{2^{-\Delta\Delta Ct}}$ method; data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the healthy control (17). For the HbSS patients, the $\mathbf{2^{-\Delta\Delta Ct}}$ evaluation indicates the fold change in gene expression relative to the control and primers efficiency curve are shown in Figure 1

Cytokine assays

IL-1β, TNF-α and IL-10 cytokines were measured in supernatant of DCs culture derived of sickle cell peripheral blood. Assays were performed by ELISA (*BD OptEIA – Biosciences, origem*) using commercially available kits, according to the manufacturer's instructions.

3. RESULTS:

The study includes a total of 7 in crisis-state HbSS patients (43% of female). Data related to patients demographic and clinical characteristics are shown on Table 1.

Immature dendritic cells show an increase of TLR2 expression after LPS stimulus

The expression of TLRs was investigated from DCs monocytes-derived from HbSS patients without and with LPS stimulus and quantities of *TLR2*, *TLR4*, *TLR5* and *TLR9* mRNA expression were determined in each sample using the real-time PCR analysis, normalized with the Hypoxanthinephophoribosyltransferase (HPRT) gene (Figure1).

The expression of *TLR2* increased post LPS stimulus. However, there were already expression of *TLR4*, *TLR5* and *TLR9*, mainly the last one, in both measure points (pre and post LPS stimulus) (Figure 2).

Different HbSS patients showed specific DCs TLR expression

The figure 2 shows specific CDs TLR gene expression from the studied HbSS patients. The patient 1 that was hospitalized by vaso-occlusive crisis (VOC) which had a clinical history showed an increase of TLR2 and TLR4 gene expression, with the highest expression of the first one. The patient 2 which also was hospitalized by VOC had an increase of TLR2 and TLR5 expression, mainly of the last one. The patient 3 was hospitalized by VOC and for leg ulcer treatment and had an increased expression of *TLR2*, *TLR4*, *TLR5* and *TLR9*, mainly of the first one. The patient 4 was hospitalized by pneumonia and exhibits an increase of *TLR4* and *TLR9* gene expression, with the highest levels of the last one. The patient 5 was hospitalized by VOC and pneumonia and had an increase of the TLR5 and TLR9 expression, mainly with the last one. However, the patients 5 showed a low expression of *TLR2* and *TLR4*. The patient 6 was hospitalized by VOC, edema and urinary infection and only shows a very low expression of *TLR2* and *TLR4.* The patient 7 was hospitalized by

VOC and pneumonia and had an expression of the TLR2 and TLR4 with a higher of the first one (Figure 3).

Cytokine levels in plasma and DCs culture supernatant

Cytokines plasma levels were measured among the seven studied in crisis–state SCA patients and all showed high levels of IL1 beta. The TNF-alpha and IL-10 plasma levels were also measured and only the patient two had a low levels of these cytokines; the others six patients had a high levels of these cytokines. The same cytokines were measured in Dcs culture pre and post LPS stimulus. Il-beta levels were measured in the CDs culture supernatants and all patients had elevated levels of this cytokine pre and post LPS stimulus.

The TNF-alpha levels were also measured before and LPS stimulus and it was found some interesting results. The patient 2,which already had low plasma level of TNF-alpha, continuous to have low levels of this cytokine in situations pre and post LPS stimulus. The patient 5 which had a high plasma levels of TNF-alpha, showed a low level of this cytokine in DCs culture supernatant pre and post LPS stimulus. IL-10 levels were measured also pre and post LPS stimulus and almost all patients showed low levels of this cytokine in the supernatant pre LPS, which increase a little or do not change after LPS challenge. However, the patient 6 showed a high level of IL-10 in the supernatant culture pre LPS which decrease substantially after LPS addition.

The pneumonia history seems to be related to the IL-beta levels pre and post LPS stimulus and with a possible viscosity state presence

A differential level of IL1-beta was associated with patients with a history of pneumonia, once the increase of this cytokine levels was strongly associated with this history post the challenge with LPS (Figure 5 A-C). Also, the increase of IL1-beta levels was associated to an increase in hemoglobin concentration (Figure 5 E).

The expression of TLR4 was increase among HbSS patients with leg ulcers history

The figure 5 (D) shows the differential expression of *TLR4* among patients with legs ulcers, showing that the ones with legs ulcers history had a very high expression of this receptor.

Cytokine levels in DCs culture supernatant pre and post LPS stimulus

The figure 5 (F-H) shows the differential levels of IL-1β, TNF-α and IL-10 cytokines when measured in supernatant of DCs culture pre and post LPS stimulus. In general patients showed an increase of these cytokine pre and post LPS stimuli.

Differential expression of TLR2 and its association with platelets activation and with hemolysis and of TLR5 with TNF-α and IL-10

An increase of *TLR2* expression was associated to the decrease of platelets and reticulocytes numbers (Figure 6 A and B) and an increase of *TLR5* expression was associated to the decrease of TNF-α and an increase of IL-10 levels.

4- DISCUSSION

The sickle cell anemia is associated to a chronic inflammatory state. The present study investigates the *TLR2*, *TLR4*, *TLR5* and *TLR9* RNAm expression among in crisis-state HbSS patients. The *TLR* mRNA expression was investigated on dendritic cells cultures and the results were obtained pre and post LPS stimulus.

Sickle cell anemia patients have a very severe anemia, characterized by hemolysis, endothelial dysfunction, followed by a hypoxia state, the difference among these mechanistic phenomenon occurred among these patients gave to them differential phenotypes frequently associated to the clinical outcome (18, 19). Commonly, patients with sickle cell anemia have chronic inflammation, state that is characterized by a continuous increase of cytokines, and inflammatory mediators, such as (5, 18). It

was described the potentially capacity that the sickle monocytes has to activated endothelial cells (2), enhancing endothelial dysfunction, and an inflammatory response which has the participation of NF-kB–mediated up-regulation of adhesion molecules and tissue factor (2).

Ours results related to the expression of TLRs pre and post LPS stimulus in DCs monocytes derived and its association with the history of some clinical events shows that although the first description of Dc was related to as probes employed by DCs to sense pathogens there are many PRRs and PRRs ligands which also transduce potent maturation signals not related to infection, suggesting a role to maintain chronic inflammatory response present in some chronic disease such as sickle cell anemia. These observations are in accord to news insights related to DCs and TLRs (20),

The find of a differential expression of *TLR* and cytokines and its association with clinical manifestation probably are related to different phenotypes of inflammatory macrophages and dendritic cells that are modulated by lipid uptake, Toll-like receptors ligands, growth factors and chemokines and cytokines. Also the oxidative modification of the endothelium by reactive oxygen species (ROS) can activate NADPH oxidase and Nox enzymes and TLRs, which regulate innate immunity (21). There are already description previous studies TLR4 with LPS induction odf ROS and NF-kB activation, with induction of proinflammatory cytokine by endothelial cells, and also increase of adhesion molecules (21, 22).

The association of hemoglobin concentration with high levels of IL-1β and could be associated to the specific HbSS phenotype of viscosity (23). Increase of TLR2 and its association with a decrease of platelets and reticulocytes counts may be related to

the internalization of this receptor in hemolytic states and under hiperhemolysis presented by HbSS patients (6, 12)

The association of TLR5 increases and decrease of TNF-α and increase of IL-10 possibly show a role of this receptor in the homeostasis equilibrium maintains as described for DCs (24).

It is the first study showing a possible role of DCs and Toll-like receptors in maintain the inflammatory response in HbSS patients and additional studies showing the mechanisms involved in this response will warrants to explain all phenomenon's and cell types participation.

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Table 1: Patients clinical characteristics.

Patient	Sex	Age	Cause of this hospitalization	Infection (N)	Crisis (N)	Hospitalization (N)	Pneumonia (N)	AVE (N)	Leg ulcer	Blood Transfusion

									(N)	(N)
P1	M	9 ys	crisis	No	Yes (1)	No	No	No	No	No
P2	M	6 ys	crisis	Yes (1)	Yes (2)	Yes (1)	Yes (1)	No	No	No
P3	F	17ys	crisis and leg ulcer treatment	Yes (3)	Yes (>5)	Yes (>5)	No	No	Yes (2)	Yes (3)
P4	F	16 ys	pneumonia	Yes (3)	Yes (5)	Yes (3)	Yes (2)	No	Yes (1)	Yes (2)
P5	F	3 ys	crisis and pneumonia	Yes (2)	Yes (1)	Yes (3)	Yes (1)	No	No	Yes (1)
P6	M	9 ys	crisis, edema and urinary infection	Yes (1)	Yes (2)	Yes (>5)	No	No	No	Yes (1)
P7	M	5 ys	crisis and pneumonia	Yes	Yes (1)	Yes (2)	Yes (1)	No	No	No

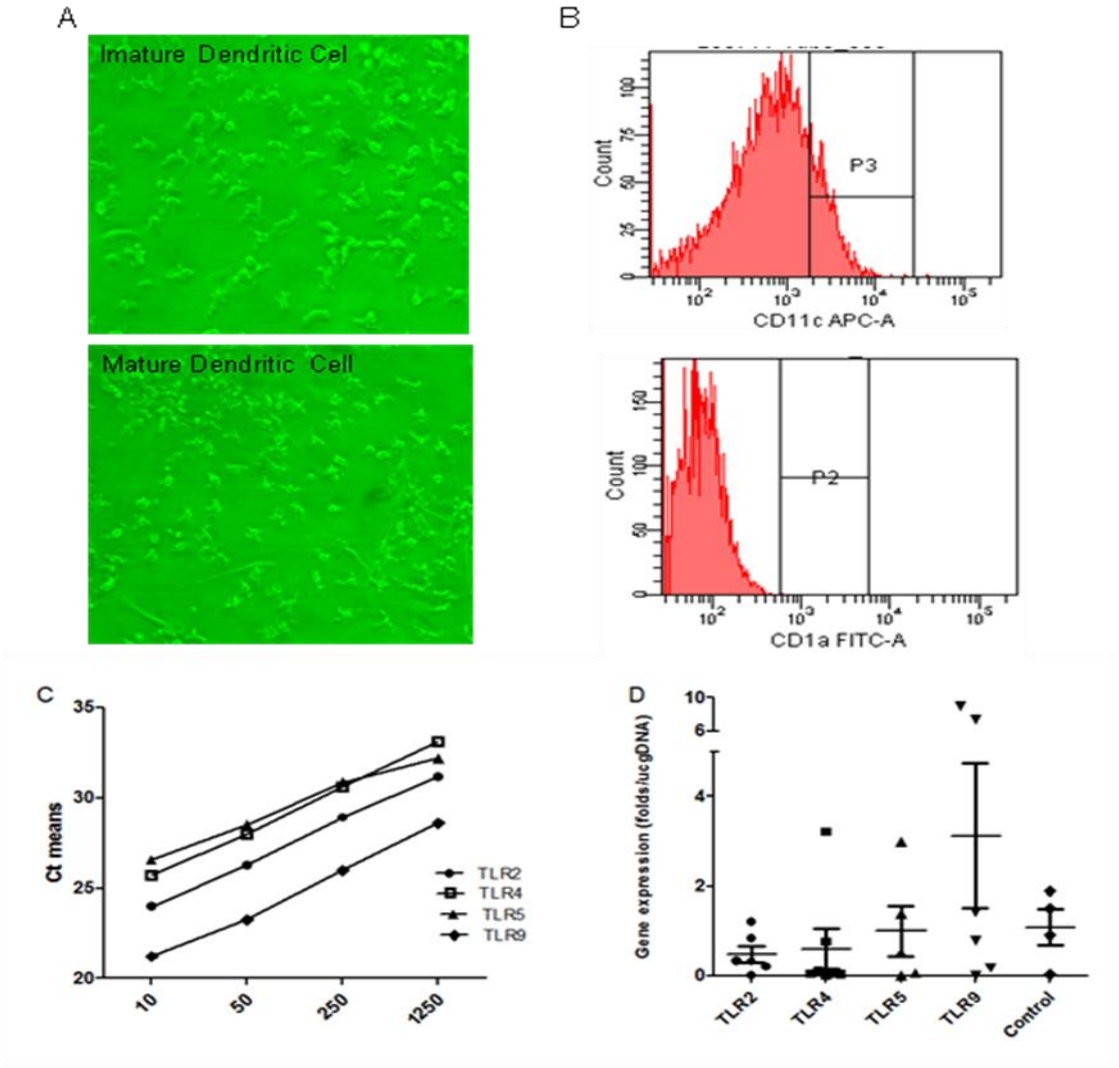


Figure 1. **The expression level of *TLR2*, *TLR4*, *TLR5* and *TLR9* genes was developed in mRNA extracted from dendritic cells (DCs) by real-time Reverse Transcriptase-PCR (RT-PCR)**. A) Immature DCs (imDCs) pre LPS stimulus, in culture with RPMI 1640 medium and mature DCs (mDCs) post 50ug/mL LPS stimulus of 48 hours incubation; B) Flow cytometer analyses of CD11c_imDCs and CD1a_mDCs; C) Primers efficiency validation of the quantitative real-time Reverse Transcriptase-PCR (RT-PCR). The mRNA abundance of each transcript was calculated as $2^{-\Delta\Delta Ct}$, as reference against the expression level of the *TLR2*, *TLR4*, *TLR5* and *TLR9* genes (TLR2 and TLR4, $R^{2=}0.999$; TLR5, $R^{2=}0.989$; TLR9, $R^{2=}0.996$) under investigation; D) Quantities of *TLR2*, *TLR4*, *TLR5* and *TLR9* mRNA expression were determined in CD1a_mDCs, using the real-time PCR analysis $2^{-\Delta\Delta Ct}$ with the relative quantification normalized using a constitutively expressed housekeeping gene, the Hypoxanthinephophoribosyltransferase (*HPRT*).

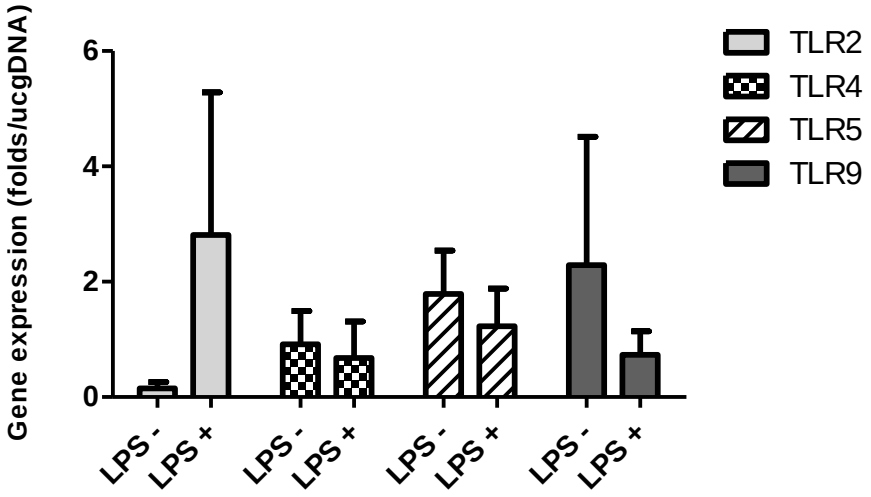


Figure 2. Analyses of *TLR* mRNA expression derived DCs treated with or without LPS stimulus from crises-state HbSS patients. mRNA expression is showing as Mean±Standard Deviation (SD) and was investigated pre and post LPS stimulus respectively for TLR2.

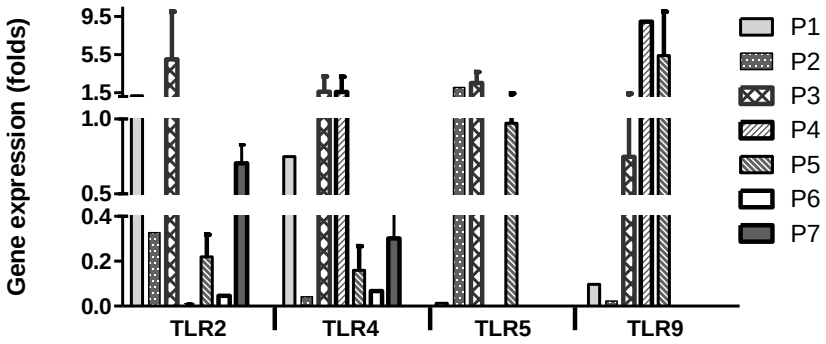


Figure 3. *TLR2*, *TLR4*, *TLR5* and *TLR9* mRNA expression were determined in vitro at monocyte-derived DC from HbSS patients post LPS stimulus. Quantities of *TLRs* mRNA expression were determined in each patients sample using the $2^{-\Delta\Delta Ct}$ methods of relative quantification real-time PCR analyses and were normalized with the control group.

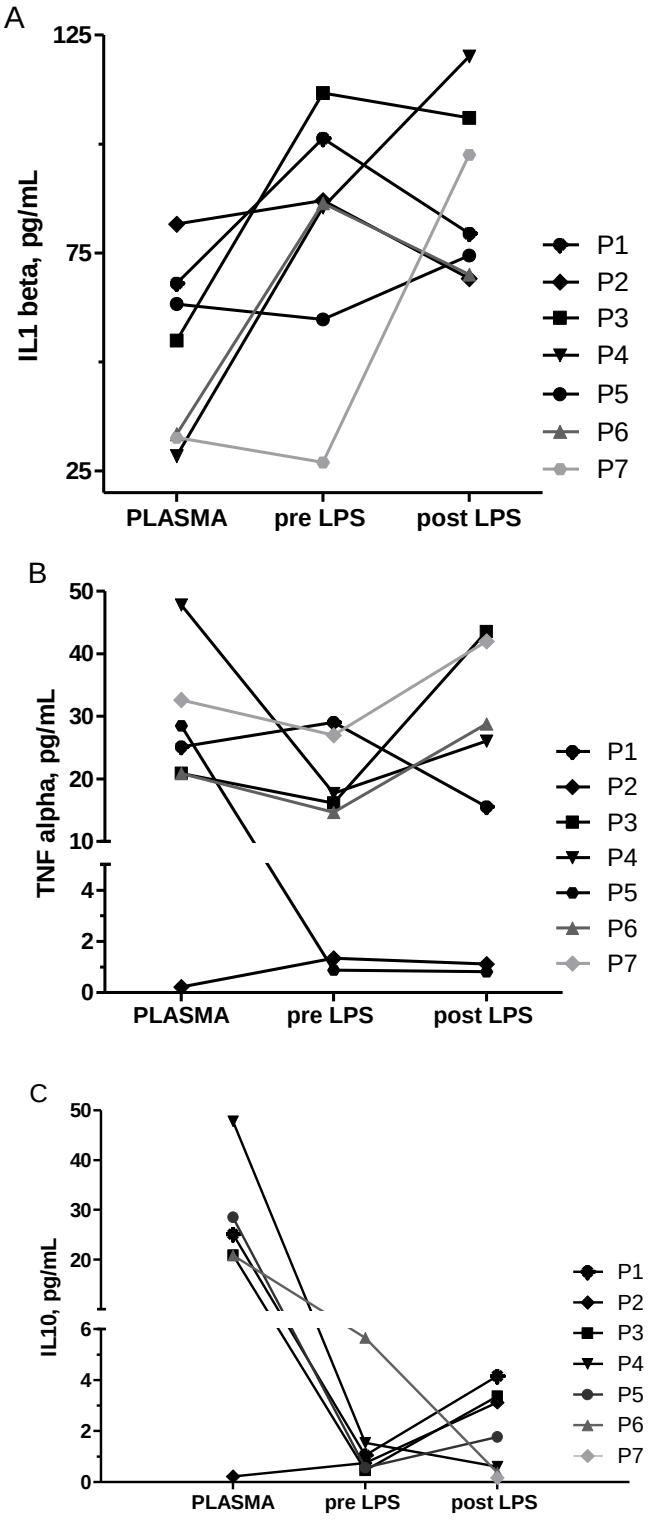
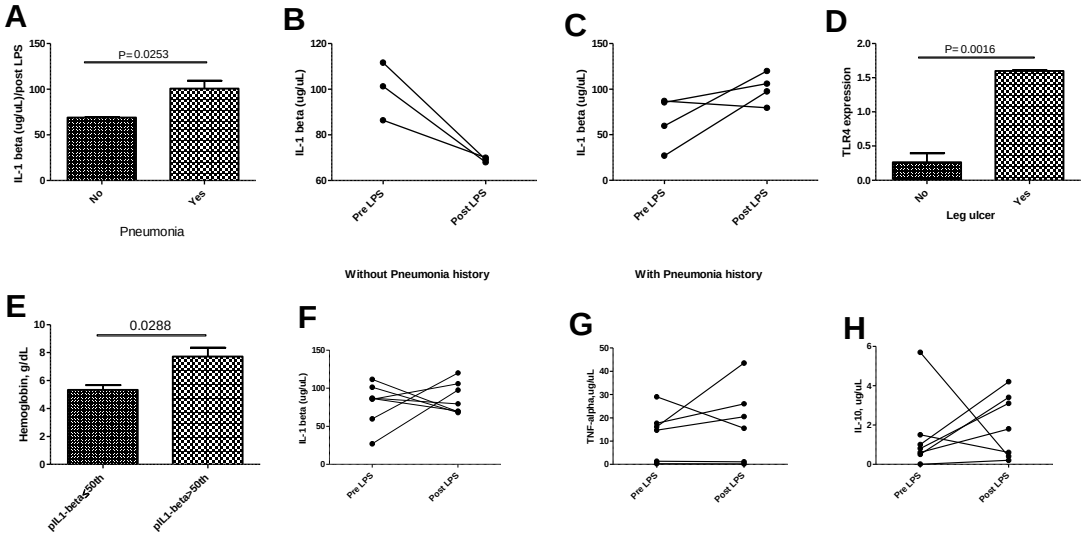


Figure 4. IL 1 beta (A), TNF alpha (B) and IL10 (C) cytokines concentration in plasma and in sobrenant of DCs culture pre (imDCs) and post (mDCs) LPS stimulus of each studied HbSS patient.

Figure 5. **Analyses of IL-1 beta concentration in DCs supernatant culture post LPS stimulus between HbSS patients without and with pneumonia history (A-C); analysis of TLR4 mRNA expression between HbSS patients without and with leg ulcer history (D); analysis of hemoglobin concentration between HbSS patients with different plasma IL-1 beta concentration (E) and analyses of IL-1 beta, TNF-alpha and IL-10 concentration pre and post LPS stimulus (D-F)**. A) IL-1 beta concentration post LPS stimulus among HbSS patients groups without and with pneumonia history (mean ± standard deviation; 69.07±0.996 and 100.8± 16.97) respectively; B) IL-1 beta concentration between HbSS patients groups without pneumonia history pre and post LPS stimulus (99.8±12.72 and 69.07+0.996) respectively; C) IL-1 beta concentration between HbSS patients groups with pneumonia history pre and post LPS stimulus (64.85±28.09 and 100.8±16.97) respectively; D) TLR4 mRNA expression between HbSS patients groups without and with leg ulcers history (0.320±0.302 and 1.60±0.014) respectively; (E) Hemoglobin concentration between HbSS patients groups with IL-1 beta concentration ≤ 50^{th} (5.333±0.577) and > 50^{th} (7.725±1.245); E) Analyses of IL-1 beta concentration between HbSS patients groups pre and post LPS stimulus (79.83±28.24 and 87.19±20.77) respectively; F) Analyses of TNF-alpha concentration between HbSS patients groups pre and post LPS stimulus (11.44±10.94 and 17.80±16.30) respectively; G) Analyses of IL-10 concentration between HbSS patients groups pre and post LPS stimulus (1.933±1.443and 1.957±1.623) respectively. Statistical analyses in A-C and E-F were developed using the Wilcoxon signed rank test (two-tailed); in D was used the unpaired T test (two-tailed).



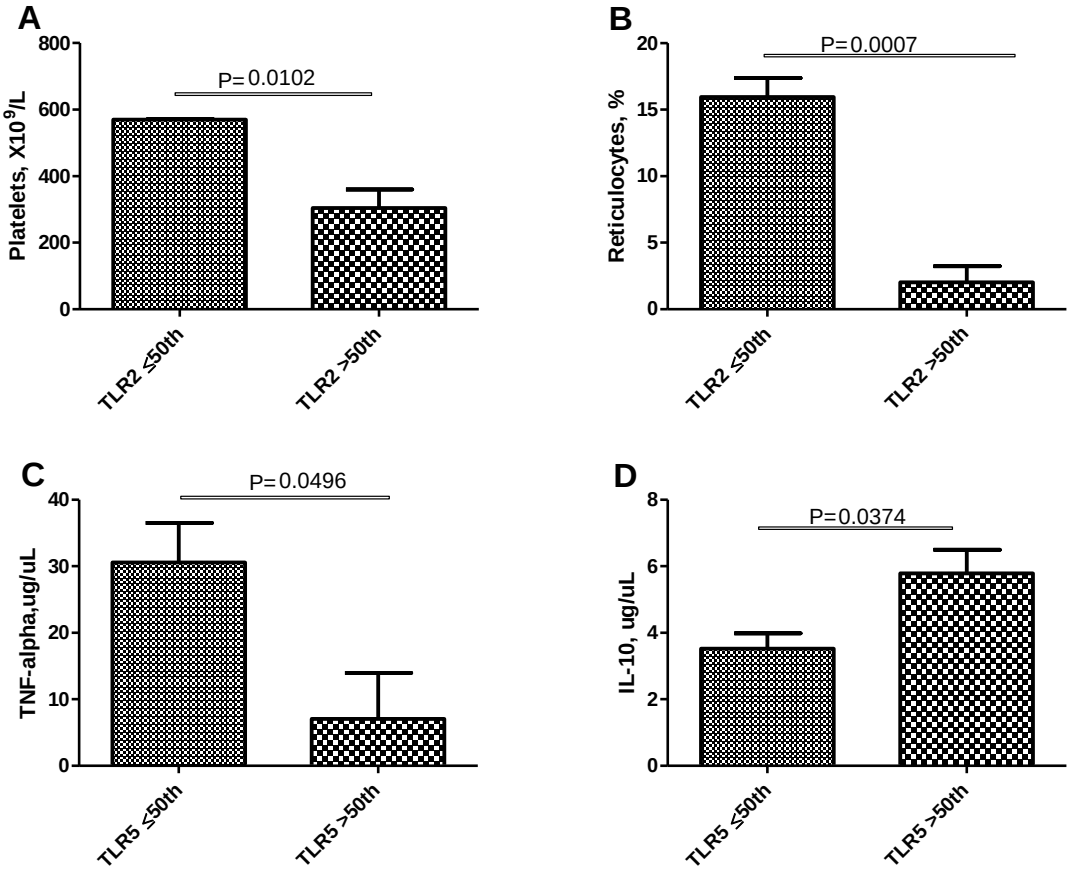


Figure 6**. Analyses of platelets and reticulocytes counts associated to *TLR2* expression and of TNF-alpha and IL-10 concentration associated to *TLR5* expression**. A) Platelets count between HbSS patients groups with *TLR2* expression $\leq 50^{th}$ (569.7±3.512) and $> 50^{th}$ (304.5±111.7); B) Reticulocytes count between HbSS patients groups with *TLR2* expression $\leq 50^{th}$ (15.97±2.450) and $> 50^{th}$ (2.025±2.417); C) TNF-alpha concentration in DCs supernatant culture among HbSS patients with *TLR5* expression $\leq 50^{th}$ (30.57±11.92) and $> 50^{th}$ (7.053±6.918); D) IL-10 concentration in DCs supernatant culture among HbSS patients with *TLR5* expression $\leq 50^{th}$ (3.525±0.921) and $> 50^{th}$ (5.790±1.226). Statistical analyses were developed using the unpaired T test (two-tailed).

6. Discussão

Nesta seção são discutidos os resultados dos três artigos que fazem parte desta tese. Para melhor compreender discutiremos os resultados de acordo com a sequência dos desenhos experimentais descritos no item 4 desta tese, que também corresponde a sequência de artigos incluídos no corpo desta tese.

O curso clínico da anemia falciforme ou HbSS apresenta substancial heterogeneidade, com a ocorrência de crises dolorosas esporádicas até a falência de órgãos, resultando em internações e em óbito precoce desses indivíduos (POWARS, 1991). Nossos resultados confirmam que algumas manifestações clínicas como a esplenomegalia são mais frequentes em crianças com até 5 anos de idade, enquanto as crises vaso-oclusivas ocorrerem durante todas as idades, confirmando a literatura (STEINBERG, 2001).

No estudo denominado CT1 os resultados encontrados concordam com trabalhos anteriores que descrevem a participação dos polimorfismos gênicos nas complicações da anemia falciforme (HOPPE, 2007; SEBASTIANI, 2010). Em nosso estudo o polimorfismo na região promotora do gene *TNF*- α aumenta 4,6 vezes o risco para ocorrência de sequestro esplênico. Além disso, a presença do alelo A do polimorfismo *TNF*- α -308G>A parece aumentar a expressão desse gene, uma vez que eleva os níveis séricos do TNF- α nos indivíduos HbSS. Esses resultados estão de acordo com resultados anteriores que demonstram que o alelo A do *TNF*-α -308G>A aumenta a atividade transcricional do TNF- α, resultando no aumento da sua expressão. O TNF-α é uma citocina potente, produzida por macrófagos e células T que apresenta atividade pró inflamatória, induzindo a ativação de células endoteliais, estimulando a inflamação, induzindo a cascata de coagulação e a produção de proteínas de fase aguda, além de participar da ativação e da adesão de neutrófilos (ABBAS, 2007).

Desta forma os níveis séricos elevados de TNF parecem representar um fator importante de risco para a anemia falciforme. Banerjee e cols (2011) descreveram a associação do alelo mutante do *TNF- α* com os níveis séricos elevados desta citocina. Nossos resultados estão de acordo com os dados descritos anteriormente que associa o alelo A do TNF- α com níveis elevados desta citocina no soro de indivíduos HbSS.

Os níveis elevados de Il-8 têm sido descritos em indivíduos HbSS em estado estável da doença (GONÇALVES *et al.*, 1998). O polimorfismo -251A>T na região promotora do gene *IL-8* parece estar relacionado com a atividade transcricional desse gene (HACKING *et al*., 2004). Entretanto, este é o primeiro estudo que demonstra o impacto do polimorfismo -251A>T na gravidade clinica da anemia falciforme, uma vez que o alelo T parece estar relacionado com a atividade transcricional desse gene (HACKING *et al*., 2004).Desta forma, enfatizamos a importância do alelo A do gene *TNF- α* e o alelo T do gene *IL8* no perfil clínico do indivíduo HbSS, identificando possíveis biomarcadores de crise nesta doença. Além disso, os níveis elevados de IL-8 podem ser considerados marcador de mal prognóstico, uma vez que esta citocina parece promover o dano oxidativo, a ativação celular, a oclusão vascular e a inflamação como evento cíclico nos indivíduos HbSS.

Na introdução desta tese já descrevemos que a heterogeneidade clinica da anemia falciforme é bastante complexa e parece influenciar o sistema imune na manutenção da condição inflamatória crônica apresentada por esses indivíduos (HIGGS & WOOD, 2008). Assim, o estudo desenvolvido no desenho experimental CT2 que investiga a presença dos polimorfismos *TLR4* -896A>G, *TLR9* -1237T>C*, MPO* -463G>A*, IL-8* -251A>T*, TNF-*α -308G>A *e Fc*γ*RIIA H/R131 e* sua relação com

a gravidade da anemia falciforme poderá trazer dados sobre o efeito desses polimorfismos e a clínica dos pacientes.

Estudos anteriores mostram a relação entre os polimorfismos de TLR e doenças inflamatórias agudas e crônicas, tais como a *TLR4*+896A>G com infecções por bactérias gram negativas e risco de choque séptico (LORENZ *et. al*., 2002), outros trabalhos mostram o efeito protetor desse polimorfismo para ocorrência de isquemia cerebral (REISMANN *et a*l., 2004). Contudo, na população estudada foi encontrada associação do *TLR4*+896A>G com a ocorrência de AVE. Esses resultados estão sendo descritos inicialmente, mas gostaríamos de salientar o pequeno número de eventos encontrados neste grupo de indivíduos HbSS.

O alelo mutante do polimorfismo *TLR9*-1237 T>C tem sido associado à ocorrência de infecções respiratórias e asma na anemia falciforme (BOYD *et al.* 2006). Neste estudo, nós não observamos a associação deste polimorfismo com asma, mas encontramos associação entre o polimorfismo *TLR9*-1237 T>C e infecção respiratória. Ainda encontramos associação do alelo mutante *TLR4* +896A>G com a ocorrência de AVE, sendo que a literatura apresenta divergência sobre a associação deste SNP com isquemia (BALISTRERI, et al, 2004; LORENZ, et al, 2002). Porém, neste estudo, não foi possível determinar como o polimorfismo *TLR4* +896A>G influencia a ocorrência de AVE, sendo necessário a realização de estudo envolvendo uma casuística maior, bem como a ampliação do leque de moléculas investigadas, visando o encontro do mecanismo envolvido.

A mieoloperoxidase apossui papel importante na resposta imune destacando sua atividade microbicida (CASCORBI *et al*. 2000). O polimorfismo -463G>A tem sido descrito como marcador de disfunção endotelial (FORGIONE *et al*., 2000) e como indutor de arterioesclerose (NICHOLLS & HAZEN, 2005). Nossos achados

concordam com estudos anteriormente descritos que mostram a associação da MPO com a ocorrência de infecção.

O alótipo FcgRIIa-H131 está associado a imunidade mediada por anticorpos, pois é capaz de promover uma ligação eficiente com a IgG2. Em infecções bacterianas causadas por microrganismos encapsulados, tais como *Neisseria meningitidis* e *Haemophilus influenzae* a IgG aparece como principal mediador da resposta imune (NORRIS *et al.1996*). Alguns trabalhos mostram a associação do polimorfismo *FcγRIIA* H/R131 com as infecções e um desses trabalhos descreve a influência desse polimorfismo com infecção por mecanismos encapsulados em crianças HbSS *(*KARASSA *et. al*., 2002). Neste estudo, nós observamos a associação do alelo H com efeito protetor para hospitalização e a ocorrência de crises vaso-oclusivas. Esses dados estão de acordo com resultados anteriores que demonstram a relação entre o polimorfismo *FcγR2A H/R131* e a ocorrência de desordens vasculares *(*RAAZ *et al*., 2009; YUAN *et al*, 2009).

Quando analisamos concomitantemente o polimorfismo *FcγRIIA* H/R131 com o *TLR4 +896A>G ou* o *MPO -463G>A ou TNFα-306G>A* observamos o aumentando da ocorrência de complicações da doença. Nós observamos o efeito protetor do alelo H para ocorrência de hospitalização, AVE e crise quando associado a outro polimorfismo. Desta forma, o FcγRIIA parece exercer influencia na doença inflamatória e na função do endotélio vascular em indivíduos HbSS, uma vez que a combinação de mais de um polimorfismo parece exacerbar a gravidade da doença, uma vez que aumentando a ocorrência de crises vaso-oclusivas, a frequência de hospitalizações, bem como a ocorrência de sequestro esplênico também aumenta.

Os TLRs são receptores que reconhecem uma ampla diversidade de famílias de ligantes produzidos por bactérias, vírus ou fungos. Mais recentemente os TLRs

têm sido associados também à sinalização de processos inflamatórios não infecciosos (HORNER, 2006). Entender os mecanismos de sinalização é o grande desafio da imunologia. Assim, em nosso terceiro artigo nós investigamos a expressão do *TLR2*, *TLR4*, *TLR5* e *TLR9* em pacientes com anemia falciforme em crise. A expressão foi investigada em células dendríticas derivadas de monócitos e esses resultados foram obtidos pré e após o estímulo com LPS. Apesar de não ter encontrado associação entre as condições pré e pós estímulo com LPS, observamos que existe uma variação na expressão dos *TLRs.* Becher e cols (2000) descreveram que a participação dos monócitos na ativação do endotélio vascular está relacionada a participação de fatores transcricionais como o NF-kB como mediador da expressão de moléculas de adesão e fator tecidual. Trabalhos atuais mostram que a expressão do TLR nas CD exerce um amplo papel na apresentação de antígenos através da indução do processo inflamatório como um estímulo endógeno e não apenas como molécula apresentadora (CHEN & NUÑEZ, 2010). Desta forma, as células dendríticas podem contribuir como fator que influencia a ativação do estado inflamatório crônico da anemia falciforme, não somente por sua ação no endotélio vascular, mas também pela sua participação na expressão diferenciada de TLR.

As citocinas exercem papel fundamental nos processos de sinalização celular da resposta imune, promovendo inclusive a ativação de CDs e a expressão de TLRs (HAMMER & MA, 2013; BEUTLER, 2004). Quando investigamos os níveis de citocinas IL-1β, IL10 e TNF-α nos pacientes e em sobrenadante de cultura de CDs, nós observamos que parece haver uma cinética na expressão dessas citocinas, onde o tratamento com LPS promove o aumento das diferentes citocinas, comparando com as amostras não tratadas. A pneumonia foi um fator indutor da produção de IL-1β ($p<0.05$). Os indivíduos com níveis elevados de hemoglobina também apresentaram níveis mais altos de IL-1β ($p<0.05$). O aumento na expressão

do *TLR2* foi associado com a ocorrência de úlcera maleolar. Esses resultados concordam com estudos anteriores que descrevem a participação da IL-1β e do TNF-α in vitro na ativação do endotélio vascular (LANARO *et al.*, 2009; BELCHER *et al.*, 2000).

No contexto do ambiente inflamatório crônico podemos levantar algumas questões: que maneira essas citocinas induzem nos monócitos a expressão do CD1? Será que é a expressão das moléculas CD1 na superfície da CD que promovem a ativação vascular e a liberação das citocinas? Essas questões precisam ser mais amplamente estudadas, uma vez que as crises vaso-oclusivas podem ter um papel regulador no aumento ou na diminuição dos níveis plasmáticos de citocina (BELCHER *et al.*, 2000).

Em nossos resultados a expressão dos *TLR2, TLR4, TLR5* e *TLR9* nos diferentes indivíduos evidencia esses achados, uma vez que cada indivíduo internado tem uma expressão própria dos TLRs, Assim, não somente a expressão dos TLRs como também o microambiente gerado pela vaso-oclusão ou pela infecção através do aumento dos níveis de citocinas/interleucinas parecem corroborar com esse papel do TLR na fisiopatologia da anemia falciforme.

Os resultados apresentados neste estudo não devem ser analizados separadamente, mas levando-se em conta a associação entre os marcadores genéticos, fenotípicos e fatores ambientais, uma vez que o conjunto destes fatores tem sido associado aos diferentes fenótipos descritos entre estes pacientes. Estudos adicionais são necessários visando esclarecer a participação dos TLRs nos processos infecciosos e de vaso-oclusão, além da influência dos fatores genéticos na resposta imune e no desenvolvimento das manifestações clínicas presentes na anemia falciforme.

7. Conclusões

- O alelo A do *TNF-α* e o alelo T do gene *IL-8* sugerem que estes genes são fatores influenciam o fenótipo da anemia falciforme;
- As citocinas IL-8 e TNF-α apresentam níveis circulantes elevados, mesmo nos pacientes em estado estável da doença;
- O polimorfismo *FcγRIIA* H131R está associado com as complicações clínicas da anemia falciforme;
- A associação do polimorfismo *FcγRIIA* H131R com os polimorfismos estudados nos genes *TLR4*, *MPO e TNF-α* podem modular o perfil clinico da anemia falciforme e esses genes parecem exercem um efeito sinérgico.
- Existe um padrão de expressão diferenciada dos *TLR2*, *TLR4*, *TLR5* e *TLR9* em células dendríticas dos pacientes HbSS, que parece estar relacionado com a gravidade da doença;
- As citocinas IL1-β, TNF-α e IL-10 apresentaram níveis elevados em sobrenadante de cultura de células dendríticas dos indivíduos HbSS;
- A participação das células dendriticas através da expressão diferenciada dos TLRs, bem como a manutenção de um microambiente rico em citocinas parece favorecer a manutenção do estado inflamatório crônico da anemia falcforme, podendo influenciar a evolução clínica da doença.

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9. Apêndices

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Sickle cell disease: Only one road, but different pathways for inflammation

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ABSTRACT

Sickle cell disease (SCD) is a genetic disorder characterized by a chronic inflammatory process, and new biomarkers have been studied as promising molecules for understanding the inflammation in its pathophysiology. The hemolysis and the release of molecules associated to the hemoglobin (Hb) catabolism, such as free Hb, iron, and heme, generate an oxidant environment with production of reactive oxygen and nitrogen species. The immune system plays a very important role in the inflammation, with cells secreting pro-inflammatory cytokines and chemokines. There is also a nitric oxide (NO) resistance state, with an impaired NO bioactivity, leading to a vascular dysfunction; activation of platelet, leukocytes, erythrocytes, and endothelial cells, with expression of adhesion molecules and its ligands, and several receptors, that altogether participate at inflammatory process. During inflammation, there is an increase of dendritic cells (DCs) expressing toll like receptors (TLR), but the role of DCs and TLR in SCD pathogenesis is unclear. Also, there are molecules contributing for enhance the endothelium dysfunction, such as homocysteine that has been associated with vascular complications in the pathology of other diseases and it may contribute to the vascular complications presented by SCD patients. Circulating microparticules (MPs) levels are augmented in several diseases and have been de- scribed in SCD, where cells membrane compounds are associated to cell's thrombotic and coagulation state, such as tissue factor and phosphatidylserine (PS), which may contribute to endothelial dysfunction. The knowledge of all these biomarkers may contribute to new therapeutic approach discover, improving SCD patient life quality.

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Keywords: Sickle Cell Disease; Inflammation; Oxidative Stress; Cells Activation

1. INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder, and the sickle cell anemia (HbSS) is the more severe genotype. The disease is characterized by the presence of the hemoglobin S (HbS), where valine replace glutamic acid ($\beta^{S\ 6\ Glu\rightarrow Val}$) at the beta globin chain, that has a single point mutation (GAG →GTG) at the sixth codon of the β-globin (*HBB*) gene [1].

Sickle cell disease clinical outcome vary widely from mild to severe and has been associated with multi-organ damage and risk of early mortality [1], with acute and chronic clinical manifestations, including vaso-occlusive episodes (VOE), painful crisis, tissue ischemia/reperfusion injury, hemolysis, impaired blood flow as a result of intravascular sickling in capillary and vessels, inflammation processes and high susceptibility to infection, encephalic vascular accident (EVA), dactylitis, leg ulceration, pulmonary hypertension, acute chest syndrome, and priapism [1,2].

Moreover, the disease pathogenesis comprehends a complex network of mechanisms, involving the vaso-occlusive phenomenon and tissue ischemia, with surface and ligands molecules activation from stressed reticulocytes, sickled erythrocytes, leukocytes, platelets and endothelial cells [1-3]; there is also an increase of oxidative stress, secondary to the hemolysis episodes and heme cytotoxicity, electron donation from the iron atom when yet inside the protoporphyrin IX ring, with the generation via Fenton reaction of reactive oxygen and nitrogen species (ROS; RNS), that has a very strong pro-oxidant capacity. Also, there is an increase of nitric oxide (NO) scavenger molecules, a vasodilator that play important

role as regulators of vascular homeostasis in SCD pathogenesis [1,4].

Acute and chronic inflammatory phenomenon's can contribute to activate several cells types and may play important role in the steady- and crisis-states of SCD patients. The immune system pathway, has several mechanisms and needs to be better understood, including the participation of inflammation and cells markers, activation of molecules related to hemolysis, nitric oxide resistance, and some very important inflammatory mediators involved in the arachidonic acid pathway, including the synthesis of molecules such as, prostaglandin E2 (PGE2), thromboxane, and leukotrienes B4 (LTB4) [1].

There are many chemistry and genetic markers, which can modulate symptoms presented by SCD patients, such as alpha-thalassemia presence, reticulocytes count, lactate dehydrogenase (LDH) and bilirubin serum levels [5]. Currently, new biomarkers have been studied as promising molecules for understanding inflammation process in SCD, and have been highlighted the role of lipids metabolism and its participation in vascular injury, C-reactive protein (CRP) and inflammation, and the myeloperoxidase (MPO), a enzyme that had been related with patients susceptibility to infection [6-8].

Bilirubins are resulted of protoporphyrin IX metabolism, which in turn is a heme component. Sickle cell disease patients, particularly those HbSS, are at risk for bile pigment cholelithiasis due to the association of this disease with hemolysis, which produces an unconjugated hyperbilirubinemia [9]. Cholecystitis presents with abdominal pain, nausea and vomiting, fever, and/or jaundice, a constellation of symptoms that has multiple possible etiologies in SCD [10].

C-reactive protein (CRP), an acute-phase protein, increases significantly in inflammatory disorders and nowadays CRP has been used for evaluation of cardiac risk. CRP is produced not only by the liver but also in atherosclerotic lesions by vascular smooth muscle cells and macrophages in response to stimulation by the pro-inflammatory cytokine interleukin-6 (IL-6) [11]. SCD is associated with elevated cardiac output and cardiomegaly to partly compensate for the reduced oxygen-carrying capacity associated to hemolysis and oxidative stress. The combination of these events has been associated with increased levels of CRP in SCD since the childhood [12-14].

Myeloperoxidase is a lysosomal enzyme and plays an important role in the host defense system. MPO deficiency was associated with a higher occurrence of severe and chronic inflammatory processes in SCD patients, and the −463G > A *MPO* gene polymorphism may be a significant genetic modulator that makes HbSS patients more susceptible to infection [13].

An association between increased low-density lipoprotein cholesterol (LDL-C) and low plasma levels of high-density lipoprotein cholesterol (HDL-C) is an important risk factor for coronary disease. Actually it has been showed an association between coronary heart disease, high levels of LDL-C and MPO, since MPO catalyses the conversion of chloride and hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl), resulting in LDL-C oxidation and conversion into high-uptake forms, such as ox-LDL for macrophages, leading to cholesterol deposition and foam cell formation *in vivo* [14]. Data from our research group showed that some SCD patients can have a specific dyslipidemic subphenotype, characterized by low HDL-C with hypertriglyceridemia and high very low density lipoprotein cholesterol (VLDL-C) in association with other biomarkers, including those related to inflammation like ferritin and CRP [15]. These biomarkers may help in understanding the inflammatory mechanism associated with SCD as well as be used as predictor tests for severe events.

2. VASCULAR DYSFUNCTION AND INFLAMMATION: NITRIC OXIDE SCAVENGING AND ARGININE METABOLISM

In regard to the vascular complication of the HbSS, the decrease of nitric oxide (NO) bioavailability is now associated with the intravascular hemolysis [16], that participate in several important complication of HbSS patients, including pulmonary hypertension, leg ulcers, priapism and different types of stroke [16-18].

The nitric oxide is a diatomic gas produced by vascular endothelial cells that act as a potent vasodilator on smooth muscle cells. The NO synthesis is from the amino acid L-arginine, via an oxidation reaction catalyzed by the enzyme nitric oxide synthase (NOS) [19]. Nitric oxide also tonically inhibits platelet activation and the expression of endothelial adhesion molecules, thus participating in health endothelial function and in the maintenance of blood flow [20,21]. The reaction involveing vascular NO can have a beneficial antioxidant effect. Moreover, NO has been demonstrated to have a cytoprotective effect by scavenging reactive oxygen species (ROS) [22]. Solovey *et al*. [23] examined the hypothesis that enhanced endothelial tissue factor (TF) expression is modulated by endogenous NO produced by endothelial nitric oxide synthase (eNOS) in animal models. The mechanism by which NO exerts its inhibiting effect on TF have not been completely defined, although it is accompanied by parallel changes in amount of TF mRNA. Because NO exerts the same regulatory influence on vascular cellular adhesion molecule-1 (VCAM-1) expression, current results also have implications beyond inflammation and coagulation system, but also with VCAM-1 inflammatory expression.

Chronic elevated level of cell-free hemoglobin in SCD patients with intravascular hemolysis range from 2 to 20 uM per heme during steady-state and increase to approximately 20 to 40 uM during vaso-occlusive crises [24]. HbSS patients present a NO resistance state, associated with an impaired NO bioactivity that can be due by cell-free hemoglobin accumulation in plasma, intensifying the NO endogenous consumption that contribute for several cellular dysfunction, such as vasoconstriction and inflammation by leukocytes, platelets and endothelial cells activation [25]. Also, ROS are generated by hemolysis, and can react with NO, limiting its bioavailability and contributing to its state of resistance in HbSS patients [18]. The heme that is released from hemolysis also induces the expression of adhesion molecules from leukocytes, such as intercellular adhesion molecules-1 (ICAM-1), and from endothelium, such as VCAM-1.

Another mechanism of NO depletion during the hemolysis is the release of arginase-1 from lysed RBC that converts arginine to ornithine, which competes with the substrate, the eNOS, for L-arginine synthesis [26]. In recent research by our team, we confirm an increased levels of serum arginase-1 in HbSS patients when compared to health controls, as well as an association of serum arginase-1 with biochemical hemolysis markers and cytokines involved in Th17 response, levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) [27]. A very new insight in this metabolism lays on a shift in arginine catabolism, where transforming grow factor beta (TGF-beta) may induces the arginase pathway instead of the NO pathway, with a possible involvement of the vascular activation and the increase of serum arginase in chronic hemolysis among HbSS patients.

3. HOMOCYSTEINE AND SICKLE CELL DISEASE

Homocysteine (Hcy), a sulfur-containing amino acid, is found at low concentration in blood and cells and is an important intermediate molecule involved in the biosynthesis of methionine and cysteine [28]. The high plasma concentration of Hcy is a well-established risk factor for several disorders, including cardiovascular disease (CVD) and stroke [29], venous thrombosis and arteriosclerosis [30].

Hyperhomocysteinemia play an important role in vascular disorders and may act through increase cytotoxic activity, especially for endothelial cells; elevating H_2O_2 levels and decreasing NO synthesis, with pro-inflammatory cytokines synthesis, pro-coagulant factors activetion, and lipid metabolism dysregulation, characterized by LDL-C oxidative modification, enhancing of atherogenesis [31]. High levels of Hcy have also been implicated in changes in the rheological properties of blood, such as decreasing antithrombin III and tissue plasminogen activator (TPA), and increasing factor VII and CRP [32]. Additionally, Hcy is reported to enhance endothelial leukocyte interactions [33].

A chronic inflammatory state in vascular tissue is recognized to contribute to thrombotic and vaso-occlusive events in HbSS patients [34]. Since Hcy has been associated with vascular complications in the pathophysiology of other disease, it may contribute to vascular complications presented by HbSS patients.

The possibility that Hcy may contribute to the ischemic phenomena present in HbSS has attracted some interest in plasma total Hcy. Lowenthal *et al*. [31] showed that the median plasma concentration of Hcy among HbSS subjects was approximately 1.5-fold higher than that of healthy controls. Additionally, SCD patients have higher plasma Hcy concentration in spite of elevated plasma folate levels and vitamin B12 concentration similar to those observed in controls. In a recent study (2012) from our team, we found significant associations between Hcy levels and increased expression of pro-inflammatory cytokines and adhesion molecules (data not published) in HbSS patients, supporting the hypothesis that Hcy levels contribute to the vascular activation and with the inflammatory state presented by HbSS patients, and probably has an important role in vaso-occlusive mechanism.

4. INNATE IMMUNITY AND INFLAMMATION IN SCD

Individuals with SCD have transient and periodic painful vaso-occlusive episodes with exposure of organ to ischemia and reperfusion, which may activate inflammatory response in other organs, leading to multiple organ failure [35,36]. Despite ischemia and reperfusion occur in a sterile environment, and activation of innate and adaptive immune responses contribute to injury, including activetion of pattern-recognition receptors, such as Toll like receptors (TLRs) and inflammatory cell trafficking into the damaged organ [37]. Moreover, is well known that the presence of immunodeficiency should be associated with SCD, but no directly deficiency has been related to the immune system was observed to explain the amount of recurrent infections presented by these individuals [34]. On the basis of these, the immune response system seems to be related with health and inflammation in SCD.

The innate immune system is the first line of protection against invading microbial pathogens and of responses to inflammatory stimuli that are mediated by phagocytes, polymorphonuclear leukocytes (PMN), monocytes, macrophages, dendritic cells (DCs), and inflammatory T cell subsets (Th1 cells and Natural Killer T cells). This immune response relies on recognition of

evolutionarily conserved structures on pathogens, named pathogen associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs), of which the family of TLRs has been studied most extensively [38].

TLRs are integral glycoprotein characterized by an extracellular or luminal ligand binding domain containing leucine rich repeat (LRR) motifs and a cytoplasm signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain [38]. Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. To date, 10 TLRs have been identified in humans, and they can recognize distinct PAMPs derived from various microbial pathogens, including viruses, bacteria, fungi, and protozoa. TLRs can be divided into subfamilies primarily recognizing related PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids [39].

Cell types expressing TLRs are APCs, including macrophages, DCs, and B lymphocytes. TLRs have been identified in most cell types, expressed either constitutively or in an inducible manner in the course of infection [39]. During inflammation, increased numbers of DCs are rapidly recruited and efficiently capture antigens due to their high phagocytic ability. Subsequent to antigen capture DCs become activated and the mature DCs by pathogens can migrate into draining lymph nodes or the spleen and transforming to powerful antigen-presenting cells that are capable of activating naive T cells. The transition from immature to mature state is followed by production of several cytokines and chemokines by the DCs that regulate their ability to interact with naïve T cells to direct T cell differentiation [40].

Two major DCs subsets can be detected in the peripheral blood, with distinct, but overlapping functions. Myeloid DCs (mDCs) express human leukocyte antigens (HLA) DR, CD11c, and CD1c and are the main producers of interleukin-12 (IL-12), while plasmacytoid DCs (pDCs) express HLA DR, CD123, and blood dendritic cell antigen 2 (BDCA2), and are the main producers of interferon-α (IFN-α) [41]. However, HbSS patients in steady-state seems to have a particular CD1-positive phenotype expression of CD1a, b, and c molecules at DCs, while the classical phenotype found among the general population, in which only 15% of the individuals express the CD1 molecules at the surface of their monocytes [42]. According to Sloma *et al*. [43], the elevated concentration of cytokines associated with monocyte activation in HbSS patients can contribute for their activated status and it may be hypothesized that CD1 expression on DCs from HbSS patients is a consequence of the elevated plasma levels of endothelin.

The role of DCs in malaria affected population and its association with hemoglobinopathies have been described and show that the activation of mDCs and pDCs during acute malaria may be faster or deeper in children with α+thalassemia than in children with normal hemoglobin profile [44]. Other studies shows the *Plasmodium falciparum* glycosylphosphatidylinositol (GPI) anchors can bind to TLR2 and TLR4 expressed on mDCs and monocytes [45], whereas a component of schizont lysate as well as hemozoin can bind to TLR9 and activate pDCs [46].

Single-nucleotide polymorphisms (SNPs) have been described for *TLR*-4 and *TLR*-9 genes. For *TLR*-4, the polymorphism (Asp299Gly) has been related to Gram-negative infections susceptibility and septic shock [47]. More recently, the Asp299Gly has been involved as a protective allele against malaria, explaining its high prevalence in sub Saharan Africa [41].

The immune response in SCD is poorly understood. It is knows that the immune system has a close relationship with health and morbidity in SCD, although the complex network involved in the mechanisms of pathogenesis present in this disease, is difficult to understand, once it is a chronic inflammatory condition. Additional studies need to be warranted to elucidate the immunologic processes in SCD.

5. OXIDATIVE STRESS AND INFLAMMATION IN SICKLE CELL ANEMIA

Oxidative stress is a physiological condition that occurs when there is imbalance between the amount of free radicals (ROS; RNS) generated by physiological processes and antioxidant mechanisms. Free radicals are defined as chemical species that contains a pair of electrons unpaired, and this gives the high-capacity reactive free radical [48]. The reactive oxygen of species includes free radicals and non-free radicals, such as hydroxyl, superoxide (O_2^-) and H_2O_2. In biological systems, the most common source of free radicals is oxygen, and ROS that can be produced from both endogenous and exogenous cellular products [49,50].

The endogenous sources of ROS include mitochondria, cytochrome P450, peroxisomes, and inflammatory cells activated [51]. Mitochondria generates significant quantities of H_2O_2 and use ~90% of cellular O_2. During the process of reducing mitochondrial oxygen for production of water, several short-lived intermediates are produced, including H_2O_2, O_2^- and the hydroxyl radical [OH], which are toxic to the cell. Another molecule is the peroxynitrite ($ONOO^-$), an anion and an unstable isomer of nitrate (NO_3^-). The peroxynitrite can be formed in vivo by the reaction of the free O_2^- with free NO, and is a potentially cytotoxic molecule [52]. Cell destruction also causes further free radical generation [53]. Neutrophils,

eosinophils and macrophages are additional endogenous sources of cellular ROS. Activated macrophages initiate increase in oxygen uptake and give rise to a variety of ROS, including O_2, NO and H_2O_2 [54].

In addition, intracellular formation of free radicals can occur by environmental sources including ultraviolet light, ionizing radiation, and pollutants such as paraquat and ozone. All of these sources of free radicals, both enzymatic and non-enzymatic have the potential to inflict oxidative damage on a wide range of biological macromolecules [55]. Membranes are target of free radicals forming due to its lipid composition, lipid peroxide, thus compromising the characteristics of fluidity and elasticity, leading to cell rupture. Other target tissues to ROS are proteins, which may lose their functionality enzyme and cell signaling and DNA; the interaction of ROS with DNA can lead to the DNA strand breaks, point mutations, gene deletions, or gene rearrangement, such changes can be lethal to the cell, with DNA lesion, that accumulate with age, and can be an important etiology of aging processes [56]. The endogenous antioxidant system responsible for neutralize free radicals, include enzymes, such as glutathione peroxidase, superoxide dismutase (SOD) and catalase. The non-enzymatic antioxidants that participate in oxidative stress defense include ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione (GSH), carotenoid, and flavonoids [57-59]. The ROS occur under physiological conditions and in many diseases cause direct or indirect damage in different organs; thus, it is known that oxidative stress (OS) is involved in pathological processes such as obesity, diabetes, cardiovascular disease, and atherogenic processes [60].

Inflammation is an immune system reaction aiming to contain and eliminate pathogens or foreign elements to the body [61,62]. After activation, innate immune system cells secrete pro-inflammatory cytokines and chemokines that induce ROS/RNS production [63]. In the innate immune system, macrophages generate ROS, including O_2^-, NO, H_2O_2, hydroxyl radical, $ONOO^-$ and HOCl to play pathogen elimination [62,63]. Chronic inflammation can lead to cellular damage, hyperplasia and, consequently, to the overproduction of ROS by inflammatory cells [61].

In the HbSS, the chronic inflammatory state promotes the production of ROS and predicts the disease severity [64-68]. In the HbSS some events contribute to the maintenance of oxidative stress such as the excessive levels of cell-free hemoglobin with its catalytic action on oxidative reactions; the characteristic recurrent ischemia-reperfusion injury, a chronic pro-inflammatory state with higher autoxidation of HbS [69-71].

Iron is a chemical element that participates in the reaction of electron transfer between molecules in the process of cellular respiration (redox reactions), and it is deposited in the form of ferritin and hemosiderin. Patients receiving multiple blood transfusions, such as patients with chronic anemia, thalassemia, and HbSS, they can exceed the storage and detoxification capacity of ferritin. Consequently, the free iron begins to accumulate into tissues; this can catalyze the formation of very injurious compounds, such as [OH] by Fenton reaction [71]. Compared with normal red blood cells (RBC) membranes, those from sickle RBC have abnormally increased Fenton reactivity, once that the instability of HbS results in generation of $.O^-$ and H_2O_2, the combination of which potentially forms the .OH [70,71]. The phenomenon of sickling and vaso-occlusive events in HbSS are directly associated with its pathogenesis, and there is evidence that several inflammatory events occur with increased levels of inflammatory and anti-inflammatory cytokines, such as IL1β, IL4, IL6, TNFα, the expression of cell adhesion molecules, such as ICAM-1, VCAM-1, P-selectin and integrins; the adhesion of activated PMN to the endothelium; the participation of activated platelets, and the presence of inflammatory biomarkers, such as CRP and prostaglandins. These factors contribute to the occurrence of vaso-occlusion and chronic organ damage, favoring an increased production of ROS [65,72-77].

6. CYTOKINE IN SICKLE CELL ANEMIA: BREAK THE BALANCE

Pro-inflammatory cytokines mainly expressed by monocytes from stimulation of bacterial components, ROS and growth factors have important role in immune innate response and inflammatory state [78]. These effects are modulated by anti-inflammatory cytokines, such as IL-4 and IL-10, necessary to down regulate leukocytes and the vascular endothelium activation [79]. This delicate balance is broken when inflammatory cytokine increase oxidative stress and overcoming antioxidant barrier by activation of complex transporter of electron in mitochondria [78,80,81], stimulating the transcription of the trans factor NF-kB with degradation of IkB, up regulating the expression of selectins, integrins, ICAM-1, VCAM-1, resulting on pre-activation of leukocytes and with the interaction of these molecules with the activated endothelium [81-83].

Cytokine levels in HbSS patients are elevated not only during crisis-state, but also in different pathophysiologic mechanisms of the disease, like hypoxia and reperfusion rate; beyond clinical inflammatory history generate an increased concentration of these molecules also in steady-state patients. Pro-inflammatory cytokines like IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-alpha are increased on basal state and during vascular occlusion events unaccompanied of an increased level of anti-inflammatory cytokines,

such as IL-4 and IL-10 [3,20,79,84-88]. This inflammatory profile is not consensus; maybe it is associated with a high inter-individual variation, mainly due difference of genetic background and environmental aspects that can generate these molecules plasma levels fluctuation.

Soluble vascular cell adhesion molecule-1 (sVCAM-1) amount increase in plasma and change accord to the profile expression of selectins and integrins on leukocytes, such as CD62L and CD11b indirectly reflecting the rate of inflammation, and oxidative stress and have been associated as promising markers of HbSS prognosis [27, 89-91]. Based on these, oxidative stress and inflammatory profile may have complementary and symbiotic effects, mainly in a chronic inflammatory and oxidative disease, such as HbSS.

Inflammatory and oxidative profile, therefore, are extremely connect. This link is also confirmed when it is analyzed the conversion of purines to uric acid by the xanthine oxidase action [92,93]. This reaction generate free radicals, like intermediary products, and is up regulated by pro-inflammatory cytokines due many potential cytokine responsive elements in the *xanthine oxidase* (*XO*) gene regulatory region [94].

Therefore, up regulation of *XO* gene generates more uric acid and contact of this molecule with free sodium driving the monosodium urate that is consider to be a biologically active structure [95]. The monosodium urate is an active form act as a danger signal by activation of inflammasome, resulting on the production of pro-inflammatory cytokines, such as IL-1 and IL-18 [96]. In recent research by our team, inflammasome pathway was observed in HbSS patients, where uric acid, considered as a danger signal was associated with high serum levels of IL-18, further connect to cytokines levels and oxidative stress markers [91]. These effects, accordingly, are amplified on a chronic hemolytic and inflammatory disease, like HbSS, where the intravascular hemolysis contribute to release of cell free hemoglobin, heme and iron, consequently increase of oxygen radicals, limiting NO bioavailability, attracting more leukocytes, activating endothelial cells, contributing to the vascular occlusion [2,15,17,27,91].

7. PLATELETS AND INFLAMMATION IN SICKLE CELL DISEASE

Platelets are small enucleated structures derivatives from megakaryocyte fragmentation and are important to homeostasis process, primary function originally described to platelets. Platelets undergo activation, adhesion and aggregation binding to damage blood vessel, producing a platelet plug and contributing to the generation of thrombin. Furthermore, platelets produce and store a variety of molecules that affect platelet function and modified the vascular tone, the fibrinolysis [97]. Leukocytes and endothelial cells are associated as a critical player in the microvascular alteration induced by inflammation. Actually, have been thought about the role of platelets in inflammatory states through leukocytes interaction, release proteins, chemokines and endothelial disfunction [98].

Some studies suggest that circulating platelets in HbSS patients are chronically activated, both during steady-state and vaso-occlusive crises, which may result of the overall hypercoagulable state or with the vaso-occlusive process or with the pro-inflammatory characteristics of the microvasculature in HbSS [20,99,100].

Platelets have important organelles to performance its function, such as alpha-granules, lysosomes, peroxisomes, dense bodies and a complex membranous system that contribute to store and rapidly release several factors and proteins [99,101]. Among released products by platelets, there are secretion of adhesion proteins, such as fibrinogen, von Willebrand factor (vWF), thrombospondin, P-selectin, GPIIb/IIIa; there are important chemokines, including RANTES and platelet factor-4; cytokine-like factors as IL1-β, CD40L, β-thromboglobulin or factors essentials for the coagulation process, as plasminogen activator inhibitor (PAI-1), protein S and factors V and XI, and also expression of innate receptors of the Toll-like receptor family, such as TLR2 and TLR4 [102-105].

During inflammatory process, activated endothelial cells and others perivascular cells and leukocytes, release several soluble mediators, such as lipids, IFN-γ, IL-2, and CXCL12, which bind to platelet receptors, leading to degranulation of platelets dense and alpha-granules, promoting self adhesion and activation. Furthermore, the OS actives phospholipase A2 and the generation of the arachidonic acid pathway metabolites and platelet activator factor (PAF), and also contribute for platelet activation [103,105]. On the other hand, the platelet activetion can induce several inflammatory responses in monocytes, neutrophils, endothelial cells or endothelial progenitor cells; product released by platelets are potent inflammatory and mitogenics substances, modifying the chemostatic, adhesive and proteolytic properties of cellular microenvironment, mainly of endothelial cells and leukocytes, resulting in an increase of transmigration of leukocytes to the site of inflammation, suggesting that platelets-leukocytes interaction may be a key role in the initiation of inflammation [98,103,104].

Many mechanisms of platelets-leukocytes interactions have been described, but the initial interaction appears to be mediated by P-selectin expressed on the surface activated platelets and P-selectin ligand glycoprotein-1 **(**PSGL-1) on the surface of neutrophils and monocytes and, subsequently, firmly adhere by binding of Mac-1 to GPIb or other receptors of the platelet membrane [98, 104]. The P-selectin was found in sickle cell transgenic mice with high constitutive levels, which could be attri-

buted to platelet activation, contributing to inflammatory response [98,104]; study suggest the P-selectin-mediated platelet-neutrophil aggregate formation, which activates neutrophils in SCD mouse model and human been carriers [104]. The sCD40L level is increased and biologically active in HbSS patients due to platelet activation, mainly, in patients in crises and positively correlates with an increase of TF and ICAM-1 expression, suggesting that an increase of CD40L may contribute to the chronic inflammation and with the increased pro-coagulant activity in HbSS patients [105].

Thus, several studies suggest that in addition to pro-coagulant role, platelets contribute directly to constant vascular inflammation state in HbSS patients by activateing neutrophils and monocytes and further research about therapies targeting function and interaction of platelets and endothelial cells and leukocytes may help to control inflammation and vaso-occlusive events among these patients.

8. CIRCULATING MICROPARTICLES: NEW INFLAMMATION BIOMARKERS IN HBSS

Microparticles (MPs) are heterogeneous group of membrane-bound vesicles described as vesicles smaller than 10^{-15} m in diameter [106]. MPs are shedding from plasma membranes, after cell activation or apoptosis of several cellular types. Essentially any cell type (e.g., leucocytes, and endothelial cells), but also platelets and RBC can release MPs [107,108]. They have been implicated to play a role in inflammation, coagulation and vascular function.

During blebbing, the lipid bilayer forms cytoplasmatic protrusions, culminating in the release of MPs [109]. This process involve an increase in intracellular calcium which affects many important enzymes for the maintaining of the cytoskeletal and membrane structure, such as gelsolin, calpain, flippase, floppase and scramblase [108].

As the lipid and protein composition of the MPs membrane resembles that from the releasing cell, analysis of MPs surface markers by flow cytometry can identify the MPs origin. Internally, MPs contain a variety of cytoplasmatic and nuclear components of their precursor cells [110]. Circulating MPs levels result from the balance between their rates of release from cells and their clearance from the circulation.

MPs levels are augmented in several diseases, such HbSS. Patients have elevated MPs, both in steady-state and crisis, implying that even patients in steady-state are fundamentally different from healthy subjects [111,112]. Total circulating MPs are more elevated in the crisis phase of the disease than in steady-state [111,113]. During crisis, endothelial damage and coagulation activation increase dramatically, and those condition are accompanied by an increases of circulating MPs [113]. Thus, it is worth considering circulating MPs as biomarkers of HbSS.

Sickle cell anemia patients have an increased risk of vascular thrombotic occlusion [114]. In addition, there is strong *in vitro* evidence that circulating MPs are involved in the coagulation system activation in HbSS [115,112]. The pro-coagulant activity depends of some molecules presence, such as phosphatidylserine (PS) and TF, both exposed on several MPs types outer membranes [110]. Therefore, this pro-coagulant activity may be relevant clinically as MPs concentrations with this phenotype are elevated in HbSS.

The majority of circulating MPs in HbSS has RBC and platelets origin [116]. The report by van Beers *et al*. [116] showed a strong association between erythrocyte-derived MPs and markers of *in vivo* coagulation and fibrinolysis activation status as well as endothelial activation [116]. In addition, MPs may support coagulation activation by exposure of PS [115,116], which offers multiple binding sites for the coagulation factors II, Va, and Xa [117]. Sickle cell anemia patients have elevated plasma levels of annexin A5- and PS-exposing MPs [111,112]. Thus, MPs can provide a platform for the assembly of the prothrombinase complex and accelerate the conversion of prothrombin into thrombin.

Importantly to vascular homeostasis has been the discovery of TF, the principal initiator of coagulation, in MPs from HbSS. Sickle blood contains a fraction of MPs originating from platelets [114], endothelial cells and monocytes [113], which are TF positive. Furthermore, once initiated by TF, thrombin generation is greatly accelerated in the presence of PS [67] and co-expression of these molecules can contribute to thrombotic events frequently observed in patients with HbSS [118]. Importantly, MPs could be capable to initiate blood coagulation.

Microparticles are emerging as important biomarker of inflammation, coagulation and thrombosis in HbSS. Linked to crucial steps of HbSS, MPs can now be viewed "partners in disease", especially in patients in crisis-state. MPs provide a vehicle to couple inflammation and coagulation, contributing to thrombotic tendencies in this disease. This increasingly close relationship between MPs and HbSS demonstrates the need for more studies on this subject. Thus, it is necessary additional research to define the precise role of circulating MPs in HbSS and allow the development of new therapeutic strategies either blocking the release of MPs or modifying their activity.

9. CONCLUSION

It is very well known that SCD is a group of genetic disorders, with 101 years of its first medical relate, but still has several pieces of the puzzle to be solved. The search for pathways and biomarkers involved in the pathophysiology of the disease are still need to be exhausted search, and it will bring the knowledge of molecules that

may contribute to increase SCD patients life quality, given opportunity for new therapeutic approaches and clinical management modalities. This review just point several mechanisms associated with SCD, and may contribute to give some ideas about the very complex molecules network involved in the inflammatory process associated with the disease pathogenesis.

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LIST OF ABBREVIATIONS

BDCA 2: blood dendritic cell antigen 2;
CRP: C-reactive protein;
CVD: cardiovascular disease;
DC: dendritic cells;
EVA: encephalic vascular accident;
GPI: glycosylphosphatidylinositol;
GSH: glutathione;
Hb: hemoglobin;
HBB: β-globin gene;
HBSS: sickle cell anemia;
Hcy: Homocysteine;
HDL-C: high-density lipoprotein cholesterol;
HLA: human leukocyte antigen;
H_2O_2: hydrogen peroxide;
HOCl: hypochlorous acid;
ICAM-1: intercellular adhesion molecule-1;
IL-1: interleukin-1;
IL-12: interleukin-12;
IL-6: interleukin-6;
INF-α: interferon-α;
LDH: lactate dehydrogenase;
LDL-C: low-density lipoprotein cholesterol;
LRR: leucine rich repeat;
LTB4: leukotriene B4;
mDC: myeloid dendritic cells;
MPO: myeloperoxidase;
MPs: Microparticles;
NO: nitric oxide;
NO_3^-: nitrate;
NOS: nitric oxide synthase;
[OH]: hydroxyl radical;
$ONOO^-$: peroxynitrite;
OS: oxidative stress;
O_2^-: superoxide;
PAF: platelet activator factor;
PAI: plasminogen activator inhibitor;
PAMP: pathogen associated molecular patterns;
pDC: plasmacytoid dendritic cells;
PGE2: prostaglandin E2;
PMN: polymorphonuclear leukocytes;
PRR: pattern recognition receptors;
PS: phosphatidylserine;
PSGL-1: P-selectin ligand glycoprotein-1;
RBC: red blood cells;
RNS: reactive nitrogen species;
ROS: reactive oxygen species;
SCD: sickle cell disease;
SNP: Single-nucleotide polymorphisms;
SOD: superoxide dismutase;
sICAM-1: soluble intercellular adhesion molecule-1;
sVCAM-1: soluble vascular cellular adhesion molecule-1;
TF: tissue factor;
TGF-beta: transforming grow factor beta;
TLR: toll like receptor;
TPA: tissue plasminogen activator;
VCAM-1: vascular cellular adhesion molecule-1;
VLDL-C: very low density lipoprotein cholesterol;
VOE: vaso-occlusive episodes;
vWF: von Willebrand factor;
XO: xanthine oxidase.

IL-8 E TNF-ALFA: MARCADORES IMUNOLÓGICOS NO PROGNÓSTICO DA ANEMIA FALCIFORME

IL-8 AND TNF-ALPHA: IMMUNOLOGICAL MARKERS IN SICKLE CELL ANEMIA PROGNOSTIC

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A hemoglobina S (HbS) possui frequência elevada no Brasil, sendo a Bahia o Estado com maior incidência da doença falciforme. A doença falciforme possui quadro clínico heterogêneo, caracterizado por episódios de vaso-oclusão e eventos infecciosos, aspectos que contribuem para a condição pró-inflamatória crônica descrita entre os seus portadores. O objetivo desse estudo foi investigar aspectos imunológicos e fenotípicos em indivíduos com anemia falciforme provenientes de Salvador, Bahia. O estudo foi desenvolvido em 126 pacientes, sendo 103 em estado clínico estável da doença e 23 hospitalizados por vaso-oclusão e/ou infecção. Foram investigados os polimorfismos -251T>A e -308G>A, localizados respectivamente nos genes da *IL-8* e do *TNF-alfa,* por PCR e PCR-RFLP; os níveis séricos da IL-8 foram detectados por ELISA e a história clínica dos pacientes foi obtida dos prontuários médicos. Houve associação entre a presença do alelo mutante para o polimorfismo -308G>A e a ocorrência de sequestro esplênico (p<0,05). Interessantemente, os níveis séricos de IL-8 foram 32,3 ± 43,3pg/ml no grupo de pacientes em crise permanecendo elevados após a alta hospitalar, diminuindo gradualmente. O alelo A do polimorfismo -251 foi associado aos níveis elevados de IL-8, independente do fator indutor. Juntos, nossos resultados mostraram a importância da interação dos marcadores de prognóstico no monitoramento dos portadores com anemia falciforme e a participação da IL-8 no processo de vaso-oclusão. Estudos adicionais são necessários para estabelecer o papel dos níveis de IL-8 e de TNF-alfa, bem como a presença de polimorfismos nesses genes, visando a sua utilização como marcadores de prognóstico no acompanhamento clínico desses indivíduos.

Palavras-chave: citocinas, prognóstico, anemia falciforme.

Hemoglobin S (HbS) has a high prevalence in Brazil, the Bahia state with the highest incidence of sickle cell disease. Sickle cell disease is a clinical heterogeneous conditions characterized by episodes of vaso-occlusion and infectious events, aspects that contribute to the pro-inflammatory chronic state described among its carriers. The aim of this study was to investigate phenotypic and immunological aspects of patients with sickle cell anemia from Salvador, Bahia. The study was conducted in 126 patients, 103 in steady-state of the disease and 23 hospitalized for vaso-occlusion and / or infection. We investigated the polymorphisms -251T>A and-308G>A respectively located in the genes of IL-8 and TNF-alpha by PCR and PCR-RFLP; serum levels of IL-8 were detected by ELISA and the clinical history of patients was obtained from medical records. There was an association between the presence of the mutant allele A for the polymorphism -308G>A and occurrence of splenic sequestration (p<0.05). Interestingly, serum levels of IL-8 were 32.3 + 43.3 pg / ml. The A allele of -251 polymorphism of IL-8 gene was associated with elevated levels of IL-8, independent of the inducing factor. Together, our results showed the importance of the interaction of prognostic markers in the monitoring of patients with sickle cell anemia and the involvement of IL-8 at the process of vaso-occlusion. Additional studies are warranted to establish the role of interleukin-8 and TNF-alpha, and the presence of polymorphisms in these genes, in order to confirm their use as prognostic markers in the clinical follow up of these individuals.

Keywords: Cytokines, prognostic, sickle cell anemia.

A doença falciforme compreende grupo de distúrbios hereditários caracterizado pela presença da hemoglobina S (HbS), que é decorrente da mutação pontual no sexto códon do gene da globina β, levando a substituição do ácido glutâmico por valina na sexta posição da cadeia polipeptídica beta ($\beta^{S\ 6\ Glu \rightarrow Val}$)[27]. Os homozigotos para a HbS são portadores da anemia falciforme (AF) e apresentam anemia hemolítica grave. Outras hemoglobinas variantes podem estar associadas à HbS, como as hemoglobinas C, D e E, dentre outras, podendo haver também interações com as talassemias. A associação dessas hemoglobinas determina a gravidade da doen-ça falciforme e os portadores apresentam quadro clinico heterogêneo variando entre formas intermediárias a graves[12 31].

Os indivíduos com AF apresentam quadro clínico heterogêneo, com gravidade elevada, podendo apresentar

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retardo no crescimento e desenvolvimento, bem como alterações em vários órgãos, sempre em decorrência da hemólise contínua, de fenômenos vaso-oclusivos e hospitalizações frequentes[1 2 6]. Outras complicações são o acidente vascular cerebral (AVC); a síndrome torácica aguda; úlceras de perna; sequestro esplênico; alterações pulmonares e oculares e o priapismo, entre outras[5 18 28 32]. As infecções são comuns na AF, sendo consideradas causa importante de morbidade e mortalidade em crianças. Alguns estudos referem às crises vaso-oclusivas e infecções como as principais causas de hospitalização entre esses indivíduos[21 26].

A fisiopatologia da anemia falciforme é multifatorial, e a heterogeneidade clínica da doença é decorrente da interação de diversos fatores, tais como, a quantidade elevada de hemoglobina polimerizada no interior das hemácias, contribuindo para ativação de reticulócitos, hemácias falcizadas, leucócitos e endotélio vascular, com o aumento da expressão de moléculas de adesão como a ICAM-1 e VCAM-1. Além disso, são descritas alterações na concentração de hemoglobina total e de hemoglobina fetal (HbF); o aumento no número de leucócitos, ativação de monócitos, expressão de proteínas de fase aguda, citocinas e quimiocinas[4 18 26 28].

Cumpre ressaltar que alterações no sistema imune que estão associadas a deficiências na via alternativa do complemento, bem como alterações na função de leucócitos podem contribuir para a ocorrência de infecções frequentemente descritas nesses pacientes[4 8 18].

Os aspectos imunológicos dessa doença têm sido cada vez mais estudados. Os níveis elevados de citocinas Th2 (IL-4, IL-6 e IL-10) e de citocinas pró-inflamatórias (IL-8 e TNF-alfa) no plasma de indivíduos com anemia falciforme em estado estável têm sido descritos, possivelmente relacionados ao aumento da expressão ou ativação das moléculas de adesão em neutrófilos e no endotélio vascular, apesar do papel fundamental dessas citocinas na fisiopatologia da anemia falciforme ainda não estar completamente claro[29].

Os níveis séricos elevados de IL-8 tem sido observados em indivíduos em crise vaso-oclusiva, aspecto clínico importante da patogênese da anemia falciforme[9]. O polimorfismo -251T>A presente na região promotora do gene IL-8 tem sido associado a níveis elevados desta citocina, sendo que a presença do genótipo AA foi relacionada à gravidade da doença[13].

O polimorfismo –308G>A na região promotora do gene do TNF-alfa tem sido associado a diferentes condições inflamatórias e a presença do alelo mutante A parece influenciar na expressão do TNF-alfa, sendo o genótipo AA considerado alto produtor[10 17].

Neste trabalho investigamos os níveis séricos de IL-8 em indivíduos com anemia falciforme na fase estável da doença e quando hospitalizados, visando o encontro de uma possível associação com o histórico clínico. Também foi investigada uma possível correlação dos polimorfismos -251T>A no gene da IL-8 e -308G>A no gene do TNF-alfa com a gravidade clínica da anemia falciforme.

Material e Métodos

Casuística

Foram investigados 126 indivíduos com anemia falciforme distribuídos em dois grupos distintos: grupo de pacientes em estado clínico estável da doença (PE) composto por 103 indivíduos acompanhados regularmente no ambulatório de Hematologia da Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA), da Secretaria de Estado da Saúde da Bahia (SESAB), entre Agosto de 2005 a Setembro de 2006; e o grupo de pacientes em crise (PC) formado por 23 pacientes em idade pediátrica internados no Hospital da Criança (HC) das Obras Sociais Irmã Dulce, no mesmo período (de 08/2005 a 09/2006).

No grupo PE, a coleta de sangue venoso em EDTA foi realizada durante a consulta ambulatorial e os dados clínicos foram obtidos através de busca retrospectiva nos prontuários médicos. No grupo PC, foram incluídos pacientes menores, internados por vaso-oclusão e/ou infecção e realizadas duas coletas de sangue, sendo a primeira coleta nos primeiros dias de internação e a segunda no dia da alta hospitalar, quando o grupo foi denominado paciente em alta (PA). Os dados clínicos foram obtidos a partir dos prontuários médicos dos pacientes, sendo considerada como idade pediátrica aquela inferior a 18 anos.

A frequência dos polimorfismos de citocinas foi investigada entre os portadores de anemia falciforme em um grupo de referência da população de Salvador, sendo composto por 212 indivíduos saudáveis.

Esse trabalho foi aprovado pelo Comitê de Ética em Pesquisa em Seres Humanos da Fundação Osvaldo Cruz (CAAE-0010.1.225.000-05) e está em acordo com a Declaração de Helsinki de 1975 e sua revisão de 2000.

As amostras de sangue e os dados clínicos e demográficos foram coletados após a assinatura do Termo de consentimento livre e esclarecido pelos pacientes ou responsáveis legais.

Análises hematológicas e de hemoglobinas

A determinação dos valores hematológicos e índices hematimétricos foi realizada em contador eletrônico de células (*Coulter Count T* – 890, *Beckman Coulter*) e a análise morfológica das hemácias pela observação microscópica de esfregaços sanguíneos corados pelo método de Wright. O perfil de hemoglobinas foi confirmado pela técnica de cromatografia líquida de alto desempenho (HPLC) em equipamento automatizado (*Variant II- Bio-rad*).

Ensaios de genotipagem

O DNA genômico foi isolado a partir de 200µL de sangue periférico, utilizando-se o método direto *QIAamp® DNA Mini Kit (Quiagen)* e armazenado a -20ºC. Os polimorfismos nos

genes das citocinas -251T>A no gene da *IL-8* e -308G>A no gene do TNF-alfa foram investigados pela reação de PCR e posterior digestão com enzimas de restrição (RFLP)[11, 25]. Os produtos obtidos nas reações foram analisados em gel de agarose (1%) e os produtos da RFLP em gel de poliacrilamida (7%).

Detecção dos níveis séricos de citocinas

As dosagens de IL-8 e de TNF-alfa foram realizadas pela técnica de ELISA (*Enzyme-linked Immunosorbent Assay*) (*BD OptEIA - Biosciences)* de acordo com as instruções do fabricante, sendo considerado como valor de referência normal ≤15pg/mL para IL-8 e ≤7,8pg/mL para o TNF-alfa.

Análises estatísticas

As análises estatísticas foram realizadas nos programas EPI-INFO versão 6.04 e GraphPad Prism versão 5. As análises de regressão linear foram realizadas com o programa estatístico SPSS versão 9.0. Os valores de p foram considerados significativos quando menores que 0,05.

Resultados

Os pacientes que compuseram o grupo PE apresentaram idade média de 14,5 (± 12,4) anos. Entre eles, 81(80,2%) apresentaram histórico de algum tipo de comorbidade, sendo mais frequentes as crises vaso-oclusivas em 90/103 (87,4%), as infecções em 48/103 (46,6%), dentre elas as infecções, as do trato respiratório (ITR) foram descritas em 25/48 pacientes (52%), as pneumonias em 18/48 (37,5%), broncopneumonias em 9/48 (18,7%), infecção do trato urinário (ITU) em 6/48 (12,5%) e osteomielite em 4/48 (8,3%).

No grupo PC, 78,3% apresentaram história de algum tipo de infecção, sendo as mais comuns as pneumonias (60,8%), a infecção do trato urinário (39,1%) e infecção do trato respiratório (13%). A Tabela 1 mostra o histórico das comorbidades mais frequentes nos pacientes dos grupos estudados de acordo com a idade.

Tabela 1. Distribuição por faixa etária (em anos) do histórico de manifestações clínicas entre os pacientes com anemia falciforme dos dois grupos estudados (grupo de pacientes em estado clínico estável; e grupo de pacientes em crise).

COMORBIDADES	**FAIXA ETÁRIA**, anos – n(%)				p
	2 |—| 5 (n=30)	6 |—| 12 (n=45)	13 |—| 20 (n=32)	>21 (n=19)	
Vaso-oclusão	28 (93,3)	42 (93,3)	28 (87,5)	13 (68,4)	0,011[a]
Acidente vascular cerebral	2 (6,7)	0	2 (6,3)	2 (10,5)	0,62[a]
Sequestro esplênico	5 (16,7)	2 (4,4)	0	0	0,008[a]
Infecção do trato urinário	5 (16,7	8 (17,8)	0	2 (10,5)	0,21[b]
Infecção do trato respiratório superior	6 (20)	10 (22,3)	7(21,9)	5 (26,3)	0,24[a]
Pneumonia	9 (30)	13 (28,9)	4 (12,5)	2 (10,5)	0,36[a]
TOTAL	126	75	41	24	-

[a] Teste exato de Fisher; [b] $\chi 2$ corrigido pelo Yates.

No grupo PE, a maioria (93,2%) estava em uso profilático regular de ácido fólico, 40,7% usavam analgésicos e 28,1% antibióticos. Não foi encontrada correlação entre o uso de penicilina profilática e a ausência de infecção (OR=1,33; IC=0,57–3,15). Entre os indivíduos do grupo PC observamos que 78,3% usavam regularmente ácido fólico, 39,1% utilizavam antibioticoterapia, também regular, com penicilina; e 21,7% utilizavam analgésicos.

Com relação ao estudo dos polimorfismos de citocinas observamos que o polimorfismo -251T>A no gene da IL-8 e -308G>A no gene do TNF-alfa estavam em equilíbrio de Hardy-Weinberg. A Tabela 2 mostra a distribuição dos genótipos no grupo de pacientes com anemia falciforme relacionando com as principais comorbidades.

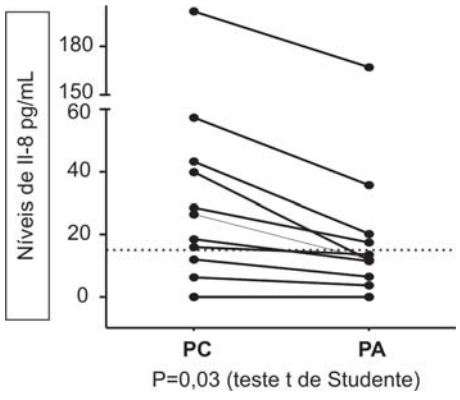
Tabela 2. Polimorfismos nos genes de citocinas e aspectos fenotípicos no grupo de pacientes com anemia falciforme.

Polimorfismos (n)	**Diagnósticos** – n (%)				
	Infecção	Pneumonia	ITRS*	SE**	AVC***
-251 da *IL-8*					
AA +AT (85)	48 (55,8)	18 (20,9)	17 (19,7)	5 (5,8)	4 (4,7)
TT (41)	20 (47,6)	7 (16,7)	12 (28,6)	2 (4,8)	3 (7,1)
Valor de p	0,49[a]	0,73[a]	0,37[a]	1[b]	0,68[b]
-308 *TNF-alfa*					
GG (101)	55 (55)	21 (21)	22 (22)	3 (3)	5 (5)
GA + AA (25)	13 (46,4)	4 (14,2)	7 (25)	4 (14,2)	2 (7,1)
Valor de p	0,55[a]	0,60[a]	0,75[a]	0,04[b]	0,64[b]

*ITRS, Infecção do trato respiratório superior; **SE, Sequestro esplênico; ***AVC, Acidente Vascular Cerebral; [a]$\chi 2$ corrigido pelo Yates; [b]Teste exato de Fisher.

Os níveis séricos de IL-8 foram estimados em 53 pacientes do grupo PE, 22 do grupo PC e 13 do grupo PA. Os pacientes pertencentes ao grupo PA foram os mesmos indivíduos do grupo PC que tiveram uma nova avaliação no momento da alta médica. A média dos níveis séricos de IL-8 foram 6,7 pg/mL (1,7 – 38,8) e de TNF-alfa 31,5 (1,3 – 68,8). A análise pareada dos níveis séricos de IL-8 no grupo PC e PA demonstrou a diminuição gradual dos níveis de IL-8, sendo que os indivíduos em alta médica ainda apresentavam níveis elevados de IL-8 (p<0,05, Teste t de Student pareado) (Gráfico 1).

Gráfico 1. Níveis séricos de IL-8 em pacientes internados (PC) e quando da alta hospitalar (PA).



A Tabela 3 mostra os resultados da associação dos diferentes genótipos dos polimorfismos nos genes da IL-8 e TNF-alfa nos pacientes pediátricos dos grupos PE e PC.

Tabela 3. Associação entre os genótipos do polimorfismo -251T>A (IL-8) e -308G>A (TNF-alfa) e níveis séricos de IL-8 no grupo de pacientes pediátricos em estado clínico estável da doença (PE) e em crise (PC).

Genótipo da citocina	Grupo do paciente n(%)		Valor de *p*	Níveis séricos de IL-8 (pg/mL)		Valor de *p*
	PE	PC		PE Média ± DP	PC Média ± DP	
IL-8						
AA	10 (14,9)	7 (30,4)	0,24[a]	1,2 ± 3,0	22,1 ± 26,5	0,06[c]
AT	37 (55,2)	8 (34,8)	0,14[a]	7,0 ± 12,	59,9 ± 89,5	0,06[c]
TT	20 (29,8)	8 (34,8)	0,85[a]	1,6 ± 4,4	17,9 ± 16,9	0,003[c]
AA+AT	47 (70,1)	15 (65,2)	0,85[a]	5,7 ± 10,9	41,0 ± 66,4	0,0091[c]
AT+TT	57 (85,0)	16(69,5)	0,93[a]	5,1 ± 10,3	37,5 ± 63,7	0,001[c]
TNF-alfa						
GG	55 (82)	20 (86,9)	0,75[b]	3,5 ± 8,4	35,6 ± 57,9	0,002[c]
GA	12 (18)	3 (13,1)	0,75[b]	8,3 ± 13,2	13,8 ± 12,6	0,55[d]
GG+GA	67 (100)	23 (100)	-[e]	4,5 ± 9,6	32,6 ± 54,3	0,0001[c]

[a]$\chi 2$ corrigido pelo Yates; [b]Teste exato de Fisher;[c]Kruskal-Wallis; [d]ANOVA; [e]Não foi realizada análise estatística, uma vez que não foi encontrado o genótipo AA para o polimorfismo no gene do TNF-alfa.

Discussão

As manifestações clínicas mais comumente encontradas nos pacientes com anemia falciforme do grupo PE foram as crises vaso-oclusivas, infecções e sequestro esplênico, confirmando a heterogeneidade fenotípica da anemia falciforme[12 24]. Quando os pacientes foram analisados de acordo com a idade, observamos que eventos clínicos como vaso-oclusão, infecções do trato respiratório e do trato urinário, sequestro esplênico e pneumonia ocorreram mais frequentemente na idade pediátrica, especialmente na faixa etária até os 12 anos. O risco de infecção em uma criança menor que 5 anos é 30 vezes maior que em crianças da população geral, concordando com nossos resultados que descreveram a prevalência elevada de infecções bacterianas na infância[8 13].

Neste estudo, nós observamos que o histórico dos indivíduos em acompanhamento ambulatorial apresentou número menor de episódios infecciosos que o histórico dos pacientes internados (PC) (p=0,014), possivelmente devido ao fato desses indivíduos estarem sob cuidado médico contínuo e em uso de medicamentos profiláticos, aspectos importantes na estabilidade clínica da doença. Além disso, o acompanhamento clínico dos pacientes com anemia falciforme tem aumentado a expectativa de vida desses indivíduos, principalmente com a realização do diagnóstico precoce, aconselhamento familiar, uso profilático de antibióticos, vacinação e mais recentemente com uso de hidroxiuréia (HU) e terapia poli-transfusional[16].

Estudos realizados na população brasileira comparando as causas de hospitalização em pacientes pediátricos da cidade de Salvador e de São Paulo descreveram a vaso-oclusão como causa principal de internação hospitalar nas duas populações[18]. As causas mais comuns de internação em crianças hospitalizadas no Congo foram as vaso-oclusões (26,7%) e infecções (36,6%) em crianças menores de 5 anos; nas crianças maiores de 5 anos a causa principal de hospitalização foi a ocorrência de colelitíase ou cardiopatia[23]. Apesar de muitos estudos relatarem a relação entre a infecção e a crise de vaso-oclusão, ainda não foi possível afirmar se a infecção pode desencadear o fenômeno vaso-oclusivo ou eventos subclínicos, bem como a sequência em que esses eventos ocorrem[19]. Desta forma, a condição inflamatória crônica da anemia falciforme e a imunidade individual podem estar diretamente relacionadas à ocorrência de infecções, apesar de não ser ainda conhecido que componente do sistema imune está diretamente envolvido na susceptibilidade elevada as infecções recorrentes apresentadas por esses pacientes. O início dos eventos de vaso-oclusão envolve fatores determinantes da obstrução dos vasos como a perfusão tecidual, diminuição do calibre dos vasos e a tendência de adesão das hemácias ao endotélio vascular[3].

A importância dos leucócitos na clínica da anemia falciforme tem sido bastante evidenciada em estudos que associam a leucocitose como fator de risco para a ocorrência de AVC e síndrome torácica aguda. A participação dos leucócitos na vaso-oclusão começa a partir do processo de rolamento e da expressão de integrinas que promovem o aumento da adesão ao endotélio vascular. Após a adesão ao endotélio vascular as hemácias diminuem o fluxo sanguíneo e facilitam a ligação de mais hemácias falciformes, iniciando a obstrução vascular[22]. Além disso, os leucócitos são estimulados a liberar proteínas citotóxicas e substâncias vaso-ativas, citocinas e quimiocinas que irão promover a atração de mais leucócitos para esses sítios. O neuropeptídio substancia P, mediador da inflamação e da dor é responsável também pela secreção de citocinas como IL-1, IL-6, TNF-alfa e IL-8 e tem sido descrito em níveis elevados em pacientes com anemia falciforme durante a crise vaso-oclusiva[20].

O aumento de citocinas do tipo Th2 (IL-4, IL-6 e IL-10), IL-8 e TNF-alfa têm sido descritas no plasma de indivíduos com anemia falciforme em estado estável, sendo que possivelmente essas alterações estão associadas ao aumento da expressão ou promovem a ativação das moléculas de adesão em neutrófilos e no endotélio vascular[20]. Nossos resultados demonstram que os indivíduos internados por crise apresentam níveis de IL-8 mais elevados que os indivíduos em estado estável, sendo que a maioria dos indivíduos em estado estável apresenta nível de IL-8 menor que 1pg/mL, concordando com estudos anteriores, sendo que alguns autores descrevem a IL-8 como marcador de crise em pacientes com anemia falciforme[7 9]. Ressaltamos que os níveis de IL-8 permaneceram elevados durante a crise e que mesmo após a alta médica esses níveis não retornaram a seu estado basal, sugerindo que o restabelecimento dos níveis de IL-8 é bastante lento. Neste ponto podemos levantar algumas

hipóteses: Os níveis de IL-8 permanecem elevados após a fase aguda (crise ou infecção) devido ao caráter crônico da anemia falciforme ou existe algum mecanismo na fisiopatologia da doença que contribui para a manutenção dessas taxas? Durante quanto tempo os níveis de IL-8 permanecem elevados, uma vez que esses indivíduos ficaram internados em média 15,6 (± 20,3) dias; esse período prolongado de níveis elevados de IL-8 pode influenciar no restabelecimento do paciente ou até mesmo desencadear nova crise? Os indivíduos com níveis mais elevados de IL-8 estão mais susceptíveis a ocorrência de crise?

Desta forma, estudos adicionais são necessários para o monitoramento dos níveis de IL-8 após a alta médica dos pacientes, visando estimar o tempo que decorre entre a crise e o restabelecimento dos níveis dessa citocina, bem como a sua influência nos processos fisiopatológicos e imunológicos presentes na doença.

As análises de frequência dos polimorfismos -251T>A e -308G>A demonstraram que esses polimorfismos estão e equilíbrio de Hardy-Weinberg em nossa população. O alelo mutante A para o polimorfismo -251T>A no gene da IL-8 tem sido relacionado com a produção elevada desta citocina, sendo classificados o genótipo AA como produtor elevado de IL-8, o AT como produtor intermediário e o TT como produtor baixo [13]. Nossos achados mostram que o genótipo AT apresentou níveis séricos de IL-8 mais elevados, porém não foi possível associar à presença do alelo A (produtor alto de IL-8) com os aspectos clínicos frequentes na doença, apesar de observarmos um número elevado de eventos em indivíduos que apresentam o alelo A. Possivelmente devido à heterogeneidade clinica e a possível ocorrência de eventos subclínicos, que muitas vezes não são informados pelo paciente.

Quando comparamos os níveis séricos de IL-8 nos diferentes grupos estudados observamos que os portadores do alelo A possuem níveis mais elevados em relação aos do alelo T, sendo esta associação mais relevante em indivíduos em alta médica ($p<0,05$). Esses resultados são ainda reforçados pela análise de regressão linear, que confirma a relação entre a presença do alelo selvagem (produtor alto) e os níveis elevados de IL-8 no grupo de pacientes no momento da crise e alta hospitalar.

Alguns estudos têm relacionado o polimorfismo -251T>A no gene da IL-8 à ocorrência de infecção pelo vírus sincicial respiratório[13 14], a susceptibilidade a esclerose múltipla e ao risco de doença gastrintestinal[15], entre outras patologias. Porém, não encontramos relação entre a presença do polimorfismo -251T>A e a ocorrência de infecções, possivelmente pelos mecanismos complexos que envolvem a fisiopatologia da anemia falciforme, ressaltando o seu caráter pró-inflamatório crônico e a associação a outros fatores que indiretamente podem estar influenciando na expressão genotípica e fenotípica da doença.

O polimorfismo -308G>A na região promotora do gene do TNF-alfa tem sido associado a níveis elevados dessa citocina. O alelo A para esse polimorfismo tem sido associado à ocorrência de choque séptico grave e óbito ou até mesmo a ocorrência de complicações neurológicas[10]. Neste estudo foi possível observar a associação entre a presença do polimorfismo -308G>A e a ocorrência de sequestro esplênico ($p<0,05$). Outro estudo demonstrou AA ocorrência de níveis elevados de TNF-alfa em indivíduos com anemia falciforme[29]. Nosso estudo não teve como objetivo realizar a dosagem dos níveis séricos de TNF-alfa, mas acreditamos que estudos adicionais serão necessários visando estabelecer a relação entre os níveis séricos de TNF-alfa e as manifestações clínicas descritas na doença, bem como estabelecer a associação dos polimorfismos estudados e os níveis dessas citocinas, visando elucidar o papel do TNF-alfa e IL-8 como fator de risco para a ocorrência de crise na anemia falciforme, destacando a sua participação nos processos de oclusão da microcirculação e após os eventos de crise[30].

A dinâmica dessas citocinas na anemia falciforme e o papel real da IL-8 e do TNF-alfa como marcadores da ocorrência de crise vaso-oclusiva ainda precisam ser esclarecidos.

Estudos adicionais são necessários visando esclarecer as questões aqui levantadas, bem como os mecanismos envolvidos nos processos infecciosos e de vaso-oclusão presentes na anemia falciforme, de maneira que possamos estabelecer o valor prognóstico tanto dos polimorfismos gênicos, como dos níveis séricos dessas citocinas no desenvolvimento das manifestações clínicas presentes nessa doença.

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SICKLE CELL DISEASE SC IN NORTHEAST OF BRAZIL: A CLINICAL AND MOLECULAR CHARACTERIZATION

DOENÇA FALCIFORME SC NO NORDESTE DO BRASIL: CARACTERIZAÇÃO CLÍNICA E MOLECULAR

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The SC disease is really prevalent in Brazil, mainly in Bahia, being that the patients present a severe anemia but with less clinical complications than SS homozygous. The patients with SC disease have less painful crisis, infections, skeletal involvement, anemia, and priapism. The aim of the present study was to investigate the hemoglobin C and S globin gene haplotypes distribution among 63 individual with SC disease from Northeast Brazil, associating with their phenotype. Our results show that the studied patients have mild anemia (hemoglobin median=10.88 g/dL) and slightly high fetal hemoglobin levels (median=3.06%). The α-thalassemia$^{3.4Kb}$ deletion was found in 18 (28%) patients. The most frequent β^C and β^S globin gene haplotypes were CAR and Benin. We have not found any association among the globin haplotypes and clinical events of the patients, however further studies need to be developed to confirm the finding related to the SC disease patients.
Keywords: Sickle cell disease, SC disease, thalassemia, haplotypes.

A doença SC é muito prevalente no Brasil, principalmente na Bahia, sendo que os pacientes apresentam anemia grave, mas com menos complicações clínicas que os homozigotos SS. Os pacientes com doença de SC têm menos crises dolorosas, infecções, comprometimento ósseo e priapismo. O objetivo do presente estudo foi realizar a caracterização clínica e molecular dos haplótipos ligados ao gene das globinas β^S e β^C e da talassemia $\alpha^{3,7Kb}$ em 63 indivíduos com doença do SC da Bahia, Brasil. Os resultados obtidos demonstram que os pacientes estudados têm moderada anemia (média de hemoglobina=10,88g/dL) e níveis discretamente elevados de hemoglobina fetal (média=3,06%). A talassemia $\alpha^{3,4Kb}$ foi encontrada em 18 (28%) pacientes. Os haplótipos ligados aos genes da globina do gene da globina β^S e β^C mais frequentes foram CAR e Benin. Não foi encontrada associação entre os haplótipos da globina e eventos clínicos dos pacientes, porém estudos adicionais poderão confirmar os resultados obtidos com relação aos pacientes com doença SC.
Palavras-chave: doença falciforme, doença SC, talassemia, haplótipos.

The hemoglobinopathies result of molecular alteration in a globin gene and may be divided in two major groups, characterized by the presence of a structurally abnormal globin chain or by a reduction or absence of globin chains synthesis named thalassemias[7].

The hemoglobin S has a single GAT→GTA at the sixth codon of the β-globin gene, conducting to the glutamic acid to valin substitution ($\beta^{S\ 6\ Glu\rightarrow Val}$) and a variant β-globin chain. Sickle cell anemia disease, the homozygous state of HbS (*HBSS*) has heterogeneous clinical picture with vasooclusive crisis, hemolysis, painful episodes and other chronic complications such as leg ulcers and priapism and others in different degrees[25].

Sickle cell disease affects million of people worldwide, in Brazil, around 4 million of people has a sickle cell trait (HbAS)[1]. In Bahia, Northeast of Brazil, studies conducted in different population groups, described a frequency of 7.4 to 15.7% for heterozygous AS[2 9], the heterozygous state is found in a frequency of 6.5% among the African population[2]. The second most common variant hemoglobin described in Brazil is the C hemoglobin in which the sixth codon of the b-globin gene, the GAG is replaced by AAG, resulting in the glutamic acid to lysine ($\beta^{6Lys\rightarrow Glu}$) substitution at globin chain[18]. The β^C homozygous (CC) has a moderate hemolytic anemia. The presence of hemoglobin S and C in Brazilian population has contributed to a high prevalence of SC disease, mainly in Bahia where the heterozygous frequency is around 3.5%. The double heterozygous SC presents a severe anemia but with less clinical complications than SS homozygous[6].

All complications that are found in patients with sickle cell disease anemia have occurred in individuals with HbSC disease. Yet, most – but not all – of these complications are seem less often and appear at later time in HbSC disease compared with sickle cell anemia[17]. The patients with SC disease have less painful crisis, infections, skeletal involvement, anemia, and priapism. However, they have more thromboembolic events, renal papillary necrosis[26] and a particular incidence of retinopathy[8], aseptic necrosis of the head of long bones, and pathological events involving the spleen during adulthood[13 14].

Five major *β^S-globin* gene haplotypes defined by the presence of restriction endonuclease polymorphics sites located throughout the *β-globin* gene cluster have been described. The haplotypes are associated with the African geographic origin of the mutation[20] with description of the Benin (BEN) type in the Midwestern Africa, the Bantu in Central Africa Republic (CAR) in South Central and Eastern

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Africa, the Senegal (SEN) in Atlantic West Africa, the Cameron along west coast and the Saudi Arabia-India on Indian subcontinent and Arabian peninsula[10 18].

The *β^C-globin* gene haplotypes have been devided among the groups: I, II and III[11]. A single origin of the mutation followed by the spread to other haplotypes by meiotic recombination of 5' to the *β-globin* gene has been proposed by Nagel and Rannel[18]**.** The *β^C-globin* gene haplotype when combined with the Benin *β^S-globin* gene haplotype, commonly presents very low HbF levels[17].

Other common hemoglobin disorder with a high worldwide distribution is caused by reduction or absence of the globin chain synthesis, known as thalassemia syndromes. The α-thalassemia has different molecular bases and the α_2-thalassemia followed by a 3.7 Kb deletion has been described in 20-25% of the black Brazilian population[23].

Based on the high prevalence of sickle cell disease and SC disease among the Northeast of Brazil population, we investigated the hemoglobin C and S globin gene haplotypes distribution among SC disease patients associating with the patients' phenotype.

Material and Methods

We studied 126 chromosomes of SC disease patients, aged 23 ± 15.3 years, after obtaining the approval of the Oswaldo Cruz Foundation Institutional Ethical Committee (protocol number 142). They attended the out patient's clinic of the Bahia Blood Center Foundation (HEMOBA) and the peripheral blood samples were obtained during a regular clinic visit. Hematological data were analyzed by an automated cell counter *(Coulter-Counter* T890), hemoglobin profile was investigated by high-performance liquid chromatography (HPLC) (*Bio-Rad VARIANT™* II, CA, USA) and DNA was isolated from the peripheral blood leukocytes by *GFX™ Genomic Blood DNA Purification KIT* (*Amersham Pharmacia Biotech.* NJ, USA). *β^S* and *β^C* globin gene haplotypes were determined by polymerase chain reaction (PCR) and RFLP techniques as previous described[5 24]. The statistical analyses were developed at the software EPI Info version 6.04.

Results

In a group of 63 (39 female and 24 male) the patients median hemoglobin concentration was 10.88 (± 1.67)g/dL; median hematocrit (Hct) 34.48 (± 7.9)%; median cell volume (MCV) 79.93 (± 11.3); median cell hemoglobin (MCH) 27,5 (± 12.80); median cell hemoglobin concentration (MCHC) of 34.66 (± 17.56) and median fetal hemoglobin concentration 3.06%. Table 1 shows the β -globins haplotypes distribution among the 63 SC disease patients.

The α-thalassemia was studied in 60 patients and we observed five (8.3%) homozygous and six (10.0%) heterozygous. The result of α-thalassemia in the different haplotypes is described on Table 2.

Table 1. The β^C and β^S globin gene haplotypes among 45 SC disease patients from Salvador, Bahia (Northeast-Brazil).

β^CHaplotypes	β Haplotypes – n(%)			Total
	Benin	CAR	Athypical	n(%)
β^C I	29 (46.1)	20 (31.7)	2 (3)	48 (80.9)
β^C II	7 (11.1)	5 (7.9)	0	12 (19.1)
Total	33 (57.2)	25 (39.7)	2 (3.1)	63 (100)

Table 2. Association between a-thalassemia and β^S globin gene haplotypes among 45 SC disease patients from Salvador, Bahia (Northeast-Brazil).

Thalassemia	Globin gene haplotypesn(%)			
	Ben I	Ben II	CAR I	CAR II
Normal	21(35)	4(6.7)	1(1.7)	18 (30)
Homozygous	3(5)	1(1.7)	0	1(1.7)
Heterozygous	3(5)	1(1.7)	1(1.7)	1(1.7)

The patients' phenotypes and hematological data are shown in Table 3. There was no statistic significance between gender and hematological data.

Table 4 shows association among the β^C/β^S haplotypes and the phenotype of 63 SC disease patients. There was not found any association between these data.

Table 5 compares the present study with other reports worldwide about SC disease clinical events.

Table 3. Hematological Data and β^C and β^S globin gene haplotypes among SC disease patients from Salvador, Bahia (Northeast-Brazil).

Haplotypes (n)	Gender (M/F)	MEANS (± SD) Age	%HbS	%HbF	%HbC
CAR I (20)	12/8	25 (15.2)	48 (2.5)	2.4 (3.1)	44.7 (1.9)
CAR II (5)	5/0	29 (18.8)	46.5 (2.7)	4.7 (4.2)	43.9 (2.6)
Ben I (29)	17/12	18.1 (13.8)	45.2 (96)	3.4 (3.9)	44.6 (2.3)
Ben II (7)	4/3	34.4 (17.8)	49.2 (3.4)	3.0 (3.5)	44.0 (3)
Athipical I (2)	1/1	23.0 (4.2)	49.7 (0.6)	0.7 (0.1)	44.0 (0.6)
p value	-	0.87*	0.22**	0.47*	0.45*

*Anova; **Kruskal-Wallis test.

Discussion

Bahia, a Northeast Brazilian state received immigrants from Portugal, Holland and France, but the major important racial group in the Bahia population is the Black African. About of 1,200,000 slaves were estimated to have been imported to Bahia from 1678 to 1851. Historical data suggest that about 90% of slaves imported into northern Brazil were from Angola, Congo and Mozambique, where the CAR or Bantu haplotype predominates[12 19]. Northeast region of Brazil (Bahia,

Table 4. The β globin gene genotypes and phenotypes among a group of 63 SC disease patients from Salvador, Bahia (Northeast-Brazil).

β^C/β^S haplotypes	N cases	Clinical features – n(%)				
		Retinopaty	Hepatomelgaly	Splenomegaly	Pain	Leg ulcer
CAR I	20	2	2	5	11	2
Ben I	29	4	5	7	18	0
P value	-	0.52*	0.68*	0.60*	<0.06*	0.17*
CAR II	5	0	1	2	3	0
Ben II	7	1	1	1	3	0
P value	-	1*	1*	0,52*	1*	-
Ben I	29	4	5	7	18	0
Ben II	7	1	1	1	3	0
P value		1*	1*	1*	<0.04*	-
CAR I	20	2	2	5	11	2
CAR II	7	0	1	2	3	0
P value		1*	0.50*	0,59*	1*	1*

*Fisher Exact Test.

Table 5. SC disease phenotype and description in several studies worldwide.

Clinical features	**Country**, Author (year)(reference) [n cases]					
	Jamaica, Serjeant et al.[22] (1973) [n=?]	**Ghana**, Konothey-Ahulu et al. (*apud* 3 15 22 27) (1974) [n=?]	**USA**, Ballas et al. [3] (1982) [n=27]	**Brazil**, Zago et al.[27] (1983) [n=26]	**Brazil**, Marmitt et al.[15] (1986) [n=32]	**Brazil**, present study [n=63]
	Percentage cases (%)					
Hepatomegaly	38	22	16	65	59	14.3
Splenomegaly	60	0	52	58	50	25.4
Bone or joint pain	82	92	0	35	72	58.7
Leg ulcers	20	2	0	0	6	3.2
Retinopaty	?	?	75	?	?	11.1
Cardiopaty	19	0	4	0	13	3.2

(?) No related.

Pernambuco and Maranhão) was heavily supplied by slaves from Central West Africa until the middle of 19th century. The Sudans composed the mayor part of Bahia's population and Pernambuco in minor part and the Bantus occupied Maranhão and center-south of Brazil[12], because of the local where arrived the slave African route in spite of economical development in Brazil.

The β^C allele is found almost exclusively among African-Americans and West Africans from Northern Ghana and the Volta territory and to a much lesser degree, Western Nigeria. The β^C allele presence in West African make up less than 10% of the haplotypes[16]. Although it has been found rarely in individuals from Italy, particularly from Sicily, its geographic distribution when compared to the other common b-globin variants such as βS, βE, βD is quite localized[19].

Haplotypes analysis is a useful tool important to describe the molecular background and is association with normal and variant β-globin alleles, providing clues about the origins of several β-globin variants[5].

This group of patients does not represent the bulk of individuals with SC hemoglobinopathy in Salvador-Bahia-Brazil, because of the small number of patients in the sample, perhaps explain with complications such as hepatomegaly, bone or join pain, retinopaty and leg ulcer were infrequent or hardly observed. RBC from these patients with HbSC disease contains comparable amounts of HbS and HbC, only one patient had increased level of HbF (16.7g/dl). Hematological and biochemical profiles of the disease were defined while the patients were in their usual steady state.

Splenomegaly is a commonly described physical finding in children with HbSC disease, other studies in Brazil found 50%[15] and 58%[27] of splenomegaly differing from our study that found 25.4%, this low number may be a special characteristic of the group in study, these authors did not made any association between age and splenomegaly. Rivera-Ruiz[21] found palpable splenomegaly in 34% of patients and was more common in males. Hepatomegaly was found only in 9 (14.3%) patients, differing from Rio de Janeiro a Southernst Brazilian State with 59% of hepatomegaly[15], Jamaica 38%[22] and US with 16%[3].

Proliferative retinopathy is more common and more severe than in sickle cell anemia and progressive loss of vision may have its onset early in the second decade. Nagel et al.[17] found that retinopathy appears in patients between 15 and 30 years old but in our group retinopathy was present in seven (11.1%) patients aged among 26 to 57, four of these presents Ben I haplotype, one Ben II and two CAR II haplotype. It was previously described that the higher Hb and Hct levels in HbSC disease may be responsible for the higher incidence of retinopathy in this disease, but these hematological data were not observed in this study with association to retinopathy. Balo et al.[4] found 84% of retinopathy SC disease. There are few publications concerning retinal complications of hemoglobinopathies.

Bone or join pain was found in 58.7 % of the patients in according with Ballas et al.[3] that found 50% of HbSC patients with painful crisis. Only 8 (17.7%) patients in the present study had some kind of infection, seven of these with respiratory infection and one osteomyelitis. These findings indicate that SC disease is characterized by a wide range of clinical severity, milder than sickle cell anemia.

The symptomatology of this group of SC disease patients seems to be peculiar. Comparing our population with others in the world, this study demonstrates some aspects of HbSC disease in Brazil that were not previous appreciated, and confirmed features described by others investigators in world. But what we are asking is: What is the influence of β^S and β^C globin gene haplotypes in the investigated SC disease group? Does the β^S haplotype is more critical from the clinical features of the HbSC disease or the β^C haplotype is responsible for better clinical of the SC disease? We found that Ben I haplotype had more painful events that Ben II and that other haplotypes. Leg ulcer appears in the CAR I haplotype. Splenomegaly was more frequent in the β^C I haplotype than in β^C II. Thalassemia was more present in Ben I haplotype. Our data suggest that there is an influence of β^S and β^C globin gene haplotypes in

the phenotype of Northeast SC disease patients. We are addressing our study to answer this question or whether chromosomes carrying the βC mutation interact differentially with the common haplotypes associated with β^S gene and affect the clinical features of HbSC disease. Further studies need to be developed to confirm the different association between haplotype and phenotype SC disease patients.

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THE LEFTWARD DELETION 4.2 KB ALPHA-THALASSEMIA IN TWO SICKLE CELL ANEMIA SIBLINGS

DELEÇÃO 4,2KB DA ALFA-TALASSEMIA EM DOIS IRMÃOS COM ANEMIA FALCIFORME

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The presence of –αthal 3.7Kb deletion is associated with better prognosis of Sickle Cell Anemia (SCA) patients, but here are not reports in the literature regarding association of –α thal 4.2Kb and its importance among SCA clinical outcome. In this report, we describe Hemoglobin profile and laboratory findings of two siblings who have SCA and are silent carriers of –α thal 4.2Kb. Both described patients have severe anemia, lower rates of Mean Corpuscular Volume (MCV) and a high leukocytes count. Further studies are required to establish a possible association between –α thal 4.2Kb and SCA severity.

Keywords: Alpha thalassemia, sickle cell anemia, hemoglobin.

A presença da deleção –α thal 3.7Kb está associada com melhor prognóstico de pacientes que possuem anemia falciforme (AF), contudo não existem estudos na literatura a respeito da associação da –α thal 4.2Kb com a evolução clínica desses pacientes. No presente relato são descritos achados laboratoriais e perfil de hemoglobina de dois irmãos que possuem AF em associação com a –α thal 4.2Kb. Ambos os pacientes apresentam anemia acentuada, baixos índices de Volume Corpuscular Médio (VCM) e contagem de leucócitos elevada. Estudos adicionais são necessários para elucidar uma possível associação entre a –α thal 4.2Kb e a gravidade da AF.

Palavras-chave: doença falciforme, doença SC, talassemia, haplótipos.

Alpha-thalassemia, the most common single-gene disease in the world, is characterized by a reduction or complete absence of α-globin gene expression. Many deletions have been described in the Alpha (α)-globin gene located at the short arm of chromosome 16, but the most prevalent are the –α thalassemia with 3.7 kilobases (Kb) deletion (–α thal 3.7Kb) and the –α thalassemia with 4.2 Kb (–α thal 4.2Kb) which are originated by homologous recombination between misaligned chromosomes[4].

Sickle cell anemia (SCA) patients have heterogeneous clinical manifestations, including hemolysis, chronic inflammation and painful crisis. The presence of –α thal 3.7Kb deletion is associated with better prognosis of SCA patients[5]. There are not reports in the literature regarding association of –α thal 4.2Kb and its importance among SCA clinical outcome.

In this report, we describe two siblings from Bahia State in Brazil, who have SCA and are silent carriers of –α thal 4.2Kb.

Whole-blood samples were collected from the *HBSS* patients attending the out-patients clinic in HEMOBA. Hematological analyses were carried out using an electronic cell counter, Coulter Count T-890 (Coulter Corporation, FL, USA). The hemoglobin (Hb) profile and HbF levels were investigated by high performance liquid chromatography (HPLC / VARIANT I; BIO-RAD, CA, USA). Biochemical markers analyses were measured in serum by immunochemistry assay (A25 system, BIOSYSTEMS SA, Barcelona, Spain).

DNA was isolated from the white blood cells (WBC) by FlexiGene DNA Kit, Qiagen (USA), according to the manufacturer's recommendations. Beta-globin gene haplotypes were investiged by PCR-RFLP[6]. The alpha-thalassemia was confirmed in the two siblings by a single-tube multiplex PCR method to detect the wide type, the –α thal 3.7Kb, and –α thal 4.2Kb alleles, using primers previously described[3].

The study was approved by the Oswaldo Cruz Research Foundation's Human Research Board (number CAAE 0024.0.225.000-06), and the study was based in accordance with Declaration of Helsinki of 1975, as revised in 2000 and all subjects or official responsible filled out a written informed consent form.

The patient number 1 is a 7-years-old afro-descendent boy, which has had recurrent hospitalizations (more than 3) with many painful crisis, meningitis episode and blood transfusion history. The patient number 2 is a 5-years-old afro-descendent girl and her clinical history indicated recurrent hospitalizations (more than 9), pneumonia episode and blood transfusion history. Moreover, this patient has been submitted to splenectomy surgery.

The laboratory findings and hemoglobin profile of both patients are described in the Table 1, which shows severe anemia, lower rates of Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCHC), a high leukocytes count and an increase of iron and ferritin serum levels that

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Table 1. Hemoglobin profile and laboratory findings of the two -$\alpha^{4.2}$/SCA patients.

	Patient 1	Patient 2
Age (years)	7	5
Gender	Male	Female
Hemoglobins (%)		
S	90.8	86.2
Fetal	5.8	10.6
A2	3.4	3.2
Beta-globin gene Haplotypes	Ben/Car	Ben/Car
Hemolysis		
Erythrocyte, million/mL	2,84	2,27
Hemoglobin, g/dL	6.9	5.5
Hematocrit, %	21.9	18.1
Mean Cell Volume, fL	77.1	79.7
Mean Cell Hemoglobin, pg	24.3	24.2
Reticulocytes Count, %	8.0	5.0
Lactate dehydrogenase,U/L	248	376
Leukocyte		
Leukocyte Count, /mL	16,300	34,300
Platelets		
Platelets cunt, thousand/mm^3	296	268
Iron metabolism		
Iron serum, mcg/dL	715	858
Ferritin, ng/mL	1,391.3	529.50
Lipidic metabolism		
Total Cholesterol, mg/dL	112	129
HDL Cholesterol, mg/dL	29	36
LDL Cholesterol, mg/dL	71	76
VLDL Cholesterol, mg/dL	12	17
Tryglicerides, mg/dL	62	86
Hemolysis plus Hepatic		
Aspartate aminotransferase, U/L	65	66
Total bilirrubin, mg/dL	1.4	0.5
Direct bilirrubin, mg/dL	0.6	0.2
Indirect bilirrubin, mg/dL	0.8	0.3
Hepatic		
Alanine aminotransferase, U/L	73	22
Renal		
Urea nitrogen, mg/dL	12	11
Creatinine, mg/dL	0.4	0.4
Total protein, g/dL	7.1	6.8
Albumin, g/dL	3.8	3.3
Globulin, g/dL	3.3	3.5
Inflammation		
C-reactive protein, mg/mL	39.2	103
Alpha 1 antitrypsin, mg/dL	222	250
ASLO (UI/mL)	132	133

could be associated with an increase of reactive oxygen species (ROS) and consequently with a increase of clinical severity[2]. The coexistence of alpha-talassemia and SCA has been related with a higher survival rates and a decreased hemolysis markers and with a frequent vaso-occlusive episodes and painful crisis[1], but the same approach is not available for the -$\alpha^{4.2Kb}$/SCA association, requiring further study.

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