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IGOR DE FARIAS DOMINGOS

INVESTIGAÇÃO DE GENES ENVOLVIDOS NA ESTABILIDADE E MANUTENÇÃO DO CITOESQUELETO ERITROCITÁRIO E SUA RELAÇÃO COM O QUADRO CLÍNICO DE PACIENTES COM ANEMIA FALCIFORME

Recife
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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

Área de Concentração: Genética

Orientador: Dr. Marcos André Cavalcanti Bezerra

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"Quando uma criatura humana desperta para um grande sonho e sobre ele lança toda a força de sua alma, todo o universo conspira a seu favor"

(Johann Wolfgang von Goethe)

RESUMO

A membrana plasmática apresenta grande importância nas propriedades estruturais das hemácias, e na anemia falciforme (AF), os eritrócitos falcizados apresentam uma maior exposição de fosfatidilserina, o que pode acelerar a hemólise e favorecer a crise vaso-oclusiva (CVO). Desse modo, o objetivo deste trabalho foi avaliar a influência de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário no quadro clínico de pacientes com AF. A amostra foi constituída por pacientes com AF, sem transfusão há 3 meses, e estratificados durante a coleta quanto ao uso de hidroxiuréia e quanto à presença de CVO. A análise da expressão dos genes *ATP11C*, *PLSCR1*, *SPHK1* e *DMTN* foi realizada por qPCR com sondas TaqMan®, e os resultados foram gerados pelo método do ΔCt e expressos utilizando a fórmula $2^{-\Delta\Delta Ct}$. Em nosso estudo, os reticulócitos de pacientes com AF apresentam baixos níveis de *ATP11C* e *SPHK1* ($P < 0,01$), embora esses achados não foram associados a um pior quadro clínico. Além disso, o uso de hidroxiuréia diminui a expressão de *SPHK1* ($P < 0,001$). Em relação à presença de CVO, altos/baixos níveis de *DMTN* e relação *ATP11C/PLSCR1* foram encontrados durante a crise de dor ($P < 0,05$), respectivamente. Desse modo, nosso trabalho demonstra que o *ATP11C*, *SPHK1* e *DMTN* estão diferencialmente expressos durante o curso clínico de pacientes com AF, embora não esteja esclarecido o impacto dessa expressão diferencial no quadro clínico dos pacientes.

Palavras-chave: Doença falciforme. Membrana eritrocitária. *ATP11C*. *SPHK1*. *DMTN*.

ABSTRACT

The plasmatic membrane presents a fundamental importance in the structural properties of erythrocytes, and in sickle cell anemia (SCA), red blood cells expose more phosphatidylserine in the membrane surface, which may accelerate hemolysis and favor vaso-occlusive crisis (VOC). Therefore, the objective of this study was to evaluate the influence of genes involved in the stability and maintenance of the erythrocyte cytoskeleton in patients with SCA. The sample consisted of patients with SCA, without transfusion for 3 months, and stratified during blood collection regarding the use of hydroxyurea and the presence of VOC. Analysis of *ATP11C*, *PLSCR1*, *SPHK1* and *DMTN* genes expression was performed by qPCR with TaqMan® probes, and the results were generated by the ΔCt method and expressed using the formula $2^{-\Delta\Delta Ct}$. In our study, reticulocytes from patients with SCA had low levels of *ATP11C* and *SPHK1* (P <0.01), although these findings were not associated with a worse clinical outcome. In addition, the use of hydroxyurea decreases the expression of *SPHK1* (P <0.001). Regarding the presence of VOC, high/low levels of *DMTN* and *ATP11C*/*PLSCR1* ratio were found during the pain crisis (P <0.05), respectively. Therefore, our work demonstrates that *ATP11C*, *SPHK1* and *DMTN* are differentially expressed during the clinical course of patients with SCA, although the impact of this differential expression on patients' clinical outcome is not clear.

Key-words: Sickle cell disease. Plasmatic membrane. *ATP11C*. *SPHK1*. *DMTN*.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

Item	**Definição**
2,3-BPG	2,3-Bifosfoglicerato
AF	Anemia Falciforme
Asp	Aspartato
ATP10A	Gene ATPase 10A
ATP11C	Gene ATPase 11C
ATP8A1	Gene ATPase 8A1
ATP8A2	Gene ATPase 8A2
ATP8B1	Gene ATP8B1
ATP8B3	Gene ATP8B3
ATPase	Adenosinatrifosfatase
AVC	Acidente vascular cerebral
BCL11A	Gene *BCL11A (*do inglês *BAF chromatin remodeling complex subunit BCL11A*)
DMTN	Gene dematina
DTC	Doppler Transcraniano
ERK 1/2	Quinases reguladas por sinal extracelular 1/2 (do inglês *extracellular signal–regulated kinases 1/2*)
FC	Fosfatidilcolina
FE	Fosfatidiletanolamina
FS	Fosfatidilserina
GMPc	Guanosina monofosfato cíclica
Glu	Ácido glutâmico ou glutamato
HBB	Gene da globina beta
HDR	Reparo dirigido por homologia (do inglês *homology-directed repair*)
HbF	Hemoglobina fetal
HBG	Gene da globina gama
HbS	Hemoglobina S
HEMOPE	Fundação de Hematologia e Hemoterapia de Pernambuco

HLA	antígeno leucocitário humano (do inglês *human leukocyte antigen*)
HU	Hidroxiuréia
ICAM-1	Molécula de adesão intercelular 1 (do inglês *Intercellular Adhesion Molecule 1*)
ICAM-4	Molécula de adesão intercelular 4 (do inglês *Intercellular Adhesion Molecule 4*)
LDH	Lactato desidrogenase
MAP-k	Proteína quinases ativadas por mitógenos (do inglês *mitogen activated protein kinases*)
NHEJ	União das extremidades não homólogas (do inglês *non-homologous end joining*)
pb	Pares de bases
PLSCR1	Gene scramblase fosfolipídica 1
hPLSCR1	Scramblase fosfolipídica 1 humana
PKA	Proteína quinase A (do inglês *protein kinase A*)
S1P	Esfingosina-1-fosfato
SCA	Anemia falciforme (do inglês *sickle cell anemia*)
SNP	Polimorfismo de base única (do inglês *Single nucleotide polymorphism*)
SPHK1	Gene esfingosina quinase 1
Sphk1	Esfingosina quinase 1
Sphk2	Esfingosina quinase 2
Val	Valina
VCAM-1	Proteína vascular de adesão celular 1 (do inglês *Vascular cell adhesion protein 1*)

SUMÁRIO

1 INTRODUÇÃO

Os eritrócitos não possuem núcleos, estruturas citoplasmáticas ou organelas. Desse modo, a membrana plasmática, formada por uma bicamada de fosfolipídios com proteínas integrais ao longo da membrana, apresenta uma grande importância nas propriedades estruturais e funcionais das hemácias. Em pacientes com anemia falciforme (AF), doença autossômica recessiva causada por uma mutação pontual no gene da globina β, a formação e o alongamento dos polímeros de HbS, característica fisiopatológica da doença, promove alterações significativas da membrana plasmática do eritrócito, como uma maior rigidez e uma maior exposição de fosfatidilserina (FS).

A exposição de FS na superfície celular é um sinal fagocítico para os macrófagos removerem essas células de circulação. Desse modo, em pacientes com AF, a aceleração da destruição pela exposição de FS resulta em um período de vida útil menor das hemácias e consequente aumento de hemólise. Além disso, essa exposição representa uma das principais causas de adesão de hemácias ao endotélio vascular, contribuindo para a crise vaso-oclusiva, manifestação clínica mais comum do paciente com AF. Sendo assim, sugere-se que uma desregulação da estabilidade e manutenção do citoesqueleto eritrocitário possa contribuir para uma maior morbidade da doença, visto que os pacientes com AF apresentam uma grande variabilidade de manifestações clínicas.

As razões para a heterogeneidade clínica de pacientes com AF ainda não são completamente entendidas. Entretanto, estudos sugerem a existência de um componente genético. Desse modo, a ação de múltiplos genes combinados pode determinar a gravidade geral da doença. Em relação à estabilidade e manutenção do citoesqueleto eritrocitário, o gene *ATP11C* surge como candidato, visto que a proteína codificada por esse gene internaliza mais de 75% da FS, e nenhuma outra enzima funcionalmente ativa compensa a falta de ATP11C. Além disso, a avaliação do gene *PLSCR1*, responsável por codificar uma proteína que externaliza a FS, também se faz necessária, visto que a exposição de FS depende de um balanço entre os mecanismos que internalizam e expõem essa molécula.

Outro gene candidato a modular o quadro clínico de pacientes com AF é o *SPHK1*, responsável por codificar uma proteína que catalisa a formação de esfingosina-1-fosfato (S1P). A S1P induz a produção de 2,3 bisfosfoglicerato (2,3-BPG) e, desse modo, reduz a afinidade da hemoglobina pelo oxigênio. Desse modo,

altos níveis de S1P em uma hemácia contendo HbS pode favorecer a falcização da hemácia e, consequentemente, a maior atividade clínica da doença.

No mesmo contexto, a dematina, proteína expressa pelo gene *DMTN*, tem a função de estabilizar o eritrócito, e a ausência desse gene em ratos fez com que os animais apresentassem uma anemia grave, além de alterações na morfologia e estabilidade da membrana eritrocitária. Ademais, no armazenamento prolongado do concentrado de hemácias ou na presença de doenças que alterem o funcionamento normal do eritrócito, como a AF, a fosforilação da dematina pela proteína quinase A compromete a estabilidade da membrana eritrocitária.

Diante do exposto, percebe-se que são poucos os dados acerca da influência desses genes na AF, e se alterações na expressão desses genes poderiam modular o quadro clínico de pacientes com AF, de modo isolado ou integradamente. Desse modo, na tentativa de melhor compreender a fisiopatologia da doença, faz-se necessário avaliar a expressão dos genes *ATP11C*, *PLSCR1*, *SPHK1* e *DMTN* em pacientes com AF, em um contexto clínico.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar a influência da expressão de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário com a modulação do quadro clínico de pacientes com anemia falciforme.

1.1.2 Objetivos Específicos

- Avaliar a expressão dos genes *ATP11C*, *PLSCR1*, *SPHK1* e *DMTN* em reticulócitos de pacientes com anemia falciforme e associar os achados com a gravidade da doença;
- Correlacionar os níveis de expressão do gene *ATP11C*, *PLSCR1*, *SPHK*1 e *DMTN* com a taxa de exposição de fosfatidilserina nas hemácias de pacientes com anemia falciforme;
- Avaliar de maneira integrada a expressão dos genes *ATP11C*, *PLSCR1*, *SPHK1* e *DMTN* e associar os resultados com a gravidade da doença.

2 REFERENCIAL TEÓRICO

2.1 ANEMIA FALCIFORME

2.1.1 Epidemiologia

A anemia falciforme (AF) é uma das desordens hereditárias mais comuns no mundo, em que 2% da população mundial apresenta a doença, além de nascerem entre 300.000-400.000 crianças falciformes a cada ano (RUSANOVA et al., 2011). No Brasil, estima-se que de 5-6% da população seja portadora do traço falciforme e que, a cada ano, nascem entre 700-1000 crianças portadoras da AF (BRASIL, 2013; LYRA et al., 2005).

No estado de Pernambuco, um em cada 23 recém-nascidos vivos possui o traço falciforme e um em cada 3000 nasce com a doença falciforme (BRASIL, 2013; CANÇADO; JESUS, 2007). Em Pernambuco, uma frequência de 5,1% de recém-nascidos portadores do traço falciforme foi encontrada ao ser realizada uma triagem em sangue de cordão umbilical (BANDEIRA et al., 1999).

2.1.2 Fisiopatologia

A anemia falciforme (AF), doença autossômica recessiva de distribuição mundial, é uma hemoglobinopatia causada por uma mutação pontual no gene da globina β, que promove a substituição do ácido glutâmico pela valina no 6º códon da cadeia polipeptídica (HBB; β^6 GAG→GTG; $glu^6 \rightarrow val^6$), levando à formação de uma hemoglobina anormal (HbS). Em condições de baixas concentrações de oxigênio, diminuição do pH e baixas concentrações de hemoglobina fetal (HbF), a HbS sofre uma polimerização devido à interação entre os resíduos hidrofóbicos dessa molécula, formando estruturas filamentosas que se depositam nas hemácias, modificando sua forma e tornando-as falciformes (REES; WILLIAMS; GLADWIN, 2010). O acúmulo de polímeros de HbS dentro das hemácias falcizadas resulta em uma lesão celular e, em larga escala, os eritrócitos danificados promovem os fenômenos hemolíticos e vaso-oclusivos da doença, além de um quadro de inflamação crônica, caracterizando o fenótipo principal da AF (CONRAN; FRANCO-PENTEADO; COSTA, 2009; REES; WILLIAMS; GLADWIN, 2010; SINGHAL et al., 2017; STEINBERG, 2008a) (Figura 1).

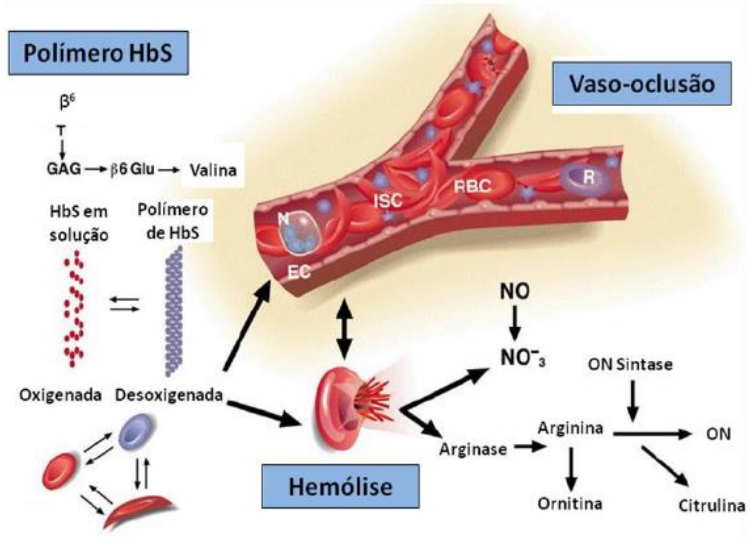

Figura 1 Fisiopatologia da anemia falciforme. Mutação no gene da globina β, na posição referente ao 6º códon, levando a formação de uma hemoglobina anômala, a HbS, que sofre uma polimerização em baixas concentrações de oxigênio. O polímero de HbS danifica o eritrócito, diminuindo sua vida útil e aumentando o consumo de óxido nítrico, além de promoverem uma vaso-oclusão (STEINBERG, 2008a).

Em pacientes com AF, a formação e o alongamento dos polímeros de HbS leva a uma distorção do glóbulo vermelho. Essa distorção é inicialmente reversível após a reoxigenação, entretanto, episódios repetidos de falcização levam a danos permanentes na membrana celular, o que diminui a elasticidade da célula e sua capacidade de retornar a uma forma de disco bicôncava normal quando as condições de oxigênio são restauradas (REES; WILLIAMS; GLADWIN, 2010). No entanto, sabe-se que outros fatores influenciam na taxa de formação do polímero, como pH, temperatura, saturação de oxigênio, além de fatores relacionados ao paciente, como haplótipo β^S, níveis de hemoglobina fetal (HbF) e co-herança com a α talassemia (STEINBERG, 2005; STUART; NAGEL, 2004). Em relação à membrana plasmática, um estudo utilizando microscopia de raio-X suave, que apresenta resolução entre a microscopia de luz e a eletrônica, evidenciou que os polímeros de HbS formam protrusões nas hemácias, danificando-as. Entretanto, é provável que a visualização de poucas protusões nas hemácias contendo HbS esteja associada a uma maior quantidade de ciclos de desoxigenação/reoxigenação, evidenciando assim um maior dano na membrana e na morfologia do glóbulo vermelho (Figura 2) (DARROW et al., 2016). Ademais, pacientes com AF apresentam uma desregulação do volume eritróide, representado por uma desidratação celular, além de alterações significativas da membrana, incluindo uma maior rigidez. Entretanto, a compreensão da regulação

desse volume em hemácias contendo HbS ainda não está completamente elucidado (MOHANDAS; GALLAGHER, 2008).

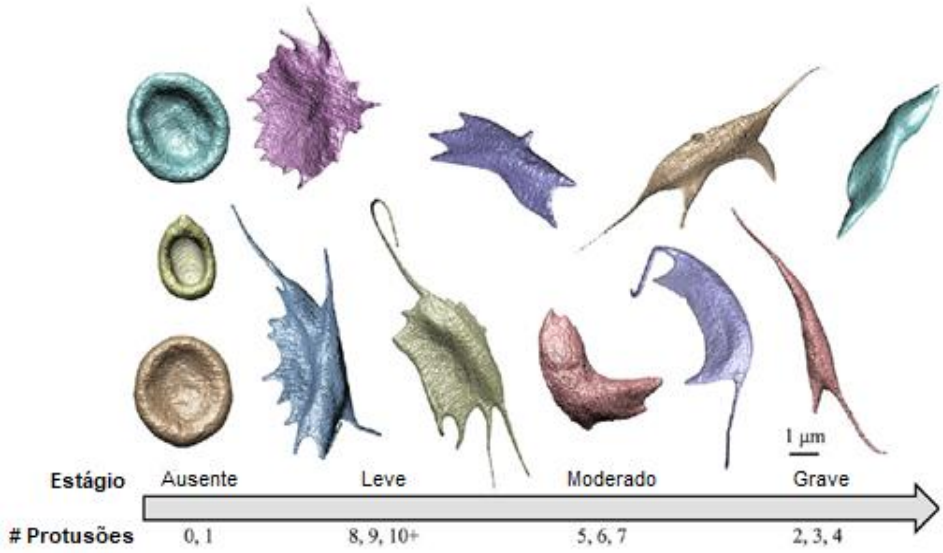

Figura 2 Estágios da falcização das hemácias (DARROW et al., 2016).

O processo de vaso-oclusão é resultado de um complexo cenário envolvendo interações de diferentes tipos celulares, incluindo células falcizadas, reticulócitos, células endoteliais, leucócitos, plaquetas, além de citocinas e fatores teciduais (CAPPELLINI, 2007; LANARO et al., 2009; MORRIS, 2008; SAKAMOTO et al., 2013). Os eritrócitos falciformes apresentam maior concentração de moléculas de adesão em sua superfície, como VCAM-1 e ICAM-1, favorecendo o processo de interação com o endotélio e com outros componentes da circulação, como leucócitos e plaquetas. Uma das moléculas de adesão exposta em grande quantidade pelo eritrócito falcizado é a fosfatidilserina (FS), que o deixa até três vezes mais aderente quando comparado aos eritrócitos normais (ZAGO; PINTO, 2007). Além dessa maior aderência, a exposição da FS foi correlacionada com a geração de trombina, substância relacionada à formação de coágulos de fibrina, que também contribuem para a oclusão vascular (SETTY; RAO; STUART, 2001).

A vaso-oclusão cria uma cascata que se retroalimenta: promove hipóxia, que gera inflamação, atraindo, assim, mais leucócitos e ativando continuamente o endotélio, facilitando a adesão dos elementos sanguíneos (ZAGO; PINTO, 2007). Desse modo, vaso-oclusões recorrentes, processos de isquemia-reperfusão e ativação do endotélio vascular induzem a contínuas respostas inflamatórias na AF, que se propagam por níveis elevados de citocinas inflamatórias, menor

biodisponibilidade do óxido nítrico (NO) e estresse oxidativo (CONRAN; FRANCO-PENTEADO; COSTA, 2009). O estresse oxidativo leva à rigidez e à instabilidade da membrana, causando danos significativos nas hemácias e aumentando a hemólise (AMER; FIBACH, 2005).

Além da vaso-oclusão, os indivíduos falciformes apresentam intensa hemólise: os eritrócitos permanecem na circulação de dois a vinte e um dias, fato este que se reflete nas baixas concentrações de hemoglobina, aumento do número de reticulócitos e aumento da concentração de marcadores de hemólise, como lactato desidrogenase (LDH) e bilirrubinas (BALLAS; MARCOLINA, 2006; KATO; STEINBERG; GLADWIN, 2017). Essa hemólise crônica tem importante papel na biodisponibilidade do NO, que está envolvido nos processos de vasodilatação, inibição da ativação e agregação plaquetária e também na diminuição da expressão de moléculas de adesão (ARMENIS et al., 2017; REITER et al., 2002).

Na AF, a hemólise ocorre extravascularmente pelo reconhecimento das hemácias danificadas por células do sistema reticuloendotelial. Entretanto, esse processo também pode ocorrer dentro dos vasos, podendo corresponder até 30% da hemólise total de um paciente com AF (STEINBERG, 2008a). Quando dentro dos vasos, libera hemoglobina livre, que consome o NO e lesa o endotélio vascular, e arginase, que promove a conversão da L-arginina (substrato da síntese do NO) em L-ornitina. Essa conversão reduz a biodisponibilidade do NO, agente vasodilatador, contribuindo para a ocorrência da vaso-oclusão, como vasoconstricção, ativação plaquetária e aumento da aderência da hemácia ao endotélio vascular (KATO; GLADWIN; STEINBERG, 2007).

Dessa forma, todo o processo vaso-oclusivo, hemolítico e inflamatório está relacionado (Figura 3), tendo papel determinante na origem das complicações clínicas apresentadas pelos indivíduos portadores de AF (ZAGO; PINTO, 2007).

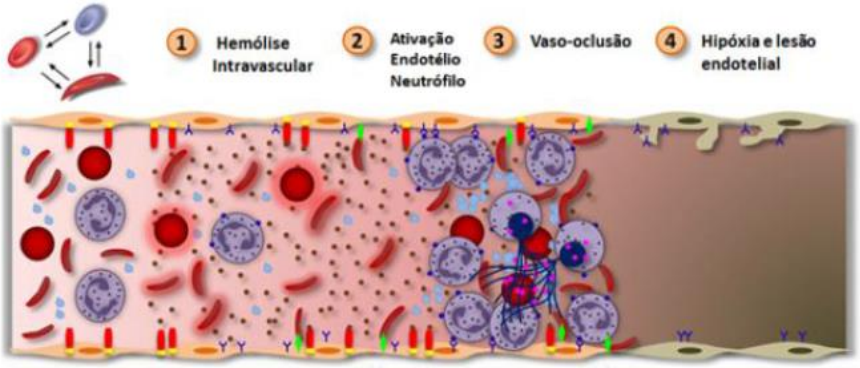

Figura 3 A hemácia falcizada induz o processo de vaso-oclusão e hemólise. (1) Eritrócitos falcizados levam à hemólise intravascular, a qual libera hemoglobina livre e arginase no plasma. (2) A ativação de neutrófilos e células endoteliais induz a expressão de moléculas de adesão. (3) A Hb livre e a arginase diminuem a biodisponibilidade de NO provocando vasoconstricção; e células endoteliais ativam a coagulação levando à adesão de plaquetas ao endotélio com participação de eritrócitos e neutrófilos. (4) Dependendo da extensão da vaso-oclusão, os tecidos podem apresentar hipóxia e necrose. (Adaptado de DUTRA; BOZZA, 2014).

2.1.3 Complicações Clínicas

O estado inflamatório crônico concomitante aos fenômenos vaso-oclusivos e à intensa hemólise leva os indivíduos com anemia falciforme a apresentarem diversas complicações clínicas, de caráter agudo ou crônico, que acometem diversos órgãos e tecidos (BALLAS et al., 2010; STEINBERG, 2008b). O quadro 1 demonstra algumas das complicações que podem ser apresentadas pelos pacientes com anemia falciforme.

Episódios de dor por vaso-oclusão
Infecções recorrentes
Exacerbações agudas da anemia Sequestro esplênico Crise aplásica
Complicações cardíacas Cardiomegalia Hipertensão arterial sistêmica Insuficiência cardíaca congestiva
Complicações gastrointestinais e hepatobiliares Colelitíase Infarto hepático Hepatomegalia Esteatose hepática
Complicações musculares, esqueléticas e dermatológicas Osteonecrose Úlceras de perna
Complicações neurológicas Acidente vascular cerebral
Complicações oftalmológicas
Complicações pulmonares Síndrome torácica aguda Hipertensão arterial pulmonar
Complicações renais
Complicações genitourinárias Priapismo
Complicações esplênicas Sequestro esplênico Autoesplenectomia

Quadro 1 Complicações clínicas que pacientes com anemia falciforme podem apresentar durante sua vida (Adaptado de BALLAS et al., 2010).

Dentre todas as complicações, a mais comum apresentada pelos indivíduos é a vaso-oclusão, que leva à isquemia tecidual, causando danos vasculares e inflamação que se refletem em episódios agudos de intensa dor, sendo a principal causa de admissão hospitalar dos pacientes com AF (LOVETT; SULE; LOPEZ, 2014). O evento vaso-oclusivo pode ocorrer em qualquer órgão, sendo mais comum na medula óssea, logo, é tido como um evento predominantemente ósseo (ALMEIDA; ROBERTS, 2005). As primeiras crises geralmente manifestam-se com dor nos pés e mãos em crianças de até 5 anos, evento este chamado de dactilite. Em crianças com idade escolar, a dor é principalmente nos ossos longos e, quando mais velhos, costumam ter crises dolorosas na coluna (HOWARD; DAVIES, 2007).

A síndrome torácica aguda é tida como a segunda maior causa de admissões hospitalares em indivíduos com AF, acometendo aproximadamente 50% deles (JAIN; BAKSHI; KRISHNAMURTI, 2017; VICHINSKY et al., 1997, 2000). Caracterizada por febre, dor no peito, infiltrado pulmonar e dificuldade respiratória, é a principal causa de morte dos adultos jovens com AF (QUINN, 2013). A gravidade desse evento é variável, mas 13% dos pacientes necessitam de ventilação mecânica e 3% morrem. O tratamento envolve antibióticos de amplo espectro, broncodilatadores e oxigênio, além do uso de transfusão de concentrado de hemácias, se os níveis de hemoglobina diminuírem substancialmente (JAIN; BAKSHI; KRISHNAMURTI, 2017).

Outro evento que acomete os pacientes com AF é o acidente vascular cerebral (AVC), que pode ser definido como um evento neurológico agudo secundário à oclusão de uma artéria ou a uma hemorragia, com consequente isquemia e/ou sinais e sintomas neurológicos. Na AF, o acidente vascular cerebral é uma das principais causas de óbito em crianças e adultos. O AVC, isoladamente, é responsável por 20% dos óbitos de crianças com doença falciforme entre 5-10 anos; além disso, 70% das crianças que desenvolvem o AVC apresentam déficit motor e significante déficit cognitivo (OHENE-FREMPONG et al., 1998; ZHOU; BEHYMER; GUCHHAIT, 2011). Ademais, crianças com anemia falciforme possuem um risco 300x maior de desenvolver um acidente vascular cerebral, tornando assim a AF a maior causadora de AVC durante a infância. (HOPPE et al., 2007; ZHOU; BEHYMER; GUCHHAIT, 2011). Para a prevenção desse evento, é realizado o exame de ultrassonografia através do Doppler Transcraniano (DTC), método não invasivo que determina as velocidades de fluxo sanguíneo das artérias cerebrais (ADAMS et al., 1992; CONNES; VERLHAC; BERNAUDIN, 2013; FLANAGAN et al., 2011). Visto que o risco do AVC é diretamente proporcional ao aumento da velocidade média nas artérias cerebrais, como as artérias carótidas internas distais e cerebrais médias proximais (ADAMS et al., 2004; FLANAGAN et al., 2011), é possível realizar, na presença de resultados alterados, a profilaxia da ocorrência do AVC com um regime de transfusão crônica (ADAMS et al., 1998).

Danos renais e cardíacos também são comuns nos indivíduos com AF. Nos rins, a baixa tensão de O_2, baixo pH e alta osmolaridade favorecem a polimerização da HbS e consequente vaso-oclusão, levando os indivíduos a apresentarem infarto renal (REES; WILLIAMS; GLADWIN, 2010; SERJEANT, 1993). Cerca de 30% dos adultos desenvolvem insuficiência renal crônica, sendo causa de morte em diversos

casos (PLATT et al., 1994). No que concerne ao sistema cardiovascular, os indivíduos com AF apresentam diversas complicações que aumentam a morbimortalidade da doença (VASCONCELOS et al., 2015). Dentre as alterações cardíacas estão cardiomegalia, valvulopatias e insuficiência cardíaca congestiva (BALLAS et al., 2010). Tais alterações ocorrem tanto em decorrência ao alto débito cardíaco, que leva ao aumento das câmaras cardíacas desde a infância, como também pela vaso-oclusão, uma vez que os eventos isquêmicos promovem lesões cardíacas como a fibrose (GUALANDRO; FONSECA; GUALANDRO, 2007).

O tecido ósseo, por apresentar microvasculatura que favorece a falcização dos eritrócitos, é bastante afetado nos indivíduos com AF. Além da já citada vaso-oclusão, os indivíduos podem apresentar danos teciduais e chegar a desenvolver a condição crônica da osteonecrose (BENNETT; NAMNYAK, 1990; SERJEANT, 1993). Essa complicação, também chamada necrose isquêmica, atinge cerca de 50% dos indivíduos AF após os 30 anos de idade, causando intensas dores e, quando na junção osteoarticular do quadril, dificuldade de locomoção, diminuindo a qualidade de vida (DA SILVA JUNIOR; DAHER; DA ROCHA, 2012). O único tratamento disponível é a artroplastia, que consiste na colocação de uma prótese na cabeça do fêmur do paciente. Tal procedimento, entretanto, apresenta altos índices de morbimortalidade e, em 50% dos casos, requer nova cirurgia após 5 ou 10 anos (AL-MOUSAWI et al., 2002).

Outra complicação comum é o desenvolvimento de úlceras em membros inferiores, que é a manifestação cutânea mais comum nos pacientes com AF e incide de 25% a 75% deles, sendo mais comum nas regiões tropicais e subtropicais devido ao clima e às baixas condições socioeconômicas (ALAVI; KIRSNER, 2015; CUMMING et al., 2008; SERJEANT et al., 2005). Essas lesões são dolorosas, podem surgir espontaneamente ou em decorrência de pequenos traumas e têm cicatrização mais lenta do que as úlceras de outras etiologias. Além disso, apresentam pouca resposta aos tratamentos, alta reincidência e são susceptíveis à infecção por microrganismos, contribuindo para diminuição na qualidade de vida do indivíduo afetado (PALADINO, 2007; POWARS et al., 2005; SERJEANT et al., 2005). Quanto à sua etiologia, os fenômenos vaso-oclusivos, ao provocarem hipóxia, inflamação e consequente necrose, levam ao dano tecidual contribuindo para a ocorrência dos ferimentos (PALADINO, 2007). A intensa hemólise, que diminui a biodisponibilidade do NO, também parece ter importante contribuição na abertura das úlceras, uma vez

que essa complicação é vista em outras formas de anemia hemolítica e os indivíduos que a apresentam mostram dados laboratoriais com altas concentrações dos marcadores de hemólise, como LDH e bilirrubinas, quando comparados aos pacientes sem a manifestação (KATO; GLADWIN; STEINBERG, 2007).

Além dessas complicações, outra que é comum nos indivíduos do sexo masculino é o priapismo, que é definido como uma ereção peniana prolongada e dolorosa que ocorre na ausência de estímulo sexual, e pode acometer até 40% dos pacientes com AF (STEINBERG, 2008a), dos quais, 90% apresentam pelo menos um episódio até os 20 anos de idade (MANTADAKIS et al., 1999). Na AF, 95% dos eventos de priapismo são do tipo isquêmico ou de baixo fluxo, que, devido à baixa oxigenação do sangue, está associado à inflamação que, na ausência de tratamento adequado, pode levar à necrose tecidual e causar insuficiência erétil (VICARI; FIGUEIREDO, 2007). Embora haja participação de evento vaso-oclusivo na sua ocorrência, o priapismo é tido como uma complicação clínica fortemente associada à intensa hemólise, pois há elevadas concentrações de marcadores hemolíticos (KATO; GLADWIN; STEINBERG, 2007).

Uma das características marcantes da doença falciforme é a variabilidade de suas manifestações clínicas, e as razões para essa heterogeneidade ainda não são completamente entendidas (LETTRE et al., 2008), variando de formas quase assintomáticas até clinicamente graves, responsáveis por alta mortalidade na infância (HIGGS; WOOD, 2008). Essa variabilidade fenotípica pode, em parte, ser explicada por fatores externos, como a condição socioeconômica do indivíduo, que determinará maior ou menor acesso a informações acerca da doença e tratamento adequado, por exemplo (CAJADO et al., 2011; CHRISTAKIS et al., 1990). Além disso, estudos sugerem a existência de um componente genético, além da mutação pontual na globina β, como indicativo do prognóstico dos pacientes com AF (STEINBERG, 2009). Desse modo, a ação de múltiplos genes combinados pode determinar a gravidade geral da doença (HOPPE et al., 2004; SEBASTIANI et al., 2010). Entre os moduladores genéticos mais conhecidos estão as variações do tipo de haplótipo ligado ao *cluster* da globina β, relacionados com as variações nos níveis de Hb Fetal (HbF), e a presença de talassemia α (HIGGS; WOOD, 2008; LETTRE et al., 2008). Além disso, alterações em genes relacionados com vias de inflamação, biologia do óxido nítrico, adesão celular, e estabilidade e manutenção do citoesqueleto eritrocitário, aparecem como possíveis candidatos para modular o quadro clínico de

pacientes com AF (ATAGA; CAPPELLINI; RACHMILEWITZ, 2007; HOPPE et al., 2007; STEINBERG, 2005; SUN et al., 2016; ZENNADI et al., 2012; ZHANG et al., 2014).

2.1.4 Tratamento

2.1.4.1 Hidroxiuréia (HU)

A hidroxiuréia (HU), agente quimioterápico inicialmente utilizado nas doenças onco-hematológicas, foi aprovada em 1998 para o tratamento da AF, por melhorar os parâmetros hematológicos e diminuir o número de crises dolorosas e hospitalizações dos pacientes, sendo considerada por muito tempo como a única droga capaz de modificar o curso clínico natural da doença (CHARACHE et al., 1995; ROSSE et al., 2000; WANG et al., 2011). Os efeitos benéficos da HU, um inibidor da fase S do ciclo celular, são atribuídos à sua capacidade de aumentar a produção de hemoglobina fetal (HbF), codificada pelo gene da globina γ (*HBG*), em células progenitoras eritróides por uma via dependente de GMPc, aumentando a concentração final de HbF na hemácia falcizada e inibindo, assim, a polimerização da HbS (COKIC et al., 2003). Além disso, alguns estudos sugerem que a HU pode promover benefícios por mecanismos não relacionados à indução de HbF, como um efeito anti-inflamatório na diminuição do número de leucócitos, citocinas e moléculas de adesão e um aumento da produção de óxido nítrico (COKIC et al., 2006; GREEN; BARRAL, 2014; PLATT, 2008; YAWN et al., 2014; ZIMMERMAN et al., 2004).

A hemoglobina fetal (HbF) é a molécula mais estudada como modulador genético na AF. Por não participar do polímero de HbS e, consequentemente, diminuir a formação deste mesmo polímero, o aumento dos níveis de HbF pode melhorar o curso clínico do paciente falciforme. Pacientes com AF apresentam índices de HbF variando entre 1-30% (média de 8%), modulados, em parte, pelos haplótipos da globina β. No entanto, ter o conhecimento do nível de HbF de um paciente falciforme é insuficiente para prever as possíveis complicações clínicas. Alguns pacientes apresentam graves complicações da doença mesmo apresentando níveis de HbF em torno de 20% (AKINSHEYE et al., 2011; WYSZYNSKI et al., 2004).

Crianças com AF apresentam uma maior sobrevida após o tratamento com HU, principalmente pela diminuição do desenvolvimento de síndrome torácica aguda

e infecções. Além disso, uma diminuição dos níveis de reticulócitos e neutrófilos, fatores de risco já estabelecidos da doença falciforme, tem sido descrita após o tratamento com essa droga (LOBO et al., 2013). Ademais, estudos prévios já demonstraram que a terapia com HU tem sido associada com uma diminuição das velocidades de fluxo sanguíneo nas artérias cerebrais, mensuradas pelo DTC, e com uma menor taxa de recorrência do AVC (LAGUNJU; BROWN; SODEINDE, 2015).

Por se tratar de um agente quimioterápico, o uso da HU foi questionado inicialmente devido aos possíveis efeitos adversos que poderiam ser causados a um longo prazo. Entretanto, vários estudos de acompanhamento de pacientes falciformes que utilizaram a droga foram realizados, não se encontrando associação entre a HU e possíveis efeitos neoplásicos (BALLAS et al., 2009; STEINBERG et al., 2003, 2010).

Sendo assim, o uso da HU tem sido cada vez mais incentivado em pacientes com AF (STEINBERG et al., 2003, 2010). Apesar de conter alguns efeitos adversos temporários, como leucopenia e plaquetopenia, que poderiam predispor os pacientes a infecções e sangramentos, o risco do uso da HU em pacientes falciformes é aceitável quando comparado com o risco de pacientes falciformes não tratados (BRAWLEY et al., 2008; NEVITT; JONES; HOWARD, 2017).

2.1.4.2 L-Glutamina

Em 2017, após quase 20 anos do início do uso da HU para o tratamento de pacientes com AF, a L-glutamina é aprovada para o tratamento de pacientes com AF maiores que 5 anos (NIIHARA et al., 2018). Apesar de ser um aminoácido não essencial, a produção de L-glutamina é insuficiente durante o período neonatal, situações de estresse ou no curso de algumas doenças crônicas, como a AF, sendo necessária uma suplementação pela dieta. Em relação a sua função, a L-glutamina é necessária para a proliferação celular e está envolvida na síntese várias moléculas, como a nicotinamida adenina dinucleotídeo (NAD) e nicotinamida adenina dinucleotídeo fosfato (NADP), importantes na produção de energia celular, além de participar na síntese de arginina e na redução da glutationa (QUINN, 2018). Em hemácias, a glutationa é um dos principais responsáveis pelo potencial redutor da célula, e níveis diminuídos dessa molécula tem sido associado à hemólise (MINNITI, 2018).

Em pacientes com AF, a L-glutamina provou-se eficaz em reduzir a frequência de crises vaso-oclusivas em 25%, além de diminuir o número de hospitalizações, a duração dos internamentos e a incidência de síndrome torácica aguda (NIIHARA et al., 2018). Entretanto, a L-glutamina não parece melhorar os níveis de hemoglobina nem a contagem de reticulócitos, ainda que o mecanismo de ação especulado envolva uma menor susceptibilidade da hemácia ao estresse oxidativo e, consequentemente, diminuição da hemólise (QUINN, 2018).

A frequência de eventos agudos também foi menor entre os pacientes que receberam concomitantemente HU, droga que apresenta um mecanismo de ação diferente da L-glutamina, o que sugere um possível efeito aditivo entre as duas drogas e, consequentemente, um maior benefício para o paciente. Além disso, para aqueles pacientes que recusem o tratamento com HU ou que apresentem efeitos colaterais, a L-glutamina surge como uma terapia alternativa (NIIHARA et al., 2018).

Apesar da eficácia da L-glutamina, ainda existem algumas barreiras para expandir o uso desse medicamento. Em relação ao custo, o tratamento com L-glutamina custa 24x mais que o tratamento com HU nos Estados Unidos, em um mesmo intervalo de tempo (MINNITI, 2018). Além disso, a taxa de abandono do estudo clínico utilizado para liberar o uso dessa droga foi considerada alta (32%), ainda que a maioria dos efeitos adversos descritos tenham sido associados à doença de base e não ao uso do medicamento. Por fim, recomenda-se precaução na prescrição de L-glutamina a pacientes com AF que apresentem alguma disfunção renal e hepática clinicamente significativa, pois pacientes com essas alterações não foram incluídos no estudo clínico inicial (NIIHARA et al., 2018).

2.1.4.3 Transfusão de Hemácias e Remoção de Ferro

A transfusão de hemácias corrige a anemia, reduz a porcentagem de HbS, suprime a síntese de HbS e reduz a hemólise, apresentando um grande benefício para o paciente com AF (REES; WILLIAMS; GLADWIN, 2010; WARE et al., 2017). Sendo assim, a transfusão tem um papel estabelecido no manejo de complicações agudas e crônicas da AF, conforme demonstrado no quadro 2.

Indicações para transfusões agudas
Exacerbação aguda da anemia Tipicamente causada pela infecção por Parvovírus B19, sequestro esplênico ou vaso-oclusão grave; necessária uma transfusão simples para aumentar as concentrações de hemoglobina para 8-9 g/dL
Síndrome torácica aguda A transfusão simples é benéfica, sendo a transfusão de troca indicada para reduzir HbS a menos de 30% apenas em casos graves
Acidente vascular cerebral ou déficit neurológico agudo Transfusão urgente para aumentar as concentrações de hemoglobina para 10 g/dL e reduzir a HbS para menos de 30%, o que normalmente requer transfusão de troca
Falha multiorgânica HbS para menos de 30% com concentração de hemoglobina de 10 g/dL
Pré-operatório Busca deixar a HbS em menos de 30% antes de cirurgia de grande porte (cardiotorácica, neurocirurgia), normalmente requerendo transfusão de trocas; cirurgia de risco médio ou de baixo risco pode precisar de transfusão simples para aumentar a concentração de hemoglobina para 10 g/dL
Indicações para transfusões regulares e de longo prazo
Prevenção de AVC primária e secundária Transfusões regulares, simples ou de troca, para manter a HbS em menos de 30%
Síndrome torácica aguda recorrente não ajudada pela hidroxiuréia Transfusões regulares, simples ou de troca, para manter a HbS em menos de 30%
Falha orgânica progressiva Inclui insuficiência hepática, renal, cardíaca e pulmonar; há poucas práticas baseadas em evidências e as estratégias de transfusão variam amplamente
Outras indicações Sequestro esplênico recorrente, gravidez complicada
Indicações controversas Dor aguda frequente, dor crônica, osteonecrose úlceras nas pernas, priapismo

Quadro 2 Indicações para transfusão de hemácias em pacientes com anemia falciforme (adaptado de MONTALEMBERT, 2009; REES et al., 2010).

O concentrado de hemácias pode ser administrado como uma simples transfusão ou por troca, no qual o sangue do paciente é removido antes da infusão. A transfusão de troca se faz necessária quando a concentração de hemoglobina inicial é alta ou quando se tem uma necessidade de reduzir rapidamente os níveis de HbS, sem alterar o hematócrito e a viscosidade sanguínea (ECKMAN, 2001).

Apesar de benéfica, a transfusão de hemácias apresenta riscos associados (VICHINSKY, 2001), como a aloimunização, que ocorre devido a diferenças entre as origens étnicas de doadores de sangue e pacientes (VICHINSKY et al., 1990). A aloimunização é a formação de anticorpos contra antígenos não presentes nas

hemácias do receptor. Esses anticorpos podem ser clinicamente significativos, levando a reações transfusionais hemolíticas tardias ou doença hemolítica do feto e recém-nascido (HENDRICKSON; TORMEY, 2016). Além disso, a transfusão crônica de hemácias é inevitavelmente associada à sobrecarga de ferro e consequente depósito de ferro no fígado e, em menor grau, no coração (WOOD et al., 2004). Desse modo, é importante associar a terapia transfusional com a quelação de ferro, utilizando desferoxamina por via parenteral ou deferasirox por via oral, principalmente para evitar danos no fígado (VICHINSKY et al., 2007).

A identificação de pacientes com risco para o desenvolvimento do AVC por um método de triagem, como o DTC, permite a administração precoce de transfusões profiláticas, beneficiando o portador de AF (STEINBERG, 2005). Manter o nível de HbS em torno dos 30% é recomendado como prevenção do AVC primário e secundário em crianças de 2-16 anos, com o uso de terapias baseadas em transfusões crônicas. Em pacientes com AF e velocidades de fluxo elevadas no DTC, transfusões crônicas e regulares de concentrado de hemácias (entre 21 e 30 dias) reduzem em 90% o risco de ocorrer um primeiro AVC, além de diminuir a taxa hemolítica e o nível de hemoglobina plasmática livre (LEZCANO et al., 2006). Entretanto, estudos têm demonstrado que a descontinuidade das transfusões, mesmo após vários anos, pode reverter as velocidades de fluxo cerebrais para valores pré-transfusionais, favorecendo o desenvolvimento do AVC (STEINBERG, 2005).

2.1.4.4 Transplante de células tronco hematopoiéticas

O transplante de células hematopoiéticas a partir de um doador saudável ou de um indivíduo com traço falciforme é único tratamento curativo da doença e começou a ser utilizado há mais de 30 anos (JOHNSON et al., 1984). Crianças com AF que recebem transplante de células tronco usando um irmão HLA compatível tem uma chance de cura de 92%, além de uma sobrevida global de 95% (KING; SHENOY, 2014; NICKEL; HENDRICKSON; HAIGHT, 2014; WALTERS, 2015). Entretanto, estima-se que menos de 30% dos indivíduos com AF tenham doadores HLA compatíveis, limitando a busca desse tratamento e estimulando o uso de doadores não relacionados, além da utilização de células provenientes do sangue de cordão umbilical (ABRAHAM et al., 2017; ALFRAIH et al., 2016; ARNOLD et al., 2016; KAMANI et al., 2012; MINIERO et al., 1998; WALTERS et al., 2016).

Apesar dos resultados excelentes associados a esse tipo de transplante, existe um debate sobre quem deve ser transplantado, e de quando esse procedimento deva ser realizado (Bender, 1993 - atualizado em 2017). Na doença falciforme, uma avaliação de 1000 transplantes realizados entre 1986 e 2013 evidenciou que a sobrevida livre de doença era de 93% e 81% para pacientes falciformes com menos e mais de 16 anos, respectivamente. Além disso, para cada ano de vida adicional, o risco de morte aumentava em 10% (GLUCKMAN et al., 2017).

O transplante com doador HLA compatível no início da vida pode subverter uma vida de complicações debilitantes e diminuir as falhas orgânicas, diminuindo os efeitos benéficos com o aumento da idade do paciente. Entretanto, os serviços pediátricos podem se preocupar demais com os riscos de transplante e não apreciar a alta morbidade das manifestações da AF na idade adulta (Bender, 1993 - atualizado em 2017). Desse modo, o transplante de células hematopoiéticas só é considerado em crianças quando complicações graves ocorrem, como na presença de doença cerebrovascular dependente de transfusões (REES; WILLIAMS; GLADWIN, 2010). No entanto, os benefícios comparativos a longo prazo dos cuidados de suporte, incluindo o uso de HU, versus a realização do transplante de células hematopoiéticas, ainda não são conhecidos (Bender, 1993 - atualizado em 2017).

O transplante de células tronco hematopoiéticas, apesar dos benefícios, sempre apresenta riscos a longo prazo associados, como doença do enxerto versus hospedeiro, infecções e infertilidade. Desse modo, metodologias que envolvam a alteração do genoma surgem como promissoras para a cura da AF, e pesquisas abordando terapia gênica para a AF tem surgido (CAVAZZANA; ANTONIANI; MICCIO, 2017; GOODMAN; MALIK, 2016; RIBEIL et al., 2017).

2.1.4.5 Terapia gênica

A edição do genoma para correção de uma mutação específica, mais conhecida como terapia gênica, pode fornecer uma opção terapêutica ideal para o tratamento de doenças sem cura pelos métodos convencionais, como as hemoglobinopatias e as imunodeficiências primárias (LOMOVA et al., 2018; MOSS, 2014). Essa alteração local de um gene específico pode ser feita através da indução da quebra da fita dupla de DNA em uma região próxima à mutação usando nucleases direcionadas, como o sistema CRISPR/Cas9, e, posteriormente, a utilização das vias

inatas de reparo celular para corrigir a quebra (LOMOVA et al., 2018). Uma vez modificadas, as células tronco hematopoiéticas, que apresentam capacidade de auto renovação, podem ser reinfundidas no paciente e fornecer, a longo prazo, um suprimento vitalício de células saudáveis (LOMOVA et al., 2018).

As duas principais vias de reparo do DNA são a união de extremidade não-homóloga (NHEJ), propensa a erros e disponível em todo o ciclo celular (LIEBER et al., 2003), e reparo dirigido por homologia (HDR), modelo preciso de reparo da dupla fita de DNA e restrito às fases S e G2 do ciclo celular (BRANZEI; FOIANI, 2008; HEYER; EHMSEN; LIU, 2010). Para a terapia gênica obter os melhores resultados possíveis, é necessário selecionar, *in vitro*, células que tenham realizado o reparo por HDR, visto que o reparo por NHEJ é mais susceptível a erros, como grandes deleções ou rearranjos genômicos (KOSICKI; TOMBERG; BRADLEY, 2018; RAN et al., 2013). No gene da globina β, essas deleções poderiam transformar o alelo β^S em um alelo β-talassêmico, provavelmente conferindo um fenótipo pior do que a doença inicial (LOMOVA et al., 2018).

Inicialmente, os ensaios clínicos em modelos murinos de AF utilizaram as abordagens de terapia gênica mediada por vírus, que foram capazes de corrigir defeitos hematológicos e danos nos órgãos dos animais (PAWLIUK et al., 2001; PERSONS, 2009). Em humanos, o primeiro relato de paciente com AF tratado por essa metodologia vem da França. Após uma quimioterapia mieloablativa, o paciente foi transplantado com 5 $x10^6$ células CD34+/Kg que apresentavam o vetor lentiviral BB305 expressando a globina $\beta^{A\text{-}T87Q}$, provenientes de 2 coletas de medula óssea. A reconstituição de todas as linhagens hematopoiéticas foi rápida e sustentada, e nenhum evento adverso relacionado ao tratamento foi observado em 15 meses de acompanhamento. Além disso, o paciente apresentava uma independência transfusional e níveis da globina $\beta^{A\text{-}T87Q}$ em torno de 50%, com um quadro clínico comparável ao de um indivíduo traço falciforme (RIBEIL et al., 2017).

A terapia gênica para hemoglobinopatias apresenta desafios adicionais. Os genes da globina estão sujeitos a uma regulação sofisticada, que confere uma expressão gênica restrita a uma linhagem, bem como a escolha de genes que sintetizam globinas específicas durante as fases embrionária, fetal e adulta (SMITH; ORKIN, 2016; WILBER; NIENHUIS; PERSONS, 2011). Estratégias futuras para a terapia gênica em AF tem surgido, e elas envolvem a correção direta da mutação β^S, já realizada por alguns grupos, mas ainda sem obter uma eficiência terapêutica

(DEVER et al., 2016; DEWITT et al., 2016; HOBAN et al., 2015, 2016), ou a ativação endógena da síntese de HbF ao silenciar o *BCL11A*, gene que codifica um fator de transcrição responsável por inibir a expressão de HbF em eritroblastos na fase adulta (CANVER et al., 2015). No entanto, essas abordagens ainda são, atualmente, menos eficientes do que a terapia gênica mediada pelo vetor lentiviral, e a segurança dessas metodologias ainda não foram testadas *in vivo* (FERRARI; CAVAZZANA; MAVILIO, 2017).

2.2 CITOESQUELETO ERITROCITÁRIO

Os eritrócitos são únicos entre as células eucarióticas, pois não possuem núcleos, estruturas citoplasmáticas e organelas. Devido a isso, as propriedades estruturais e funcionais dos eritrócitos estão intimamente associadas às suas membranas plasmáticas (MOHANDAS; GALLAGHER, 2008). A membrana plasmática em células eucariotas consiste em uma estrutura de bicamada de glicerofosfolípidos, esfingolípidos e colesterol (GORTER; GRENDEL, 1925) com proteínas embutidas. Uma das características únicas da membrana plasmática é a distribuição assimétrica de fosfolípidos específicos entre os dois folhetos da bicamada (VAN MEER; VOELKER; FEIGENSON, 2008), sendo a perda dessa assimetria um sinal de morte celular (FADOK et al., 1992). Por exemplo, a fosfatidilcolina (FC) e a esfingomielina estão predominantemente presentes no folheto exoplasmático, enquanto a fosfatidilserina (FS) e a fosfatidiletanolamina (FE) são principalmente confinadas ao folheto citoplasmático da membrana plasmática (LEVENTIS; GRINSTEIN, 2010). O estabelecimento e a manutenção dinâmica da distribuição não aleatória de fosfolípidos é importante para funções normais da membrana e tem sido implicado em muitos processos celulares, incluindo coagulação do sangue, formação de vesículas e apoptose (LEVENTIS; GRINSTEIN, 2010).

Além da bicamada de fosfolipídios, a membrana dos eritrócitos apresenta malha pseudo-hexagonal de proteínas, contendo 20 proteínas principais e pelo menos 850 menores, que podem ser divididas em proteínas integrais e periféricas (PESCIOTTA et al., 2012). As proteínas integrais estão embutidas na bicamada lipídica e são organizadas em complexos macromoleculares em torno da banda 3, o canal que permite a troca de ânions. Já a maioria das proteínas periféricas são responsáveis por formar o citoesqueleto da membrana, uma malha de proteína de 40

a 90 nm de espessura que margeia a superfície interna da hemácia (Figura 4). O citoesqueleto eritrocitário é composto principalmente de espectrina, actina, anquirina, proteína 4.1R e algumas proteínas auxiliares, como a tropomiosina, tropomodulina, aducina e dematina, e tem a função de fortalecer a bicamada lipídica e conferir durabilidade e flexibilidade para a hemácia sobreviver na circulação (LUX, 2016).

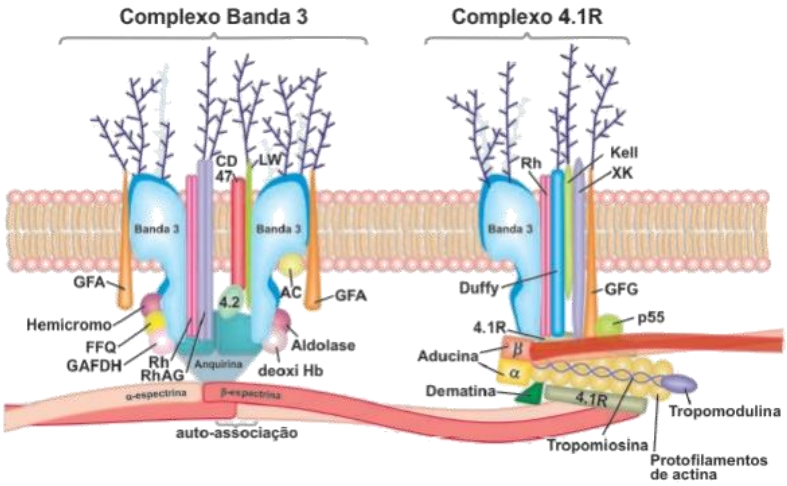

Figura 4 Representação da membrana eritrocitária (ZAGO; FALCÃO; PASQUINI, 2013).

O citoesqueleto eritrocitário é ancorado na bicamada de fosfolipídios através de duas ligações entre as proteínas; uma ligação ocorre entre a banda 3, proteína integral de membrana, e a espectrina, via anquirina, enquanto que a segunda ligação envolve o complexo juncional de actina, que une a extremidade C-terminal de espectrina a protofilamentos de actina curta, a F-actina (AZIM et al., 1995; BRUCE et al., 2003). O complexo juncional é composto pela proteína 4.1, dematina e aducina, e esse complexo é atraído para a membrana plasmática por outro complexo proteico, formado pela proteína 4.1, p55 (ou MMP1, metaloproteinase de matriz 1) e pela glicoforina transmembranar C (MARFATIA et al., 1994). Além disso, a dematina e/ou aducina se ligam diretamente ao receptor de membrana do transportador de glicose-(Glut1) e a banda 3 (ANONG et al., 2009; KHAN et al., 2008). Ademais, sugere-se que o complexo juncional da actina seja essencial para garantir a estabilidade da membrana e da forma das hemácias, e sabe-se que a sobrevivência a longo prazo dos glóbulos vermelhos depende da resistência mecânica e da deformabilidade da sua membrana plasmática. Entretanto, o mecanismo preciso que mantém a integridade dessas junções ainda é pouco compreendido (LUX, 2016).

Nos eritrócitos humanos, a fosfatidilserina (FS) está presente exclusivamente no folheto interno da bicamada lipídica de membrana como resultado do transporte

ativo dependente de ATP (*flipping*) de aminofosfolípidos do exoplasma para o citoplasma. A FS interage com a espectrina e auxilia no processo de deformabilidade da membrana da hemácia, além de ajudar a conferir uma estabilidade mecânica aos eritrócitos (MANNO; TAKAKUWA; MOHANDAS, 2002). Além disso, prevenir a exposição de FS na superfície externa é fundamental para a sobrevivência dos eritrócitos, visto que essa exposição, no final da vida útil das hemácias, é um sinal fagocítico para os macrófagos remover as células senescentes (LAUBER et al., 2004).

Apesar da FS estar restrita ao folheto interno da membrana eritrocitária, a fisiopatologia de várias doenças pode contribuir para uma maior exposição de FS. Em hemácias de pacientes com AF, o estresse oxidativo, que promove um acúmulo de grupo heme e íons ferro, pode ativar os mecanismos que expõem FS (HEBBEL et al., 1988). Além disso, o aumento de Ca2+ intracelular parece estar envolvido na exposição de FS, e a homeostase do cálcio está desregulada em hemácias contendo HbS, especialmente em situações desoxigenadas (LEW; BOOKCHIN, 2005). Na AF, cerca de 2 a 10% das hemácias apresentam uma exposição de FS, valores mais altos que o encontrado em hemácias de indivíduos saudáveis (DE JONG et al., 2001; KUYPERS, 2008).

Na presença de uma doença que altere o funcionamento normal do eritrócito, como a AF, a aceleração da destruição pela exposição de FS pode contribuir para a redução da vida útil dessas células (LANG et al., 2002). Além de ser exposta em células senescentes normais, a FS é exposta prematuramente por eritrócitos falciformes e talassêmicos, resultando em um período de vida reduzido dos glóbulos vermelhos e consequente anemia hemolítica nestes distúrbios (BOAS; FORMAN; BEUTLER, 1998; CHIU et al., 1981; KUYPERS et al., 1998). Além disso, hemácias com uma maior exposição de FS são encontradas em pacientes submetidos a esplenectomia (KRISTINSSON et al., 2014).

Além da distribuição assimétrica de fosfolípidos, alterações na fosforilação de proteínas de membrana também podem regular a morfologia e a estabilidade da membrana (DZANDU; JOHNSON, 1980; JOHNSON; DZANDU; WARTH, 1986; PANTALEO et al., 2010), e as hemácias de pacientes com AF tem apresentado um padrão distinto quando comparado a hemácias de indivíduos saudáveis. Em hemácias contendo HbS, vários trabalhos têm relatado uma diminuição da fosforilação de espectrina e anquirina, além de uma maior fosforilação da banda 3, dematina e banda 4,2 (LIU et al., 1991; SCHWARTZ et al., 1987; SICILIANO et al.,

2010; WAUGH et al., 1986). Ademais, a atividade da proteína quinase C encontra-se cerca de 50% aumentada na membrana de hemácias falciformes, quando comparadas às hemácias saudáveis (APOVO et al., 1989).

2.3 ATP11C

O *ATP11C* está localizado no braço longo do cromossomo X (Xq27.1), apresenta 36 éxons e codifica uma flipase de grande importância em eritrócitos humanos (ARASHIKI et al., 2016a). Com base na sequência de aminoácidos, as flipases são proteínas da família P-IV ATPase, e os membros dessa família são parecidos com as bombas convencionais de cátions-ATPase, como a bomba Na^+-K^+-ATPase e Ca^{2+}-ATPase, possuindo um domínio transmembranar com 10 hélices e três domínios citoplasmáticos: P (fosforilação), N (ligação de nucleotídeos) e A (atuador) (Figura 5) (VESTERGAARD et al., 2014). Além disso, de maneira similar a uma bomba de cátion, os membros da família P-IV ATPase formam um intermediário 4-aspartil fosfato, originado a partir do Asp412 na sequência de aminoácidos, e essencial para a função dessa molécula (ARASHIKI et al., 2016a; VESTERGAARD et al., 2014).

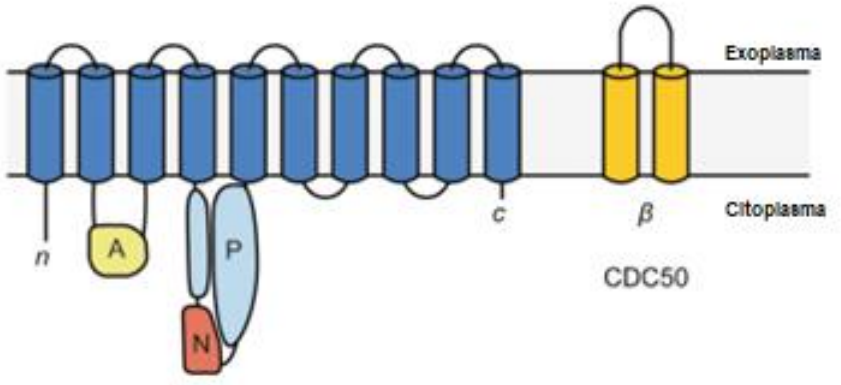

Figura 5 Representação esquemática das flipases. Os membros dessa família apresentam um domínio transmembranar com 10 hélices e três domínios citoplasmáticos: P (fosforilação), N (ligação de nucleotídeos) e A (atuador) (ADAPTADO DE LOPEZ-MARQUES et al., 2014).

A maioria dos membros da família P-IV ATPase, incluindo o ATP11C, funcionam como um heterodímero com a proteína CDC50, onde a P-IV ATPase representa a subunidade α e a CDC50 representa a subunidade β. A heterodimerização com CDC50 é essencial para a atividade adequada da ATP11C, apesar de ainda não estar claro como as flipases são capazes de mover um

fosfolípido, que é aproximadamente 10 vezes maior do que os íons transportados pelas bombas de cátions (NAIK et al., 2015; VESTERGAARD et al., 2014).

As flipases são responsáveis por transportar (flip) a fosfatidilserina (FS) e fosfatidiletanolamina (FE) da camada externa para a interna da membrana eritrocitária, através de um transporte ativo dependente de ATP de aminofosfolípidos, mantendo a assimetria da bicamada lipídica (DALEKE; LYLES, 2000). Em mamíferos, além da ATP11C, as flipases codificadas pelos genes *ATP8A1*, *ATP8A2*, *ATP8B1*, *ATP8B3* e *ATP10A* estão envolvidas na translocação de fosfolipídios entre os dois folhetos da bicamada da membrana celular (CAI et al., 2009; LEVANO et al., 2012; NAITO et al., 2015). Desse modo, esses achados sugerem que os membros da família P-IV ATPase atuam em conjunto para internalizar a FS. Entretanto, em células do sistema imune, mais de 75% da internalização de FS pelas flipases parece ser mediada pela proteína codificada pelo *ATP11C* (YABAS et al., 2016), e defeito nesse gene tem sido associado com uma menor atividade da flipase em linfócitos B, interrompendo a produção de anticorpos, além de estar relacionado com alguns tipos de câncer, como o carcinoma hepatocelular (YABAS et al., 2011).

A baixa expressão de *ATP11C* pode estar associada a uma maior taxa de exposição à FS em células de sangue periféricas normais (SIGGS et al., 2011a; YABAS et al., 2011). Em ratos, o ATP11C tem apresentado um papel importante na longevidade e morfologia dos eritrócitos (YABAS et al., 2014), bem como na secreção da bile (SIGGS et al., 2011b). Durante a apoptose, a ATP11C sofre uma proteólise e facilita a exposição da FS para o meio extracelular (SEGAWA et al., 2014). Além disso, o aumento do nível de Ca^{2+} citoplasmático em eritrócitos humanos inibe a incorporação dos aminofosfolípidos na membrana celular. Desse modo, uma baixa expressão de *ATP11C* somada a uma ativação da scramblase, enzima dependente de Ca^{2+}, pode permitir a exposição de FS na membrana celular (TAKATSU et al., 2017).

Em hemácias, a deficiência de ATP11C resulta em um acúmulo de FS na superfície celular. Apesar de estudos *in vitro* demonstrarem que a ATP11C é expressa predominantemente em células precursoras durante a eritropoiese (KINGSLEY et al., 2013), a diminuição do *flipping* de FS durante o desenvolvimento do eritrócito parece ter um efeito duradouro na membrana celular, resultando em deformabilidade reduzida das hemácias, além do aumento da destruição intravascular (YABAS et al., 2014). Além disso, a deficiência de ATP11C não confere uma fragilidade osmótica

para a hemácia, e não influencia a homeostase de Na^+ e K^+. Desse modo, a alteração do formato da hemácia parece ocorrer pela alteração na composição lipídica da membrana plasmática (YABAS et al., 2014), visto que essa alteração pode levar a uma expansão ou contração do folheto interno ou externo da membrana (DALEKE; HUESTIS, 1985).

2.4 PLSCR1

O *PLSCR1* está localizado no braço curto do cromossomo 3 (3q23) e codifica a scramblase fosfolipídica 1 (hPLSCR1), proteína composta por 318 aminoácidos e implicada em múltiplos processos celulares, incluindo o transporte de fosfolipídios, regulação gênica, proliferação celular e apoptose (LU et al., 2007). A hPLSCR1 é expressa em várias células, incluindo as plaquetas e as hemácias (ZHOU et al., 2002). Essa proteína apresenta um domínio rico em prolina, uma região de ligação ao DNA e um sítio de palmitoilação, que é a ligação covalente de ácidos graxos em resíduos de cisteína, e em menor frequência, serina e treonina. Além disso, a hPLSCR1 apresenta um sinal de localização nuclear, um sítio de ligação ao Ca^{2+}, uma α-hélice transmembranar e uma cauda extracelular curta na porção C-terminal (Figura 6) (ANDRAKA et al., 2017).

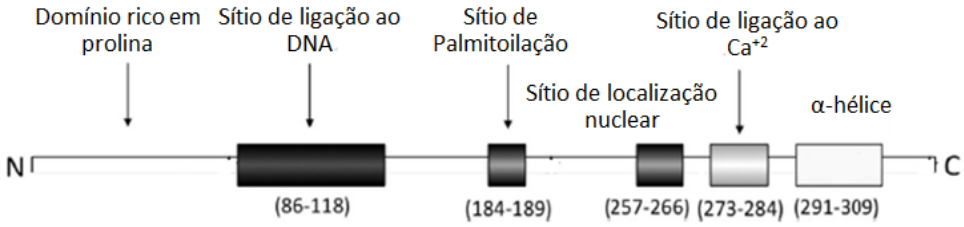

Figura 6 Representação esquemática da scramblase (Adaptado de ANDRAKA et al., 2017).

A região de ligação ao Ca^{2+} tem uma afinidade constante por esse íon em concentrações de ordem milimolar, sendo essencial para a atividade da hPLSCR1 (SAHU; ARADHYAM; GUMMADI, 2009; STOUT et al., 1998). Além disso, o domínio C-terminal apresenta uma grande importância para a hPLSCR1 exercer sua função corretamente. Em scramblases com essa porção ausente, a atividade proteica e afinidade de ligação ao cálcio ficam reduzidas, apesar da ligação à membrana ainda ocorrer (ANDRAKA et al., 2017). Por fim, o sítio de palmitoilação é importante na localização celular dessa proteína, visto que, na ausência da ligação com os ácidos graxos, todas as scramblases localizam-se no núcleo (WIEDMER et al., 2003).

Inicialmente, acreditava-se que a scramblase desempenhava apenas o papel de redistribuir, independente da presença de ATP, os fosfolipídios da membrana plasmática após uma ativação celular, lesão ou apoptose, com uma maior exposição de FS no folheto exoplasmático (BASSÉ et al., 1996; SIVAGNANAM; PALANIRAJAN; GUMMADI, 2017). Entretanto, dados recentes sugerem um papel biológico mais complexo para a hPLSCR1. Atualmente, sabe-se que o *PLSCR1* pode ser estimulado por interferons em várias linhagens celulares, sugerindo um papel da scramblase na imunomodulação (ZHOU et al., 2000). Além disso, a hPLSCR1 pode interagir com o DNA ao ser importada para o núcleo, evidenciando uma função dessa proteína na regulação gênica (BEN-EFRAIM et al., 2004).

Em humanos, a hPLSCRs já foi considerada como a causadora da síndrome de Scott, uma desordem em que o mecanismo responsável por expor FS na membrana plaquetária é defeituoso, resultando em prejuízo na formação de trombina e consequente sangramento (ZWAAL; COMFURIUS; BEVERS, 2004). Entretanto, estudos posteriores demonstraram que a síndrome de Scott é causada por mutações no *TMEM16F* (SUZUKI et al., 2010b, 2013). Além disso, o aumento de hPLSCR1 foi evidenciado em monócitos de indivíduos portadores da síndrome antifosfolípide (AMENGUAL et al., 2013) e de lúpus eritematoso sistêmico (SUZUKI et al., 2010a), indicando que altos níveis dessa proteína podem favorecer uma maior exposição de FS e contribuir, em parte, com a tendência pró-trombótica dessas patologias (SUZUKI et al., 2010a).

Em modelos murinos, a deleção do *PLSCR1* não conferiu anormalidades hematológicas, e a expansão dos progenitores eritróides pela eritropoetina não foi alterada (ZHOU et al., 2002). No entanto, hemácias com altas concentrações de scramblase apresentaram maior exposição de FS na membrana da hemácia, e são preferencialmente retiradas de circulação (KEAN et al., 2002). Desse modo, acredita-se que a exposição de FS depende de um balanço entre os mecanismos que internalizam e externalizam essa molécula, e os genes *ATP11C* e *PLSCR1* estão envolvidos nesse cenário (BARBER et al., 2009). Enquanto alguns trabalhos sugerem que o ATP11C é a proteína mais importante envolvida na exposição de FS (BEVERS et al., 1998; DE JONG et al., 2001), outros apontam um papel predominante da hPLSCR1, com a ATP11C apresentando apenas um papel modulatório (BARBER et al., 2009; BRATTON et al., 1997).

2.5 SPHK1

O *SPHK1* está localizado no braço longo do cromossomo 17 (17q25.1), apresenta 7 éxons e, devido à ocorrência de um processamento alternativo, pode dar origem a três variantes da enzima esfingosina quinase (SphK1a, SphK1b, SphK1c), que diferem no comprimento da sequência N-terminal (PYNE et al., 2009). A SphK1 apresenta dois domínios: um domínio N-terminal, que é composto de 6 folhas β e seis α-hélices organizados em um formato sanduíche (α/β/α), além de conter um sítio de ligação ao nucleotídeo; e um domínio C-terminal, que hospeda o sitio de ligação para a fosforilação da esfingosina. O centro catalítico está localizado em uma fenda formada na junção do interdomínio (ADAMS; PYNE; PYNE, 2016).

A SphK1 catalisa a fosforilação dependente de ATP da esfingosina, presente na membrana plasmática, para formar esfingosina-1-fosfato (S1P), um mediador lipídico pleiotrópico endógeno que regula muitos efeitos fisiológicos, incluindo a modulação da integridade da barreira vascular, angiogênese e tráfico de células do sistema imune (BLAHO; HLA, 2011; ENGLISH et al., 2000; NAGAHASHI et al., 2012), além de apresentar um papel fisiopatológico na disfunção autoimune, inflamação, lesão endotelial, trombose, câncer e muitas outras doenças (CAMERER et al., 2009; ENGLISH et al., 2002; MACEYKA; SPIEGEL, 2014; PAPPU et al., 2007; PYNE; PYNE, 2011; SPIEGEL; MILSTIEN, 2011). Estudos *in vitro* demonstraram que a Sphk1 tem uma alta capacidade de se ligar na membrana celular, e que essa enzima pode ser ativada na presença de FS (OLIVERA; ROSENTHAL; SPIEGEL, 1996; STAHELIN et al., 2005). Intracelularmente, o S1P regula a proliferação e a sobrevivência, e extracelularmente, essa molécula liga-se e ativa uma família de cinco receptores acoplados à proteína G específicos de S1P, $S1P_1$-$S1P_5$ (KIHARA et al., 2014; KUNKEL et al., 2013).

Níveis intracelulares elevados de SphK1 parecem desempenhar um papel importante na proliferação e metástase em alguns tipos de câncer, como câncer de pulmão e cerebral (JOHNSON et al., 2005; LI et al., 2008). Neste contexto, mais de um estudo demonstrou que a inibição de SphK1 tem um potencial considerável como estratégia anticancerígena, visto que a regulação negativa desse gene pode ser capaz de conferir sensibilidade à quimioterapia ou radioterapia (PYNE; PYNE, 2017; SHIDA et al., 2008). No plasma, por apresentar baixa solubilidade em água, a S1P se liga à chaperonas, como a apolipoproteína M e a albumina (CHRISTOFFERSEN et

al., 2011). Além disso, a S1P pode regular alguns processos, como o desenvolvimento vascular (MENDELSON; EVANS; HLA, 2014) e o tráfico de linfócitos para órgãos secundários (CYSTER; SCHWAB, 2012).

Os eritrócitos apresentam altos níveis de S1P (HÄNEL; ANDRÉANI; GRÄLER, 2007; ITO et al., 2007). Além disso, níveis elevados dessa molécula em hemácias e plasma de camundongos e humanos com AF já foram demonstrados (ZHANG et al., 2014). Intracelularmente, a S1P é gerada por 2 enzimas: esfingosina quinase 1 e 2 (SphK1 e SphK2), e os níveis intracelulares dessa molécula são geralmente determinados por um equilíbrio nas atividades de síntese e degradação de S1P. Na maioria das células, as atividades das enzimas degradantes são maiores, mantendo os níveis intracelulares de S1P baixos (PAPPU et al., 2007). Entretanto, os eritrócitos não possuem enzimas que degradam S1P e, desse modo, apresentam níveis elevados de S1P, sendo considerado o principal tipo de célula para armazenar e fornecer plasma rico em S1P (HÄNEL; ANDRÉANI; GRÄLER, 2007; ITO et al., 2007). Além disso, apenas a SphK1 é usada para gerar S1P nas hemácias (KIHARA; IGARASHI, 2008; KOBAYASHI et al., 2009), visto que os eritrócitos humanos não possuem SphK2, que se localiza predominantemente no núcleo (SANKALA et al., 2007).

2.6 DMTN

O *DMTN* está localizado no braço curto do cromossomo 8 (8p21.3), apresenta 21 éxons e codifica uma proteína de ligação a actina abundante em uma variedade de orgãos, incluindo cérebro humano, coração, sangue, músculo esquelético, rim e pulmão (KIM; AZIM; CHISHTI, 1998). A dematina, proteína codificada pelo *DMTN* e que já foi chamada de proteína 4.9, desempenha um papel estrutural nos eritrócitos, estabilizando e anexando o citoesqueleto de espectrina/actina à membrana dos eritrócitos de uma maneira dependente de fosforilação (SIEGEL; BRANTON, 1985). Além disso, estudos já demonstraram que a dematina pode modular vias de sinalização MAP-quinase (LUTCHMAN et al., 2002) e estar envolvida na patogênese do câncer de próstata (LUTCHMAN et al., 1999).

A dematina purificada dos eritrócitos se apresenta como um trímero, composta por duas cópias de um polipeptídeo de 48 kDa e uma cópia de um polipeptídeo de 52 kDa (AZIM et al., 1995). Apresenta um domínio N-terminal único,

que contém um sítio de ligação a actina, mas não apresenta uma sequência homóloga a qualquer sequência de outra proteína conhecida, além de apresentar um domínio *headpiece* com 76 aminoácidos na extremidade C-terminal, que se liga a forma filamentosa da actina (F-actina). Devido a um *splicing* alternativo, a isoforma de 52 kDa apresenta uma inserção de 22 aminoácidos perto da extremidade N-terminal do domínio *headpiece*, diferindo assim da isoforma de 48 kDa (AZIM et al., 1995; KIM; AZIM; CHISHTI, 1998).

O domínio *headpiece* da dematina é essencial para ocorrer o agrupamento de actina, e estudos *in vitro* demonstram que a fosforilação desse domínio induzida pela proteína quinase A (PKA), dependente de adenosina cíclica (AMP-c), regula negativamente esse agrupamento (HUSAIN-CHISHTI; LEVIN; BRANTON, 1988; KOSHINO; MOHANDAS; TAKAKUWA, 2012; SIEGEL; BRANTON, 1985). Especificamente, a proteína quinase fosforila a dematina na posição 74 do domínio *headpiece* e abole a atividade de agrupamento de actina (HUSAIN-CHISHTI et al., 1989; HUSAIN-CHISHTI; LEVIN; BRANTON, 1988). Entretanto, a fosforilação não elimina a atividade de ligação da actina (AZIM et al., 1995; VARDAR et al., 2002). Desse modo, como o eritrócito maduro contém muito poucos filamentos de actina, essa regulação de fosforilação deve ocorrer durante o desenvolvimento de eritrócitos (JIANG; MCKNIGHT, 2006).

O domínio *headpiece* da dematina é essencial para a função da proteína e a manutenção correta do citoesqueleto de eritrócitos, visto que os camundongos que não apresentam esse domínio são viáveis, mas desenvolvem anemia e hemácias esféricas (KHANNA et al., 2002). Sem esse domínio, a associação de espectrina e actina à membrana plasmática é enfraquecida, formando eritrócitos osmoticamente frágeis com deformabilidade reduzida (KHANNA et al., 2002). Além disso, a ausência de dematina está associada a uma perda considerável de espectrina, aducina e actina (60%, 90% e, 90%, respectivamente). Desse modo, a dematina é a primeira proteína citoesquelética auxiliar nas junções espectrina-actina cuja ausência apresenta um grande efeito prejudicial sobre a integridade e funções da membrana da hemácia (LU et al., 2016). Ademais, a deleção do *DMTN* fez com que ratos apresentassem uma anemia grave e exibissem grandes alterações na morfologia e na estabilidade da membrana dos eritrócitos (LU et al., 2016).

Em humanos, a fosforilação da dematina pela proteína quinase A rompe a ligação actina-espectrina e compromete a estabilidade da membrana eritrocitária na

presença de doenças que alterem o funcionamento normal do eritrócito, como a AF, ou no armazenamento prolongado do concentrado de hemácias (KOSHINO; MOHANDAS; TAKAKUWA, 2012; RINALDUCCI et al., 2015). Além disso, na malária, a dematina apresenta uma grande importância para a remodelação da hemácia após a infecção pelo *Plasmodium sp.* (LALLE et al., 2011).

2.7 INFLUÊNCIA DO ATP11C, PLSCR1, SPHK1 E DMTN NA AF

A exposição de FS em eritrócitos está diretamente relacionada com a idade e desidratação da hemácia, e se correlaciona inversamente com o conteúdo de hemoglobina fetal, demonstrando que a falcização está implicada no processo (YASIN et al., 2003). Sendo assim, a polimerização da HbS bem como uma ausência ou uma deficiência do mecanismo fisiológico envolvido na internalização da FS podem aumentar esse processo. Neste contexto, os membros da família P-IV ATPase, como o ATP11C, surgem como uma classe importante de enzimas para serem analisadas (YABAS et al., 2014), além de que nenhuma outra enzima funcionalmente ativa compensa a falta de atividade dos membros dessa família (TAKATSU et al., 2014).

Estudos relatam que a exposição de FS em hemácias de pacientes com AF contribua para a morbidade da doença. Em geral, essa exposição representa uma das principais causas de adesão de células sanguíneas ao endotélio vascular e pode contribuir tanto para a crise vaso-oclusiva, como para a hemólise extra vascular (KUYPERS; DE JONG, 2004; SETTY; KULKARNI; STUART, 2002). Além disso, a exposição de FS nos eritrócitos tem sido associado a um maior estado de hipercoagulabilidade, representada por um aumento da geração de trombina e maior ativação plaquetária (ATAGA; CAPPELLINI; RACHMILEWITZ, 2007; SETTY; RAO; STUART, 2001), além de aumentar o risco de acidente vascular cerebral (STYLES et al., 1997). Ademais, a atuação deficiente do baço pode contribuir para a permanência das hemácias que expõem FS na circulação sanguínea (DE JONG; KUYPERS, 2006).

Em ratos falciformes, cerca de 90% dos reticulócitos tem uma ativação da scramblase, mas apenas 42% expõe FS. Entretanto, como a ativação da hPLSCR1 ocorre antes da saída de FS, podem existir hemácias que ativaram esta via, mas ainda não apresentam FS suficiente na superfície da membrana (KEAN et al., 2002). Além disso, os mecanismos que internalizam a FS podem atuar, resultando em células com baixa exposição de FS, independente dos níveis de scramblase (KEAN et al., 2002).

Em humanos, é esperado que as hemácias de pacientes com AF apresentem uma ativação da hPLSCR1. Desse modo, prevenir a exposição de FS ao inibir a ativação do *PLSCR1* pode ser uma estratégia terapêutica para esses indivíduos (BARBER et al., 2009).

Em relação ao Sphk1, estudos *in vitro* demonstram que situações de hipóxia podem ser um estímulo para induzir a atividade dessa molécula nas hemácias de camundongos e humanos com AF (SUN et al., 2016; ZHANG et al., 2014). O aumento de S1P promove uma reprogramação metabólica nos eritrócitos, responsável por aumentar a canalização da glicose para a glicólise e não para a via das pentoses fosfato, via responsável por gerar potencial redutor. Desse modo, uma supressão da via das pentoses-fosfato compromete a homeostase da glutationa e aumenta o estresse oxidativo, enquanto que a glicólise aumentada induz a produção de 2,3-BPG e, desse modo, reduz a afinidade da hemoglobina pelo oxigênio (SUN et al., 2016).

Na presença de HbS, o favorecimento da desoxigenação favorece a polimerização da HbS e consequentemente a falcização da hemácia, a hemólise, inflamação e a progressão da doença (SUN et al., 2016; ZHANG et al., 2014). Desse modo, é provável que as hemácias falciformes tenham maior atividade de SphK1 do que as hemácias normais, visto que a AF é uma doença que apresenta uma hipóxia induzida pela anemia, além da atividade de SphK1 se encontrar aumentada em resposta a condições de hipóxia (SUN et al., 2016). Além disso, já foi demonstrado que a adenosina, uma molécula de sinalização conhecida por ser induzida em situações de hipóxia, induz a atividade de SphK1 em hemácias normais e falciforme. Ao ativar o receptor de adenosina A2B (ADORA2B), a adenosina regula a atividade de SphK1 nas hemácias, além de promover uma ativação *downstream* das vias PKA e ERK 1/2, relacionadas com a sinalização celular (SUN et al., 2016).

Com relação a dematina, análises de proteômica indicam que essa molécula, assim como a proteína 4.1, α-aducina e β-aducina, são alvos *downstream* das vias de sinalização ERK em hemácias contendo HbS. Desse modo, a fosforilação de proteínas citoesqueléticas pela via ERK pode levar à desorganização do citoesqueleto, e uma ativação anormal de ERK pode estar associada à fisiopatologia da AF. Além disso, a desorganização do citoesqueleto pode fosforilar a ICAM-4 e mediar a adesão ao endotélio, tornando a via ERK um potencial alvo terapêutico para prevenção e tratamento da vaso-oclusão na AF (ZENNADI et al., 2012).

3. THE RATIO OF *ATP11C*/*PLSCR1* MRNA TRANSCRIPTS HAS CLINICAL SIGNIFICANCE IN SICKLE CELL ANEMIA

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Red Cell Biology & its Disorders

The ratio of *ATP11C*/*PLSCR1* mRNA transcripts has clinical significance in sickle cell anemia

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Phosphatidylserine (PS) exposure in the plasma membrane represents one of the multiple mechanisms facilitating blood cell adhesion to the vascular endothelium and may contribute to both vaso-occlusive crisis (VOC) and extravascular hemolysis in beta-hemoglobinopathies, including sickle cell anemia (SCA).[1] This process may be potentiated by SS-hemoglobin polymerization,[2] with consequent PS exposure-related thrombin generation,[3] and significantly worsened if physiologic mechanisms responsible to maintain asymmetric phospholipid distribution in human erythrocyte membranes (i.e., PS internalization/externalization) are compromised. In this context, members of the P4-type ATPase family (ATP8A1, ATP11A, and ATP11C, previously proposed to act as flippases[4,5]) constitute an important class of enzymes responsible for active transport of PS from the outer to the inner leaflet.[6] ATP11C constitutes the major flippase in human erythrocytes and its defect may cause congenital hemolytic anemia.[6] In contrast to ATP11C, the calcium-mediated activation of the ATP-independent phospholipid scramblase 1 (PLSCR1) counteracts flippase activities and induces the PS surface exposure to the outer leaflet.[7,8] Aberrant expression of either or both *ATP11C* and *PLSCR1* transcripts may disrupt the PS internalization/externalization process and become clinically relevant for patients with SCA. Here, we investigated whether the imbalance between *ATP11C* and *PLSCR1* transcript (hereafter-called *ATP11C*/*PLSCR1* ratio) associates with clinical outcomes in patients with SCA. We also studied the role of *ATP11C*/*PLSCR1* ratio in PS exposure in SS-genotyped erythrocytes.

Overall, 189 patients with SCA were enrolled. According to the inclusion criteria, all patients were off hydroxyurea therapy and without blood transfusion for at least three months at the time of blood collection. Details about the baseline features and clinical complications are described in the Supplemental data. For comparisons, 18 patients with β-thalassemia (13 intermedia, and 5 major), 18 patients with HbSC genotype, and 12 patients with HbSβ (two $HbS\beta^{+(mild)}$, seven $HbS\beta^{+(severe)}$, and three $HbS\beta^{0}$) were included. The transcript levels of *APT11C* and *PLSCR1* were quantified using TaqMan Gene Expression Assay (Applied BioSystems), following the manufacturer's instructions. The comparative Ct method was applied to determine the relative *ATP11C* and *PLSCR1* transcript levels. For the reference group, peripheral blood reticulocytes from 23 age- and sex-adjusted healthy volunteers (hemoglobin profile AA) with no history of hematological diseases were included. *ATP11C* and *PLSCR1* expression were calculated relative to a reference cDNA (i.e., mean value of

the ∆Ct from the reference group). Of note, the same reference cDNA also served as an internal control throughout all experiments to ensure full comparability among experiments. The PS-exposed erythrocytes were labeled using Annexin-V binding assay kit (BD Biosciences) and their rate was analyzed via standard flow-cytometry methods. Details can be found in the Supplemental data.

Patient baseline characteristics were reported descriptively. According to survival ROC analysis and C index analyses, patients were grouped into low and high expression groups using the median values of *ATP11C/18s* and *PLSCR1/18s* expression as cutoff. Correlation analyses were accomplished using Pearson correlation test. Logarithmic values of the *ATP11C*/*PLSCR1* ratio and PS exposure rate were used in the correlations analyses to better fit the data. We dichotomized patients into two groups according to *ATP11C*/*PLSCR1* ratio expression (low ratio, <1; high ratio, ≥1). Details can be found in the Supplemental data.

Figure 1A exhibits the *ATP11C*/*PLSCR1* ratio in samples from patients with sickle cell disease (HbSC/HbSβ), β-thalassemia and SCA. Although patients with SCA and HbSβ had lower levels of *ATP11C*/*PLSCR1* ratio, this difference did not reach significance (P<0.05). Next, we restricted our analyses to patients with SCA. Baseline characteristics were similar between patients with low and high *ATP11C*/*PLSCR1* ratio (Table 1), except for lower frequency of recurrent clinical events in patients with high *ATP11C*/*PLSCR1* ratio (P=0.026). Logistic regression analysis showed that patients with high *ATP11C/PLSCR1* ratio had 2.5-fold lower risk to present clinical complications (acute or chronic organ damage) than patients with low *ATP11C*/*PLSCR1* ratio (odds ratio: 0.4, 95% confidence interval: 0.19-0.85; P=0.018). When analyzed separately, *ATP11C* and *PLSCR1* genes had no impact on clinical outcomes of patients with SCA (Details in Supplemental data). This finding prompted us to conduct an exploratory analysis to evaluate whether the *ATP11C*/*PLSCR1* ratio might modulate the PS exposure rate in SS-genotyped erythrocytes. For this purpose, we collected samples from a different subset of patients (using the same inclusion criteria as previously described) during VOC (crisis state, 13 patients) or in steady state (15 patients). Patients in steady state exhibited mean values of *ATP11C/PLSCR1* ratio significantly higher (mean value: 1.91, range, 0.3-5.6) than those who were in crisis (mean value: 0.38, range, 0.1-1.01) (P<0.001). Proportionally, the number of patients with high *ATP11C/PLSCR1* ratio was higher in steady state group (nine out of 15; 60%) than in crisis group (one out of 13; 8%) (P=0.006). Most importantly, high PS exposure

had a strong correlation with low *ATP11C*/*PLSCR1* ratio in sickle erythrocytes (Pearson correlation coefficient, r = -0.67; Figure 1B). In order to analyze the dynamic of the *ATP11C*/*PLSCR1* ratio, we evaluated the individual transcript levels of *ATP11C* and *PLSCR1* in patients assigned to the first (lowest) and fourth (highest) *ATP11C*/*PLSCR1* ratio quartiles. The median value of *ATP11C* transcript levels was significantly higher in patients assigned to the fourth quartile (P=0.017), while patients assigned to the first quartile exhibited higher median values of *PLSCR1* transcript levels (P=0.002).

A growing body of evidence highlights the importance of the encoded ATP11C to organize plasma membrane asymmetry in normal peripheral blood cells,[5,9] particularly in erythrocytes.[6] An et al. showed that normal erythroblasts constitutively express *ATP8A1*, *ATP11A*, and *ATP11C* genes at all stages of human terminal erythroid differentiation.[10] Importantly, no other functionally active enzyme compensates for the lack of activity of one of these members,[11] suggesting a functional specificity among the group. In line with these findings, emerging data suggest that low expression of the *ATP11C* gene is associated with a higher rate of PS exposure in normal peripheral blood cells.[5] This hypothesis is supported by functional studies, in which erythropoiesis evaluation of *ATP11C*-deficient mice showed a lower rate of PS translocation, abnormal morphology and a shortened lifespan of mature erythrocytes, with consequent anemia.[12] Along with the ATP11C flipping activity, the ATP-independent activity of PLSCR1 also maintains the PS exposure/internalization balance in non-apoptotic cells. Arashiki et al. demonstrated that PS surface exposure may be prevented by cholesterol-mediated suppression of PLSCR1 under physiological low calcium concentrations.[8] In senescent erythrocytes, elevated calcium concentrations activate PLSCR1 which, in turn, leads to PS exposure with consequent targeting of cells to phagocytosis by macrophages, irrespective of ATP11C activity.[7] Importantly, other mechanisms may be involved in PS exposure in patients with sickle cell disease, such as elevated levels of autophagic vesicles on circulating reticulocytes.[13] If such a process occurs in response to an imbalance between ATP11C and PLSCR1 proteins remains to be elucidated. Although it is believed that the PS distribution in human normal erythrocytes (important for both survival and death) is determined by flippase and scramblase activity, it is unknown whether these proteins are expressed in the same way in SS-genotyped erythroid progenitors, or even if other

mechanisms (microRNA modulation or other epigenetic events) could influence their functions in a sickle cell scenario.

To our knowledge, the current study represents the first evaluation of *ATP11C*/*PLSCR1* ratio in a clinical context. In a comparative analysis, our results showed that patients with SCA had a tendency of lower-than-normal *ATP11C*/*PLSCR1* ratio. Of note, we observed similar results for patients with HbSβ, most of them carrying the worst genotype combination and presenting clinical features very similar to those presented by patients with SCA. If the differential expression of *ATP11C* and *PLSCR1* retains biological significance with the sickle cell disease phenotype remains unknown. Particularly in SCA, the clinical disease severity is strongly associated with sickle cell adhesion to the vascular endothelium. Because PS exposure on the surface of erythrocytes may significantly increase adhesion to the vascular endothelium and other related events (activating clotting factors, hemolysis, consequent anemia),[14] an increased rate of VOC, and consequent poor prognosis, would be expected. As expected, a higher expression of *ATP11C* in relation to *PLSCR1* was associated with lower risk of clinical complications, probably because sickled erythrocytes with high *ATP11C*/*PLSCR1* ratio had a lower PS surface exposure during most lifespan. Of course, we cannot rule out that patients assigned to the “no complication” group (i.e., those who did not experience recurrent clinical events) may develop clinical complications over time, although those patients remained free of events during the entire study (or at least, until the last follow up). Furthermore, it is conceivable that the *ATP11C/PLSCR1* ratio may switch from high to low during a VOC, although the underlying reasons require further investigations. Based on the individual transcript levels analysis, variations in both *ATP11C* and *PLSCR1* transcripts can influence such balance, reinforcing the importance of the ratio in a clinical setting. Future studies could provide a better understanding about the balance between *ATP11C* and *PLSCR1* in SCA and how or if they influence the switch from steady state to the development of an acute clinical manifestation.

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AUTHOR CONTRIBUTIONS

D.A.P-M. and J.L.C-S. performed experiments, analyzed and interpreted data, and drafted the manuscript. P.L.F-N, I.F.D., I.W., A.S.A., R.F.F, and M.A.B. recruited patients, updated the clinical data, and reviewed the manuscript. D.A.P-M. and A.R.L-A. analyzed and interpreted data performed statistical analyses, and drafted the manuscript. D.A.P-M., J.L.C-S., and A.R.L-A. conceived and designed the study and reviewed the manuscript. A.R.L-A. gave the final approval of the version to be submitted.

CONFLICTS OF INTEREST DISCLOSURE

The authors have no competing financial interests to declare.

REFERENCES

1. Yamaja Setty BN, Kulkarni S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood*. 2002;99(5):1564–1571.

2. Setty BN, Kulkarni S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood*. 2002;99(0006–4971 (Print)):1564–1571.

3. Wang Y, Zhang S, Luo L, et al. Platelet-derived microparticles regulates thrombin generation via phophatidylserine in abdominal sepsis. *J. Cell. Physiol.* 2018;233(2):1051–1060.

4. Tanaka K, Fujimura-Kamada K, Yamamoto T. Functions of phospholipid flippases. *J.Biochem.* 2011;149(1756–2651 (Electronic)):131–143.

5. Yabas M, Teh CE, Frankenreiter S, et al. ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. *Nat.Immunol.* 2011;12(1529–2916 (Electronic)):441–449.

6. Arashiki N, Takakuwa Y, Mohandas N, et al. ATP11C is a major flippase in human erythrocytes and its defect causes congenital hemolytic anemia. *Haematologica*. 2016;101(5):559–565.

7. Arashiki N, Takakuwa Y. Maintenance and regulation of asymmetric phospholipid distribution in human erythrocyte membranes: Implications for erythrocyte functions. *Curr. Opin. Hematol.* 2017;24(3):167–172.

8. Arashiki N, Saito M, Koshino I, et al. An unrecognized function of cholesterol: Regulating the mechanism controlling membrane phospholipid asymmetry. *Biochemistry*. 2016;55(25):3504–3513.

9. Segawa K, Kurata S, Nagata S. Human Type IV P-type ATPases That Work as Plasma Membrane Phospholipid Flippases and Their Regulation by Caspase and Calcium. *J.Biol.Chem.* 2016;291(1083–351X (Electronic)):762–772.

10. An X, Schulz VP, Li J, et al. Global transcriptome analyses of human and murine terminal erythroid differentiation. *Blood*. 2014;123(1528–0020 (Electronic)):3466–3477.

11. Takatsu H, Takayama M, Naito T, et al. Phospholipid flippase ATP11C is endocytosed and downregulated following Ca2+mediated protein kinase C activation. *Nat. Commun.* 2017;8(1).

12. Yabas M, Coupland LA, Cromer D, et al. Mice deficient in the putative phospholipid flippase Atp11c exhibit altered erythrocyte shape, anemia, and reduced erythrocyte life span. *J. Biol. Chem.* 2014;289(28):19531–19537.

13. Mankelow TJ, Griffiths RE, Trompeter S, et al. Autophagic vesicles on mature human reticulocytes explain phosphatidylserine-positive red cells in sickle cell disease. *Blood*. 2015;126(15):1831–1834.

14. Yasin Z, Witting S, Palascak MB, et al. Phosphatidylserine externalization in sickle red blood cells: associations with cell age, density, and hemoglobin F. *Blood*. 2003;102(0006–4971 (Print)):365–370.

Table 1 - Baseline characteristics.

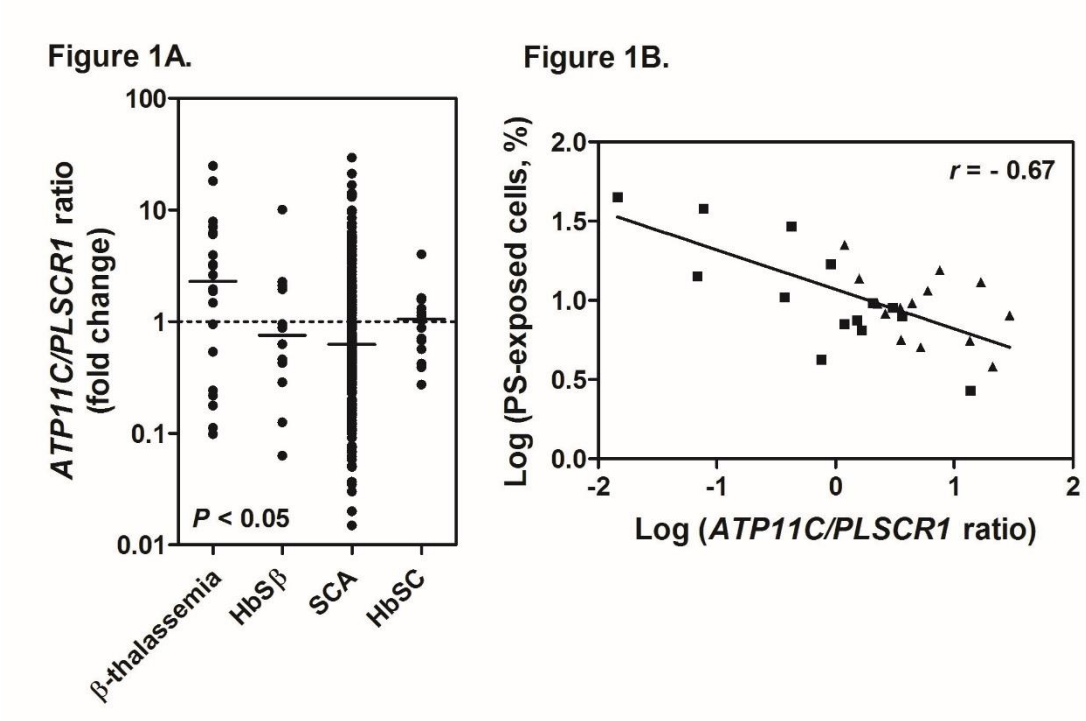
Characteristics	All patients		*ATP11C/PLSCR1* ratio				*P*-value[1]
			Low		High		
	No.	%	No.	%	No.	%	
Gender							0.88
Male	93	49.2	59	48.8	33	50	
Female	96	50.8	62	51.2	33	50	
Age (years), median (range)	20.5 (3, 63)		19 (5, 63)		23 (3, 61)		0.618
β^S haplotype							0.871
CAR/CAR	98	56.6	64	56.1	33	57.9	
Non-CAR/CAR	75	43.4	50	43.9	24	42.1	
Missing data	16	-	7	-	9		
α-thalassemia ($\alpha^{-3.7kb}$)							0.504
Mutated	30	19.2	17	16.3	11	22	
Non-mutated	126	80.8	87	83.7	39	78	
Missing data	33	-	17	-	16	-	
HbF (%), median (range)	5.8 (0.7, 25)		6.4 (0.7, 25)		5.6 (1.3, 24)		0.752
Number the VOC/year[2]							0.442
≤2	93	50.3	62	52.5	30	46.2	
3 or more	92	49.7	56	47.5	35	53.8	
Missing data	4	-	3	-	1	-	
Recurrent clinical events[3]							**0.026***
No clinical complication	**53**	**29**	**27**	**23.1**	**25**	**39.1**	
One complication or more	**130**	**71**	**90**	**76.9**	**39**	**60.9**	
Missing data	**6**	**-**	**4**	**-**	**2**	**-**	

Abbreviations: HbF, fetal hemoglobin; CAR, Central African Republican; VOC, vaso-occlusion crisis.
NOTE: * Indicate differences statistically significant.

1: Missing values were excluded from calculation of *P*-values.
2: We only considered the number of VOC documented in the last year (2017).
3: It was considered as recurrent clinical events: leg ulcer, stroke, priapism, osteonecrosis, acute chest syndrome, transcranial Doppler high-risk, cardiopathy, dactylitis, acute spleen sequestration, nephropathy, retinopathy and acute lung hypertension. Patients assigned to the “No clinical complication” group were those who did not experience the aforementioned recurrent clinical events.

FIGURE LEGEND

Figure 1 - (A) Quantitative analysis of *ATP11C*/*PLSCR1* ratio in samples from patients with β-thalassemia, HbSβ, SCA, and HbSC. The expression of *ATP11C* and *PLSCR1* was quantified by real-time quantitative PCR, separately, and then expressed as a ratio. The horizontal bars represent the median value of *ATP11C*/*PLSCR1* ratio. Comparisons were accomplished using Kruskal–Wallis test followed by a Dunn's post-test. (B) Correlation between PS exposure and *ATP11C*/*PLSCR1* ratio. These data were generated from 13 patients in crisis (square shaped marker) and 15 patients in steady state (triangle shaped marker). Correlation analyses were accomplished using Pearson correlation test. Logarithmic values of the *ATP11C*/*PLSCR1* ratio and PS exposure were used to better fit the data.



Supplemental data

METHODS

Patients

Between February 2015 and December 2017, 189 patients with SCA (median age: 31 years, range, 15-61 years, with 93 males; 49%) followed in a single reference center in northeast Brazil were enrolled. All patients were fully characterized for the β^S-globin gene haplotype and co-inheritance of alpha-thalassemia. Five patients underwent splenectomy. All patients were off hydroxyurea therapy and without blood transfusion for at least three months at the time of blood collection. Importantly, most of the patients off hydroxyurea treatment were those who did not meet the clinical criteria to be treated with hydroxyurea, according to the brazilian therapeutic guidelines for the use of hydroxyurea in sickle cell disease.[1] Informed consent was obtained from all patients or their relatives, as appropriate. For comparisons, 18 patients with β-thalassemia (13 intermediate, and 5 major), 18 patients with HbSC genotype, and 12 patients with HbSβ (two $HbS\beta^{+(mild)}$, seven $HbS\beta^{+(severe)}$, and three $HbS\beta^{0}$) were included. The local research ethics board approved this study (#035/10).

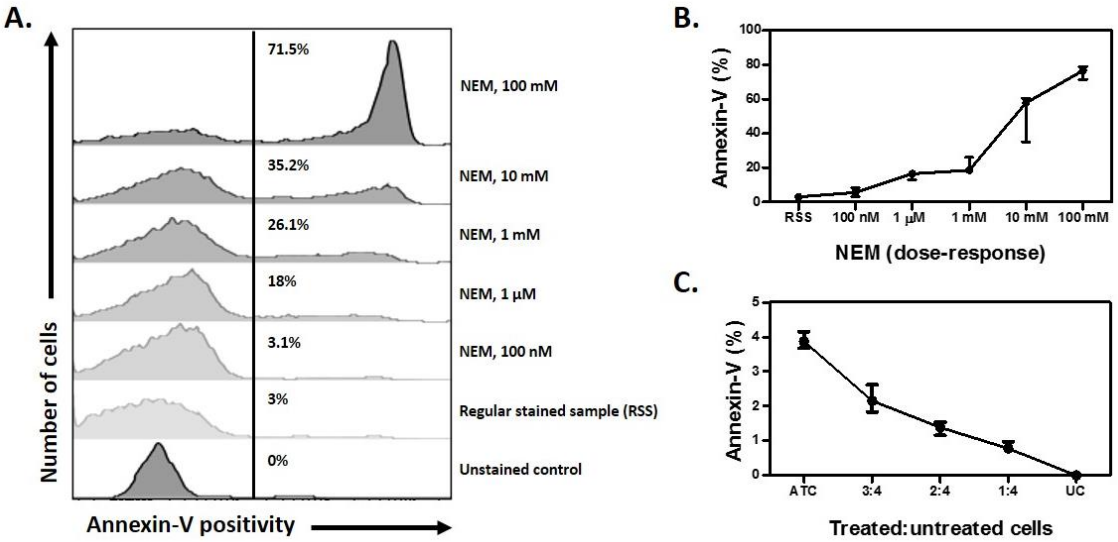
Gene expression profile of ATP11C and PLSCR1

Total RNA from peripheral blood reticulocytes[2] was isolated using Trizol reagent. Following total RNA extraction, real-time quantitative polymerase chain reaction (qPCR) assays with sample-derived cDNA were performed in duplicate on MicroAmp optical 96-well plates using a 7500 Real-Time PCR System (Applied BioSystems) using the subunit 18S from human ribosomal RNA (Hs9999901_s1, Applied Biosystems) as an endogenous control. In case of a discrepancy greater than 1 in cycle of threshold (Ct) values between duplicates, a third test was performed. Samples whose Cts of the endogenous control were lower or higher than 2 standard deviations of average were excluded from analysis. *ATP11C* (Hs_00937051_m1, Applied Biosystems) and *PLSCR1* (Hs01062171_m1, Applied Biosystems) transcript levels were quantified using TaqMan Gene Expression Assay (Applied BioSystems) according to manufacturer recommendations. The comparative Ct method was applied to determine the relative *ATP11C* and *PLSCR1* transcript levels. As such, the difference of Ct number ($\Delta Ct = Ct_{APT11C} - Ct_{18S}$ and $Ct_{PLSCR1} - Ct_{18S}$) was calculated for each replicate. Without detectable *ATP11C* or *PLSCR1* amplification within 40

cycles (standard amplification conditions for qPCR), both expression values were set to 0. For the reference group, peripheral blood reticulocytes from 23 age- and sex-adjusted healthy volunteers (hemoglobin profile AA) with no history of hematological diseases were included. *ATP11C* and *PLSCR1* expression were calculated relative to a reference cDNA (mean value of the ∆Ct from the reference group) and set to 1. The same reference cDNA also served as an internal control throughout all experiments to ensure full comparability among experiments. Relative *ATP11C* and *PLSCR1* transcript values were calculated as relative quantification using $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta Ct_{patients} - \Delta Ct_{healthy\ volunteer}$. The results were presented as a ratio between *ATP11C* and *PLSCR1* transcripts (*ATP11C*/*PLSCR1* ratio).

PS exposure detection in SS-genotyped erythrocyte

The PS exposure level was determined using the AnnexinV-fluorescein isothiocyanate binding assay (Cat. N#556419; BD Biosciences) and analyzed via standard flow-cytometry methods. To determine the PS exposure in the erythrocyte's population, we gated the cells of interest with high forward scatter and side scatter, in order to exclude microparticles and platelets (extensively recognized for having high levels of PS). Alternatively, we performed a Glycophorin A staining using the anti-CD235a-R-phycoerythrin (Clone: GA-R2; BD Biosciences). Those cells population exhibiting high forward scatter values and expressing the CD235a were defined as erythrocytes. Immunophenotypic analysis was accomplished using 1×10^6 erythrocytes stained with 5 µL of Annexin-V from 28 freshly newly collected samples from patients with SCA during follow-up appointments. Patients were included if they were off hydroxyurea therapy and without blood transfusion for at least three months, regardless of their clinical presentation (steady state or in crisis) and only afterward classified into two groups: patients in steady state (15 patients) and patients in vaso-occlusive crisis (13 patients). All experiments were performed in triplicate and in each sample, a minimum of 100,000 events was acquired and the analyses was performed using the log scale for the Annexin-V. Unlabeled cells were used as negative controls to determine the best threshold for each sample. In addition, increasing doses of N-ethylmaleimide (NEM, an ATP11C inhibitor) were used to determine the sensibility of the method (Supplemental figure 1).



Supplemental figure 1 - Distribution of PS exposure on plasma membrane according to N-ethylmaleimide (NEM) and calcium concentrations. (A) Normal erythrocytes treated with increasing doses of NEM. Annexin-V positivity is displayed on single parameter histograms (after the black line) and PS is shown on the x-axis (log scale) and event frequency is shown on the y-axis for RBC. (B) Dose-response curve for normal erythrocytes treated with increasing doses of NEM. (C) A ratio between treated cells with calcium media and untreated cells. ATC: all treated cells, UC: untreated cells.

Statistical analysis

Patient baseline characteristics were reported descriptively. Fisher's exact test or Chi-square test, as appropriate, was used to compare categorical variables, and Kruskal-Wallis test was used to compare continuous variables. According to survival ROC analysis[3] and C index analyses,[4] patients were grouped into low and high expression groups using the median values of *ATP11C/18s* and *PLSCR1/18s* expression as cutoff. Correlation analyses were accomplished using Pearson correlation test. Logarithmic values of the *ATP11C*/*PLSCR1* ratio and PS exposure rate were used in the correlations analyses to better fit the data. We dichotomized patients into two groups according to *ATP11C*/*PLSCR1* ratio expression (low ratio, <1; high ratio, ≥1). All calculations were performed using Stata statistical data analysis software version 14.1 (StataCorp, College Station, TX, USA), statistical package for the social sciences (SPSS) 19.0, and R 3.3.2 (The CRAN project, www.r-project.org) software. All *P* values were two-sided with a significance level of 0.05.

Supplemental data

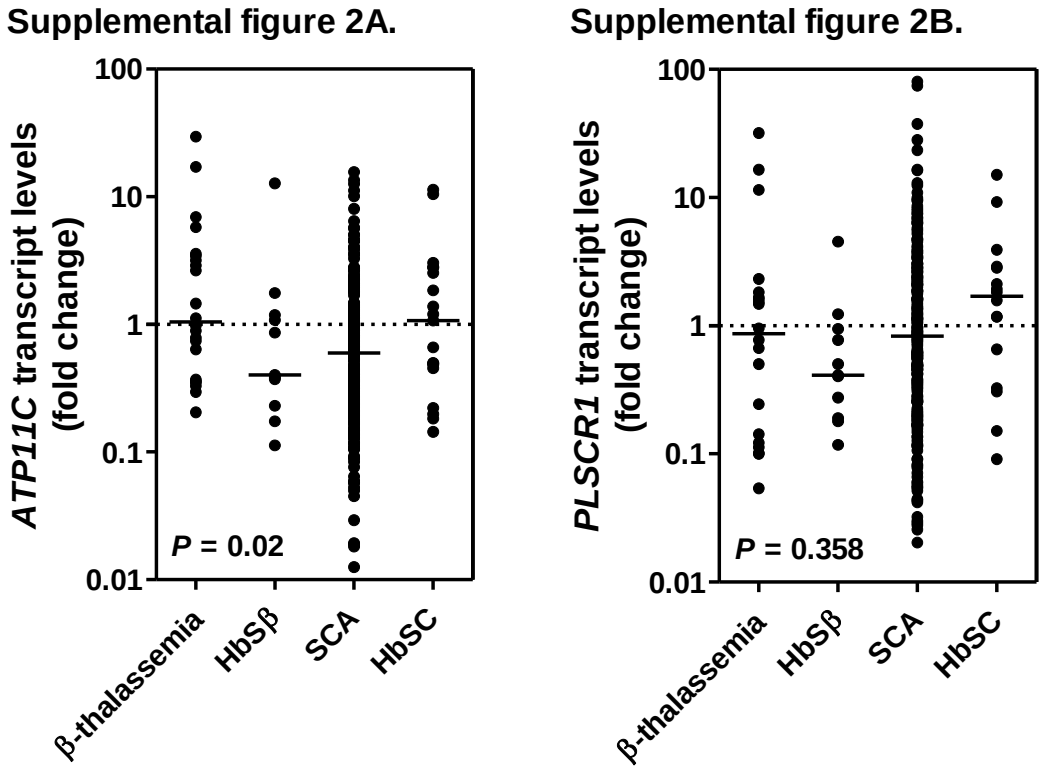
RESULTS

The relative quantification of *ATP11C* and *PLSCR1* genes were successfully performed in all 189 patients. Supplemental Table 1 summarizes the central tendency and dispersion measurements of primary data for *ATP11C*, *PLSCR1*, and the *ATP11C*/*PLSCR1* ratio in patients with SCA. The *ATP11C* transcript levels ranged from 0.12 to 17.1, while *PLSCR1* transcript levels ranged from 0.1 to 80.1 expression values of *ATP11C* (relative to the reference 18s rRNA) or *PLSCR1* (relative to the reference 18s rRNA) in patients with SCA. *ATP11C*/*PLSCR1* ratio ranged from 0 to 18.1. For the cohort comprehending the patients with SCA in steady state and in crisis, the *ATP11C* transcript levels (mean value: 1.4, range, 0.1 to 16.45), *PLSCR1* transcript levels (mean value: 4.88, range, 0.2 to 74.3) and the *ATP11C*/*PLSCR1* ratio (mean value: 1.2, range, 0 to 15.6) exhibited similar distribution compared to the main cohort.

Supplemental table 1A - Primary data for *ATP11C*, *PLSCR1*, and *ATP11C*/*PLSCR1* ratio in patients with SCA

Central tendency and dispersion measurements	*ATP11C/18s*	*PLSCR1/18s*	*ATP11C*/*PLSCR1*
Mean value (range)	1.48 (0.1 to 17.1)	3.42 (0.1 to 80.1)	1.42 (0 to 18.1)
25th percentile	0.26	0.29	0.18
Median value	0.63	0.83	0.54
75th percentile	1.35	2.3	1.51

Supplemental figure 2 exhibits the expression values of *ATP11C* (1A) and *PLSCR1* (1B) genes in samples from patients with sickle cell disease (HbSC/HbSβ), β-thalassemia and SCA. Patients with SCA and HbSβ had lower levels of *ATP11C* (*P*=0.02), while no difference was found for *PLSCR1* expression (*P*=0.358).



Supplemental figure 2 - Relative expression of *ATP11C* and *PLSCR1* genes. Quantitative analysis of (A) *ATP11C* and (B) *PLSCR1* genes in samples from patients with β-thalassemia, HbSβ, SCA, and HbSC. The expression of *ATP11C* and *PLSCR1* was quantified by real-time quantitative PCR, relative to the reference 18s rRNA. The horizontal bars represent the median value of *ATP11C/18s* and *PLSCR1/18s* (fold change). Comparisons were accomplished using Kruskal–Wallis test followed by a Dunn's post-test.

Baseline features of patients according to *ATP11C* and *PLSCR1* transcript levels are summarized in Supplemental table 2. Except for a higher frequency of female in high *PLSCR1* group (*P*=0.042), no significant differences were observed. According to the clinical complications described in our patients, 32 out of 189 patients with SCA (17%) had cerebrovascular disease, followed by 14 patients with leg ulcer (7%). Twenty-seven patients (14%) exhibited at least one of the most common clinical complications in SCA, including priapism, osteonecrosis, acute chest syndrome, cardiopathy, dactylitis, acute spleen sequestration, nephropathy, retinopathy and acute lung hypertension. Fifty-eight patients (31%) had two or more complications combined. Fifty-two patients (27%) had no documented clinical complication (at least, until the last follow up, which occurred in December 2017). Six patients had no information on their medical record, and therefore, were not considered for further analyses. Neither

ATP11C (*P*=0.519) nor *PLSCR1* (*P*=0.196) expression was associated with clinical complications (acute or chronic organ damage) in SCA.

Supplemental table 2 - Baseline characteristics.

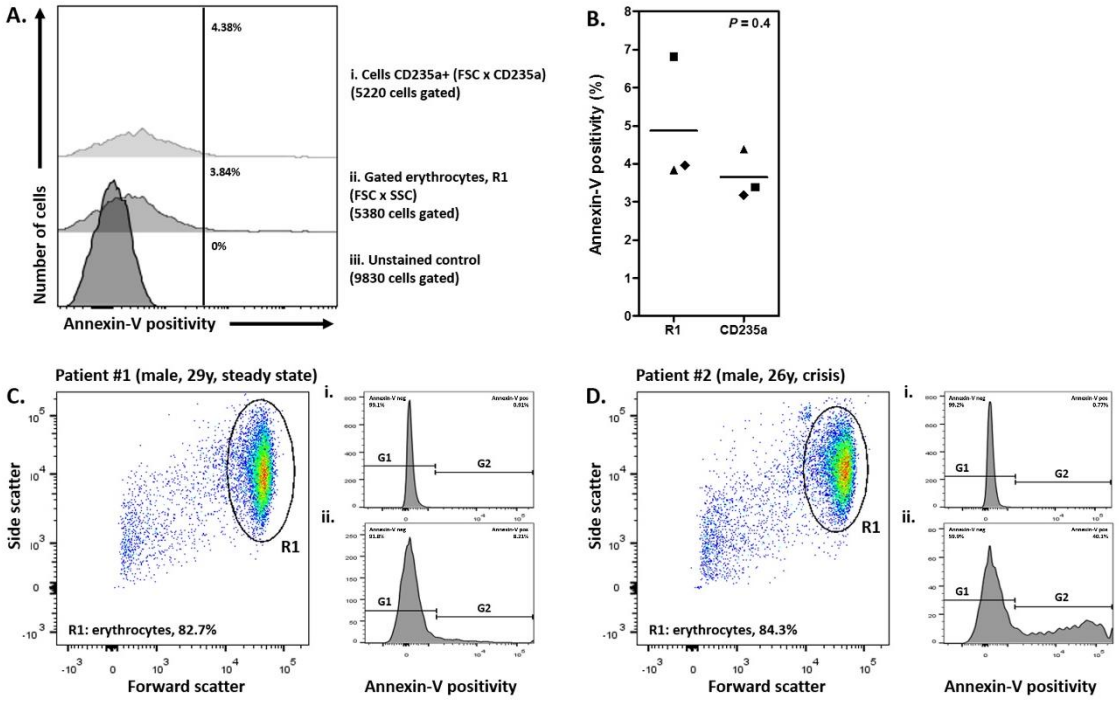
Characteristics	All patients		*ATP11C* transcript levels				*P*-value[1]	*PLSCR1* transcript levels				*P*-value[1]
			Low		High			Low		High		
	No.	%	No.	%	No.	%		No.	%	No.	%	
Gender							0.191					0.042*
Male	93	49.2	51	54.3	42	44.2		54	56.8	39	41.5	
Female	96	50.8	43	45.7	53	55.8		41	43.2	55	58.5	
Age (years), median	20.5		19.5		21		0.761	23		19		0.302
(range)	(3, 63)		(4, 56)		(3, 63)			(3, 63)		(3, 61)		
β^S haplotype							0.54					0.544
CAR/CAR	98	56.6	51	59.3	47	54		53	58.9	45	54.2	
Non-CAR/CAR	75	43.4	35	40.7	40	46		37	41.1	38	45.8	
Missing data	16	-	8	-	8	-				11	-	
α-thalassemia ($\alpha^{-3.7kb}$)							0.839					0.54
Mutated	30	19.2	14	17.9	16	20.5		17	21.5	13	16.9	
Non-mutated	126	80.8	64	82.1	62	79.5		62	78.5	64	83.1	
Missing data	33	-	16	-	17	-		16	-	17	-	
HbF (%), median	5.8		5.65		6.1		0.682	5.4		6.7		0.175
(range)	(0.7, 25)		(1.2, 25)		(0.7, 22.4)			(1, 22.4)		(0.7, 25)		
Number the VOC/year[2]							1.00					0.463
≤2	93	50.3	45	50	48	31.2		43	47.3	50	53.2	
3 or more	92	49.7	45	50	64	68.8		48	52.7	44	46.8	
Missing data	4	-	4	-	2	-		4	-	-	-	
Recurrent clinical events[3]							0.519					0.196
No clinical complication	53	29	24	26.7	29	31.2		22	24.4	31	33.3	
One complication or more	130	71	66	73.3	64	68.8		68	75.6	62	66.7	
Missing data	6	-	4	-	2	-		5	-	1	-	

Abbreviations: HbF, fetal hemoglobin; CAR, Central African Republican; VOC, vaso-occlusion crisis.
NOTE: * Indicate differences statistically significant.

1: Missing values were excluded from the calculation of *P*-values.
2: We only considered the number of VOC documented in the last year (2017).

3: It was considered as recurrent clinical events: leg ulcer, stroke, priapism, osteonecrosis, acute chest syndrome, transcranial Doppler high-risk, cardiopathy, dactylitis, acute spleen sequestration, nephropathy, retinopathy and acute lung hypertension. Patients assigned to the “No clinical complication” group were those who did not experience the aforementioned recurrent clinical events.

Supplemental figure 3A shows a representative example of one out of three independent experiments using peripheral blood from healthy individuals to determine the best strategy for isolating the erythrocytes populations for subsequent PS quantification. Supplemental figure 3B exhibits the percentage of annexin-V on the plasma membrane of healthy individuals using gated erythrocytes (R1) or CD235a (Glycophorin A) in a paired samples analysis. Since there was no difference between samples, we opted to perform subsequent analyses using the gated erythrocytes strategy (Supplemental figure 3C and 3D).

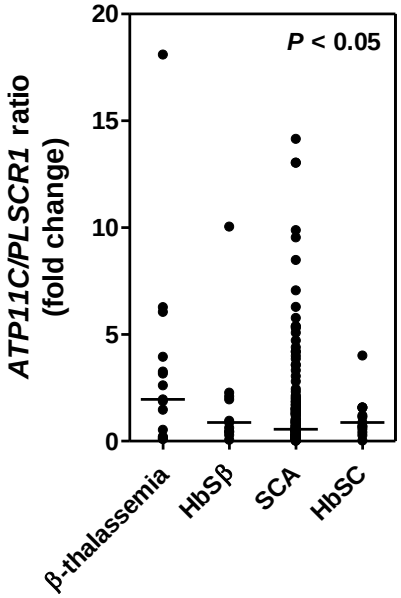


Supplemental figure 3 - PS exposure on the plasma membrane. (A) Histograms showing the annexin-V positivity in peripheral blood samples from healthy individuals using (A-i) cells labeled with anti-CD235a-R-phycoerythrin, (A-ii) gated cells in erythrocytes population (R1), (A-iii) unstained control. The percentage of positive cells using CD235a labeling or gated in erythrocytes are displayed in the figure. (B) Paired samples analysis of the percentage of annexin-V on the plasma membrane of healthy individuals between gated erythrocytes (R1) and CD235a labeled cells. Similar symbol shapes in R1 and CD235a labeling indicates that the samples are the same (C-D) Representative analysis of PS exposure in erythrocytes from patients with sickle cell anemia. Dot plot (forward scatter *versus* side scatter) of peripheral blood samples from patients with SCA collected while in steady state (C) and during a vaso-occlusive crisis (D). Region R1 represents the erythrocytes population. Annexin-V positivity is displayed on single parameter histograms (C-i and D-i, negative control) and (C-ii and D-ii, stained Annexin-V samples). PS exposure is directly proportional to Annexin-V binding to the erythrocytes. PS exposure is shown on the x-axis and event frequency is shown on the y-axis for the erythrocytes. Gated regions G1

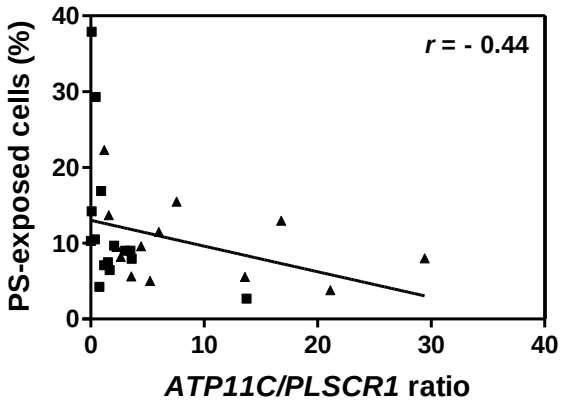
and G2 show, respectively, the PS-negative and PS-positive regions for erythrocytes.

Supplemental figure 4A shows the primary data (i.e., in linear scale) of the *ATP11C*/*PLSCR1* ratio in samples from patients with sickle cell disease (HbSC/HbSβ), β-thalassemia and SCA. *ATP11C*/*PLSCR1* ratio was similar among all groups (*P*<0.05). The mean level of PS-positive RBC in the SS-genotype patients group in steady-state and with VOC were 10.05 ± 4.75% and 13.4 ± 10.19%, respectively. Supplemental figure 4B exhibits the correlation (primary data) between *ATP11C*/*PLSCR1* ratio and PS exposure in samples from patients with SCA (Pearson correlation coefficient, *r* = -0.44).

Supplemental figure 4A.



Supplemental figure 4B.



Supplemental figure 4 - Primary data for both *ATP11C*/*PLSCR1* ratio and PS exposure. (A) Quantitative analysis of *ATP11C*/*PLSCR1* ratio in samples from patients with β-thalassemia, HbSβ, SCA, and HbSC. The expression of *ATP11C* and *PLSCR1* was quantified by real-time quantitative PCR, separately, and then expressed as a ratio. The horizontal bars represent the median value of *ATP11C*/*PLSCR1* ratio. Comparisons were accomplished using Kruskal–Wallis test followed by a Dunn's post-test. (B) Correlation between PS exposure and *ATP11C*/*PLSCR1* ratio. These data were generated from 13 patients in crisis (square shaped marker) and 15 patients in steady state (triangle shaped marker). Correlation analyses were accomplished using Spearman correlation test.

Supplemental data

REFERENCES

1. Cancado, RD, Lobo C, Ângulo IL, et al. Clinical protocol and therapeutic guidelines for the use of hydroxyurea in sickle cell disease. *Rev. Bras. Hematol. Hemoter*. 2009; 31(1516-8484) (online):361-366.

2. Goossens M, Kan YY. DNA analysis in the diagnosis of hemoglobin disorders. *Methods Enzym*. 1981;76(0076–6879 (Print):805–817.

3. Heagerty PJ, Zheng Y. Survival model predictive accuracy and ROC curves. *Biometrics*. 2005;61(1):92–105.

4. Harrell Jr. FE, Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. *JAMA*. 1982;247(0098–7484 (Print)):2543–2546.

Supplemental data

RESPONSE TO REVIEWERS: R1

REVIEWER #1: COMMENTS FOR THE AUTHOR

The article is a commentary that aims to relate the ratio of ATP11C/PLSCR1 mRNA expression in reticulocytes (and by extension erythrocytes) to clinical presentation in sickle cell patients. The authors show that a lower ATP11C/PLSCR1 ratio relates to increased levels of PS externalization on erythrocytes and in turn higher levels of clinical complications. They go on to suggest that a change in the ATP11C/PLSCR1 ratio from high to low may be relevant in vaso-occlusive crisis. There is some information of interest in this commentary, however, in its current form I think the data shown is difficult to interpret (because of the numerous steps in its generation) and the authors could do more with their data to enhance the overall message of the commentary.

Comments and Concerns

Minor

1. The title is not descriptive enough. Something along the lines "The ratio of ATP11C/PLSCR1 mRNA transcripts has clinical significance in sickle cell anemia" would be better?

RESPONSE: We thank the reviewer for the constructive comments about the title. In fact, your title suggestion captures the essence of our results in a better manner; also, it was well received by the authors. Following your recommendations, we are now presenting the revised version with the new suggested title.

2. Reference 2 and reference 15 are identical. I suspect that reference 2 is an error and the authors meant to refer to another publication.

RESPONSE: We thank the reviewer for these observations and apologize for this misunderstanding. As you correctly mentioned, we inserted the study of Mankelow et al. (*Blood*. 2015;126(15):1831–1834) by mistake, which was properly substituted by Setty et al. (*Blood. 2002;99(5):1564–1571*) in the present version.

3. Figure 1A. It is unclear why the authors have gone for a log scale. Personally, I prefer the figure as it is in the supplemental material with just the direct ATP11C/PLSCR1 ratio given.

RESPONSE: We thank the reviewer for the observation and respect your opinion about the data presentation. As requested, we are now presenting our results using the primary (linear) data of *ATP11C*/*PLSCR1* ratio. Of note, we opted to present the Y-axis in log10 scale to improve the visibility of data. For comparison purpose, Supplemental figure 4A displays the same data from Figure 1 presented in linear scale. With respect to data presentation of the Figure 1B, we opted to use the log scale in both in Y-axis and X-axis because it makes the tendency line clearer to see (as your own correctly assumed in question #6). This strategy can be valuable both for making patterns in the data more interpretable and for helping to meet the assumptions of inferential statistics. This information was properly highlighted in the Figure legend of the main document (sentence in red) and in the Supplemental data (Statistical analysis section, page #4, sentence in blue) of the revised version of the manuscript. We must highlight that, depending on the choice of the scale, and consequently, the distribution of the data, statistical tests should be properly used (Pearson or Spearman correlation tests). Considering that both correlation analyses (using the linear or log scales) supported exactly the same conclusions (i.e., the lower the *ATP11C/PLSCR1* ratio, the higher the PS-exposure on the plasma membrane), we respectfully ask for your permission to present the Figure 1B as it is currently presented (i.e., in log scale). We thank you in advance for your comprehension.

4. Figure 1B. What are the units in both the main chart and supplemental on the y-axis "PS expression levels" is it % cells positive, mean fluorescence or another?

RESPONSE: We thank the reviewer for the observation. In the present version, we are now presenting the units of the Figure 1B. We also take the opportunity to replace the label of Y-axis from "*PS exposure levels*" to "*PS-exposed cells (%)*" (Please, see comments from Reviewer #2, first question).

5. Figure 1B legend. It should state that this is data from 13 crisis patients and 15 steady state patients. It would be useful to indicate (different shaped markers) which of the 28 dots are from steady state and which are in crisis. In addition, as stated earlier, it should be stated what is actually being measured to give "PS exposure".

RESPONSE: We thank the reviewer for the observation. As requested, we included in the Figure legend a sentence explaining that the data from the correlation analysis were generated from patients in crisis and in steady state (Figure legend, sentence in red). In addition, we are now using different shaped markers to discriminate patients in crisis (square marker) and in steady state (triangle marker).

6. Figure 1B. It is unclear why the authors have gone for a log scale on the X-axis. I assume it was used on the Y-axis as it makes the line of best fit clearer to see.
RESPONSE: Please, refer to the question #3.

7. Table 1. I would highlight the "recurrent clinical events" section in bold to clearly show that it is here that the "low" and "high" ATP11C/PLSCR1 ratio have a significant difference
RESPONSE: As requested, we highlighted in bold the aforementioned section.

8. Authors use the term "low" and "high" ATP11C/PLSCR1 ratio throughout without ever stating the numerical range of ratios defined as "low" and "high".
RESPONSE: We apologize if this information was not clear in the previous version, but the strategy for cohort dichotomization was clearly presented in the Supplemental data (Statistical analysis section, page #5, sentence in red) from the previous version. To avoid further misunderstanding, we are now presenting details about the central tendency and dispersion measurements of the *ATP11C/PLSCR1* ratio, as well as a complete analysis of the primary data from *ATP11C* and *PLSCR1* (separately) regarding their association with clinical outcomes in SCA (Supplemental data, Results section, sentences in red).

9. In the reference list, An et al cited as "Cells RED, An X, Schulz VP, et al". I assume a typographical error. Another typographical error at the bottom of page 4, it should read "Importantly, other mechanisms may be involved in PS exposure in patients with sickle cell disease.
RESPONSE: We apologize for these typographical errors and thank the reviewer for them pointing out. As requested, we properly corrected the aforementioned reference and the typographical error (page #5, sentence in red). In addition, we take the opportunity to double-check the Reference list to avoid further mistakes.

11. Reference in Supplemental material (Top of Page 2). I assume it should be reference 2 after "Total RNA from peripheral blood reticulocytes"

RESPONSE: We apologize for this mistake. In the present version, the aforementioned reference was properly adjusted (Supplemental data, Gene expression profile section, sentence in red).

Major

1. In the description of what has been performed (top of page 3) not much detail is given. My first reaction was that no "normal" samples had been analyzed and it wasn't until reading the supplemental material that I was able to work out that this was incorrect and that this had been performed and incorporated into the data analysis. I feel this section needs to be re-written (despite the restrictive word limit) to incorporate this information in the main text rather than having it in the supplementary material.

RESPONSE: As requested, we properly included in the main document of the revised version all information regarding samples from healthy subjects (control group) (page #3, sentence in red and underlined). Of note, we maintained the description of healthy subjects in Supplemental data unaltered.

2. Personally, I don't like the use of a ATP11C/PLSCR1 ratio. I can see it is useful in certain circumstances to give generic differences in expression levels of these two genes, however, the description of "high" and "low" ratios makes no account of which of the partners has an altered expression in the patient groups. For example, some patients have a "high" ATP11C/PLSCR1 ratio and some have a "low". Is the difference between these groups an increase in ATP11C expression (relative to 18s rRNA), a decrease in PLSCR1 expression (relative to 18s rRNA) or a combination of the two? If only the expression of one of the partners is altered then it makes a difference to the overall biology of the cell and the hypothesis of the commentary. Indeed, early in the paper (page 2) the authors state "Aberrant expression of either or both ATP11C and PLSCR1 transcripts may disrupt the PS internalisation/externalisation process and become clinically relevant for patients with SCA". It would be interesting to know if this is the case and a re-analysis of the data to show expressions of ATP11C and PLSCR1 (relative to the reference 18s rRNA) rather than a ratio would show this.

RESPONSE: We thank the reviewer for these constructive comments. As requested, we are now providing an extra analysis showing the clinical importance of *ATP11C and PLSCR1* genes separately. Supplemental table 2 summarize baseline features of patients according to *ATP11C* and *PLSCR1* transcript levels (Supplemental data, Results section, sentences in red). We are aware that this information was not included in the previous version of the manuscript but, before we presented the *ATP11C/PLSCR1* ratio as an option to predict recurrent clinical events in patients with SCA, we first examined the prognostic value of each variable separately and found that the ratio was the most prognostic among the three metrics. We apologize for not including this information earlier. In contrast to the ratio analysis, the separate analysis of *ATP11C* and *PLSCR1* genes had no impact on clinical outcomes of patients with SCA. We would like to point out that these data were not included in the main text, in order to not drastically exceed the text word count and thus were included in the Supplemental data instead (Supplemental data, Results section, sentences in red).

3. Figure 1A. Given the variability in the ATP11C/PLSCR1 ratio, and thus I assume expression of the individual genes, in all patient groups it would be useful to know how variable this is in the control "healthy" group of 23 individuals. Again, by showing the expression levels relative to the 18s rRNA reference for both ATP11C and PLSCR1 the data for the "healthy" individuals could be shown alongside that of the SCA groups and direct comparisons made.

RESPONSE: We thank the reviewer for the suggestion. We agree that showing the expression levels of *ATP11C* and *PLSCR1* (or even the *ATP11C*/*PLSCR1* ratio) for the healthy control group alongside to the hemoglobinopathies groups could provide an idea on the variability of the data and the intersection rate between patients and healthy individuals. Nevertheless, it was not our intention to compare healthy individuals *versus* patients, because we believe that such analysis would be little informative in term of biological significance (mainly if we consider that healthy individuals have no recurrent clinical event). The main reason to include healthy volunteers in our study was to create a reference group for the comparative Ct method, strengthening the statistical analyses and providing reliable data. Consequently, the control "reference" group could not be used in the comparative analyzes, because it is now part of from the "fold change" final value. We thank you in advance for your comprehension.

4. Following on from this on Page 4 the authors comment, "emerging data suggests that low expression of the ATP11C gene is associated with a higher rate of PS exposure. The authors could use their data of the 28 patients that they have with both expression ATP11C levels and PS exposure to comment further on this point.

RESPONSE: We thank the reviewer for the suggestion. As requested, we performed a correlation analysis using individual *ATP11C* transcript levels and PS exposure. *ATP11C* transcript levels were not correlated with surface PS accumulation (Pearson correlation coefficient, r = -0.058). We opted to not include this information in the manuscript because of the limited number of words. Most important, it was not our intention to evaluate *ATP11C* or *PLSCR1* transcript levels individually, but rather the balance between them. Based on our results, we highlighted the importance of the *ATP11C*/*PLSCR1* ratio in a clinical setting (page #7, final paragraph, sentence in red).

REVIEWER #2: COMMENTS FOR THE AUTHOR

This manuscript deals with an important topic to suggest a possible cause for facilitating PS exposure to erythrocyte surface in severe SCA and demonstrates that patients who had low transcripts ratio ATP11C/PLSCR1 tended to show complicated clinical symptoms. The experiments, the results, and the conclusion seem to be consistent, but I have some concerns that should be addressed.

1. It is difficult to understand the mean of PS exposure level (Y-axis in Fig. 1B). I speculate from the Y-axis values of supplementary Fig. 1B that it represents the percentage of PS-exposed cells in total 100,000 counts. If so, the authors should replace PS exposure level with PS-exposed cells (%) both in Fig. 1B and supplemental Fig. 1B. Additionally, the sentence, "The PS exposure rate..." in the text (p. 3; L. 6~), should be changed for more proper explanation with "The PS-exposed erythrocytes were labeled using Annexin V binding assay kit (BD Biosciences) and their rate was analyzed via standard flow-cytometry methods.". If my speculation is wrong, a comprehensible explanation is required.

RESPONSE: We appreciate the reviewer's positive feedback and wish to address all the concerns. As requested, Y-axis values of both Figure 1B and Supplementary figure 1B were properly adjusted. In addition, we are now presenting in the revised version

the sentence “T*he PS exposure rate*…” properly adjusted (page #3, end of the first paragraph, sentence in red).

2. The balance between flippase and scramblase activities is certainly important for the determination of the distribution of PS. However, ATP11C/PLSCR1 ratio masks valuable information about individual transcript levels. For enhancing the contribution of this manuscript to this field, I would like to recommend that individual transcript levels especially in the very high ratio and very low ratio groups be additionally described in the text without showing the data.

RESPONSE: We thank the reviewer for the constructive and valuable observation. In the present version, we compared the median values of *ATP11C* and *PLSCR1* transcript levels (individually) in patients assigned to the first and fourth quartile of *ATP11C/PLSCR1* ratio (page #4, sentence in red). Of note, such analysis was performed in those 13 crisis patients and 15 steady state patients (Please, see Reviewer #1, major comment #4).

Supplemental data

RESPONSE TO REVIEWERS: R2

REVIEWER #1: COMMENTS FOR THE AUTHOR

This is a revised version of a previously submitted commentary. The authors have clearly shown the changes they have made in response to the reviewers. The authors have either made the changes directed by the reviewers or given a reasonable response if they have not. Unfortunately, one of the changes the authors have made gives major cause for concern (see below).

Comments and Concerns

Minor

I thank the author for clarification on "high" and "low" ratios; however, as the terms are referred to throughout the article, I feel that the explanation should be in the main text rather than Supplemental.

RESPONSE: We appreciate the reviewer's positive feedback, which we truly believe that helped us to improve our manuscript. As requested, we transfer part of the *Statistical analysis section* from the Supplemental data to the main document (page #3, sentence in blue).

Major

Figure 1B and supplemental figure 2B. The authors have now given a descriptive label to the y-axis. The data presented in these charts show the "% of PS exposed cells vs the ATP11C/PLSCR1 ratio", however, the percentage levels of PS exposed cells is much higher than previously published data for SCA. The authors present percentage levels of PS exposed cells in both steady state and crisis SCA patients from approximately 61% down to about 10% with the majority seemingly clustered between 15-25%. A quick scan through the literature reveals percentage levels of PS exposed cells in SCA samples are detected at much lower levels.

1. *Wood et al 1996 (Blood 88(5):1873-80.) - 2.86% ± 2% (205 SCA samples) (Annexin V FITC)*
2. *Setty et al 2001 (Blood 98(12):3228-33.) – 4.12% ± 2.41% (± SD) (25 SCA samples) (Annexin V FITC)*

3. *de Jong et al 2001 (Blood 98(3):860-7.) - 2.1% ± 1.8% (mean ± SD; range, 0.1% to 11.1%) (147 SCA samples) (Annexin V FITC)*
4. *Setty et al 2002 (Blood 99(5):1564-71.) - 1.44% to 5.59% SCA (87 SCA samples) (Annexin V FITC)*

The authors need to include an explanation of the disagreement between their data and that previously reported.

RESPONSE: We thank the reviewer for these constructive comments. Indeed, after reading the aforementioned references and others suggested by the reviewer (Nur et al., Ann Hematol. 91(7):1097-105; Westerman et al., Br J Haematol. 142(1):126-35; Whelihan et al., J Thromb Haemost. 14(10):1941-1952), we concluded that our results did not represent most of the studies in the current literature. Therefore, we decided to reanalyze our dataset (patient by patient) in order to find a plausible explanation for this disagreement. Unfortunately, we found a primary mistake in our analysis: the entire flow cytometric dataset was analyzed without gating the erythrocytes population. As such, part of the samples had a PS exposure rate (particularly in those considered outliers) overestimated by the presence of platelets and debris, a well-known source of PS in peripheral blood. After a careful reanalysis of the data (i.e., using a proper gate in erythrocytes population), we conclude that the highest value of PS exposure on the erythrocyte's plasma membrane was 37.9%, instead 63.2% presented in the previous version of the manuscript. It is important to highlight that most of the patients who had higher (or lower) PS exposure rate in the previous analysis retained their results in the present version, which means that the main conclusions of the manuscript remained unchanged.

In order to exclude further mistakes and guarantee the quality of our study, we performed three independent experiments using peripheral blood from healthy individuals. The main goals of these experiments were: 1) to evaluate the sensibility/specificity of our method using increasing doses of a specific inhibitor of ATP11C flipping activity (N-ethylmaleimide, NEM) as previously stated in the last version. We are now presenting these data in the Supplemental data (Method section, page #3, second paragraph, sentence in blue; Supplemental figure 1A and 1B). Our second goal was determining the best strategy to isolate erythrocytes for subsequent PS quantification. Briefly, this strategy constituted in determining the percentage of PS-

exposed cells via flow cytometry methods using samples labeled with anti-CD235a-R-phycoerythrin and unlabeled cells gated in erythrocytes population (applying a log acquisition setup and selecting cells with high FCS and high SSC). Subsequently, the percentage of Annexin-V was compared in a paired analysis. As demonstrated in the Supplemental figure 3B (Supplemental data, Results section, page #10), no significant difference was observed between labeled (CD235a) and gated (R1) cells. Consequently, we opted to perform subsequent analyses using the gated erythrocytes strategy (Supplemental data, Results section, Supplemental figure 3C and 3D, page #10).

Overall, we are now presenting a proper validation of our method and a correct presentation of our data. Yet, our patients still present higher values of PS exposure (mean value of PS in patients at steady state: 10%, ± 1.2%) than those reported by others (references below). These intriguing results prompted us to looking for a plausible explanation for this disagreement, especially considering the methodological similarity among studies. After a careful analysis of the references suggested by the reviewer, we noticed that the acquisition process, analysis and presentation of data could underlie these discrepancies between studies. Although most studies cited by the reviewer did not show the strategy of acquisition and analysis, those who did, reported the PS values analysis applying the linear scale for Annexin-V. This is an important issue and deserve particular attention. If we present our data using a linear scale, the data of Annexin V will be underestimated, and thus, high values could be converted into values of signifier smaller magnitude. If we present our data using a linear scale, the values will be much closer to that described in the literature (please, see Table 1). We take the opportunity to perform two simple analysis. First, we sampled an SS-patient in steady state. If the levels of labeled for Annexin-V were analyzed on the log scale, the resulting value is 9.64%. On the other hand, if analyzed on the linear scale, the same sample will present values around 1.1% (significantly lower and different values than that previously observed), and much closer to that described in the literature. In the same context, we used the same approach to a second example: an SS-patient with the sample collected during an episode of VOC. Applying the log scale, the value of Annexin V was 37.9%. However, if analyzed using the linear scale, the levels fall drastically to 17.4% (2 fold less).

The reason underlying this discrepancy lies in the determination of the threshold. If we apply a threshold to define the positivity of the Annexin-V positive cells

in the linear scale and apply the same threshold in the log scale, we will observe cells with values of up to 10^4 fluorescence (considered negative), which would be an underestimation of the actual labeled of cells. Of note, if we apply the linear scale to analyze the Annexin-V staining in the healthy volunteers, all values are now above 1%, similar to the previous studies referred by reviewer. Nevertheless, we respectfully disagree with such analysis. The linear scale is based on the principle that the channels are distributed equally along the scale. The problem behind this analysis it is that two populations, with very different levels of intensity, could be plotted together. This is a common situation in several publications involving flow cytometry data, in which non-fluorescent cells are visualized on the same plot because of their own fluorescence. For this reason, linear scaling plots has become less useful, as it will be very difficult to see both fluorescent and non-fluorescent at the same time, no matter what PMT voltage we use. On the other hand, log scales facilitate the visualization of data with very different medians. Importantly, even though each channel itself contains the same number of digital values, data channels are not distributed equally across the scale. On the log scale, data is compressed to a much greater degree at the high end than it is at the low end, and it is this very property that makes it so good for visually representing data with very different medians.

In summary, the linear scale must be used for light scattering measurements (where particles differ subtly in signal intensity) and log scale must be used for fluorescence (where particles differ quite starkly in the signal) (Herzenberg LA et al. Nature Immunology, 2006 Jul;7(7):681-685) (attached). Therefore, applying the linear scale to our data is completely wrong, and I (on behalf of all authors) would like to express our disappointment with the lack of knowledge of the reviewer; in fact, after a careful evaluation of all his/her comments, it seems to us that could have a conflict of interest

Table 1. Summary of studies reporting the percentage levels of PS exposed cells in SCA samples.

Disease	Patient status	PS levels (%; Mean ± SD)	Analyses (Log *versus* Linear)	References
SCD (HbSS or HbSβ^0-thalassemia)	Steady-state	2.44 (1.12 – 3.53)	Only erythrocytes with more than one log greater than the PS negative erythrocytes	Nur et al 2012, 91:1097-1105
SCA	Steady-state	1.6 ± 0.4	Log scale	Westerman et al 2007, 142:126-135
SCD (No information about the genotypes)	Steady-state	2.86 ± 2.0	Linear scale	Wood et al 1996, 88:1873-1880
SCD (HbSS, HbSC and HbSβ$^+$-thalassemia)	Steady-state	1.44 ± 0.42; 4.38 ± 1.56	Linear scale	Setty et al 2002, 99:1564-1571
SCD (HbSS or HbSβ^0-thalassemia)	Steady-state (19/25 in hydroxyurea therapy)	4.8 ± 3.9 – 7.6 ± 4.5 (Non-hydroxyurea)	Log scale	Whelihan et al 2016, 14:1941-1952
SCD (HbSS and HbSC)	Steady-state	4.12 ± 2.41	Log scale	Setty et al 2001, 98:3228-3233
SCD (HbSS or HbSβ^0-thalassemia)	Steady-stare	2.96 ± 3.27 – 6.27 ± 3.33 (Before hydroxyurea)	No information available	Covas et al 2004, 89:273-280
SCA	Steady-state	2.1 ± 1.8	Log scale	De Jong et al 2001, 98:860-867
SCA	Steady-state	31.1 ± 3.9	No information available	Hannemann et al 2018, 182(4):567-578
SCD (HbSS and HbSC)	Steady-state	PS levels in MFI values (arbitrary units)	Log scale	Garnier Y et al 2017, 12(5):e0177397

Abbreviations: SCA, Sickle cell anemia; SCD, Sickle cell disease, PS, Phosphatydylserine; MFI, Mean fluorescence intensity.

We would like to point out that this explanation regarding genetic background was not included in the main text to not drastically exceed the text word count and because this issue is not related to the main goal of the study.

REFERENCES SUGGESTED BY THE REVIEWER:

1. *Wood et al 1996: 2.86% ± 2%*
2. *Setty et al 2001: 4.12% ± 2.41%*
3. *de Jong et al 2001: 2.1% ± 1.8%*
4. *Setty et al 2002: 1.44% to 5.59%*
5. *Nur et al 2012: 1.64% (11 patients with age range 20–47)*
6. *Westerman et al 2008: 1.6±0·4 % (31 patients with age range 18-56)*
7. *Whelihan et al 2016: 4.8 ± 3.9% (25 patients mean age 33.3)*

REVIEWER #2: COMMENTS FOR THE AUTHOR

I have no comment to the author.

RESPONSE: We appreciate the reviewer's positive feedback and by the opportunity to resubmit our study to Blood Advances.

Supplemental data

RESPONSE TO REVIEWERS: R3

REVIEWER #1: COMMENTS FOR THE AUTHOR

Major

The authors have explained the discrepancy between results from theirs and others as being "methodological" and "criteria for patient inclusion", theirs being predominantly adult patients with 46% experiencing occlusive crisis. To this reviewer this seems unlikely. In the articles referenced (2, 18-20) all have used Annexin V FITC in a flow cytometric assay in a similar method to that used by the authors and in the Wood et al 1996 study, 205 adult patients were examined giving a PS-positive red cell population of 2.86% ± 2%. In addition, other studies using adult sickle patients and an Annexin V flow cytometric assay report similar results.

1. *Nur et al 2012 (Ann Hematol. 91(7):1097-105.) - 1.64% (11 patients with age range 20–47, Annexin V FITC – Flow cytometry).*
2. *Westerman et al 2008 (Br J Haematol. 142(1):126-35.) - 1·6±0·4 % (31 patients with age range 18-56, Annexin V FITC - Flow cytometry)*
3. *Whelihan et al 2016 (J Thromb Haemost. 14(10):1941-1952.) - 4.8 ± 3.9% (25 patients mean age 33.3, Annexin V FITC - Flow cytometry)*

Whereas I concur with the authors that PS exposure is likely to be elevated during occlusive crisis I doubt it would reach the limits that they are reporting in the 13 patients who are in crisis. It also does not take account the 15 patients in steady state in their study who, as expected, do have lower percentages of cells that are PS-positive than those in crisis but that are reported at much higher percentages than observed in previous studies (see above). The authors could try and validate their methodology by using erythrocytes from a healthy individual (who should have a very low level of PS-positive cells). Treating these cells with a calcium ionophore in a calcium media should result in 100% of the cells being PS-positive. After washing, these cells could be mixed with untreated cells in different ratios, all treated cells, 1:4, 2:4, 3:4 and all untreated cell and testing them in their flow assay to see if it corresponds with levels of 100%,

75%, 50%, 25% and 0% PS-positive cells. In the supplemental, the authors state that treating cells with "increasing doses of N-ethylmaleimide were used to determine the sensibility of the test (data not shown)". It is impossible to evaluate unseen data; however, they may have already performed a similar experiment to the one I have suggested above.

RESPONSE: We thank the reviewer for these comments. We completely agree that "unseen data" is impossible to evaluate and apologize for this misunderstanding. In the present version of the manuscript, we included the data relative to N-ethylmaleimide experiments (Supplemental data, Methods section, Supplemental figure 1A and 1B, page #4). We also included the suggested validation experiment with increasing concentration of cells treated with calcium media (Supplemental data, Methods section, Supplemental figure 1C, page #4).

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MS ID#: HAEMATOL/2019/216721
MS TITLE: The ratio of ATP11C/PLSCR1 mRNA transcripts has clinical significance in sickle cell anemia

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Thank you very much for your submission.

Best wishes,

The Editorial Staff

4. DIFFERENTIAL EXPRESSION OF *SPHK1* AND *DMTN* IN PATIENTS WITH SICKLE CELL ANEMIA

Artigo a ser submetido à revista "Blood" - Fator de Impacto: 13,164; Qualis/CAPES Ciências Biológicas I: A1

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RED CELLS, IRON, and ERYTHROPOIESIS

Differential expression of *SPHK1* and *DMTN* in patients with sickle cell anemia

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Running head: *SPHK1* and *DMTN* expression in SCA.

Text word count: 1.990 words.
Number of figures: 2 figures.
Number of tables: 1 table.
Number of references: 18 references.
Number of manuscript pages: 16 pages.

KEY POINTS

- *SPHK1* expression is reduced in patients with SCA on HU therapy.
- Differential expression of *DMTN* is elevated in patients with SCA during VOC.

ABSTRACT

The plasmatic membrane presents a great importance in the structural properties of erythrocytes, and in sickle cell anemia (SCA), red blood cells present a short lifespan and altering normal metabolism of erythrocyte membrane may contribute to a more serious phenotype in SCA. Therefore, the objective of this work was to evaluated *SPHK1* and *DMTN* transcript levels in reticulocytes from patients with SCA and associated these findings with clinical disease severity. The sample consisted of patients with SCA, without transfusion for 3 months, and stratified during blood collection regarding the use of hydroxyurea and the presence of VOC. Analysis of *SPHK1* and *DMTN* genes expression was performed by qPCR with TaqMan® probes, and the results were generated by the ΔCt method and expressed using the formula 2-ΔΔCt. Our data showed that reticulocytes from patients with SCA had low levels of *SPHK1* ($P < 0.01$), although these findings were not associated with a worse clinical outcome. Additionally, the use of hydroxyurea decreases the expression of *SPHK1* ($P < 0.001$) and high levels of *DMTN* were found during painful crisis ($P = 0.043$). Therefore, our work demonstrates that *SPHK1* and *DMTN* are differentially expressed during the clinical course of patients with SCA, although the impact of this differential expression on patients' clinical outcome is not clear.

KEY-WORDS: Sickle cell disease, plasmatic membrane, *SPHK1*, *DMTN*.

INTRODUCTION

Red blood cells from patients with sickle cell anemia (SCA), a multisystem disease associated with episodes of acute illness and progressive organ damage, present a short lifespan and altering normal metabolism of erythrocyte membrane may contribute to a more serious phenotype in SCA [1,2]. Additionally, disease severity in SCA is associated with adhesion of sickled RBCs to the vascular endothelium [3].

It is clear that sphingosine-1-phosphate (S1P), a bioactive lipid enriched in erythrocytes and generated intracellularly exclusively by sphingosine kinase 1 (SPHK1), is significantly elevated in erythrocytes and plasma of mice and humans with sickle cell anemia (SCA) [4,5]. In SCA, an increased erythrocyte S1P promotes oxidative stress and induces production of 2,3-bisphosphoglycerate (2,3-BPG), and thus increasing deoxygenated sickle Hb (deoxyHbS), deoxyHbS polymerization, sickling, hemolysis and disease progression. Additionally, S1P functioning intracellularly binds to deoxyHbS and facilitates deoxyHbS anchoring to the membrane [5]. In view of these important findings, identifying specific factors and signaling pathways related to the increase of S1P in SCA is important [6].

In the same context, dematin, which was previously called protein 4.9, plays an important structural role in erythrocytes [7]. Without this protein, the association of spectrin and actin to the plasma membrane is weakened, resulting in osmotically fragile erythrocytes with reduced deformability. Additionally, the absence of dematin is associated with a considerable loss of spectrin, adducin and actin (60%, 90% and, respectively, 90%) [8]. Furthermore, sickled red blood cells contain abundant ERK1/2, which can promote ICAM-4-mediated adhesion to endothelium, and proteomic analysis has already revealed that phosphorylation of cytoskeletal proteins, such as dematin and protein 4.1, via the ERK pathway may lead to disorganization of red blood cell cytoskeleton [9].

To date, little is known about the differential expression of *SPHK1* and *DMTN* in SS-genotyped cells and its impact on clinical outcomes in SCA. In the present study, we evaluated *SPHK1* and *DMTN* transcript levels in reticulocytes from patients with SCA and associated these findings with clinical disease severity.

MATERIAL and METHODS

Patients

The present study was drawn from a cohort of 68 unrelated adult patients with SCA (median age: 30 years, range, 18-54 years, with 28 males; 41%), who had been attending at the Hematology and Hemotherapy Foundation of Pernambuco (HEMOPE), Brazil, since January 2000. Between February 2017 and October 2017, peripheral blood (PB) samples were collected. To enter in the study, all patients should be off hydroxyurea therapy and not receive blood transfusions in the last 3 months prior blood collection. All patients were fully characterized for the β^S-globin gene haplotype and co-inheritance of alpha-thalassemia. Twenty-five adult patients with SCA on hydroxyurea therapy and 15 age- and sex-matched volunteers (hemoglobin profile AA) with no history of hematological disease were included for comparisons. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients. This study was approved by the local Research Ethics Board (#2.727.352).

Laboratory data

Hemoglobin (Hb) and reticulocyte counts were done on a Coulter STKS (Coulter Electronics, Hialeah, FL). The quantification of hemoglobin F (HbF) was performed by high performance liquid chromatography (HPLC) using the Bio-Rad Variant II HPLC System (Bio-Rad Laboratories, Hercules, CA, USA).

Gene expression profile of *SPHK1* and *DMTN*

Total RNA from reticulocytes [10] was isolated using Trizol reagent. After total RNA extraction, real-time quantitative polymerase chain reaction (qPCR) assays with sample-derived cDNA were performed in duplicate on MicroAmp optical 96-well plates using a 7500 Real-Time PCR System (Applied BioSystems) using the subunit 18S from human ribosomal RNA (Hs9999901_s1, Applied Biosystems) and actin beta (Hs01060665_g1, Applied Biosystems) as an endogenous control. In case of a discrepancy greater than 1 in cycle of threshold (Ct) values between duplicates, a third test was performed. Samples whose Cts of the endogenous control were lower or higher than 2 standard deviations of average were excluded from analysis. *SPHK1* (Hs00184211_m1, Applied Biosystems) and *DMTN* transcript levels (Hs00157387_m1, Applied Biosystems) were quantified using TaqMan Gene

Expression Assay (Applied BioSystems) according to manufacturer recommendations. The comparative Ct method was applied to determine the relative *SPHK1* and *DMTN* transcript levels. As such, the difference of Ct number ($\Delta Ct = Ct_{SPHK1}$ – geometric mean of Ct_{18S} + Ct_{ACTB} and Ct_{DMTN} – geometric mean of Ct_{18S} + Ct_{ACTB}) was calculated for each replicate. Without detectable *SPHK1* or *DMTN* amplification within 40 cycles (standard amplification conditions for qPCR), both expression values were set to 0. For the reference group, peripheral blood reticulocytes from age 15 and sex-adjusted healthy volunteers (hemoglobin profile AA) with no history of hematological diseases were included. *SPHK1* and *DMTN* expression were calculated relative to a reference cDNA (mean value of the ΔCt from the reference group) and set to 1. The same reference cDNA also served as an internal control throughout all experiments to ensure full comparability among experiments. Relative *SPHK1* and *DMTN* transcript values were calculated as relative quantification using $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta Ct_{patients} - \Delta Ct_{healthy\ volunteer}$.

PS exposure detection in SS-genotyped erythrocyte

The PS exposure level was determined using the AnnexinV-fluorescein isothiocyanate binding assay (BD Biosciences) and analyzed via standard flow-cytometry methods. To determine the PS exposure in the erythrocyte's population, we gated the cells of interest with high forward scatter and side scatter, in order to exclude debris and platelets. Alternatively, we performed a Glycophorin A staining using the anti-CD235a-R-phycoerythrin (Clone: GA-R2; BD Biosciences). Those cells population exhibiting high forward scatter values and expressing the CD235a were defined as erythrocytes. Immunophenotypic analysis was accomplished using 1×10^6 erythrocytes from 22 newly collected samples from patients with SCA during follow-up appointments. Patients were included if they were off hydroxyurea therapy and without blood transfusion for at least three months. All experiments were performed in triplicate and in each sample, a minimum of 100,000 events was acquired. Unlabeled cells were used as negative controls to determine the best threshold for each sample. In addition, increasing doses of N-ethylmaleimide (NEM, an ATP11C inhibitor) were used to determine the sensibility of the test (data not shown).

Statistical analysis

Patient baseline characteristics were reported descriptively. Fisher's exact test or Chi-square test, as appropriate, was used to compare categorical variables, and Kruskal-Wallis test was used to compare continuous variables. Correlation analyses were accomplished using Pearson correlation test. Logarithmic values of *SPHK1* and *DMTN* transcript levels and PS exposure rate were used in the correlations analyses to better fit the data. Median values were used to dichotomize patients into two groups according to *SPHK1* and *DMTN* expression (for *SPHK1*: low expression, < 1.10; high expression, ≥1.10; for *DMTN*: low expression, <1.61; high expression, ≥1.61). All calculations were performed using Stata statistical data analysis software version 14.1 (StataCorp, College Station, TX, USA), statistical package for the social sciences (SPSS) 19.0, and R 3.3.2 (The CRAN project, www.r-project.org) software. All *P* values were two-sided with a significance level of 0.05.

RESULTS

All the main clinical and laboratory features are summarized in Table 1 Baseline features were similar between patients with low and high expression levels of *SPHK1* and *DMTN* ($P>0.05$). Figure 1 exhibit transcript levels of *SPHK1* and *DMTN* in peripheral blood from healthy volunteers and in samples from patients with SCA. *SPHK1* expression was significantly lower in patients with SCA on hydroxyurea (HU) therapy ($P<0.001$). No significant changes in *DMTN* expression were found ($P=0.207$).

Next, we evaluate whether deregulation of *SPHK1* or *DMTN* gene expression modulates the occurrence of VOC or the PS exposure in patients with SCA, since sickled red blood cells present a huge exposure of PS [11]. Of the 68 adult patients with SCA included in the study, 30 samples were obtained during VOC (crisis state) and 38 samples in steady state. Patients in crisis presented high expression of *DMTN* (median value: 2.14, range, 0.32-19.83) compared to steady state patients (median value: 1.24, range, 0.32-12.23) ($P = 0.043$). Although patients in crisis exhibited high levels of *SPHK1* (median value: 1.24, range, 0.47-5.00) compared to steady state patients (median value: 0.92, range, 0.26-10.84), this difference did not reach significance ($P = 0.248$) (Figure 2).

Furthermore, we correlated the PS exposure rate with the differential expression of *SPHK1* and *DMTN* in newly collected erythrocytes from patients with SCA. No

correlation was found between *SPHK1* or *DMTN* transcript levels and surface PS accumulation (for *SPHK1*: Pearson correlation coefficient, r= -0.12; for *DMTN*: Pearson correlation coefficient, r= 0.15).

At last, in attempt to globally assess the impact of dysregulation from erythrocyte membrane, we evaluated whether high levels of *SPHK1, DMTN* and *ATP11C*, another protein already associated with a worse prognosis in SCA [12,13], can together modulate the occurrence of VOC in a small set of patients (19 patients in steady state and 9 patients during crisis state). Interestingly, patients in crisis state exhibited high levels of *SPHK1*, *DMTN* and *ATP11C* (median value for *SPHK1*: 1.77, range, 0.48-3.45; median value for *DMTN*: 3.41, range, 0.67-19.84; median value for *ATP11C*: 3.30, range, 0.35-12.05) compared to steady state patients (median value for *SPHK1*: 0.90, range, 0.26-10.84; median value for *DMTN*: 1.15, range, 0.32-12.23; median value for *ATP11C*: 1.52, range, 0.33-13.11), although this difference did not reach significance (*P*=0.103).

DISCUSSION

Sickled red blood cells present several features, such as an accumulation of heme and iron ions, which can promote oxidative stress, and increased intracellular calcium. Altogether, these features can activate mechanisms for PS exposure and improve hemolysis [1,14,15]. Although early diagnosis, penicillin prophylaxis, blood transfusion, transcranial Doppler imaging and hydroxyurea can dramatically improve survival and quality of life for patients with SCA, our understanding of the role of genetic and nongenetic factors in explaining the remarkable phenotypic diversity of this mendelian disease is still limited [16]. Furthermore, it is clear that a better prediction of SCA severity disease could lead to more precise treatment and management [17].

Increasing evidence has shown that SPHK1-mediated elevation of S1P contributes to sickling, promotes disease progression and highlight potential therapeutic opportunities for SCA [4-6], albeit data about the differential expression of *SPHK1* in SCA are limited. Additionally, the loss of dematin weakens the bounding of spectrin and actin to the plasmatic membrane, reducing red blood cells stability and deformability [8]. To our knowledge, the current study represents the first evaluation of *SPHK1* and *DMTN* transcript levels in patients with SCA in a clinical context. Our results demonstrated that patients with SCA on hydroxyurea therapy had reticulocytes with significantly lower *SPHK1* transcript levels, when compared with healthy

volunteers and patients with SCA off HU therapy, although the reason for this modulation remains unclear. However, it must be pointed out that elevated S1P increases sickling [5], and that HU has an established role in increasing HbF production and inhibiting HbS polymerization [18]. Therefore, inhibition of SPHK1 is likely to be another route of action for this drug.

Additionally, patients during painful crisis expressed high levels of DMTN compared to steady state patients. Although data about the role of dematin in SCA are limited, this protein is a substrate for extracellular signal-regulated kinase (ERK1/2). Therefore, triggering these kinases, which are abundant in red blood cells, may promote activation of signaling pathways, phosphorylate ICAM-4, and consequent increase RBC adhesion to the endothelium followed by painful crisis [9]. Unfortunately, we cannot draw this conclusion from our data. Further studies specifically designed for this purpose could confirm this hypothesis.

According to our data, another possibility is that differential expression of *SPHK1* and *DMTN* may not retain biological significance with SCA prognosis alone, but in an integrative manner with other genetic modifications. In this context, albeit with no mathematical significance, our results showed that patients in crisis state exhibited high levels of *SPHK1, DMTN* and *ATP11C*, another gene related as a potential candidate to modulate SCA phenotype [12,13]. Therefore, we support the idea that an integrative approach of differential gene expression and hydroxyurea therapy might improve patient outcomes.

In summary, we have provided the first evidence that *SPHK1* and *DMTN* are differentially expressed in reticulocytes during the clinical course of patients with SCA. Nevertheless, the underlying reasons remain to be elucidated. Future studies could provide a better understanding about development of clinical manifestation in SCA and differential expression of *SPHK1* and *DMTN*.

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AUTHOR CONTRIBUTIONS

I.F.D. performed experiments, analyzed and interpreted data, and drafted the manuscript. D.A.P-M., D.A.F., J.L.C-S., P.L.F-N., R.F.F., A.S.A., and M.A.B. recruited patients, updated the clinical data, and reviewed the manuscript. A.R.L-A. analyzed and interpreted data, performed statistical analyses, and drafted the manuscript. I.F.D., M.A.B. and A.R.L-A. conceived and designed the study and reviewed the manuscript. A.R.L-A. gave the final approval of the version to be submitted.

CONFLICTS OF INTEREST DISCLOSURE

The authors have no competing financial interests to declare

REFERENCES

1. Lang KS, Roll B, Myssina S, et al. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell. Physiol. Biochem.* 2002;12(5–6):365–72.

2. Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: Reappraisal of the role of hemolysis in the development of clinical subphenotypes. *Blood Rev.* 2007;21(1):37–47.

3. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N. Engl. J. Med.* 1994;330(23):1639–44.

4. Zhang Y, Berka V, Song A, et al. Elevated sphingosine-1-phosphate promotes sickling and sickle cell disease progression. *J. Clin. Invest.* 2014;124(6):2750–2761.

5. Sun K, Zhang Y, D'Alessandro A, et al. Sphingosine-1-phosphate promotes erythrocyte glycolysis and oxygen release for adaptation to high-altitude hypoxia. *Nat. Commun.* 2016;7(May):1–13.

6. Sun K, Zhang Y, Bogdanov M V., et al. Elevated adenosine signaling via adenosine A2B receptor induces normal and sickle erythrocyte sphingosine kinase 1 activity. *Blood*. 2015;125(10):1643–1652.

7. Siegel DL, Branton D. Partial purification and characterization of an actin-bundling protein, band 4.9, from human erythrocytes. *J. Cell Biol.* 1985;100(3):775–85.

8. Khanna R, Chang SH, Andrabi S, et al. Headpiece domain of dematin is required for the stability of the erythrocyte membrane. *Proc. Natl. Acad. Sci. U. S. A.* 2002;99(10):6637–42.

9. Zennadi R, Whalen EJ, Soderblom EJ, et al. Erythrocyte plasma membrane – bound ERK1 / 2 activation promotes ICAM-4 – mediated sickle red cell adhesion to endothelium. *Blood*. 2012;119(5):1217–1227.

10. Goossens M, Kan YY. DNA analysis in the diagnosis of hemoglobin disorders. *Methods Enzymol.* 1981;76:805–17.

11. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc. Natl. Acad. Sci. U. S. A.* 1998;95(6):3077–81.

12. Setty BNY, Kulkarni S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood*. 2002;99(5):1564–71.

13. Kuypers FA, de Jong K. The role of phosphatidylserine in recognition and removal of erythrocytes. *Cell. Mol. Biol. (Noisy-le-grand).* 2004;50(2):147–58.

14. Hebbel RP, Morgan WT, Eaton JW, Hedlund BE. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* 1988;85(1):237–41.

15. Lew VL, Bookchin RM. Ion transport pathology in the mechanism of sickle cell dehydration. *Physiol. Rev.* 2005;85(1):179–200.

16. Piel FB, Steinberg MH, Rees DC. Sickle Cell Disease. *N. Engl. J. Med.* 2017;376(16):1561–1573.

17. Steinberg MH. Genetic Etiologies for Phenotypic Diversity in Sickle Cell Anemia. *Sci. World J.* 2009;9:46–67.

18. Cokic VP, Smith RD, Beleslin-Cokic BB, et al. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. *J. Clin. Invest.* 2003;111(2):231–9.

Table 1 - Baseline characteristics.

Characteristics	All patients (n = 68)		*SPHK1* expression				*DMTN* expression				
			Low expression		High expression		*P*-value[1]	Low expression		High expression		*P*-value[1]
	No.	%	No.	%	No.	%		No.	%	No.	%	
Gender							0.218					0.806
Male	28	41.2	11	32.4	17	50.0		13	38.2	15	44.1	
Female	40	58.8	23	67.6	17	50.0		21	61.8	19	55.9	
Age (years), median	30		29		31		0.684	28		31		0.287
(range)	(18 - 54)		(18 - 47)		(19 - 54)			(18 - 54)		(18 – 52)		
β^S haplotype							0.790					0.789
CAR/CAR	29	52.7	13	50.0	16	55.2		14	50.0	15	55.6	
Non-CAR/CAR	26	47.3	13	50.0	13	44.8		14	50.0	12	44.4	
Missing data	13	-	8	-	5	-		6	-	7	-	
α-thalassemia ($\alpha^{-3.7kb}$)							0.752					0.205
Mutated	13	23.6	7	26.9	6	20.7		9	32.1	4	14.8	
Non-mutated	42	76.4	19	73.1	23	79.3		19	67.9	23	85.2	
Missing data	13	-	8	-	5	-		6	-	7	-	
Hb (g/dL), median	8.4		8.5		8.4		0.430	8.6		8.4		0.331
(range)	(4.3 - 11.0)		(6.4 – 11.0)		(4.3 - 10.7)			(4.3 - 10.7)		(5.0 - 11.0)		
Ret (x $10^3/mm^3$), median	11.7		12.8		11.4		0.287	11.0		13.1		0.368
(range)	(5.6 - 23.5)		(5.6 – 23.5)		(4.6 - 18.4)			(6.1 – 18.6)		(4.6 – 23.5)		
HbF (%), median	5.6		5.5		6.2		0.856	5.7		5.6		0.949
(range)	(1.7 - 21.9)		(1.7 – 21.9)		(2.6 - 13.7)			(1.7 – 21.9)		(2.2 – 13.7)		
Number the VOC/year[2]							0.615					0.802
≤2	27	45.0	14	48.3	13	41.9		16	48.5	11	40.8	
3-5	24	40.0	12	41.4	12	38.7		12	36.4	12	44.4	
≥6	9	15.0	3	10.3	6	19.4		5	15.1	4	14.8	
Missing data	8	-	5	-	3	-		1	-	7	-	

Abbreviations: HbF, fetal hemoglobin; CAR, Central African Republican; VOC, vaso-occlusion crisis.

[1]Missing values were excluded from calculation of *P*-values

[2]We only considered the number of VOC documented in the last year (2018).

FIGURE LEGEND

Figure 1 - Quantitative analysis of *SPHK1* (A) and *DMTN* (B) transcript levels in samples from healthy volunteers and patients with SCA on/off hydroxyurea therapy. The expression of *SPHK1* was quantified by real-time quantitative PCR. The horizontal bars represent the median value of *SPHK1* expression relative to the *18s* subunit and *ACTB*. Reticulocytes from patients with SCA on hydroxyurea therapy had lower *SPHK1* transcript levels compared to healthy volunteers and patients with SCA of hydroxyurea therapy (Kruskal–Wallis test followed by a Dunn's post-test). These data were generated from 13 patients in crisis (square shaped marker) and 15 patients in steady state (triangle shaped marker). Correlation analyses were accomplished using Pearson correlation test. Logarithmic values of the *ATP11C*/*PLSCR1* ratio and PS exposure were used to better fit the data.

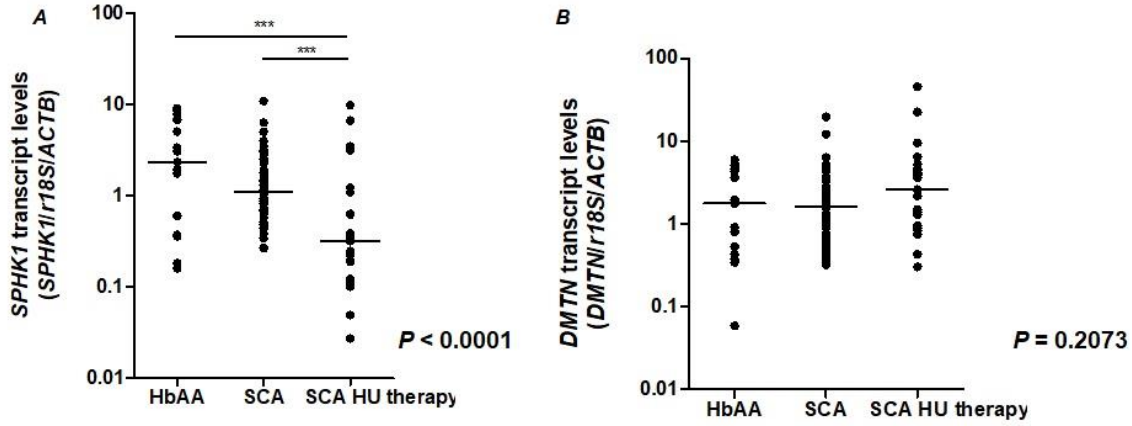
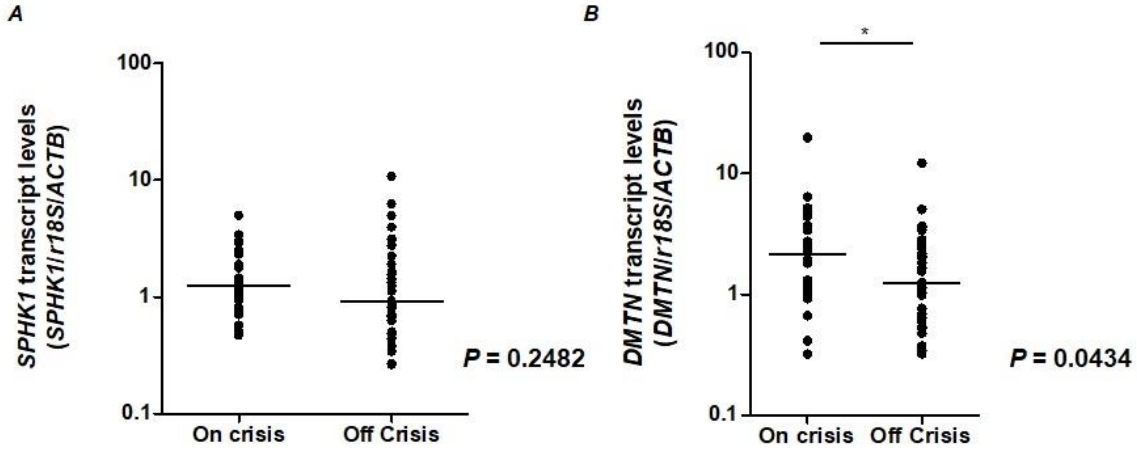


FIGURE LEGEND

Figure 2. Quantitative analysis of *SPHK1* (A) and *DMTN* (B) transcript levels in samples from SCA during vaso-occlusive crisis and steady state. The expression of *SPHK1* was quantified by real-time quantitative PCR. The horizontal bars represent the median value of *SPHK1* expression relative to the *18s* subunit and *ACTB*. Reticulocytes from patients with SCA on hydroxyurea therapy had lower *SPHK1* transcript levels compared to healthy volunteers and patients with SCA of hydroxyurea therapy (Kruskal–Wallis test followed by a Dunn's post-test).



5. DISCUSSÃO GERAL

As hemácias, por não apresentarem núcleo, estruturas citoplasmáticas e organelas, têm suas propriedades estruturais e funcionais intimamente associadas às suas membranas plasmáticas (MOHANDAS; GALLAGHER, 2008). Na anemia falciforme (AF), alterações no metabolismo normal da hemácia podem reduzir a sua vida útil e contribuir para um fenótipo mais grave. Essas alterações, como o acúmulo de íons heme e ferro, presente em hemácias contendo HbS, podem promover estresse oxidativo e aumento do cálcio intracelular. Em conjunto, essas características podem ativar mecanismos de exposição à fosfatidilserina (FS) e aumentar a hemólise (HEBBEL et al., 1988; LANG et al., 2002; LEW; BOOKCHIN, 2005). Embora o diagnóstico precoce, além de algumas abordagens terapêuticas, como a transfusão de sangue e o uso de hidroxiuréia, possam melhorar a sobrevida e a qualidade de vida de pacientes com AF, o papel de fatores genéticos não relacionados a mutação da β globina na diversidade fenotípica da doença ainda não é totalmente compreendido (PIEL; STEINBERG; REES, 2017).

Vários estudos já demonstraram a importância da flipase codificada pelo *ATP11C* na manutenção da assimetria da membrana plasmática em diversos tipos celulares, incluindo os eritrócitos (AN et al., 2014; ARASHIKI et al., 2016a; ARASHIKI; TAKAKUWA, 2017; TAKATSU et al., 2017). Ademais, dados recentes sugerem que a baixa expressão do *ATP11C* aumenta a exposição de FS na superfície da membrana (ARASHIKI et al., 2016a; YASIN et al., 2003), e camundongos deficientes para essa flipase apresentaram uma menor taxa de translocação de FS, além de anemia e morfologia eritróide anormal (AN et al., 2014). Além da ATP11C, a atividade da scramblase codificada pelo gene *PLSCR1* é necessária para manter o balanço de exposição/internalização de FS, visto que a supressão da hPLSCR devido a baixos níveis de Ca^{2+} previne a externalização de FS (ARASHIKI et al., 2016b; YABAS et al., 2011). Em nosso estudo, os pacientes com AF apresentavam uma tendência de menor relação *ATP11C*/*PLSCR1* quando comparados com indivíduos saudáveis. Além disso, uma maior expressão de *ATP11C* em relação a *PLSCR1* foi associada com um menor risco de complicações clínicas. Desse modo, é provável que os pacientes com alta relação *ATP11C*/*PLSCR1* tenham exposto menos FS na membrana eritrocitária ao longo do curso clínico da doença e, consequentemente, menor adesão ao endotélio vascular, além de diminuição da hemólise e da anemia

(KEAN et al., 2002; KUYPERS et al., 1998; YASIN et al., 2003). Por fim, é possível que a relação *ATP11C*/*PLSCR1* possa alterar, em um mesmo paciente, entre o estado basal e na presença de crise vaso-oclusiva. Entretanto, nosso trabalho não testou essa hipótese, sendo necessários novos estudos para elucidar esse cenário.

Em relação ao *SPHK1*, vários trabalhos já demonstraram que a elevação da esfingosina-1-fosfato (S1P) mediada pela SPHK1 contribui para a falcização das hemácias (SUN et al., 2015, 2016; ZHANG et al., 2014), embora os dados sobre a expressão diferencial do *SPHK1* na AF sejam limitados. Nossos dados demonstraram que os reticulócitos de pacientes com AF em uso de hidroxiuréia (HU) apresentavam uma menor expressão de *SPHK1* quando comparados com indivíduos saudáveis e pacientes que não fazem uso dessa terapia, embora a razão para essa modulação permaneça incerta. Desse modo, a inibição da SPHK1 é provavelmente mais uma via de ação desse medicamento, visto que a elevação da S1P aumenta a falcização (SUN et al., 2016), e que o uso de HU aumenta a produção de HbF e, consequentemente, inibe a polimerização da HbS (COKIC et al., 2003).

Sobre o *DMTN*, sabe-se que a perda da proteína codificada por esse gene enfraquece a ligação da espectrina e actina à membrana plasmática, reduzindo a estabilidade e a deformação dos eritrócitos (KHANNA et al., 2002). Em nosso estudo, pacientes com AF apresentaram altos níveis de *DMTN* durante a crise vaso-oclusiva, quando comparados aos pacientes com AF em estado basal. Embora os dados sobre o papel da dematina na AF sejam escassos, esta proteína é um substrato para a via regulada por ERK1/2. Desse modo, a ativação dessas quinases, que são abundantes em hemácias, pode promover a ativação de vias de sinalização, fosforilar ICAM-4 e, consequentemente, aumentar a adesão das hemácias ao endotélio vascular e favorecer o desenvolvimento de uma crise vaso-oclusiva (ZENNADI et al., 2012).

Na tentativa de avaliar integradamente o impacto da desregulação desses genes na membrana eritrocitária, nosso estudo verificou se altos níveis de *SPHK1*, *DMTN* e *ATP11C* podem, em conjunto, favorecer o desenvolvimento de crise vaso-oclusiva. Embora sem significância matemática, nossos resultados mostraram que pacientes com AF durante a crise vaso-oclusiva apresentavam altos níveis de expressão de *SPHK1*, *DMTN* e *ATP11C*. Entretanto, fazemos uma ressalva para essa análise, pois a mesma foi realizada em um pequeno conjunto de pacientes. Desse modo, novos estudos em coortes maiores e independentes são necessários para melhor compreendermos essa abordagem integrativa.

Na AF, a formação e o alongamento dos polímeros de HbS leva a uma distorção do glóbulo vermelho, promovendo alterações estruturais na membrana do eritrócito (REES; WILLIAMS; GLADWIN, 2010). Além disso, um maior estresse oxidativo e uma maior concentração intracelular de cálcio podem favorecer a exposição de FS na superfície das hemácias, acelerando a destruição celular (HEBBEL et al., 1988; LEW; BOOKCHIN, 2005). Desse modo, é importante compreender os mecanismos que favoreçam a falcização eritróide, além dos mecanismos que regulam a exposição de FS nas hemácias de pacientes portadores de AF. Até onde sabemos, o presente estudo é o primeiro a demonstrar, em um contexto clínico, a expressão diferencial de *ATP11C*, *PLSCR1*, *SPHK1* e *DMTN* em reticulócitos de pacientes com AF. No entanto, como as causas dessa expressão diferencial e o real impacto dessas variantes no quadro clinico de pacientes com AF ainda não estão completamente esclarecidas, estudos futuros são fundamentais para fornecer uma melhor compreensão sobre o tema.

6. CONCLUSÕES

A partir dos resultados obtidos, foi possível concluir que:

- Os reticulócitos de pacientes com AF apresentam níveis diminuídos de *ATP11C* e SPHK1, e uma maior relação *ATP11C/PLSCR1* foi associada a um menor número de complicações clínicas;
- Os níveis de expressão de *SPHK1* estão diminuídos em pacientes com AF em uso de hidroxiuréia quando comparados à indivíduos saudáveis e pacientes que não fazem uso dessa terapia;
- Pacientes com AF durante a crise vaso-oclusiva apresentaram altos níveis de *DMTN* e uma menor relação *ATP11C/PLSCR1*;
- A exposição de fosfatidilserina na superfície da membrana eritróide de pacientes com AF encontra-se correlacionada apenas à relação *ATP11C/PLSCR1*;
- A expressão de *SPHK1*, *DMTN* e *ATP11C*, de modo integrado, apresenta-se aumentada em pacientes com AF durante a crise de dor, embora essa diferença não apresente diferença estatística.

REFERÊNCIAS

ABRAHAM, A. et al. Unrelated Umbilical Cord Blood Transplantation for Sickle Cell Disease Following Reduced-Intensity Conditioning: Results of a Phase I Trial. **Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation**, v. 23, n. 9, p. 1587–1592, set. 2017.

ADAMS, D. R.; PYNE, S.; PYNE, N. J. Sphingosine Kinases: Emerging Structure-Function Insights. **Trends in Biochemical Sciences**, v. 41, n. 5, p. 395–409, 2016.

ADAMS, R. et al. The use of transcranial ultrasonography to predict stroke in sickle cell disease. **The New England journal of medicine**, v. 326, n. 9, p. 605–10, 27 fev. 1992.

ADAMS, R. J. et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. **The New England journal of medicine**, v. 339, n. 1, p. 5–11, 2 jul. 1998.

ADAMS, R. J. et al. Stroke and conversion to high risk in children screened with transcranial Doppler ultrasound during the STOP study. **Blood**, v. 103, n. 10, p. 3689–3694, 2004.

AKINSHEYE, I. et al. Fetal hemoglobin in sickle cell anemia. **Blood**, v. 118, n. 1, p. 19–27, 7 jul. 2011.

AL-MOUSAWI, F. et al. Total hip replacement in sickle cell disease. **International orthopaedics**, v. 26, n. 3, p. 157–61, 2002.

ALAVI, A.; KIRSNER, R. S. Hemoglobinopathies and Leg Ulcers. **The international journal of lower extremity wounds**, v. 14, n. 3, p. 213–6, set. 2015.

ALFRAIH, F. et al. Alternative donor allogeneic hematopoietic cell transplantation for hemoglobinopathies. **Seminars in hematology**, v. 53, n. 2, p. 120–8, abr. 2016.

ALMEIDA, A.; ROBERTS, I. Bone involvement in sickle cell disease. **British Journal of Haematology**, v. 129, n. 4, p. 482–490, 2005.

AMENGUAL, O. et al. Phospholipid scramblase 1 expression is enhanced in patients with antiphospholipid syndrome. **Modern rheumatology**, v. 23, n. 1, p. 81–8, jan. 2013.

AMER, J.; FIBACH, E. Chronic oxidative stress reduces the respiratory burst response of neutrophils from beta-thalassaemia patients. **British journal of haematology**, v. 129, n. 3, p. 435–41, maio 2005.

AN, X. et al. Global transcriptome analyses of human and murine terminal erythroid differentiation. **Blood**, v. 123, n. 22, p. 3466–77, 29 maio 2014.

ANDRAKA, N. et al. The conformation of human phospholipid scramblase 1, as studied by infrared spectroscopy. Effects of calcium and detergent. **Biochimica et**

biophysica acta. Biomembranes, v. 1859, n. 5, p. 1019–1028, maio 2017.

ANONG, W. A. et al. Adducin forms a bridge between the erythrocyte membrane and its cytoskeleton and regulates membrane cohesion. **Blood**, v. 114, n. 9, p. 1904–12, 27 ago. 2009.

APOVO, M. et al. Alteration in protein kinase C activity and subcellular distribution in sickle erythrocytes. **Biochimica et biophysica acta**, v. 984, n. 1, p. 26–32, 21 ago. 1989.

ARASHIKI, N. et al. ATP11C is a major flippase in human erythrocytes and its defect causes congenital hemolytic anemia. **Haematologica**, v. 101, n. 5, p. 559–565, 2016a.

ARASHIKI, N. et al. An Unrecognized Function of Cholesterol: Regulating the Mechanism Controlling Membrane Phospholipid Asymmetry. **Biochemistry**, v. 55, n. 25, p. 3504–3513, 2016b.

ARASHIKI, N.; TAKAKUWA, Y. Maintenance and regulation of asymmetric phospholipid distribution in human erythrocyte membranes: Implications for erythrocyte functions. **Current Opinion in Hematology**, v. 24, n. 3, p. 167–172, 2017.

ARMENIS, I. et al. Prognostic value of T786C and G894T eNOS polymorphisms in sickle cell disease. **Nitric oxide : biology and chemistry**, v. 62, p. 17–23, 30 jan. 2017.

ARNOLD, S. D. et al. Haematopoietic stem cell transplantation for sickle cell disease - current practice and new approaches. **British journal of haematology**, v. 174, n. 4, p. 515–25, ago. 2016.

ATAGA, K. I.; CAPPELLINI, M. D.; RACHMILEWITZ, E. A. Beta-thalassaemia and sickle cell anaemia as paradigms of hypercoagulability. **British journal of haematology**, v. 139, n. 1, p. 3–13, out. 2007.

AZIM, A. C. et al. Isoform cloning, actin binding, and chromosomal localization of human erythroid dematin, a member of the villin superfamily. **The Journal of biological chemistry**, v. 270, n. 29, p. 17407–13, 21 jul. 1995.

BALLAS, S. K. et al. Exposure to hydroxyurea and pregnancy outcomes in patients with sickle cell anemia. **Journal of the National Medical Association**, v. 101, n. 10, p. 1046–51, out. 2009.

BALLAS, S. K. et al. Definitions of the phenotypic manifestations of sickle cell disease. **American Journal of Hematology**, v. 85, n. 1, p. 6–13, 2010.

BALLAS, S. K.; MARCOLINA, M. J. Hyperhemolysis during the evolution of uncomplicated acute painful episodes in patients with sickle cell anemia. **Transfusion**, v. 46, n. 1, p. 105–10, jan. 2006.

BANDEIRA, F. M. G. C. et al. Características de recém-nascidos portadores de hemoglobina “ S ” detectados através de triagem em sangue de cordão umbilical . **Jornal de Pediatria**, v. 75, n. 3, p. 167–171, 1999.

BARBER, L. A. et al. Aminophospholipid translocase and phospholipid scramblase activities in sickle erythrocyte subpopulations. **British Journal of Haematology**, v. 146, n. 4, p. 447–455, 2009.

BASSÉ, F. et al. Isolation of an erythrocyte membrane protein that mediates Ca2+-dependent transbilayer movement of phospholipid. **The Journal of biological chemistry**, v. 271, n. 29, p. 17205–10, 19 jul. 1996.

BEN-EFRAIM, I. et al. Phospholipid scramblase 1 is imported into the nucleus by a receptor-mediated pathway and interacts with DNA. **Biochemistry**, v. 43, n. 12, p. 3518–26, 30 mar. 2004.

BENDER, M. Sickle Cell Disease. **In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A, editors. SourceGeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2018**, p. updated 2017 Aug 17, 1993.

BENNETT, O. M.; NAMNYAK, S. S. Bone and joint manifestations of sickle cell anaemia. **The Journal of bone and joint surgery. British volume**, v. 72, n. 3, p. 494–9, maio 1990.

BEVERS, E. M. et al. Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. **Biological chemistry**, v. 379, n. 8–9, p. 973–86, 1998.

BLAHO, V. A.; HLA, T. Regulation of mammalian physiology, development, and disease by the sphingosine 1-phosphate and lysophosphatidic acid receptors. **Chemical reviews**, v. 111, n. 10, p. 6299–320, 12 out. 2011.

BOAS, F. E.; FORMAN, L.; BEUTLER, E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. **Proceedings of the National Academy of Sciences of the United States of America**, v. 95, n. 6, p. 3077–81, 17 mar. 1998.

BRANZEI, D.; FOIANI, M. Regulation of DNA repair throughout the cell cycle. **Nature reviews. Molecular cell biology**, v. 9, n. 4, p. 297–308, abr. 2008.

BRASIL. **Doença Falciforme**. Disponível em: <http://u.saude.gov.br/index.php/o-ministerio/principal/secretarias/955-sas-raiz/dahu-raiz/sangue-e-hemoderivados/l2-sangue-e-hemoderivados/13335-doenca-falciforme>. Acesso em: 28 jan. 2018.

BRATTON, D. L. et al. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. **The Journal of biological chemistry**, v. 272, n. 42, p. 26159–65, 17 out. 1997.

BRAWLEY, O. W. et al. National Institutes of Health Consensus Development Conference statement: hydroxyurea treatment for sickle cell disease. **Annals of internal medicine**, v. 148, n. 12, p. 932–8, 17 jun. 2008.

BRUCE, L. J. et al. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. **Blood**, v. 101, n. 10, p. 4180–8, 15 maio 2003.

CAI, S.-Y. et al. ATP8B1 deficiency disrupts the bile canalicular membrane bilayer structure in hepatocytes, but FXR expression and activity are maintained. **Gastroenterology**, v. 136, n. 3, p. 1060–9, mar. 2009.

CAJADO, C. et al. TNF-alpha and IL-8: Serum levels and gene polymorphisms (-308G>A and -251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia. **Cytokine**, v. 56, n. 2, p. 312–317, 2011.

CAMERER, E. et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. **The Journal of clinical investigation**, v. 119, n. 7, p. 1871–9, jul. 2009.

CANÇADO, R. D.; JESUS, J. A. A doença falciforme no Brasil. **Revista brasileira de hematologia e hemoterapia**, v. 29, n. 3, p. 204–206, 2007.

CANVER, M. C. et al. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. **Nature**, v. 527, n. 7577, p. 192–7, 12 nov. 2015.

CAPPELLINI, M. D. Coagulation in the pathophysiology of hemolytic anemias. **Hematology. American Society of Hematology. Education Program**, p. 74–8, 2007.

CAVAZZANA, M.; ANTONIANI, C.; MICCIO, A. Gene Therapy for β-Hemoglobinopathies. **Molecular therapy : the journal of the American Society of Gene Therapy**, v. 25, n. 5, p. 1142–1154, 3 maio 2017.

CHARACHE, S. et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. **The New England journal of medicine**, v. 332, n. 20, p. 1317–22, 18 maio 1995.

CHIU, D. et al. Sickled erythrocytes accelerate clotting in vitro: an effect of abnormal membrane lipid asymmetry. **Blood**, v. 58, n. 2, p. 398–401, ago. 1981.

CHRISTAKIS, J. et al. Comparison of homozygous sickle cell disease in northern Greece and Jamaica. **Lancet (London, England)**, v. 335, n. 8690, p. 637–40, 17 mar. 1990.

CHRISTOFFERSEN, C. et al. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 23, p. 9613–8, 7 jun. 2011.

COKIC, V. P. et al. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. **The Journal of clinical investigation**, v. 111, n. 2, p. 231–9, jan. 2003.

COKIC, V. P. et al. Hydroxyurea induces the eNOS-cGMP pathway in endothelial cells. **Blood**, v. 108, n. 1, p. 184–91, 1 jul. 2006.

CONNES, P.; VERLHAC, S.; BERNAUDIN, F. Advances in understanding the pathogenesis of cerebrovascular vasculopathy in sickle cell anaemia. **British Journal of Haematology**, v. 161, n. 4, p. 484–498, 2013.

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

CUMMING, V. et al. Venous incompetence, poverty and lactate dehydrogenase in Jamaica are important predictors of leg ulceration in sickle cell anaemia. **British journal of haematology**, v. 142, n. 1, p. 119–25, jul. 2008.

CYSTER, J. G.; SCHWAB, S. R. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. **Annual review of immunology**, v. 30, p. 69–94, 2012.

DA SILVA JUNIOR, G. B.; DAHER, E. D. F.; DA ROCHA, F. A. C. Osteoarticular involvement in sickle cell disease. **Revista brasileira de hematologia e hemoterapia**, v. 34, n. 2, p. 156–64, 2012.

DALEKE, D. L.; HUESTIS, W. H. Incorporation and translocation of aminophospholipids in human erythrocytes. **Biochemistry**, v. 24, n. 20, p. 5406–16, 24 set. 1985.

DALEKE, D. L.; LYLES, J. V. Identification and purification of aminophospholipid flippases. **Biochimica et biophysica acta**, v. 1486, n. 1, p. 108–27, 26 jun. 2000.

DARROW, M. C. et al. Visualizing red blood cell sickling and the effects of inhibition of sphingosine kinase 1 using soft X-ray tomography. **Journal of Cell Science**, v. 129, n. 18, p. 3511–3517, 2016.

DE JONG, K. et al. Characterization of the phosphatidylserine-exposing subpopulation of sickle cells. **Blood**, v. 98, n. 3, p. 860–7, 1 ago. 2001.

DE JONG, K.; KUYPERS, F. A. Sulphydryl modifications alter scramblase activity in murine sickle cell disease. **British Journal of Haematology**, v. 133, n. 4, p. 427–432, 2006.

DE MONTALEMBERT, M. Current strategies for the management of children with sickle cell disease. **Expert review of hematology**, v. 2, n. 4, p. 455–63, ago. 2009.

DEVER, D. P. et al. CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. **Nature**, v. 539, n. 7629, p. 384–389, 2016.

DEWITT, M. A. et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. **Science translational medicine**, v. 8, n. 360, p. 360ra134, 2016.

DUTRA, F. F.; BOZZA, M. T. Heme on innate immunity and inflammation. **Frontiers in pharmacology**, v. 5, p. 115, 2014.

DZANDU, J. K.; JOHNSON, R. M. Membrane protein phosphorylation in intact normal and sickle cell erythrocytes. **The Journal of biological chemistry**, v. 255, n. 13, p. 6382–6, 10 jul. 1980.

ECKMAN, J. R. Techniques for blood administration in sickle cell patients. **Seminars in hematology**, v. 38, n. 1 Suppl 1, p. 23–9, jan. 2001.

ENGLISH, D. et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 14, n. 14, p. 2255–65, nov. 2000.

ENGLISH, D. et al. Lipid mediators of angiogenesis and the signalling pathways they initiate. **Biochimica et biophysica acta**, v. 1582, n. 1–3, p. 228–39, 23 maio 2002.

FADOK, V. A. et al. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. **Journal of immunology (Baltimore, Md. : 1950)**, v. 148, n. 7, p. 2207–16, 1 abr. 1992.

FERRARI, G.; CAVAZZANA, M.; MAVILIO, F. Gene Therapy Approaches to Hemoglobinopathies. **Hematology/Oncology Clinics of North America**, v. 31, n. 5, p. 835–852, 2017.

FLANAGAN, J. M. et al. Genetic predictors for stroke in children with sickle cell anemia. **Blood**, v. 117, n. 24, p. 6681–6684, 2011.

GLUCKMAN, E. et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. **Blood**, v. 129, n. 11, p. 1548–1556, 2017.

GOODMAN, M. A.; MALIK, P. The potential of gene therapy approaches for the treatment of hemoglobinopathies: achievements and challenges. **Therapeutic advances in hematology**, v. 7, n. 5, p. 302–315, out. 2016.

GORTER, E.; GRENDEL, F. On Bimolecular Layers of Lipoids on the Chromocytes of the Blood. **The Journal of experimental medicine**, v. 41, n. 4, p. 439–43, 31 mar. 1925.

GREEN, N. S.; BARRAL, S. Emerging science of hydroxyurea therapy for pediatric sickle cell disease. **Pediatric research**, v. 75, n. 1–2, p. 196–204, jan. 2014.

GUALANDRO, S.; FONSECA, G.; GUALANDRO, D. Complicações

cardiopulmonares das doenças falciformes. **Rev Bras Hematol Hemoter**, v. 29, p. 291–298, 2007.

HÄNEL, P.; ANDRÉANI, P.; GRÄLER, M. H. Erythrocytes store and release sphingosine 1-phosphate in blood. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 21, n. 4, p. 1202–9, abr. 2007.

HEBBEL, R. P. et al. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. **Proceedings of the National Academy of Sciences of the United States of America**, v. 85, n. 1, p. 237–41, jan. 1988.

HENDRICKSON, J. E.; TORMEY, C. A. Understanding red blood cell alloimmunization triggers. **Hematology. American Society of Hematology. Education Program**, v. 2016, n. 1, p. 446–451, 2 dez. 2016.

HEYER, W.-D.; EHMSEN, K. T.; LIU, J. Regulation of homologous recombination in eukaryotes. **Annual review of genetics**, v. 44, p. 113–39, 2010.

HIGGS, D. R.; WOOD, W. G. Genetic complexity in sickle cell disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 105, n. 33, p. 11595–6, 19 ago. 2008.

HOBAN, M. D. et al. Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. **Blood**, v. 125, n. 17, p. 2597–604, 23 abr. 2015.

HOBAN, M. D. et al. CRISPR/Cas9-mediated correction of the sickle mutation in human CD34+ cells. **Molecular Therapy**, v. 24, n. 9, p. 1561–1569, 2016.

HOPPE, C. et al. Gene interactions and stroke risk in children with sickle cell anemia. **Blood**, v. 103, n. 6, p. 2391–6, 15 mar. 2004.

HOPPE, C. et al. Confirmation of an association between the TNF(-308) promoter polymorphism and stroke risk in children with sickle cell anemia. **Stroke; a journal of cerebral circulation**, v. 38, n. 8, p. 2241–2246, 2007.

HOWARD, J.; DAVIES, S. C. Sickle cell disease in North Europe. **Scandinavian journal of clinical and laboratory investigation**, v. 67, n. 1, p. 27–38, 2007.

HUSAIN-CHISHTI, A. et al. Purification of erythrocyte dematin (protein 4.9) reveals an endogenous protein kinase that modulates actin-bundling activity. **The Journal of biological chemistry**, v. 264, n. 15, p. 8985–91, 25 maio 1989.

HUSAIN-CHISHTI, A.; LEVIN, A.; BRANTON, D. Abolition of actin-bundling by phosphorylation of human erythrocyte protein 4.9. **Nature**, v. 334, n. 6184, p. 718–21, 25 ago. 1988.

ITO, K. et al. Lack of sphingosine 1-phosphate-degrading enzymes in erythrocytes. **Biochemical and biophysical research communications**, v. 357, n. 1, p. 212–7, 25 maio 2007.

JAIN, S.; BAKSHI, N.; KRISHNAMURTI, L. Acute Chest Syndrome in Children with Sickle Cell Disease. **Pediatric Allergy, Immunology, and Pulmonology**, v. 30, n. 4, p. 191–201, 2017.

JIANG, Z. G.; MCKNIGHT, C. J. A phosphorylation-induced conformation change in dematin headpiece. **Structure (London, England : 1993)**, v. 14, n. 2, p. 379–87, fev. 2006.

JOHNSON, F. L. et al. Bone-marrow transplantation in a patient with sickle-cell anemia. **The New England journal of medicine**, v. 311, n. 12, p. 780–3, 20 set. 1984.

JOHNSON, K. R. et al. Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. **The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society**, v. 53, n. 9, p. 1159–66, set. 2005.

JOHNSON, R. M.; DZANDU, J. K.; WARTH, J. A. The phosphoproteins of the sickle erythrocyte membrane. **Archives of biochemistry and biophysics**, v. 244, n. 1, p. 202–10, jan. 1986.

KAMANI, N. R. et al. Unrelated donor cord blood transplantation for children with severe sickle cell disease: results of one cohort from the phase II study from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). **Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation**, v. 18, n. 8, p. 1265–72, ago. 2012.

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

KATO, G. J.; STEINBERG, M. H.; GLADWIN, M. T. Intravascular hemolysis and the pathophysiology of sickle cell disease. **Journal of Clinical Investigation**, v. 127, n. 3, p. 750–760, 2017.

KEAN, L. S. et al. Comparison of mechanisms of anemia in mice with sickle cell disease and β-thalassemia: Peripheral destruction, ineffective erythropoiesis, and phospholipid scramblase-mediated phosphatidylserine exposure. **Experimental Hematology**, v. 30, n. 5, p. 394–402, 2002.

KHAN, A. A. et al. Dematin and adducin provide a novel link between the spectrin cytoskeleton and human erythrocyte membrane by directly interacting with glucose transporter-1. **The Journal of biological chemistry**, v. 283, n. 21, p. 14600–9, 23 maio 2008.

KHANNA, R. et al. Headpiece domain of dematin is required for the stability of the erythrocyte membrane. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 10, p. 6637–42, 2002.

KIHARA, A.; IGARASHI, Y. Production and release of sphingosine 1-phosphate and

the phosphorylated form of the immunomodulator FTY720. **Biochimica et biophysica acta**, v. 1781, n. 9, p. 496–502, set. 2008.

KIHARA, Y. et al. Lysophospholipid receptor nomenclature review: IUPHAR Review 8. **British journal of pharmacology**, v. 171, n. 15, p. 3575–94, ago. 2014.

KIM, A. C.; AZIM, A. C.; CHISHTI, A. H. Alternative splicing and structure of the human erythroid dematin gene. **Biochimica et biophysica acta**, v. 1398, n. 3, p. 382–6, 9 jul. 1998.

KING, A.; SHENOY, S. Evidence-based focused review of the status of hematopoietic stem cell transplantation as treatment of sickle cell disease and thalassemia. **Blood**, v. 123, n. 20, p. 3089–94; quiz 3210, 15 maio 2014.

KINGSLEY, P. D. et al. Ontogeny of erythroid gene expression. **Blood**, v. 121, n. 6, p. e5–e13, 7 fev. 2013.

KOBAYASHI, N. et al. Characterization of the ATP-dependent sphingosine 1-phosphate transporter in rat erythrocytes. **The Journal of biological chemistry**, v. 284, n. 32, p. 21192–200, 7 ago. 2009.

KOSHINO, I.; MOHANDAS, N.; TAKAKUWA, Y. Identification of a novel role for dematin in regulating red cell membrane function by modulating spectrin-actin interaction. **Journal of Biological Chemistry**, v. 287, n. 42, p. 35244–35250, 2012.

KOSICKI, M.; TOMBERG, K.; BRADLEY, A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. **Nature biotechnology**, v. 36, n. 8, p. 765–771, set. 2018.

KRISTINSSON, S. Y. et al. Long-term risks after splenectomy among 8,149 cancer-free American veterans: a cohort study with up to 27 years follow-up. **Haematologica**, v. 99, n. 2, p. 392–8, fev. 2014.

KUNKEL, G. T. et al. Targeting the sphingosine-1-phosphate axis in cancer, inflammation and beyond. **Nature reviews. Drug discovery**, v. 12, n. 9, p. 688–702, set. 2013.

KUYPERS, F. A. et al. Membrane phospholipid asymmetry in human thalassemia. **Blood**, v. 91, n. 8, p. 3044–51, 15 abr. 1998.

KUYPERS, F. A. Red cell membrane lipids in hemoglobinopathies. **Current molecular medicine**, v. 8, n. 7, p. 633–8, nov. 2008.

KUYPERS, F. A.; DE JONG, K. The role of phosphatidylserine in recognition and removal of erythrocytes. **Cellular and molecular biology (Noisy-le-Grand, France)**, v. 50, n. 2, p. 147–58, mar. 2004.

LAGUNJU, I.; BROWN, B. J.; SODEINDE, O. Hydroxyurea lowers transcranial Doppler flow velocities in children with sickle cell anaemia in a Nigerian cohort. **Pediatric blood & cancer**, v. 62, n. 9, p. 1587–91, set. 2015.

LALLE, M. et al. Dematin, a component of the erythrocyte membrane skeleton, is internalized by the malaria parasite and associates with Plasmodium 14-3-3. **Journal of Biological Chemistry**, v. 286, n. 2, p. 1227–1236, 2011.

LANARO, C. et al. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. **Journal of leukocyte biology**, v. 85, n. 2, p. 235–242, 2009.

LANG, K. S. et al. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. **Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology**, v. 12, n. 5–6, p. 365–72, 2002.

LAUBER, K. et al. Clearance of apoptotic cells: getting rid of the corpses. **Molecular cell**, v. 14, n. 3, p. 277–87, 7 maio 2004.

LETTRE, G. et al. DNA polymorphisms at the BCL11A, HBS1L-MYB, and -globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. **Proceedings of the National Academy of Sciences**, v. 105, n. 33, p. 11869–11874, 2008.

LEVANO, K. et al. Atp8a1 deficiency is associated with phosphatidylserine externalization in hippocampus and delayed hippocampus-dependent learning. **Journal of neurochemistry**, v. 120, n. 2, p. 302–13, jan. 2012.

LEVENTIS, P. A.; GRINSTEIN, S. The distribution and function of phosphatidylserine in cellular membranes. **Annual review of biophysics**, v. 39, p. 407–27, 2010.

LEW, V. L.; BOOKCHIN, R. M. Ion transport pathology in the mechanism of sickle cell dehydration. **Physiological reviews**, v. 85, n. 1, p. 179–200, jan. 2005.

LEZCANO, N. E. et al. Regular transfusion lowers plasma free hemoglobin in children with sickle-cell disease at risk for stroke. **Stroke**, v. 37, n. 6, p. 1424–1426, 2006.

LI, J. et al. Clinical significance of sphingosine kinase-1 expression in human astrocytomas progression and overall patient survival. **Clinical cancer research : an official journal of the American Association for Cancer Research**, v. 14, n. 21, p. 6996–7003, 1 nov. 2008.

LIEBER, M. R. et al. Mechanism and regulation of human non-homologous DNA end-joining. **Nature reviews. Molecular cell biology**, v. 4, n. 9, p. 712–20, set. 2003.

LIU, S. C. et al. Uncoupling of the spectrin-based skeleton from the lipid bilayer in sickled red cells. **Science (New York, N.Y.)**, v. 252, n. 5005, p. 574–6, 26 abr. 1991.

LOBO, C. L. DE C. et al. The effect of hydroxcarbamide therapy on survival of children with sickle cell disease. **British journal of haematology**, v. 161, n. 6, p.

852–60, jun. 2013.

LOMOVA, A. et al. Improving Gene Editing Outcomes in Human Hematopoietic Stem and Progenitor Cells by Temporal Control of DNA Repair. **Stem cells (Dayton, Ohio)**, 29 out. 2018.

LOPEZ-MARQUES, R. L. et al. P4-ATPases: lipid flippases in cell membranes. **Pflugers Archiv : European journal of physiology**, v. 466, n. 7, p. 1227–40, jul. 2014.

LOVETT, P. B.; SULE, H. P.; LOPEZ, B. L. Sickle cell disease in the emergency department. **Emergency medicine clinics of North America**, v. 32, n. 3, p. 629–47, ago. 2014.

LU, B. et al. Expression of the phospholipid scramblase (PLSCR) gene family during the acute phase response. **Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids**, v. 1771, n. 9, p. 1177–1185, 2007.

LU, Y. et al. Gene disruption of dematin causes precipitous loss of erythrocyte membrane stability and severe hemolytic anemia. **Blood**, v. 128, n. 1, p. 93–103, 2016.

LUTCHMAN, M. et al. Loss of heterozygosity on 8p in prostate cancer implicates a role for dematin in tumor progression. **Cancer genetics and cytogenetics**, v. 115, n. 1, p. 65–9, nov. 1999.

LUTCHMAN, M. et al. Dematin interacts with the Ras-guanine nucleotide exchange factor Ras-GRF2 and modulates mitogen-activated protein kinase pathways. **European journal of biochemistry**, v. 269, n. 2, p. 638–49, jan. 2002.

LUX, S. E. Anatomy of the red cell membrane skeleton: unanswered questions. **Blood**, v. 127, n. 2, p. 187–99, 14 jan. 2016.

LYRA, I. M. et al. Clinical , hematological , and molecular characterization of sickle cell anemia pediatric patients from two different cities in Brazil. **Caderno Saúde Pública**, v. 21, n. 4, p. 1287–1290, 2005.

MACEYKA, M.; SPIEGEL, S. Sphingolipid metabolites in inflammatory disease. **Nature**, v. 510, n. 7503, p. 58–67, 5 jun. 2014.

MANNO, S.; TAKAKUWA, Y.; MOHANDAS, N. Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 4, p. 1943–8, 19 fev. 2002.

MANTADAKIS, E. et al. Prevalence of priapism in children and adolescents with sickle cell anemia. **Journal of pediatric hematology/oncology**, v. 21, n. 6, p. 518–22, 1999.

MARFATIA, S. M. et al. In vitro binding studies suggest a membrane-associated

complex between erythroid p55, protein 4.1, and glycophorin C. **The Journal of biological chemistry**, v. 269, n. 12, p. 8631–4, 25 mar. 1994.

MENDELSON, K.; EVANS, T.; HLA, T. Sphingosine 1-phosphate signalling. **Development (Cambridge, England)**, v. 141, n. 1, p. 5–9, jan. 2014.

MINIERO, R. et al. Cord blood transplantation (CBT) in hemoglobinopathies. Eurocord. **Bone marrow transplantation**, v. 22 Suppl 1, p. S78-9, jul. 1998.

MINNITI, C. P. l-Glutamine and the Dawn of Combination Therapy for Sickle Cell Disease. **The New England journal of medicine**, v. 379, n. 3, p. 292–294, 2018.

MOHANDAS, N.; GALLAGHER, P. G. Red cell membrane: Past, present, and future. **Blood**, v. 112, n. 10, p. 3939–3948, 2008.

MORRIS, C. R. Mechanisms of vasculopathy in sickle cell disease and thalassemia. **Hematology. American Society of Hematology. Education Program**, p. 177–85, 2008.

MOSS, J. A. Gene therapy review. **Radiologic technology**, v. 86, n. 2, p. 155- 80; quiz 181–4, 2014.

NAGAHASHI, M. et al. Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. **Cancer research**, v. 72, n. 3, p. 726–35, 1 fev. 2012.

NAIK, J. et al. ATP8B1 and ATP11C: Two Lipid Flippases Important for Hepatocyte Function. **Digestive diseases (Basel, Switzerland)**, v. 33, n. 3, p. 314–8, 2015.

NAITO, T. et al. Phospholipid Flippase ATP10A Translocates Phosphatidylcholine and Is Involved in Plasma Membrane Dynamics. **The Journal of biological chemistry**, v. 290, n. 24, p. 15004–17, 12 jun. 2015.

NEVITT, S. J.; JONES, A. P.; HOWARD, J. Hydroxyurea (hydroxycarbamide) for sickle cell disease. **The Cochrane database of systematic reviews**, v. 4, p. CD002202, 2017.

NICKEL, R. S.; HENDRICKSON, J. E.; HAIGHT, A. E. The ethics of a proposed study of hematopoietic stem cell transplant for children with “less severe” sickle cell disease. **Blood**, v. 124, n. 6, p. 861–6, 7 ago. 2014.

NIIHARA, Y. et al. A Phase 3 Trial of l-Glutamine in Sickle Cell Disease. **New England Journal of Medicine**, v. 379, n. 3, p. 226–235, 2018.

OHENE-FREMPONG, K. et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. **Blood**, v. 91, n. 1, p. 288–294, 1998.

OLIVERA, A.; ROSENTHAL, J.; SPIEGEL, S. Effect of acidic phospholipids on sphingosine kinase. **Journal of cellular biochemistry**, v. 60, n. 4, p. 529–37, 15 mar. 1996.

PALADINO, S. F. Leg ulcers in sickle cell disease. **Revista Brasileira de Hematologia e Hemoterapia**, v. 29, n. 3, p. 288–290, 2007.

PANTALEO, A. et al. Current knowledge about the functional roles of phosphorylative changes of membrane proteins in normal and diseased red cells. **Journal of proteomics**, v. 73, n. 3, p. 445–55, 3 jan. 2010.

PAPPU, R. et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. **Science (New York, N.Y.)**, v. 316, n. 5822, p. 295–8, 13 abr. 2007.

PAWLIUK, R. et al. Correction of sickle cell disease in transgenic mouse models by gene therapy. **Science (New York, N.Y.)**, v. 294, n. 5550, p. 2368–71, 14 dez. 2001.

PERSONS, D. A. Hematopoietic stem cell gene transfer for the treatment of hemoglobin disorders. **Hematology. American Society of Hematology. Education Program**, p. 690–7, 2009.

PESCIOTTA, E. N. et al. A label-free proteome analysis strategy for identifying quantitative changes in erythrocyte membranes induced by red cell disorders. **Journal of proteomics**, v. 76 Spec No, p. 194–202, 5 dez. 2012.

PIEL, F. B.; STEINBERG, M. H.; REES, D. C. Sickle Cell Disease. **New England Journal of Medicine**, v. 376, n. 16, p. 1561–1573, 2017.

PLATT, O. S. et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. **The New England journal of medicine**, v. 330, n. 23, p. 1639–44, 9 jun. 1994.

PLATT, O. S. M. D. Hydroxyurea for the Treatment of Sickle Cell Anemia. **N Engl J Med 2008**, p. 1362–1369, 2008.

POWARS, D. R. et al. Outcome of sickle cell anemia: a 4-decade observational study of 1056 patients. **Medicine**, v. 84, n. 6, p. 363–76, nov. 2005.

PYNE, N. J.; PYNE, S. Sphingosine Kinase 1: A Potential Therapeutic Target in Pulmonary Arterial Hypertension? **Trends in Molecular Medicine**, v. 23, n. 9, p. 786–798, 2017.

PYNE, S. et al. Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate signalling in health and disease. **Cellular signalling**, v. 21, n. 1, p. 14–21, jan. 2009.

PYNE, S.; PYNE, N. J. Translational aspects of sphingosine 1-phosphate biology. **Trends in molecular medicine**, v. 17, n. 8, p. 463–72, ago. 2011.

QUINN, C. T. Sickle cell disease in childhood: from newborn screening through transition to adult medical care. **Pediatric clinics of North America**, v. 60, n. 6, p. 1363–81, dez. 2013.

QUINN, C. T. l-Glutamine for sickle cell anemia: more questions than answers. **Blood**, v. 132, n. 7, p. 689–693, 16 ago. 2018.

RAN, F. A. et al. Genome engineering using the CRISPR-Cas9 system. **Nature protocols**, v. 8, n. 11, p. 2281–2308, nov. 2013.

REES, D. C.; WILLIAMS, T. N.; GLADWIN, M. T. Sickle-cell disease. **The Lancet**, v. 376, n. 9757, p. 2018–2031, 2010.

REITER, C. D. et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. **Nature medicine**, v. 8, n. 12, p. 1383–9, dez. 2002.

RIBEIL, J.-A. et al. Gene Therapy in a Patient with Sickle Cell Disease. **New England Journal of Medicine**, v. 376, n. 9, p. 848–855, 2017.

RINALDUCCI, S. et al. Targeted quantitative phosphoproteomic analysis of erythrocyte membranes during blood bank storage. **Journal of Mass Spectrometry**, v. 50, n. 2, p. 326–335, 2015.

ROSSE, W. F. et al. New Views of Sickle Cell Disease Pathophysiology and Treatment. **Hematology. American Society of Hematology. Education Program**, p. 2–17, 2000.

RUSANOVA, I. et al. β-globin gene cluster haplotypes in sickle cell patients from Panamá. **American Journal of Human Biology**, v. 23, n. 3, p. 377–380, 2011.

SAHU, S. K.; ARADHYAM, G. K.; GUMMADI, S. N. Calcium binding studies of peptides of human phospholipid scramblases 1 to 4 suggest that scramblases are new class of calcium binding proteins in the cell. **Biochimica et biophysica acta**, v. 1790, n. 10, p. 1274–81, out. 2009.

SAKAMOTO, T. M. et al. Increased adhesive and inflammatory properties in blood outgrowth endothelial cells from sickle cell anemia patients. **Microvascular research**, v. 90, p. 173–9, nov. 2013.

SANKALA, H. M. et al. Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. **Cancer research**, v. 67, n. 21, p. 10466–74, 1 nov. 2007.

SCHWARTZ, R. S. et al. Protein 4.1 in sickle erythrocytes. Evidence for oxidative damage. **The Journal of biological chemistry**, v. 262, n. 32, p. 15666–72, 15 nov. 1987.

SEBASTIANI, P. et al. Genetic modifiers of the severity of sickle cell anemia identified through a genome-wide association study. **American Journal of Hematology**, v. 85, n. 1, p. 29–35, 2010.

SEGAWA, K. et al. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. **Science**, v. 344, n. 6188, p. 1164–1168,

2014.

SERJEANT, G. R. The clinical features of sickle cell disease. **Bailliere's clinical haematology**, v. 6, n. 1, p. 93–115, mar. 1993.

SERJEANT, G. R. et al. Leg ulceration in sickle cell disease: Medieval medicine in a modern world. **Hematology/Oncology Clinics of North America**, v. 19, n. 5, p. 943–956, 2005.

SETTY, B. N.; RAO, A. K.; STUART, M. J. Thrombophilia in sickle cell disease: the red cell connection. **Blood**, v. 98, n. 12, p. 3228–33, 1 dez. 2001.

SETTY, B. N. Y.; KULKARNI, S.; STUART, M. J. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. **Blood**, v. 99, n. 5, p. 1564–71, 1 mar. 2002.

SHIDA, D. et al. Targeting SphK1 as a new strategy against cancer. **Current drug targets**, v. 9, n. 8, p. 662–73, ago. 2008.

SICILIANO, A. et al. Deoxygenation affects tyrosine phosphoproteome of red cell membrane from patients with sickle cell disease. **Blood Cells, Molecules, and Diseases**, v. 44, n. 4, p. 233–242, 2010.

SIEGEL, D. L.; BRANTON, D. Partial purification and characterization of an actin-bundling protein, band 4.9, from human erythrocytes. **The Journal of cell biology**, v. 100, n. 3, p. 775–85, mar. 1985.

SIGGS, O. M. et al. The P4-type ATPase ATP11C is essential for B lymphopoiesis in adult bone marrow. **Nature immunology**, v. 12, n. 5, p. 434–40, maio 2011a.

SIGGS, O. M. et al. X-linked cholestasis in mouse due to mutations of the P4-ATPase ATP11C. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 19, p. 7890–5, 10 maio 2011b.

SINGHAL, R. et al. Development of pro-inflammatory phenotype in monocytes after engulfing Hb-activated platelets in hemolytic disorders. **Clinical immunology (Orlando, Fla.)**, v. 175, p. 133–142, fev. 2017.

SIVAGNANAM, U.; PALANIRAJAN, S. K.; GUMMADI, S. N. The role of human phospholipid scramblases in apoptosis: An overview. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1864, n. 12, p. 2261–2271, 2017.

SMITH, E. C.; ORKIN, S. H. Hemoglobin genetics: recent contributions of GWAS and gene editing. **Human molecular genetics**, v. 25, n. R2, p. R99–R105, 2016.

SPIEGEL, S.; MILSTIEN, S. The outs and the ins of sphingosine-1-phosphate in immunity. **Nature reviews. Immunology**, v. 11, n. 6, p. 403–15, jun. 2011.

STAHELIN, R. V et al. The mechanism of membrane targeting of human sphingosine kinase 1. **The Journal of biological chemistry**, v. 280, n. 52, p. 43030–8, 30 dez.

2005.

STEINBERG, M. et al. Effect of hydroxyurea on mortality and morbidity in sickle cell anaemia: risk and benefits up to 9 years of treatment. **Jama**, v. 289, n. 13, p. 1645–1651, 2003.

STEINBERG, M. H. Predicting clinical severity in sickle cell anaemia. **British Journal of Haematology**, v. 129, n. 4, p. 465–481, 2005.

STEINBERG, M. H. Sickle cell anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches. **TheScientificWorldJournal**, v. 8, p. 1295–324, jan. 2008a.

STEINBERG, M. H. SNPing away at sickle cell pathophysiology. **Blood**, v. 111, n. 12, p. 5420–1, 15 jun. 2008b.

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

STEINBERG, M. H. et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. **American Journal of Hematology**, v. 85, n. 6, p. 403–408, 2010.

STOUT, J. G. et al. Change in conformation of plasma membrane phospholipid scramblase induced by occupancy of its Ca2+ binding site. **Biochemistry**, v. 37, n. 42, p. 14860–6, 20 out. 1998.

STUART, M. J.; NAGEL, R. L. Sickle-cell disease. **Lancet (London, England)**, v. 364, n. 9442, p. 1343–60, 2004.

STYLES, L. et al. Increased RBC phosphatidylserine exposure in sickle cell disease patients at risk for stroke by transcranial Doppler screening. **Blood**, v. 90, p. 604a, 1997.

SUN, K. et al. Elevated adenosine signaling via adenosine A2B receptor induces normal and sickle erythrocyte sphingosine kinase 1 activity. **Blood**, v. 125, n. 10, p. 1643–1652, 2015.

SUN, K. et al. Sphingosine-1-phosphate promotes erythrocyte glycolysis and oxygen release for adaptation to high-altitude hypoxia. **Nature Communications**, v. 7, n. May, p. 1–13, 2016.

SUZUKI, E. et al. Increased expression of phospholipid scramblase 1 in monocytes from patients with systemic lupus erythematosus. **The Journal of rheumatology**, v. 37, n. 8, p. 1639–45, 1 ago. 2010a.

SUZUKI, J. et al. Calcium-dependent phospholipid scrambling by TMEM16F. **Nature**, v. 468, n. 7325, p. 834–8, 9 dez. 2010b.

SUZUKI, J. et al. Calcium-dependent phospholipid scramblase activity of TMEM16

protein family members. **The Journal of biological chemistry**, v. 288, n. 19, p. 13305–16, 10 maio 2013.

TAKATSU, H. et al. Phospholipid flippase activities and substrate specificities of human type IV P-type ATPases localized to the plasma membrane. **The Journal of biological chemistry**, v. 289, n. 48, p. 33543–56, 28 nov. 2014.

TAKATSU, H. et al. Phospholipid flippase ATP11C is endocytosed and downregulated following Ca2+-mediated protein kinase C activation. **Nature communications**, v. 8, n. 1, p. 1423, 10 nov. 2017.

VAN MEER, G.; VOELKER, D. R.; FEIGENSON, G. W. Membrane lipids: where they are and how they behave. **Nature reviews. Molecular cell biology**, v. 9, n. 2, p. 112–24, fev. 2008.

VARDAR, D. et al. Villin-type headpiece domains show a wide range of F-actin-binding affinities. **Cell motility and the cytoskeleton**, v. 52, n. 1, p. 9–21, maio 2002.

VASCONCELOS, M. C. M. et al. Left ventricular remodeling in patients with sickle cell disease: determinants factors and impact on outcome. **Annals of hematology**, v. 94, n. 10, p. 1621–9, out. 2015.

VESTERGAARD, A. L. et al. Critical roles of isoleucine-364 and adjacent residues in a hydrophobic gate control of phospholipid transport by the mammalian P4-ATPase ATP8A2. **Proceedings of the National Academy of Sciences of the United States of America**, v. 111, n. 14, p. E1334-43, 8 abr. 2014.

VICARI, P.; FIGUEIREDO, M. S. Priapismo na doença falciforme. **Revista Brasileira de Hematologia e Hemoterapia**, v. 29, n. 3, set. 2007.

VICHINSKY, E. et al. A randomised comparison of deferasirox versus deferoxamine for the treatment of transfusional iron overload in sickle cell disease. **British journal of haematology**, v. 136, n. 3, p. 501–8, fev. 2007.

VICHINSKY, E. P. et al. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. **The New England journal of medicine**, v. 322, n. 23, p. 1617–21, 7 jun. 1990.

VICHINSKY, E. P. et al. Acute chest syndrome in sickle cell disease: clinical presentation and course. Cooperative Study of Sickle Cell Disease. **Blood**, v. 89, n. 5, p. 1787–92, 1 mar. 1997.

VICHINSKY, E. P. et al. Causes and outcomes of the acute chest syndrome in sickle cell disease. National Acute Chest Syndrome Study Group. **The New England journal of medicine**, v. 342, n. 25, p. 1855–65, 22 jun. 2000.

VICHINSKY, E. P. Current issues with blood transfusions in sickle cell disease. **Seminars in hematology**, v. 38, n. 1 Suppl 1, p. 14–22, jan. 2001.

WALTERS, M. C. Update of hematopoietic cell transplantation for sickle cell disease. **Current opinion in hematology**, v. 22, n. 3, p. 227–33, maio 2015.

WALTERS, M. C. et al. Indications and Results of HLA-Identical Sibling Hematopoietic Cell Transplantation for Sickle Cell Disease. **Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation**, v. 22, n. 2, p. 207–211, fev. 2016.

WANG, W. C. et al. Hydroxycarbamide in very young children with sickle-cell anaemia: a multicentre, randomised, controlled trial (BABY HUG). **Lancet (London, England)**, v. 377, n. 9778, p. 1663–72, 14 maio 2011.

WARE, R. E. et al. Sickle cell disease. **Lancet (London, England)**, v. 390, n. 10091, p. 311–323, 2017.

WAUGH, S. M. et al. Heinz bodies induce clustering of band 3, glycophorin, and ankyrin in sickle cell erythrocytes. **The Journal of clinical investigation**, v. 78, n. 5, p. 1155–60, nov. 1986.

WIEDMER, T. et al. Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane. **Biochemistry**, v. 42, n. 5, p. 1227–33, 11 fev. 2003.

WILBER, A.; NIENHUIS, A. W.; PERSONS, D. A. Transcriptional regulation of fetal to adult hemoglobin switching: new therapeutic opportunities. **Blood**, v. 117, n. 15, p. 3945–53, 14 abr. 2011.

WOOD, J. C. et al. Myocardial iron loading in transfusion-dependent thalassemia and sickle cell disease. **Blood**, v. 103, n. 5, p. 1934–6, 1 mar. 2004.

WYSZYNSKI, D. F. et al. Polymorphisms near a chromosome 6q QTL area are associated with modulation of fetal hemoglobin levels in sickle cell anemia. **Cellular and molecular biology (Noisy-le-Grand, France)**, v. 50, n. 1, p. 23–33, fev. 2004.

YABAS, M. et al. ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. **Nature Immunology**, v. 12, n. 5, p. 441–449, 2011.

YABAS, M. et al. Mice deficient in the putative phospholipid flippase Atp11c exhibit altered erythrocyte shape, anemia, and reduced erythrocyte life span. **Journal of Biological Chemistry**, v. 289, n. 28, p. 19531–19537, 2014.

YABAS, M. et al. ATP11C facilitates phospholipid translocation across the plasma membrane of all leukocytes. **PLoS ONE**, v. 11, n. 1, p. 1–12, 2016.

YASIN, Z. et al. Phosphatidylserine externalization in sickle red blood cells: associations with cell age, density, and hemoglobin F. **Blood**, v. 102, n. 1, p. 365–70, 1 jul. 2003.

YAWN, B. P. et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. **JAMA**, v. 312, n. 10, p. 1033–48,

10 set. 2014.

ZAGO, M. A.; PINTO, A. C. S. Fisiopatologia das doenças falciformes: da mutação genética à insuficiência de múltiplos órgãos. **Revista Brasileira de Hematologia e Hemoterapia**, v. 29, n. 3, set. 2007.

ZAGO, M.; FALCÃO, R.; PASQUINI, R. **Tratado de Hematologia**. 1ª Ed ed. Rio de Janeiro: Atheneu, 2013.

ZENNADI, R. et al. Erythrocyte plasma membrane – bound ERK1 / 2 activation promotes ICAM-4 – mediated sickle red cell adhesion to endothelium. **Blood**, v. 119, n. 5, p. 1217–1227, 2012.

ZHANG, Y. et al. Elevated sphingosine-1-phosphate promotes sickling and sickle cell disease progression. **Journal of Clinical Investigation**, v. 124, n. 6, p. 2750–2761, 2014.

ZHOU, Q. et al. Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon-alpha. **Blood**, v. 95, n. 8, p. 2593–9, 15 abr. 2000.

ZHOU, Q. et al. Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. **Blood**, v. 99, n. 11, p. 4030–4038, 2002.

ZHOU, Z.; BEHYMER, M.; GUCHHAIT, P. Role of extracellular hemoglobin in thrombosis and vascular occlusion in patients with sickle cell anemia. **Anemia**, v. 2011, p. 918916, 2011.

ZIMMERMAN, S. A. et al. Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. **Blood**, v. 103, n. 6, p. 2039–45, 15 mar. 2004.

ZWAAL, R. F. A.; COMFURIUS, P.; BEVERS, E. M. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. **Biochimica et biophysica acta**, v. 1636, n. 2–3, p. 119–28, 22 mar. 2004.

ANEXO A - NORMAS DA REVISTA "HAEMATOLOGICA"

Normas da revista "Haematologica" – Fator de Impacto: 9,090; Qualis/CAPES Ciências Biológicas I: A1

A. Manuscript submission

Manuscripts should be submitted through the online manuscript processing system: http://submit-haematol.highwire.org.

*At **initial submission**, these files will be requested:* one pdf file with the text, tables, and figures (please make sure the file size is below 5 Mb); one pdf file with all supplemental data; Information on AUTHOR CONTRIBUTION and DISCLOSURES should be provided during online submission.

We are **very flexible on the format of the first submission** we believe that the data is important, not the format.

*At submission of a **revised manuscript**, these files will be requested:* one-word document with text and tables (indicate in color the changes that were made compared to the first submission); single high-quality files for each figure (jpg is preferred); one pdf file with all supplemental data; **at revision, we request that authors strictly follow our guidelines to ensure fast publication.**

Submission fees

A nonrefundable fee of 50 Euro (including VAT) is due on submission of original articles. There is no submission fee for letters, case reports, comments, editorials, guideline articles and review articles. The publication fee covers the cost of the manuscript processing through the journal's online submission system, and is not refundable once the manuscript has been submitted. This is not a review or publication fee, and the payment of the submission fee is in no way related to the outcome of the in-house or external peer-review process.

Review process

All manuscripts submitted to Haematologica are critically assessed by external and inhouse experts in accordance with the principles of Peer Review (http://www.icmje.org/ethical_3peer.html), which is fundamental to the scientific publication process and the dissemination of sound science. Each paper is first evaluated by one or more editors, who will assess the overall quality and novelty of the work and the article's appropriateness for the scope of Haematologica. Articles that

are not found to be relevant for Haematologica will not be send out for external review and will be returned to the authors. The remaining articles are reviewed by external referees (second step of classical peer-review). We aim to provide feedback to the authors in less than 4 weeks after submission.

Publications charges

Once an article is accepted and in press, authors are required to pay publication charges. These fees cover the cost for Open Access and for publication of the article in print, online and through various other applications. All Haematologica articles are also uploaded to PubMed Central as a digital preservation service to our authors.

Fees (2018):

Articles: For all the papers the publication charge is € 1600 (plus VAT when needed).

Letters/Case reports: a publication charge of € 300 (plus VAT when needed) is due before publication.

Editorials, Review Articles, Guideline Articles, Comments: Free of charge

These fees include all publication charges, including page charges, costs for color images, open access, and costs for online supplements. No other costs will be charged.

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Ethical considerations

Protection of human individuals in research

When reporting experiments on human individuals, authors should indicate whether the procedures followed were 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 2008. If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Patients have a right to privacy that should not be violated without *informed consent*. Identifying information, including names, initials, or hospital numbers, should not be published in written descriptions, photographs, or pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication.

Protection of animals in research

When performing experiments on animals or animal tissues, authors should seek approval by an institutional ethics committee and should strictly follow the institutional and national guide for the care and use of laboratory animals. At time of manuscript submission, authors should provide information on the study approval by an institutional ethical committee. We can only consider manuscripts reporting on studies on animals or animal tissues if ethical committee approval of the study can be documented.

B. Manuscript preparation

1. Manuscript style

Manuscripts should be prepared according to the *Uniform Requirements* established by the *International Committee of Medical Journal Editors* (ICMJE): http://www.icmje.org/manuscript_1prepare.html

Manuscripts should be prepared using **American English spelling**, and **should be submitted as Word files.** Submit one manuscript file with the main text, figure legends and tables. Save your file in .doc format (not as .docx file).

Scientific nomenclature should be used without Saxon Genitive (for example: use 'Hodgkin Lymphoma' and not 'Hodgkin's Lymphoma'). Saxon Genitive should be maintained in references.

Units and measurements: The SI system should be used for all scientific units. Please refer to http://www.bloodindex.com/normal_laboratory_values.php for hematological measures. Authors can also refer to http://www.unc.edu/~rowlett/units/scales/clinical_data.html for conversions from conventional units to SI units. Please adopt standardized abbreviations and define full forms in footnotes, e.g. “NA” (not applicable) ; “ND” (not determined).

The *use of commercial names of drugs* should be avoided. Drugs should only be referred to under their generic names unless different products are being compared. (for example: Use deferiprone, not Ferriprox)

Use the *official gene symbols* when referring to genes, transcripts, proteins. (for example: Use ABL1, not ABL or c-ABL ; use MYC, not c-Myc). Use this database as a reference: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene. Human Genes' names have to be indicated in Italic and capital, Human Proteins' names only in capital letter. Mouse and Rat Genes' names have to be indicated in italic, with only the first letter in capital and the remaining letters in lowercase (*Shh*). Mouse and Rat Proteins' names have to be indicated only in capital letter (SHH). Zebrafish and Frog (xenopus sp.) Genes' names have to be indicated in italic and all in lowercase (*shh*). Zebrafish and Frog (xenopus sp.) Proteins' names must have the first letter in capital and the remaining letters in lowercase.

References: Please follow the recommendations of ICMJE for the style of the references available at http://www.nlm.nih.gov/bsd/uniform_requirements.html. An Endnote style is available for download: **Haematologica Endnote style** (updated in 2015).

Image size and layout: Image layout should be simple, clear and precise. In order to promote good management of the space, images must take up the least space possible without compromising clarity. Figures can be either one column width (8 cm) or 2 column widths (16 cm). If the figure contains different panels their content should be identified (use capital letters to identify each panel) and described in the order in which they are presented. Please ensure that a description is provided for all parts of the figure. Please ensure that different parts of the image are shown in proportion to each other, e.g. axis scales and labels, internal descriptive text. Lines should be black (not grey) and sufficiently thick. Data which has no graphic significance to any part of the figure content and form should be presented as a separate table.

Figure quality: When a revised version of a manuscript is submitted, high quality figures will be required and will be asked to the authors. High quality figures should be submitted in jpg or tiff format. Note that we **cannot** accept figures as presentation slides (Microsoft Powerpoint, Apple Keynote, or similar) as these cannot be printed in high quality. The resolution of figures should be sufficiently high to allow clear sharp printing. The best way to determine if a figure is of sufficient quality is to print it in its final size: if all lines, letters, images are sharp and clear (not blurry or unfocused) and sufficiently large to read, then the figure is most likely fine. It is not possible to increase the quality of low quality images: be sure to capture high quality images at all times during experimental procedures. When making figures in adobe illustrator or similar

software, export your figures as 300 dpi or 600 dpi jpg files (for text 600 dpi is likely to be the best option).

We cannot accept low quality figures. A manuscript cannot be accepted if the authors fail to provide high quality figures.

We are flexible at initial submission, but at submission of a revised manuscript these guidelines need to be followed carefully.

2. Types of manuscripts

Articles are the main type of manuscripts, reporting both clinical and experimental hematology research studies that are of excellent quality and report novel findings that are of high importance for the field. These articles are to be divided into sections under the following subheadings: Abstract, Introduction, Methods, Results, Discussion, References. Results and Discussion can be combined in one section if appropriate.

The **abstract** should be a summary of the work in **maximum 250 words**. State clearly the rationale for the study, how the study was performed, the most important results obtained, and the conclusions that can be drawn from this study. Articles reporting clinical trials should provide the trial registration number at the end of the abstract.

The **main text** (introduction, methods, results, discussion) can be **maximum 4000 words.**

The **methods** section in the article should be a *brief summary of the most important methods* (**maximum 500 words**), and is placed after the introduction. Describe basic material (patient samples, cell lines, specific methods, special reagents). **If more space is needed for the methods, this can be provided in the online supplement** that will be published with the online version of the article. There is no restriction to the length of the online methods section, but avoid making this part unnecessary long.

References should be given in the correct style (see the latest issue of Haematologica for an example), with a **maximum of 50 references**

Figures and tables: Original articles can contain 8 display items (figures and/or tables). Authors are requested to provide the tables as text files in the manuscript file (word file). Tables should only contain data that is essential for the manuscript. Large additional tables (for example with patient characteristics, primer sequences, etc.) should be placed in the supplement. Authors are requested to provide the figures as high-resolution images (jpg or tiff – power point figures are not accepted) in the final size in which they will be published, taking the relative size of all the graphs into account, as well as the size of letters and symbols in the graphs.

All supplementary material should be prepared and submitted as one single pdf file. This file will also be reviewed by the reviewers. In case of acceptance of the manuscript, this supplemental pdf file will be published as provided by the authors (this file will not be edited by the journal office, it is the responsibility of the authors to ensure the supplement is complete and correct).

Review articles are invited articles, direct submission without invitation is not possible. Review articles are maximum 5000 words long (excluding figure legends and references) and do not have a specific structure. The articles should contain an abstract (short summary of the topic without specific structure) of maximum 250 words, may contain several display items in color, and have a reference list of maximum 100 references. These articles should not simply go over or summarize general information which is already known, but also discuss the importance of the data and provide a clear view on how these insights have transformed or will transform the hematology field. Authors of review articles are encouraged to include 4 to 6 figures. Authors should only provide a draft of the figures (all formats acceptable), as the final high-quality figures for review articles will be generated by Somersault1824 (www.somersault1824.com).

Guideline articles should consist of meta-analyses and/or guidelines carried out and prepared by an international group of experts. These articles are up to 5000 words long (excluding figure legends and references) and do not have a specific structure. The articles should contain an unstructured abstract (short summary/ introduction) of maximum 200 words, may contain several display items in color, and have a reference list of maximum 100 references.

Editorials are commentaries or opinions on specific articles or on general concepts in hematology practice or research. These articles are usually invited articles, but proposals are welcome and should be addressed to the editorial office (office@haematologica.org). These articles have a free structure (no abstract, no specific subheadings), contain about 1,500 words (excluding references), up to 20 references and may contain two display items (figures and/or tables). Haematologica has a wide international readership both in the field of hematology and in other sectors of the medical profession. Its aim is to reach also those without specialized knowledge. Authors are asked to use simple, straightforward language to promote the understanding by all readers.

Letters to the Editor: We encourage submission of important new findings that fit into the format of a letter. Start the letter with a paragraph summarizing the rationale for the study and the major conclusions. Letters have no abstract, no headings, and a maximum of 1500 words in the text (excluding title, authors, affiliations, figures legends or references), a maximum of 3 tables and/or figures, and only 15 essential references. Letters will appear in the online version of the journal, will appear in the table of contents, and are indexed in PubMed.

Case Reports & Case Series: Detailed reports of special cases that contribute to a better mechanistic understanding of hematological diseases or to patient care will be considered for publication under 'Case Reports & Case Series'. These reports need to be conclusive and will need to describe various data (molecular data, biology data, etc.) in addition to the description of the clinical data. Only few case reports can be published and a strict selection is in place. Case Reports have no abstract, no headings, and a maximum of 1500 words in the text (excluding title, authors, affiliations, figure legends or references), a maximum of 3 tables and/or figures, and only 15 essential references. Case Reports will appear in the online version of the journal, will appear in the table of contents, and are indexed in PubMed.

Comments are the format to discuss a recently published article in Haematologica, or to discuss cutting edge ideas or developments in the broad field of hematology. Comments follow the same structure as *Letters*, but cannot contain new data. Comments have no abstract, a maximum of 1000 words in the text (excluding title, affiliations, figures legends or references), and only 10 essential references. Please start the comment with a paragraph summarizing the rationale for the comment, citing the article or articles that form the basis for the comment. Comments will appear in the online version of the journal, will appear in the table of contents, and are indexed in PubMed.

Conflict of interest note:

Conflict of interest regarding papers that do not report original research (primary data). As detailed under Policies and Practices (http://www.haematologica.org/misc/policies.dtl) authors must disclose all relationships that could be viewed as potential conflicts of interest both in the online manuscript submission system and in the manuscript. These disclosures are expected to help readers in establishing whether the reported relationships may influence the authors' judgment. Haematologica believes that this procedure is appropriate with

respect to papers reporting original research (original articles, brief reports and research letters) as primary data speak for themselves. This procedure may be insufficient with respect to papers that do not report primary data, such as editorials, perspective articles, commentaries, review articles, guidelines, consensus papers and position papers. As stated by Kassirer & Angell [Kassirer JP, Angell M. Financial conflicts of interest in biomedical research. N Engl J Med. 1993 Aug 19;329(8):570-1. PubMed PMID: 8204121] “unlike reports of original research, these articles represent the judgment of their authors, based on their evaluation of the literature. What studies they select to discuss and their analysis of them are necessarily subjective. Bias may be extremely difficult to detect because these articles contain no primary data to speak for themselves.” Nonetheless, disclosing relationships that could be viewed as potential conflicts of interest may be acceptable in many of these papers. *Haematologica, however, no longer considers for publication papers not reporting primary data - such as those listed above - whose preparation has been promoted, sponsored or supported in any way by a company whose product is discussed in the paper.* In fact, the clear conflict of interest is very likely to influence judgment in these cases, and there are no primary data that can speak for themselves. This point is detailed in the online manuscript processing system; if doubts exist about this issue, the authors are invited to contact the editorial office (office@haematologica.org) before proceeding with submission.

3. Manuscript structure

3.1. Title Page

Information provided on the title page should correspond exactly with the information provided in the online system.

The **title** should consist of a phrase or a sentence; question forms should be avoided. Capitalize the first letter of the sentence only, and do not use abbreviations. Study group names may be presented in the title, however, all members’ names should be listed in an appendix and presented at the end of the main text; Add for example: A complete list of the members of the European Prospective Investigation into Cancer and Nutrition Group appears in a supplement. Acronyms such as EBMT, GOELAMS, GEIL, are acceptable. Commercial names of drugs should be avoided (use only the generic names), unless different products are being compared.

Names of authors should be presented as full first name, initial of middle name (if applicable) and full last name. Use a comma between each author, use “and” before the final author. Number authors’ affiliation in superscript. Do not include professional titles or abbreviations of qualifications or positions held.

Authors’ affiliations should be provided on a new line immediately after the authors’ names. Every affiliation on a new line. Provide only the city and country (acronym for the USA countries) of each institute, not full address, the postal code is not needed.

Statement of equal authors’ contribution: In cases in which authors share equal responsibility for the study, a statement of equal contribution can be stated here. Use authors’ initials rather than full names, e.g. ***“***GGH and FS contributed equally to this work.”

Running heads: *please provide* a shortened title as running head ***of maximum 50 characters.*** The use of acronyms and abbreviations is permitted.

Contact information for correspondence should confirm the name and e-mail address of the corresponding author or authors.

Word count: Provide a word count for the abstract and for the main text (= introduction + methods + results + discussion). Indicate how many tables and figures are present in the manuscript. Indicate how many supplemental files are associated with the manuscript (we request 1 supplemental pdf file; only in specific circumstances we can accept more than 1 supplemental file).

Trial registration: Confirmation and details of trial registration should be given on the first page; please use the following form: “clinicaltrials.gov identifier: NCT00123456.”

Acknowledgments should refer to secretarial and editorial assistance, technical and intellectual input and advice, funding, fellowships and grants. The form to be used is “The authors would like to thank...”

3.2. Abstract

Summary of the work, word limit is dependent on the type of article.

Letters, Case reports and editorials do not have an abstract.

3.3. Main text

Word limit is dependent on the type of article.

Products that are commercially available should be clearly described with specific information, non-commercial products should be completely described in the manuscript or in the supplement. Sequences, chemical structures, datasets, etc. should be fully disclosed and made available to other researchers.

Tables and Figures: The presentation of Tables and Figures should always follow the same order in which they are presented in the main text. All references to Tables and Figures should be presented in brackets and should only specify “Table” or “Figure” and the relevant identification number.

When reference is made to more than one Table or more than one Figure, please separate the identification numbers with a hyphen and use “and” to present Tables or Figures that are not consecutive.

Please pay particular attention to spacing (for example: Figures 1-2; Tables 1 and 3; Figures 2-4 and 6; Tables 2, 4 and 6). References referring to Figure panels and subpanels should be presented by adding a capital letter in alphabetic order immediately after the identification number (for example: Figure 1A, Figure 1B). When reference is made to more than one Figure panel or subpanel, please separate the capital letters with a hyphen and use a comma followed by a space to separate capital letters that are not consecutive (for example: Figure 2 B-C ; Figure 3 B, D).

3.4. References

We use a new style since March 2015. Please see the latest issue of Haematologica for an example.

In the main text, refer to published litterature by using a number, for example:

Melick and colleagues previously reported IDH1 mutations in AML.[5]

Several groups previously reported the presence of IDH1 mutations in AML.[5-9]

Two studies previously reported IDH1 mutations in AML.[5,6]

To generate the reference list, follow these new guidelines:

For references with 6 or less than 6 authors, list all authors:

Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matripase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Met. 2008;8(6):502-511.

For references with more than 6 authors, list the first 3 authors followed by "et al."

Rose ME, Huerbin MB, Melick J, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res. 2002;935(1-2):40-46.

For articles published ahead of print:

Those articles which have already been accepted for publication and which have been published ahead of print should follow the same reference format as a journal article, using the date on which the article was published ahead of print, e.g. Bonci D, Musumeci M, Coppola V, Addario A, Conticello C, et al. Blocking the APRIL circuit

enhances acute myeloid leukemia cell chemosensitivity. Haematologica 2008 Oct 6. [Epub ahead of print]

For unpublished data:

Authors should provide the names of all persons responsible for the resource, a short description, and the relevant date.

3.5. Tables *(tables should be placed after the references)*

Provide tables in a simple format, without specific layout. The final layout will be given by journal staff at time the final manuscript pdf file is prepared. Tables should have a short clear title. Provide a legend to the table (if needed) directly below the table. Explain all abbreviations that are used in the table in the legend.

3.6. Figure legends *(figure legends should be placed after the tables)*

Figures should be numbered consecutively in the order in which they are presented in the main text, e.g. Figure 3. Give every figure a short clear title, followed by a brief description of figure content. Provide sufficient detail in the figure legend, but do not repeat what is discussed in the text.

3.7. Supplements

In addition to the main Word file, one additional pdf file can be submitted containing supplemental material. Please prepare one single pdf file containing all supplemental data (supplemental methods, supplemental data, supplemental figures, supplemental tables). Additional files that cannot be incorporated in a pdf file (such as video, large excel tables, etc.) can be provided separately.

Please submit the manuscript with the tables and figure legends placed after the references.

ANEXO B - NORMAS DA REVISTA "BLOOD”

Normas da revista "Blood” – Fator de Impacto: 15,132; Qualis/CAPES Ciências Biológicas I: A1

Instructions for Authors

Regular Articles

Maximum length for a Regular Article is 4,000 words of text - counting only the Introduction, Methods, Results, and Discussion. Submissions are limited to a total of 7 figures, and digital images are required. We recommend a limit of 100 references. The sections of a Regular Article should be ordered as follows: Abstract; Introduction; Methods (must include sufficient information to allow readers to understand the article content); Results; Discussion; Acknowledgements; Authorship Contributions; Disclosure of Conflicts of Interest; References; Tables; Figure Legends; Figures.

Supplemental data - to be published online only - may include additional information regarding methodology, supplemental figures or tables, or primary data sets; it must be submitted with the original manuscript submission, so it can be peer reviewed. (See "Supplemental data")

Any involvement of medical writers/researchers, particularly those employed or supported by the pharmaceutical industry, in the writing of an article must be clearly defined and disclosed in the Authorship and/or the Acknowledgements section(s) as appropriate. This type of involvement must also be disclosed to the Editor-in-Chief in the Cover Letter. For more information, see the journal Conflict of interest disclosure and the editorial policies for authors.

Brief Reports

Short manuscripts definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Single-case reports or case series cannot be accommodated unless they elucidate very novel and important disease biology or approaches to therapy. Brief Reports are not intended to allow publication of incomplete or preliminary findings. The review process is equally rigorous as for Regular Articles and the acceptance rate is lower. Brief Reports may not exceed 1,200 words of text -counting only the Introduction, Methods, Results, and Discussion. Abstracts must not exceed 200 words and should be a single paragraph with no subheadings. Only 2 figures/tables and 25 references may be included. The

sections of a Brief Report should be ordered as follows: Abstract; Introduction; Methods (must include sufficient information to allow readers to allow reproduction of the data); A combined Results and Discussion section; Acknowledgements; Authorship Contributions; Disclosure of Conflicts of Interest; References; Tables; Figure Legends; Figures.

Manuscript Submission

Before submitting your manuscript online at Blood Bench>Press, please read and carefully follow the guidelines below. Any deviations could result in significant delay in the submission and review process.

Please note that Blood adheres to the criteria of the International Committee of Medical Journal editors, which has established Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

Manuscript length restrictions

Please adhere to the length, figure/table and reference number restrictions described on the previous page for each article type. On the title page, include a text word count, abstract word count, the number of figures and tables, and the number of references. Submissions exceeding these limits will not be considered for review and will be returned to the author.

Manuscript organization

Organize the content of your manuscript file as follows: Title Page, Key Points, Abstract, Introduction, Methods, Results, Discussion, Acknowledgments, Authorship Contributions, Conflict of Interest Disclosures, References, Tables, Figure Legends, and Figures. For Brief Reports, Results and Discussion must be combined. Your text document must include page numbers, meaning there must be page numbers inserted into the header or the footer of your document.

Article title and title page

The title should succinctly and effectively convey to non-specialists the content of the article with no more than 120 characters, including spaces. Titles should be in active rather than passive voice, without the use of punctuation or abbreviations. If commonly-understood abbreviations are included in the title, they must be defined in the abstract. If the article reports on results utilizing solely non-human model systems, the species must be indicated in the title.

Title page must contain the following: article title; short title for the running head (not to exceed 50 characters, including spaces between words); full and accurate names

of all authors (as you want them to appear in online searches and citations); affiliations of institutions where the research was done, reflecting the order of authorship by using superscripted numbers; corresponding author's full name, address, e-mail address, and phone and fax numbers; word counts for text and abstract, figure/table count and reference count.

Regular Articles and Brief Reports should also include on the title page an appropriate scientific category chosen during submission.

Key Points

Blood now publishes 1 to 2 Key Point summaries of research papers - specifically, Regular Articles, Brief Reports, and e-Blood articles. The purpose of these short, bullet-pointed statements is to identify the most relevant outcomes of the paper and to provide a synopsis encapsulating the significance of the research and its implications for readers.

Key Points should be written clearly and succinctly. Avoid using scientific jargon whenever possible. Each Key Point should be no more than 140 characters, including spaces. Key Points are required upon manuscript submission, immediately preceding the Abstract in both the submission form metadata and the text document, and they will be reviewed by the assigned Editor.

Key Points are published online, in First Edition, and in print immediately preceding the Abstract and will be freely available upon publication. They will not be indexed by PubMed, but will be searchable via Google and other search engines.

Abstract

The abstract should contain 250 words or fewer (200 words or fewer for Brief Reports; check the word count limit in the description for other article types) and succinctly, in a logical progression state the rationale/hypothesis, objectives, findings/results, and conclusions of the study. Abstracts should be a continuous narrative and not broken up into subheadings, and should not contain references.

Authors need to ensure that abstracts are easily readable and understandable to a broad readership. The abstract should accurately reflect the content of the article, be written in plain and succinct language and, as much as possible, avoid jargon and acronyms.

The abstract of a research paper should preferably contain the following elements (per ICMJE recommendations): The context or background for the study. The authors should consider that a vast majority of readers have either no or limited knowledge of

the article context: one or two plain-language sentences should clearly describe this background; The study's purpose, i.e., why the study was done. The objectives of the research should be explicitly provided, rather than in general statements; Methods/procedures (selection of study participants, settings, measurements, analytical methods); Main findings, giving specific effect sizes and their statistical and clinical significance, if possible; Main conclusions and interpretation of findings with emphasis on new and important aspects of the study and/or observations.

Methods

The materials and methods section should be detailed enough to provide clear information on what was done experimentally, including all major experimental plans and procedures. The Journal will not consider manuscripts that include significant portions of the methods section as supplemental data.

Include in the Methods section as appropriate: A statement that the research was approved by the relevant institutional review boards or ethics committees and that all human participants gave written informed consent; A statement regarding the identity of those who analyzed the data and confirming access of all authors to primary clinical trial data; The clinical trial registration number and approved registry name for all clinical trials; For phase 3 randomized clinical trials, we request that the authors provide a flow diagram in CONSORT format and include all of the information required by the CONSORT checklist within the body of the manuscript. When restrictions on length prevent the inclusion of some of this information in the manuscript, it may be provided instead as supplemental data. The CONSORT statement, checklist, and flow diagram are available at http://www.consort-statement.org.

Acknowledgments

Support received from individuals, organizations, grants, corporations, and/or any other sources must be acknowledged. For work involving a biomedical product or potential product partially or wholly supported by corporate funding, a note must be included stating: This study was supported (in part) by research funding from [company name] to [author’s or authors’ initials]. Grant support, if received, needs to be stated and the specific granting institution(s) name(s) and grant numbers provided when applicable. Any individuals involved in the writing/editing/researching of the paper not named as authors should be identified, their role specified, and their funding source specified; for example, “Joseph Smith, a medical writer supported by funding from [company name], provided drafts and editorial assistance to the authors during

preparation of this manuscript." Prior to submission of the manuscript, we recommend that authors notify all individuals being included in the acknowledgments section to ensure their names and roles are being identified accurately.

Authorship and conflict-of-interest statements

For each author, include in this section his or her category of contribution and list any potential conflicts of interest. These statements will be printed and posted online in the First Edition and in the final version in the Authorship section.

If the author(s) declare no competing financial interests, this must be explicitly stated and will be included in all versions of the article. Contributions and COI must appear both in the metadata and in the manuscript text.

References

Include references in numerical order at the end of the article according to the order of citation in the manuscript text. Text citations of reference should consist of superscript numbers. Format references per the instructions of the Blood Style Guide. If you use citation software, check it carefully to ensure that it formats your references according to the current Blood style.

Authors can now have Medline links in their HTML references for citations that have only been published via prepublication in Blood First Edition or in other prepublished articles. Since prepublished articles have PubMed records and a PubMed ID (PMID) is listed at the bottom of every PubMed record as the citation identifier, an author can include the PMID within his or her manuscript references to link the prepublication citation to its PubMed record. Citation of a paper prepublished in First Edition must also include its DOI number, as shown in the prepublished article.

Figures

When submitting a manuscript for review, image file formats accepted for uploading include: GIF, JPEG (.jpg), PDF, TIFF, and EPS. PowerPoint (.ppt) files are acceptable but are strongly discouraged due to conversion issues and poor resolution in the published article.

High-resolution image files are not preferred for initial submission as the file sizes may be too large. The total file size of the PDF for peer review should not exceed 5 MB. However, high-resolution figures are required for accepted articles entering into prepublication and print production. To prepare print-quality figures, see Figure preparation and sizing for the final print publication. Note that no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. If groupings

of images from different parts of the same gel or microscopic field, or from different gels, fields, or exposures are used, they must be made explicit by the arrangement of the figure (i.e., by inserting black dividing lines) and in the text of the figure legend, explaining what steps were taken to produce the final image and for what reason. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and as long as they do not obscure, eliminate, or misrepresent any information present in the original, including backgrounds. Without background information, it is not possible to evaluate how much of the original gel is shown. Nonlinear adjustments (e.g., changes to gamma settings) must be disclosed in the figure legend. The use of special software tools (e.g., erasing, cloning) available in popular image-editing software is strongly discouraged unless absolutely necessary, and any such manipulations must be explained in the figure legend. Cases of deliberate misrepresentation of data will result in revocation of acceptance and will be reported to the corresponding author's home institution or funding agency. All figure legends must begin with a short, descriptive sentence that summarizes the intent and content of the figure. This sentence should be in boldfaced font. A more detailed explanation of the data contained in the figure and/or its parts should follow in standard (non-boldfaced) font.

Tables

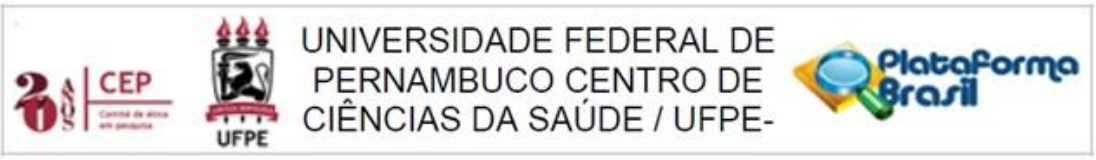
Each table should have a brief, specific, descriptive title, giving sufficient explanation to make the data intelligible without reference to the text. Number all tables and cite in numerical order in the text, using Arabic numerals.

Supplemental data

The Journal encourages the submission of supplemental data linked to primary research articles, including videos and short movies, that enhance the understanding of the science discussed in the manuscript. Supplemental data must be included during the initial submission of the parent manuscript. All supplemental data, other than videos, must be contained in a single PDF or Microsoft Word (.doc or .docx) file — not as separate files for each individual component. Do not include any supplemental data in the main manuscript text document, including appendices (e.g., lists of contributors to a consortium), methods, tables, figures, and legends of any kind. The Editors will review the supplemental material along with the manuscript, but acceptance of the manuscript does not guarantee ultimate acceptance of the supplement.

Supplemental data may or may not appear alongside an accepted article at the time of its publication in First Edition, depending on the time needed to process the supplemental material. Blood instituted a publication fee of $105 for each standard data supplement accompanying an accepted paper. Any supplement exceeding 5 MB will incur an additional $105 (USD) fee; exceptions are possible for certain video files at the Editor's discretion. The fee is waived for Review Articles, How I Treat, Perspectives, and e-Blood articles. For more information, please see Supplemental data in Blood. Any information necessary for a reader to fully evaluate and understand an article must be included in the main text of a paper — not included solely in supplemental data.

ANEXO C - PARECER CONSUBSTANCIADO DO CEP



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Investigação de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário na modulação do quadro clínico de pacientes com anemia falciforme

Pesquisador: Igor de Farias Domingos
Área Temática:
Versão: 2
CAAE: 88290618.3.0000.5208
Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.727.352

Apresentação do Projeto:
Projeto de pesquisa intitulado "Investigação de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário na modulação do quadro clínico de pacientes com anemia falciforme", apresentado ao Curso de Doutorado em Genética da UFPE pelo discente, regularmente matriculado, Igor de Farias Domingos, sob orientação do Prof. Dr. Marcos André Cavalcanti Bezerra. Estudo do tipo corte transversal analítico, que será realizado no período de julho de 2018 a fevereiro de 2019. O trabalho irá avaliar a influência de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário e associar esses achados com a modulação do quadro clínico de pacientes com anemia falciforme. A amostra será constituída de 100 pacientes portadores de anemia falciforme, maiores de 18 anos, não aparentados, e acompanhados regularmente no ambulatório do hospital de hematologia da Fundação HEMOPE. A expressão dos genes nas amostras testes e referências serão avaliadas pela técnica de RQ-PCR. Alterações nos genes ATP11C, SPHK1 e DMTN podem estar associadas com o quadro clínico, e contribuir para a heterogeneidade clínica dos pacientes com anemia falciforme. Desse modo, a identificação dessasalterações fornecerá subsídios para um melhor atendimento e acompanhamento médico.

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RECIFE, 21 de Junho de 2018

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

ANEXO D - CURRICULUM VITAE (LATTES)

Igor de Farias Domingos
Curriculum Vitae

Dados Pessoais

Nome Igor de Farias Domingos
Filiação Ricardo Domingos e Irene Maria de Farias Domingos
Nascimento 28/06/1990 - Recife/PE – Brasil
E-mail domingos_if@hotmail.com

Formação acadêmica/titulação

2015 - Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Investigação de genes envolvidos na estabilidade e manutenção do citoesqueleto eritrocitário e sua relação com o quadro clínico de pacientes com anemia falciforme, Ano de início: 2015
Orientador: Marcos André Cavalcanti Bezerra
Co-orientador: Antonio Roberto Lucena de Araújo
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil

2013 - 2015 Mestrado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Investigação de Polimorfismos em Genes Relacionados ao Estresse Oxidativo e Inflamação no Desenvolvimento da Doença Cerebrovascular em Pacientes com Anemia Falciforme, Ano de obtenção: 2015
Orientador: Marcos André Cavalcanti Bezerra
Co-orientador: Antonio Roberto Lucena de Araújo
Bolsista do(a): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, FACEPE

2008 - 2011 Graduação em Biomedicina.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Características Moleculares de Pacientes com Anemia Falciforme e Acidente Vascular Cerebral
Orientador: Marcos André Cavalcanti Bezerra

Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

2015 - 2016 Vínculo: Servidor público, Enquadramento funcional: Professor Substituto, Carga horária: 20, Regime: Parcial
Coordenador e docente da disciplina de graduação Produtos Hemoterápicos (FA321) no semestre 2015.1, 2016.1 e

2016.2, tendo ministrado uma carga horária de 115 h/a por semestre.

Coordenador e docente da disciplina de graduação Interpretação do Hemograma (BR267) no semestre 2016.2, tendo ministrado uma carga horária de 30 h/a.

Docente da disciplina de graduação Exames Hematológios I (BR256) no semestre 2016.2, tendo ministrado uma carga horária de 30 h/a.

2. Secretária Estadual de Saúde de Pernambuco - SES-PE

2016 - Atual Enquadramento funcional: Analista Clínico, Carga horária: 24, Regime: Parcial

Analista clínico lotado no laboratório do Hospital Correia Picanço, vinculado a Secretaria Estadual de Saúde de Pernambuco.

3. Centro Universitário Dr. Leão Sampaio - UNILEAO

2018 - 2018 Vínculo: Professor Visitante, Enquadramento funcional: Professor convidado, Carga horária: 20, Regime: Parcial

Professor convidado no curso de Especialização Lato Sensu em Hematologia Clínica, ministrando a disciplina de Hemoterapia e Controle de Qualidade, perfazendo um total de 20 horas aula.

2017 - 2017 Vínculo: Professor Visitante, Enquadramento funcional: Professor convidado, Carga horária: 20, Regime: Parcial

Professor convidado no curso de Especialização Lato Sensu em Hematologia Clínica, ministrando a disciplina de Biologia Molecular Aplicada ao Diagnóstico em Hematologia Clínica, perfazendo um total de 20 horas aula.

4. Faculdades Integradas de Patos - FIP

2017 - 2017 Vínculo: Professor Visitante, Enquadramento funcional: Professor convidado, Carga horária: 20, Regime: Parcial

Professor convidado no curso de Especialização Lato Sensu em Hematologia Clínica, ministrando a disciplina de Imunohematologia (Módulos I e II), perfazendo um total de 40 horas aula.

5. Universidade de Pernambuco - UPE

2018 - 2018 Vínculo: Professor Visitante, Enquadramento funcional: Docente contratado, Carga horária: 6, Regime: Parcial

Docente contratado pela Universidade de Pernambuco - campus Serra Talhada, ministrando aulas teóricas na área de hematologia para o 8º período do curso de Medicina (Módulo Prática Médica VIII), com carga horária total de 24h.

Produção bibliográfica
Artigos completos publicados em periódicos

1. ROMANELLO, KAREN S.; TEIXEIRA, KARINA K. L.; SILVA, JOÃO PEDRO M. O.; NAGAMATSU, SHEILA T.; BEZERRA, MARCOS ANDRÉ C.; DOMINGOS, I. F.; MARTINS, DIEGO A. P.; ARAUJO, ADERSON S.; LANARO, Carolina; BREYER, CARLOS A.; FERREIRA, REGIANE A.; FRANCO-PENTEADO, CARLA; Costa, Fernando F.; MALAVAZI, IRAN; NETTO, LUIS E. S.; DE OLIVEIRA, MARCOS A.; CUNHA, A. F.
Global analysis of erythroid cells redox status reveals the involvement of Prdx1 and Prdx2 in the severity of beta thalassemia. PLoS One., v.13, p. e0208316 -, 2018.

2. **DOMINGOS, IGOR F.**; PEREIRA-MARTINS, DIEGO A.; COELHO-SILVA, JUAN L.; BORGES-MEDEIROS, RAYSSA L.; FALCÃO, DIEGO A.; AZEVEDO, RENATA C.; ANJOS, ANA C.; COSTA, FERNANDO F.; MENDONÇA, TACIANA F.; CAVALCANTI, MARIA S.; ARAUJO, ADERSON S.; LUCENA-ARAUJO, ANTONIO R.; BEZERRA, MARCOS A.
Interleukin-6 G-174C polymorphism predicts higher risk of stroke in sickle cell anaemia. BRITISH JOURNAL OF HAEMATOLOGY., v.182, p.294 - 297, 2018.

3. ALAGBE, A.E.; JUSTO JUNIOR, A.S.; RUAS, L.P.; TONASSÉ, W.V.; SANTANA, R.M.; BATISTA, T.H.C.; **DOMINGOS, I.F.**; ARAUJO, A.S.; BEZERRA, M.A.C.; SANTOS, M.N.N.; BLOTTA, M.H.S.L.
Interleukin-27 and interleukin-37 are elevated in sickle cell anemia patients and inhibit in vitro secretion of interleukin-8 in neutrophils and monocytes. CYTOKINE. , p.85 - 92, 2017.

4. LEONARDO, FLÁVIA C.; BRUGNEROTTO, ANA F.; DOMINGOS, I. F.; FERTRIN, KLEBER Y.; DE ALBUQUERQUE, DULCINÉIA M.; Bezerra, Marcos A. C.; ARAÚJO, ADERSON S.; Saad, Sara T. O.; Costa, FF; MENZEL, STEPHAN; CONRAN, Nicola; THEIN, SWEE LAY
Reduced rate of sickle-related complications in Brazilian patients carrying HbF-promoting alleles at the BCL11A and HMIP-2 loci. British Journal of Haematology (Print)., v.173, p.456 - 460, 2016.

5. PARAHYM, ANA MARIA RABELO DE CARVALHO; ROLIM NETO, PEDRO JOSÉ; SILVA, CAROLINA MARIA DA; **DOMINGOS, IGOR DE FARIAS**; GONÇALVES, SARAH SANTOS; LEITE, EDINALVA PEREIRA; MORAIS, VERA LÚCIA LINS DE; MACÊDO, DANIELLE PATRÍCIA CERQUEIRA; LIMA NETO, REGINALDO GONÇALVES DE; NEVES, REJANE PEREIRA
Invasive infection due to Saprochaete capitata in a young patient with hematological malignancies. Brazilian Journal of Microbiology (Online)., v.46, p.527 - 530, 2015.

6. ARAUJO, NARA B.; **DOMINGOS, IGOR F.**; MEDEIROS, FERNANDA S.; HATZLHOFER, BETÂNIA L.; MENDONÇA, TACIANA F.; VASCONCELOS, LUYDSON R.; CAVALCANTI, MARIA DO SOCORRO M.; ARAUJO, ADERSON S.; OLIVEIRA, MARIA DO CARMO V.; LUCENA-ARAUJO, ANTONIO R.; BEZERRA, MARCOS A.
Lack of association between the Duffy antigen receptor for chemokines (DARC) expression and clinical outcome of children with sickle cell anemia. Immunology

Letters. , v.166, p.140 - 142, 2015.

7. **DOMINGOS, I. F.**; HATZLHOFER, B. L.; OLIVEIRA, F. B.; ARAUJO, F. R.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A.
Prevalence and molecular defect characterization of glucose-6-phosphate dehydrogenase deficiency in Brazilian blood donors. International Journal of Laboratory Hematology (Print)., v.--, p.n/a - n/a, 2015.

Trabalhos publicados em anais de eventos (resumo)

1. TONASSE, W. V.; CHENOU, F.; **DOMINGOS, I. F.**; BATISTA, T. H. C.; SANTANA, R. M.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Correlations between hemolysis and hypercoagulability markers and effects of hydroxyurea therapy in sickle cell patients In: 23rd Congress of the European Hematology Association, 2018, Estocolmo. **23rd Congress of the European Hematology Association**., 2018.

2. TONASSE, W. V.; CHENOU, F.; **DOMINGOS, I. F.**; BATISTA, T. H. C.; SANTANA, R. M.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Hemolysis and coagulation markers in sickle cell anemia patients with and without hydroxyurea therapy In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2018, 2018, São Paulo. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2018**., 2018.

3. TONASSE, W. V.; CHENOU, F.; **DOMINGOS, I. F.**; BATISTA, T. H. C.; SANTANA, R. M.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Hemolysis and hypercoagulability markers in sickle cell anemia patients with and without hydroxyurea therapy In: 11ª semana de pesquisa da FCM - UNICAMP, 2018, Campinas. **11ª semana de pesquisa da FCM - UNICAMP**., 2018.

4. **DOMINGOS, I. F.**; MARTINS, D. A. P.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Influência do SPHK1 na modulação do quadro clínico de pacientes com anemia falciforme acompanhados em Pernambuco In: VIII Jornada de Pós-Graduação em Genética - UFPE, 2018, Recife. **VIII Jornada de Pós Graduação em Genética - UFPE**., 2018.

5. XAVIER, A. L.; BATISTA, J. V. G. F.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; FALCAO, D. A.; SA, A. F.; ANJOS, A. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. WRN and BLM RecQ-Helicase genes are hyperexpressed in patients with SCA In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2018, 2018, São Paulo. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2018**., 2018.

6. XAVIER, A. L.; MARTINS, D. A. P.; BATISTA, J. V. G. F.; SILVA, E. N.; **DOMINGOS, I. F.**; FALCAO, D. A.; ANJOS, A. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Associação do SNP rs6601330 no gene TNKS1 com dados clínicos e laboratoriais de pacientes com anemia falciforme oriundos da fundação HEMOPE In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

7. ALAGBE, A. E.; JUSTO JUNIOR, A. S.; RUAS, L. P.; TONASSE, W. V.; **DOMINGOS, I. F.**; SANTANA, R. M.; ARAUJO, A. S.; BEZERRA, M. A. C.; SANTOS, M. N. N.; BLOTTA, M. H. S. L. Evaluation of interleukin-27 and interleukin-37 in sickle cell anemia patients and their effect on neutrophils and monocytes In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

8. BATISTA, J. V. G. F.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; FALCAO, D. A.; OLIVEIRA, J. M. F.; XAVIER, A. L.; ANJOS, A. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Expressão diferencial de Klotho em indivíduos com e sem anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

9. HOUNKPE, B. W.; CHENOU, F.; **DOMINGOS, I. F.**; BEZERRA, M. A. C.; ARAUJO, A. S.; ALBUQUERQUE, D. M.; OLIVEIRA, R. T. D.; BLOTTA, M. H. S. L.; SONATI, M. F.; COSTA, F. F.; SANTOS, M. N. N.; PAULA, E. V. Expression of netosis regulators and correlation with inflammatory and coagulation biomarkers in sickle cell anemia patients In: 59th ASH Annual Meeting & Exposition, 2017, Atlanta. **59th ASH Annual Meeting & Exposition**., 2017.

10. TONASSE, W. V.; CHINEDU, O.; FABER, E. W.; ALBUQUERQUE, D. M.; **DOMINGOS, I. F.**; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Heme oxygenase-1 gene promoter rs2071746 A>T polymorphism is associated with increased estimated glomerular filtration rate in brazilian sickle cell anemia patients In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

11. ARCANJO, G. S.; **DOMINGOS, I. F.**; MEDEIROS, R. L. B.; BATISTA, J. V. G. F.; BATISTA, T. H. C.; SANTANA, R. M.; ANJOS, A. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Influência dos polimorfismos T-786C e VNTR (4A/B) no gene eNOS com o desenvolvimento de doença cerebrovascular em pacientes pediátricos com anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

12. CHENOU, F.; HOUNKPE, B. W.; ALBUQUERQUE, D. M.; **DOMINGOS, I. F.**; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; PAULA, E. V.; SANTOS, M. N. N. Polimorfismo do gene PADI4 como fator de risco para a síndrome torácica aguda na anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

13. TONASSE, W. V.; ALBUQUERQUE, D. M.; **DOMINGOS, I. F.**; MARTINS, D. A. P.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Rs2000999 (A/G) allele frequencies and genotype distribution in brazilian sickle cell anemia patients In: Brazilian International Congress of Genetics, 2017, Águas de

Lindóia. **Brazilian International Congress of Genetics**., 2017.

14. TONASSE, W. V.; ALBUQUERQUE, D. M.; **DOMINGOS, I. F.**; MARTINS, D. A. P.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Rs2000999 G>A allele frequencies and genotype distribution in brazilian sickle cell anemia patients In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017, 2017, Curitiba. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2017**., 2017.

15. FALCAO, D. A.; **DOMINGOS, I. F.**; MARTINS, D. A. P.; LARANJEIRA, L. P. M.; BATISTA, T. H. C.; ARCANJO, G. S.; PERES, J. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Análise do perfil de expressão gênica da SMAD7 no desenvolvimento de úlceras maleolares em pacientes com anemia falciforme In: XXI Encontro de Genética do Nordeste, 2016, Recife. **XXI Encontro de Genética do Nordeste**., 2016.

16. MEDEIROS, R. L. B.; MARTINS, D. A. P.; HATZLHOFER, B. L. D.; **DOMINGOS, I. F.**; AZEVEDO, R. C.; ANJOS, A. C.; PERES, J. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Associação do polimorfismo IVS-14-1046 C>T no gene ANXA2 com o desenvolvimento do acidente vascular cerebral em pacientes pediátricos com anemia falciforme In: XXI Encontro de Genética do Nordeste, 2016, Recife. **XXI Encontro de Genética do Nordeste**., 2016.

17. **DOMINGOS, I. F.**; MARTINS, D. A. P.; SILVA, J. L. C.; MEDEIROS, R. L. B.; FALCAO, D. A.; AZEVEDO, R. C.; ANJOS, A. C.; MENDONCA, T. F.; CAVALCANTI, M. S. M.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Association between interleukin-6 G-174C polymorphism and stroke development in sickle cell anemia In: XXI Encontro de Genética do Nordeste, 2016, Recife. **XXI Encontro de Genética do Nordeste**., 2016.

18. ROMANELLO, K. S.; LOPES, K. K.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; BEZERRA, M. A. C.; COSTA, F. F.; NETTO, L. E. S.; OLIVEIRA, M. A.; MALAVAZI, I.; CUNHA, A. F. Aumento na oxidação de PRDX2 revela um importante alvo envolvido na deficiência de glicose-6-fostato desidrogenase In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

19. MEDEIROS, R. L. B.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; HATZLHOFER, B. L. D.; AZEVEDO, R. C.; ANJOS, A. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Avaliação de polimorfismos no gene ANXA2 com o desenvolvimento do acidente vascular cerebral em pacientes com anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

20. MARTINS, D. A. P.; **DOMINGOS, I. F.**; ALBUQUERQUE, D. M.; LANARO, C.; ARAUJO, A. S.; BLOTTA, M. H. S. L.; COSTA, F. F.; SONATI, M. F.; BEZERRA, M. A. C.; SANTOS, M. N. N. High levels of pro-inflammatory cytokines is associated a poor clinical outcome in sickle cell anemia In: Congresso Brasileiro de Hematologia,

Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

21. **DOMINGOS, I. F.**; MARTINS, D. A. P.; MEDEIROS, R. L. B.; FALCAO, D. A.; HATZLHOFER, B. L. D.; MENDONCA, T. F.; CAVALCANTI, M. S. M.; CUNHA, A. F.; AZEVEDO, R. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Influence of SOD2 Val16Ala polymorphism on stroke development in a sickle cell anemia Brazilian population In: XXI Encontro de Genética do Nordeste, 2016, Recife. **XXI Encontro de Genética do Nordeste**., 2016.

22. HATZLHOFER, B. L. D.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; FALCAO, D. A.; MEDEIROS, R. L. B.; BATISTA, J. V. G. F.; SOUZA, M. B. S.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Influência dos haplótipos beta S e da talassemia alfa no perfil clínico da anemia falciforme em Pernambuco, Brasil In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

23. BATISTA, J. V. G. F.; MARTINS, D. A. P.; FALCAO, D. A.; **DOMINGOS, I. F.**; CALLADO, F. M. R. A.; PERES, J. C.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Klotho gene polymorphisms and their association with clinical manifestations in patients with sickle cell anemia In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

24. PIELLUSCH, B. F.; FERREIRA, R. A.; ALBUQUERQUE, D. M.; **DOMINGOS, I. F.**; MARTINS, D. A. P.; ARAUJO, A. S.; BEZERRA, M. A. C.; COSTA, F. F.; SONATI, M. F.; SANTOS, M. N. N. Perfil de expressão do microRNA-155 em células mononucleares de pacientes com anemia falciforme com e sem úlcera de perna In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

25. ROMANELLO, K. S.; LOPES, K. K.; OLIVEIRA, J. P. M.; NAGAMATSU, S. T.; BEZERRA, M. A. C.; **DOMINGOS, I. F.**; MARTINS, D. A. P.; ARAUJO, A. S.; FRANCO-PENTEADO, C.; NETTO, L. E. S.; COSTA, FERNANDO F.; MALAVAZI, I.; OLIVEIRA, M. A.; CUNHA, A. F. Peroxirredoxinas são diferencialmente reguladas e podem estar envolvidas na fisiopatologia da anemia falciforme e da beta talassemia In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016, 2016, Florianópolis. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2016**., 2016.

26. BATISTA, J. V. G. F.; MARTINS, D. A. P.; **DOMINGOS, I. F.**; FALCAO, D. A.; HATZLHOFER, B. L.; ARAUJO, A. S.; ANJOS, A. C.; CALLADO, F. M. R. A.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Associação de polimorfismos genéticos em Klotho com o desenvolvimento de priapismo em pacientes com anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2015, 2015, São Paulo. **Revista Brasileira de Hematologia e Hemoterapia**., 2015.

27. LARANJEIRA, L. P. M.; MARTINS, D. A. P.; FALCAO, D. A.; FARIAS, I. C. C.; **DOMINGOS, I. F.**; MEDEIROS, R. L. B.; CALLADO, F. M. R. A.; ARAUJO, A. S.; LUCENA-ARAUJO, A. R.; BEZERRA, M. A. C. Investigação dos polimorfismos em genes da via do TGF beta na ocorrência de úlceras maleolares em pacientes com anemia falciforme In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2015, 2015, São Paulo. **Revista Brasileira de Hematologia e Hemoterapia**., 2015.

28. MACHADO, L. M. S.; PIELLUSCH, B. F.; REZENDE, N. C.; SANTOS, M. N. N.; ALBUQUERQUE, D. M.; MOTA, N. O.; BEZERRA, M. A. C.; **DOMINGOS, I. F.**; ARAUJO, A. S.; COSTA, FERNANDO F.; SONATI, M. F.; RIBEIRO, D. M. olimorfismo do gene da hemopexina (HPX) em pacientes com anemia falciforme do estado de Pernambuco In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular - HEMO 2015, 2015, São Paulo. **Revista Brasileira de Hematologia e Hemoterapia**., 2015.

Apresentação de trabalho e palestra

1. **Evaluation of genetic modulators of patients with sickle cell anemia from Pernambuco, a Brazilian northeast state**, 2018. Local: Auditório FCM Unicamp; Cidade: Campinas; Evento: Brazil-UK Workshop on Haemoglobinopathies; Inst.promotora/financiadora: UNICAMP

2. **Fatores genéticos associados ao desenvolvimento de acidente vascular cerebral em pacientes portadores de anemia falciforme**, 2018 Local: Faculdade Unissau; Cidade: João Pessoa; Evento: Ciclo de Palestras; Inst.promotora/financiadora: UNINASSAU

3. **Anemias Hemolíticas**, 2017. Local: Auditório Jayme Sherb do campus Santo Amaro, Universidade de Pernambuco; Cidade: Recife; Evento: I Simpósio de Hematologia; Inst.promotora/financiadora: Liga Acadêmica de Hematologia de Pernambuco (LAHEPE)

4. **Imunohematologia básica e Sistema ABO**, 2017. Local: Auditório do Centro Integrado de Saúde Amaury de Medeiros; Cidade: Recife; Evento: Capacitação em Agência Transfusional para Técnicos de Laboratório; Inst.promotora/financiadora: Universidade de Pernambuco

5. **Obtenção e Uso de Hemocomponentes e Hemoderivados**, 2017. Local: Faculdade Boa Viagem; Cidade: Recife; Evento: I Jornada de Biomedicina da FBV/DeVry; Inst.promotora/financiadora: FBV/DeVry

6. **Polimorfismo IL-6 G-174C promove maior risco de AVC em pacientes com anemia falciforme**, 2017. Local: Auditório Luiz Gonzaga, HEMOPE; Cidade: Recife; Evento: Reunião Técnico-científica; Inst.promotora/financiadora: Fundação de Hematologia e Hemoterapia de Pernambuco

7.**Ações e Oportunidades no Laboratório de Hematologia**, 2016. Local: Universidade Federal de Pernambuco; Cidade: Recife; Evento: Disciplina Introdução

as Análises Clínicas (FA670); Inst.promotora/financiadora: Departamento de Ciências Farmacêuticas

8. **Diagnóstico Laboratorial das Hemoglobinopatias**, 2015. Local: Centro de Ciências Biológicas; Cidade: Recife; Evento: XXII Semana de Biomedicina: “Perspectivas no avanço científico e diagnóstico”; Inst.promotora/financiadora: Universidade Federal de Pernambuco

9. **Investigação de polimorfismos em genes relacionados à resposta ao estresse oxidativo e inflamação na susceptibilidade à ocorrência da doença cerebrovascular em pacientes com anemia falciforme**, 2015. Local: Auditório Luiz Gonzaga, HEMOPE; Cidade: Recife; Evento: Reunião técnico-científica; Inst.promotora/financiadora: Fundação de Hematologia e Hemoterapia de Pernambuco

Orientações e supervisões concluídas

Monografias de conclusão de curso de aperfeiçoamento/especialização

1. Everton Diego Carvalho Silva. **A influência da anemia ferropriva no período gestacional**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

2. Ian Marcel de Medeiros Martins Pontes. **Análise laboratorial para diagnóstico das hemoglobinopatias**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

3. Sílvia Cecilia Brandão Bezerra. **Anemia aplástica: Uma revisão da literatura**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

4. Alba Virginia Aguiar de Carvalho Cardoso. **Anemia ferropriva em lactentes: Causas, diagnóstico e tratamento**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

5. Jeniffer Maria Assis dos Santos. **Anemia ferropriva na infância: Fatores associados, estratégias de prevenção e tratamento**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

6. Nathália Monique da Silva Coêlho. **Anemia ferropriva no período gestacional - importância do diagnóstico e tratamento**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

7. João Henrique Alves da Cunha. **Doença falciforme, tratamentos atuais e novas abordagens terapêuticas**. 2017. Monografia (Hematologia Clínica e Laboratorial) - Universidade Federal de Pernambuco

Trabalhos de conclusão de curso de graduação

1. Camila Mabel de Albuquerque Ramos. **Influência do polimorfismo do gene**

UGT1A1 na hiperbilirrubinemia e incidência de colelitíase em pacientes com anemia falciforme. 2018. Curso (Biomedicina) - Universidade Federal de Pernambuco

2. Eduarda Ruth de Oliveira Lira. **Uso da transfusão e aloimunização em pacientes com anemia falciforme**. 2017. Curso (Biomedicina) - Universidade Federal de Pernambuco

3. Carlos Eduardo Charamba de Araújo. **Uso da transfusão no tratamento de pacientes com anemia falciforme**. 2017. Curso (Biomedicina) - Universidade Federal de Pernambuco

4. Marlon Fagundes Ribeiro. **Associação do polimorfismo no gene NOS3 T-786C no desenvolvimento de síndrome torácica aguda em pacientes com anemia falciforme**. 2016. Curso (Biomedicina) - Universidade Federal de Pernambuco

5. Wendy Anushika Alves Cavalcanti. **Uso de hidroxiureia na anemia falciforme**. 2016. Curso (Biomedicina) - Universidade Federal de Pernambuco

Bancas

Participação em banca de trabalhos de conclusão de curso de graduação

1. Participação em banca de Alberto de Lima Xavier. **Análise da expressão relativa de genes do metabolismo telomérico em pacientes com anemia falciforme**, 2018 (Biomedicina) Universidade Federal de Pernambuco

2. Participação em banca de Amanda Caroliny Santos de Freitas. **Fatores de risco para o desenvolvimento de inibidores do fator VIII na hemofilia A**, 2018 (Farmácia) Universidade Federal de Pernambuco

3. Participação em banca de Everton Neri da Silva. **Hemoglobinopatias SC: Panorama clínico, laboratorial e análise da influência da talassemia alfa e dos haplótipos do agrupamento de genes da globina beta como moduladores fenotípicos da doença**, 2018 (Biomedicina) Universidade Federal de Pernambuco

4. Participação em banca de Gabriela da Silva Arcanjo. **Análise da influência de polimorfismo no gene eNOS com o desenvolvimento de doença cerebrovascular em pacientes pediátricos com anemia falciforme**, 2017 (Biomedicina) Universidade Federal de Pernambuco

5. Participação em banca de Aimeé Diacuí de Almeida Barrêto. **Aspectos clínicos e laboratoriais da trombocitemia essencial**, 2017 (Biomedicina) Universidade Federal de Pernambuco

6. Participação em banca de Daiany Gabrielly Lima Barros. **Avaliação da eficácia do tratamento com ácido transretinóico e trióxido de arsênico em pacientes com leucemia promielocítica aguda: Revisão sistemática e metanálise**, 2017 (Biomedicina) Universidade Federal de Pernambuco

7. Participação em banca de Marcondes José de Vasconcelos Costa Sobreira. **Avaliação da influência do polimorfismo IVS-14-1046 C>T no gene ANXA2 com o desenvolvimento de doppler transcraniano de alto risco em pacientes com anemia falciforme**, 2017 (Biomedicina) Universidade Federal de Pernambuco

8. Participação em banca de Gabriela Vitória Menezes Castelo Branco. **Influência da carga alélica JAK2V617F em pacientes com neoplasias mieloproliferativas**, 2017 (Biomedicina) Universidade Federal de Pernambuco

9. Participação em banca de Luanna Soares Fernandes do Nascimento. **Significado clínico e laboratorial de mutações no gene do NMP1 da leucemia mielóide aguda**, 2017 (Biomedicina) Universidade Federal de Pernambuco

10. Participação em banca de Amanda Luíze Melo Tavares Ramos. **Alterações epigenéticas na leucemia mielóide aguda**, 2016 (Biomedicina) Universidade Federal de Pernambuco

11. Participação em banca de Thais Helena Chaves Batista. **Análise da expressão do gene SMAD7 na ocorrência de úlceras maleolares em pacientes com anemia falciforme**, 2016 (Biomedicina) Universidade Federal de Pernambuco

12. Participação em banca de Isabela Cristina Cordeiro Farias. **Associação de polimorfismos do gene MBL2 com úlcera de membros inferiores em pacientes com anemia falciforme**, 2016 (Biomedicina) Universidade Federal de Pernambuco

13. Participação em banca de Jobson Ferraz do Nascimento. **Estudo citogenético em pacientes com LMA de novo**, 2016 (Biomedicina) Universidade Federal de Pernambuco

14. Participação em banca de Guilhermy Victor Sousa de Araújo. **Investigação do perfil mutacional do gene DNMT3A em pacientes com leucemia mielóide aguda**, 2016 (Biomedicina) Universidade Federal de Pernambuco

15. Participação em banca de Elaine Cristina Fontinele Martins. **O papel do polimorfismo TP53 R72P na susceptibilidade e prognóstico da leucemia mielóide aguda**, 2016 (Biomedicina) Universidade Federal de Pernambuco

16. Participação em banca de Daniel Soares de Mendonça. **Revisão dos critérios diagnósticos e estadiamento clínico de pacientes com leucemia mielóide crônica**, 2016 (Biomedicina) Universidade Federal de Pernambuco